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Inhibition of MEPE cleavage by *Phex*

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Abstract

X-linked hypophosphatemia (XLH) and the *Hyp*-mouse disease homolog are caused by inactivating mutations of *Phex* which results in the local accumulation of an unknown autocrine/paracrine factor in bone that inhibits mineralization of extracellular matrix. In these studies, we evaluated whether the matrix phosphoglycoprotein MEPE, which is increased in calvaria from *Hyp* mice, is a substrate for *Phex*. Using recombinant full-length *Phex* (r*Phex*WT) produced in *Sf*9 cells, we failed to observe *Phex*-dependent hydrolysis of recombinant human MEPE (rMEPE). Rather, we found that r*Phex*-WT inhibited cleavage of rMEPE by endogenous cathepsin-like enzyme activity present in *Sf*9 membrane. *Sf*9 membranes as well as purified cathepsin B cleaved MEPE into two major fragments of ~50 and ~42 kDa. r*Phex*WT protein in *Sf*9 membrane fractions, co-incubation of r*Phex*WT and cathepsin B, and pre-treatment of *Sf*9 membranes with leupeptin prevented the hydrolysis of MEPE in vitro. The C-terminal domain of *Phex* was required for inhibition of MEPE cleavage, since the C-terminal deletion mutant r*Phex* (1–433) [r*Phex*3′M] failed to inhibit *Sf*9-dependent metabolism of MEPE. *Phex*-dependent inhibition of MEPE degradation, however, did not require *Phex* enzymatic activity, since EDTA, an inhibitor of r*Phex*, failed to block r*Phex*WT inhibition of MEPE cleavage by *Sf*9 membranes. Since we were unable to identify interactions of *Phex* with MEPE or actions of *Phex* to metabolize cathepsin B, *Phex* may be acting to interfere with the actions of other enzymes that degrade extracellular matrix proteins. Although the molecular mechanism and biological relevance of non-enzymatic actions of *Phex* need to be established, these findings indicate that MEPE may be involved in the pathogenesis defective mineralization due to *Phex* deficiency in XLH and the *Hyp*-mouse.

Keywords: M13 endopeptidase; Mineralization; Bone; XLH; *Hyp*

Phex is a zinc-dependent type II cell-surface membrane metalloprotease that is involved in regulating phosphate and mineral homeostasis [1–4]. Inactivating mutations of *Phex* lead to defective calcification of bone and cartilage and renal phosphate wasting in the clinical disorder X-linked hypophosphatemic rickets (XLH) [3,5,6] and the *Hyp* and *Gy* mice disease homologs [7–9]. The pathogenesis of phosphate wasting and impaired mineralization in these disorders remain poorly understood, but may represent the accumulation of distinct *Phex* substrates or the accumulation of factors as an indirect consequence of inactivating *Phex* mutations. Related endopeptidases have multiple substrates that are often co-expressed with the enzyme in different tissues [10].

Parabiosis experiments [11] and kidney cross-transplantations [12] in the *Hyp*-mouse homolog of XLH indicated that defective *Phex* function leads to the accumulation of a circulating phosphaturic factor, called phosphatonin [6]. Recent investigations have identified FGF23, a phosphaturic factor that is expressed by tumors causing oncogenic hypophosphatemic osteomalacia (OHO) [13,14], and mutated in autosomal dominant hypophosphatemic rickets (ADH) [15], as a possible *Phex* substrate and candidate for phosphatonin [16,17].

Increased circulating FGF23 levels, however, do not fully explain the impaired mineralization associated with inactivation of *Phex*. First, inhibition of mineralization

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is observed ex vivo in *Phex* deficient osteoblast cultures [18], which lack FGF23 [19]. Moreover, the mineralization defect in *Hyp*-derived osteoblasts is not totally dependent on hypophosphatemia, since partial correction of defective mineralization but not hypophosphatemia can be achieved by the targeted expression of *Phex* in bone of *Hyp* mice [19]. Rather, the impaired mineralization appears to result from the autocrine/paracrine secretion by osteoblasts of a mineralization inhibitory factor [8,20–22]. Recently, several peptides have been identified that are cleaved by *Phex* in vitro, including ZAAL-pNA, leu[enkephalin], and human parathyroid hormone related peptide [18,23], but none of these substrates account for the mineralization defects associated with inactivating mutations of *Phex*.

Another candidate for the putative bone derived *Phex* substrate is the matrix extracellular phosphoglycoprotein (called MEPE or OF45) [24,25]. MEPE, which was originally isolated and cloned from mesenchymal tumors derived from OHO patients, is expressed in bone and has molecular similarities with several extracellular matrix RGD-containing phosphoglycoproteins involved in mineralization, including dentin sialophosphoprotein (DSPP), osteopontin (SPP1), and dentin matrix protein-1 (DMP1). Preliminary studies in MEPE deficient mice indicate that the absence of MEPE is associated with accelerated mineralization [26]. Conversely, MEPE transcripts are increased in poorly mineralizing bone derived from *Hyp*-mice [27]. These associations suggest that the *Phex* gene product might control bone matrix mineralization by directly or indirectly regulating the metabolism of MEPE. To date, no studies have examined whether MEPE is a substrate for *Phex*.

In the present investigation, we investigated whether MEPE is a substrate for *Phex*. Using recombinant epitope tagged MEPE and *Phex* proteins, we were unable to show that MEPE is a substrate for *Phex*; however, we found that *Phex* inhibited the proteolytic cleavage of MEPE by cathepsin B-like activity present in *Sf*9 cell membranes. These findings support an indirect role for *Phex* in the regulation of MEPE metabolism through inhibition of factors involved in degradation of extracellular matrix proteins. This action of *Phex* may be of physiological importance in regulating mineralization of extracellular matrix.

Materials and methods

Materials. C-terminal V5-His epitope tagged recombinant active wild-type *Phex* (1–749) [designated r*Phex*WT] and inactive 3′ truncated *Phex* (1-433) [designated r*Phex*3′M] were expressed in *Sf*9 membranes as previously described [18]. *PhexWT* and *Phex*3′M expressing membranes were solublized with 1% *n*-dodecyl-β-D maltoside (DDM) and collected by centrifugation. The protein content of each sample was determined by the NanoOrangeTM Protein Quantitation Kit (Molecular Probes, Eugene OR, USA). Purified recombinant calmodulin binding peptide (CBP), N-terminal tagged human MEPE protein comprising amino acids 95–525, and anti-human MEPE antisera were generated as previously described [24]. Cathepsins B and D were obtained from CalBiochem (San Diego, CA, USA). Leupeptin, aprotinin, and pefabloc, a protease inhibitor cocktail containing all three of these inhibitors were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). The anti-V5 monoclonal antibody was obtained from Invitrogen (Carlsbad, CA, USA). Z-Ala-Ala-Leu-*p*-nitroanilide (ZAAL-pNA) was purchased from Bachem Biosciences (King of Prussia, PA, USA). Leucine aminopeptidase was purchased from Sigma (St. Louis, MO, USA).

Assessing Phex enzyme activity. We assessed *Phex* activity by modifications of previously described methods [18]. We used membrane fractions from the *Sf*9 cells (50 μg) expressing vector or the V5-His epitope tagged *Phex* constructs. Protein samples were incubated with 50 μM ZAAL-pNA in 100 μl of 100 mM MES (pH 6.5) for 1 h at 37 °C. After completion of the initial incubation, the reaction mixture was further incubated with 0.4 mU leucine aminopeptidase for 20 min at 37 °C. The reaction was stopped by the addition of 100 mM EDTA and the absorbency was measured at 405 nm after centrifugation. In some studies, membrane fractions were preincubated with 100 mM EDTA for 30 min before the addition of ZAAL-pNA.

Assessing MEPE cleavage. Purified recombinant human MEPE (100 ηg) was incubated with various enzymes preparations, including 30 μg solubilized membranes derived from *Sf*9 cell infected with either wild-type baculovirus, *rPhex*WT or *rPhex*3′M. In addition, rMEPE was incubated with 5 μg cathepsin B or 0.5 U cathepsin D. For all studies, the reaction mixture contained 50 mM NaCl, 20 mM Tris pH 7.4 for 1 h at 37 °C. The reactions were stopped by adding protein loading buffer (Invitrogen Carlsbad, CA, USA). In some studies, either leupeptin (1 μM), aprotinin (0.3 μM), and pefabloc (4 μM), a proteinase inhibitor cocktail containing all three of these inhibitors, or 200 mM EDTA pre-incubated with 100 ηg CBP-tagged MEPE for 20 min at 37 °C, followed by addition of the various enzymes. All the reactions were analyzed by Western blot using anti-MEPE antisera.

SDS–PAGE and Western blot analyses. Immunoblot analysis was carried out by modifications of previously described methods [28]. The specified amount of membrane proteins was dissolved in SDS–gel loading buffer (Invitrogen Carlsbad, CA, USA). Separated proteins were transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad, Chicago, IL USA) over a 30 min period at 2.5 mA/cm^2 at room temperature using a Semi-dry blotting system (Millipore, Chicago, IL USA). Immunoblotting was performed using anti-MEPE antisera diluted 1:2000 in TBST and 1% bovine albumin (BSA) or using antiV5 monoclonal antibody (Invitrogen Carlsbad, CA, USA) for 1 h at room temperature. The blots were washed with TBST for 60 min and incubated with anti-rabbit IgG conjugated with HRP (diluted to 1:5000) for polyclonal antibody or anti mouse IgG for monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA USA) at room temperature using for 60 min. After washes with TBST, immunoreactivity was detected by ECL (Amersham Pharmacia Biotech., UK, HP79NA).

Quantitative real-time PCR. Total (1.5 μg) RNA was denatured for 5 min at 65 °C in the presence of 0.5 η mols random hexamer, snap cooled in ice water, and then reverse transcribed in 100 μl using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. PCR reactions contained 50 ηg template (cDNA or RNA), 300 ηM each forward and reverse primer, and 1× SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in 50 μl. Samples were amplified for 40 cycles in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with an initial melt at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to dsDNA. The partial cycle at which a statistically significant increase in MEPE product was first detected

(threshold cycle, Ct) was normalized to the Ct for GAPDH. A passive reference dye, ROX, was used to normalize for variations in volume and/or dye concentration between sample wells. Post-amplification melting curves were performed to confirm that a single PCR product was produced in each reaction. The contribution of contaminating genomic DNA to the observed product was determined from the Ct given by the RNA template. This quantity was usually less than 0.1%. The primer sequences used include MEPE-F primer ATGCAGGG AGAGCTGGTTAC and MEMP-R primer TGGTTCCCTTTGG ACTCTTC and cyclophilin A-F primer CTGCACTGCCAAGACT GAAT and cyclophylin A-R primer CCACAATGTTCATGCC TTCT.

Two-Yeast hybrid analysis. The extracellular domain of mouse *Phex* containing residues 46–749 was subcloned into the yeast expression vector (pGBKT7; Clontech, Palp Alto, CA) encoding a GAL4 DNA binding domain (BD) to generate the PGBKT7-Phex bait construct. This was accomplished by PCR with amplification of the *Phex* cDNA using a *Nco*I-containing forward primer 5′-ATTCTTC CATGGAGAGTTTCCAAGCTAAACAGGAG-3′ and a *Sal*I-containing reverse primer5′-ATTCTTGTCGACCCAGAGTCGGCAAG AATCTGC-3′. The human MEPE cDNA containing residues 18–531 was subcloned into the yeast expression vector (pGADT7; Clontech) encoding a GAL4 DNA transcriptional activation domain (AD) to create the pGADT7-MEPE prey construct. This construct was obtained by PCR with amplification of the pBlueBac4.5-MEPE cDNA using a *Eco*RI-containing forward primer 5′-ATTCTTGAATTCC CAACATTTCAACCACAG-3′and a *Bam*HI-containing reverse primer 5′-ATTCTTGGATCCCTAGTCACCATCGCTCTC-3′. The orientation and alignment of the reading frame for the subcloned cDNAs were confirmed by DNA sequencing. To examine the ability of the extracellular domain of *Phex* to interact with MEPE, the bait construct was introduced into the yeast strain PJ69-2A and the prey construct was introduced into the yeast strain Y187. The LiAc-mediated method was used to introduce plasmid DNA into yeast [29]. The following yeast mating pairs were set up: pGBKT7-*Phex* vs. pGADT7-MEPE, pGBKT7-53 (murine p53 cDNA; Clontech) vs. pGADT7-T (SV40 large T antigen cDNA; Clontech) as a positive protein–protein interaction control and PGBKT7-Lam (human lamin C cDNA, Clontech) vs. pGADT7-T (SV40 large T antigen cDNA) as a negative protein–protein interaction control. The mating culture was then spread on SD/-trp,-leu/X-α-Gal (5-Bromo-4-chloro-3indolyl-α-D-galactopyranoside; Clontech) and SD/-trp,-leu,-his/X-α-Gal,+2.5 mM 3AT (3-amino-1,2,4-triazole; Sigma) plates to select for transformants containing the plasmid(s) that expressed β-galactosidase and histidine-3 genes. The plates were incubated at 30 °C for 4–5 days or until diploid cells appeared. A colony of each the pairs cultured in the SD selection medium was assayed for β-galactosidase activity using ONPG (*o*-nitrophenyl β-D-galactopyranoside; Sigma) as substrate [30].

Statistical analysis. Analysis of variance was performed with the Statgraphics software package (Statistical Graphics, Princeton, NJ, USA).

Results

Increased MEPE expression in Hyp mice

We investigated whether inactivating *Phex* mutations in *Hyp* mice is associated with increased MEPE expression. Consistent with previous reports [27], we found that MEPE transcripts are increased in calvaria derived from *Hyp*-mice (Fig. 1). Using the available anti-MEPE antibodies that readily detect recombinant MEPE, we were unable to detect MEPE protein in bone from normal and *Hyp*-mice by Western blot analysis (data not shown).

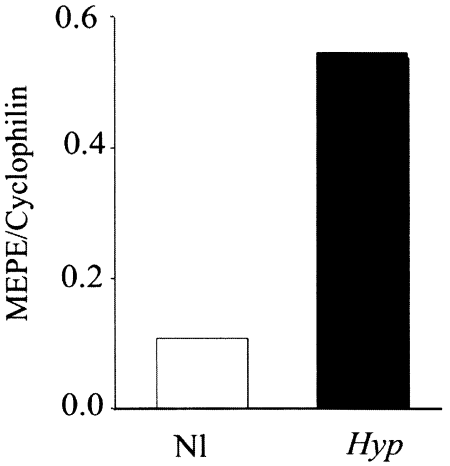

Fig. 1. Expression of MEPE in normal and *Hyp* mouse calvaria. Real-time PCR was performed as described in Materials and methods. Values are expressed as a ratio of MEPE to cyclophilin and represent the mean of two separate determinations in calveria derived from 6-week-old normal and *Hyp* mice.

rPhex-WT but not rPhex-3′M cleave Z-Ala-Ala-Leu-pNA

We [19] and others [31] have previously reported that recombinant mouse *Phex* protein cleaves the synthetic chromagenic peptide, Z-Ala-Ala-Leu-pNA. In these studies, we confirmed that theV5-His N-terminal tagged *Phex* produced in *Sf*9 cell membrane cleaved Z-Ala-Ala-Leu-pNA (Fig. 2) and that the inactive mutant, r*Phex* 3′M, which lacks the C-terminal catalytic domain, fails to hydrolyze Z-Ala-Ala-Leu-pNA (Fig. 2). Moreover, addition of the chelating agent EDTA (100 mM) inhibited *rPhex* hydrolysis of Z-Ala-Ala-Leu-pNA (Fig. 2).

rPhex does not cleave MEPE

Next, we compared the activity of membranes derived from *Sf*9 cells expressing vector with *Sf*9 membranes expressing *rPhex*WT and the inactive *rPhex* 3′M, to cleave human recombinant MEPE. Purified recombinant MEPE was identified as a predominant 68 kDa band (Fig. 3A). After incubation with *Sf*9 membranes, the MEPE protein was cleaved into at least two major fragments of apparent molecular weights of 50 and 42 kDa (Fig. 3A). In some studies, the 50 kDa product could be resolved into two distinct bands and other products were observed in low abundance. Unexpectedly, incubation of rMEPE protein with *Sf*9 membranes expressing *rPhex*WT resulted in disappearance of the 50 and 42 MEPE cleavage products (Fig. 3A). It is unlikely that *Phex* degrades the MEPE fragments, thereby explaining their absence, since the amount of uncleaved MEPE is the same in the presence and absence of *Phex*.

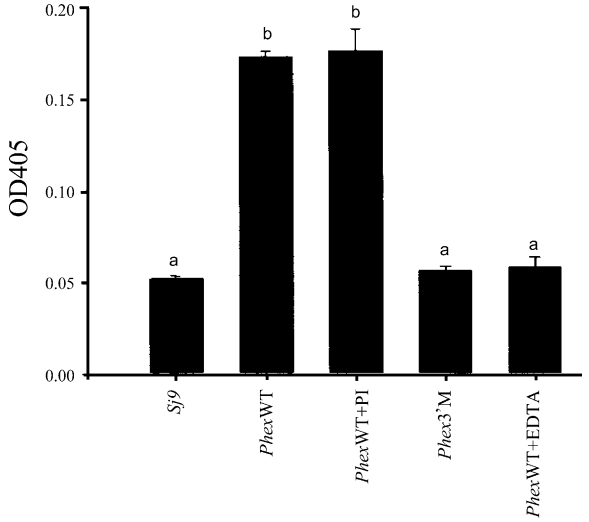

Fig. 2. Comparison of enzymatic activity of wild-type and mutated *rPhex* constructs. Membrane fractions (50 μg) derived from *Sf*9 cells after baculovirus-mediated transduction with vector alone, *rPhex*WT or *rPhex*3′M were analyzed for peptidase activity using ZAAL-pNA as a substrate in the absence or presence of EDTA and protease inhibitor cocktail (PI) as described in Materials and methods. Data are representative of at least three independent experiments and shown with means ± SEM.

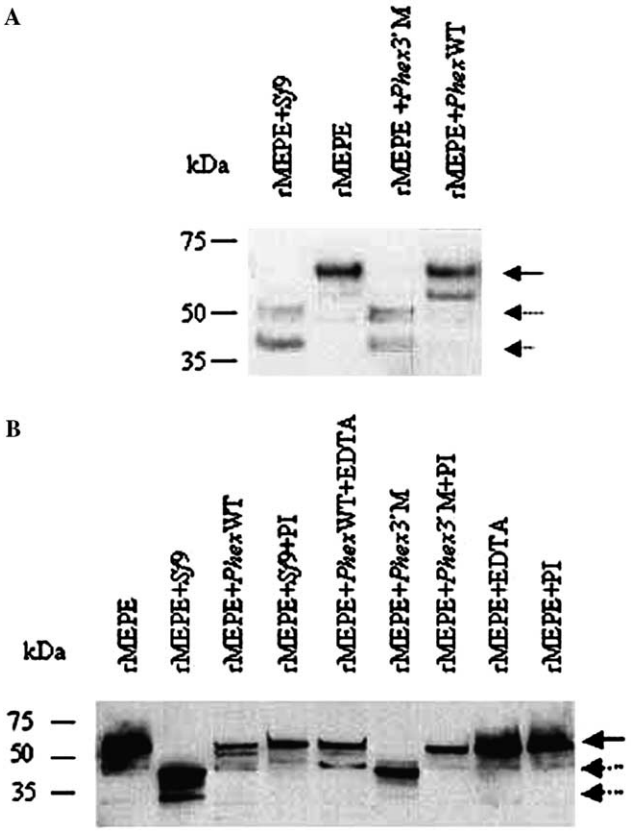

Fig. 3. Assessment of MEPE hydrolysis by Western blot analysis. (A) Inhibition of MEPE cleavage by *rPhex*. rMEPE (96–525) (100 ηg) was incubated with cell membrane preparations (30 μg) from vector alone, *rPhex*3′M and *rPhex*WT infected *Sf*9 cells as described in Materials and methods. Endogenous enzymes in *Sf*9 membranes hydrolyzed rMEPE (96–525) as evident by the disappearance of the 68 kDa rMEPE (lane 2; solid arrow) and the appearance of two major bands of 50 and 42 kDa after incubation with *Sf*9 membranes (lane 1; broken arrows). Incubation of rMEPE with *rPhex*WT inhibited hydrolysis of MEPE (lane 4), whereas *rPhex*3′M (lane 3) did not block *Sf*9 cleavage of rMEPE. (B) Effect of inhibitors on *Sf*9 cleavage of MEPE. rMEPE (96–525) (100 ηg) was incubated with cell membrane preparations (30 μg) from vector alone and *rPhex*3′M in the presence and absence of a complete EDTA-free protease inhibitor cocktail (containing leupeptin [1 μm], aprotinin [0.3 μm], and pefabloc [4 mM]) or *rPhex*WT in the presence and absence of 100 mM EDTA as described in Materials and methods. rMEPE hydrolysis was monitored by Western blot analysis using anti-MEPE polyclonal antibody. The addition of *Phex* consistently altered the degradation of MEPE in five separate experiments.

The effect of *rPhex*WT to inhibit degradation of rMEPE by endogenous *Sf*9 membrane derived enzymes was dependent on the C-terminus, since the mutant *rPhex*-3′M did not block *Sf*9 membrane cleavage of rMEPE (Fig. 3A). In addition, we incubated *Sf*9 membranes with a protease inhibitor cocktail to establish that endogenous enzymes in *Sf*9 cells were responsible for the observed degradation of rMEPE (Fig. 3B). Similar to *rPhex*WT, incubation of *Sf*9 membranes with a mixture of leupeptin, aprotinin, and pefabloc blocked degradation of rMEPE. Moreover, the inhibition of *Sf*9 mediated cleavage of rMEPE by *rPhex* did not appear to require *rPhex* activity, since pre-treatment of *rPhex*WT with EDTA did not alter the capacity of *Phex* to prevent MEPE cleavage. Also, native *Sf*9 cell membranes not infected with the baculovirus cleaved MEPE (data not shown).

Sf9 membranes contain cathepsin B-like proteolytic activity against MEPE

To further characterize the endogenous enzymatic activity in *Sf*9 membranes responsible for MEPE degradation, we tested the ability of individual inhibitors in the protease cocktail to block MEPE degradation by *Sf*9 membranes (Fig. 4A). We found that leupeptin was responsible for the inhibitor activity of the protease cocktail (Fig. 4A). In contrast, the other components of the cocktail, aprotinin, and prefabloc failed to inhibit *Sf*9 cleavage of MEPE (Fig. 4A).

The effects of leupeptin suggested that the responsible endogenous enzymatic activity in *Sf*9 membranes is a cysteine protease such as cathepsin B. This was confirmed by evaluating the ability of cathepsin B to cleave MEPE (Fig. 4B). Similar to endogenous enzyme activity in *Sf*9 membranes, incubation of purified cathepsin B with rMEPE (but not cathepsin D, data not shown) resulted in cleavage of MEPE into two major bands of 50 and 42 kDa (Fig. 4B), consistent with its ability to degrade extracellular matrix components. In addition, pre-treatment with leupeptin inhibited cleavage by

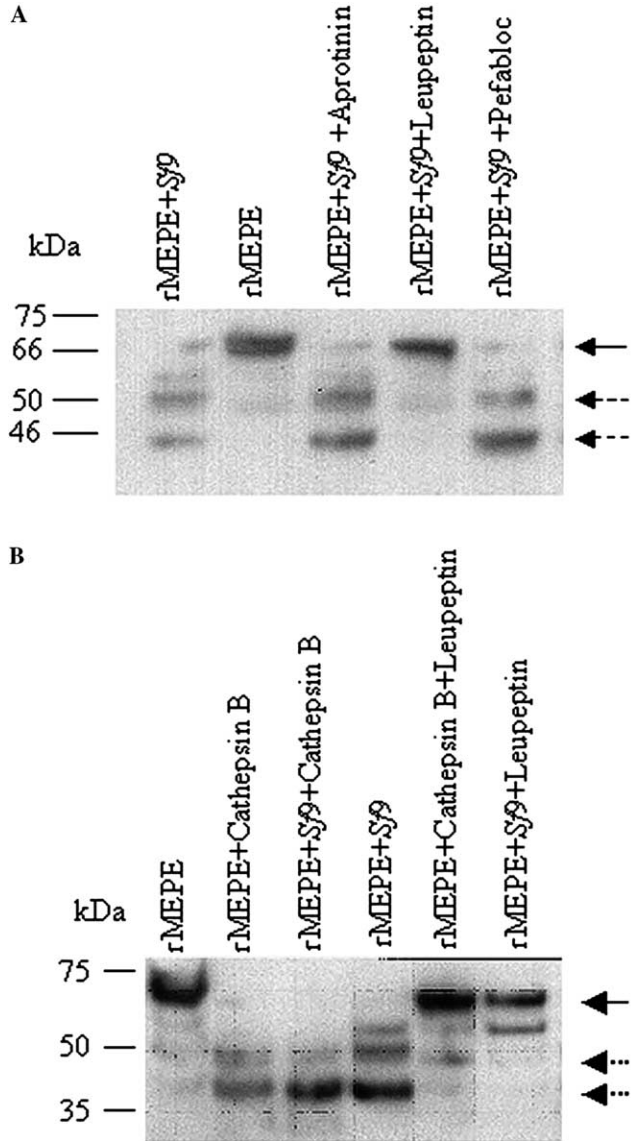

Fig. 4. Characterization of the endogenous enzymatic activity in *Sf*9 membranes responsible for MEPE cleavage. (A) Effects of individual serine and cystine protease inhibitors on *Sf*9 membrane cleavage of MEPE. Thirty μg of *Sf*9 membrane infected with Bac-WT, *Phex*WT, or *Phex*3′M was preincubated with protease inhibitor cocktail, 1 μm leupeptin, 0.3 μm aprotinin or 4 mM pefabloc 20 min at 37 °C and then 100 ηg recombinant MEPE added for an additional 1 h at 37 °C. (B) Comparison of MEPE cleavage by purified cathepsin B and *Sf*9 membranes. rMEPE (96–525) (100 ηg) was incubated with cathepsin B (5 μg) or *Sf*9 cell membranes (30 μg) in the presence or absence of leupeptin (1 μM). rMEPE hydrolysis was monitored by Western blot analysis using anti-MEPE polyclonal antibody. Both cathepsin B and endogenous protease activity in*Sf*9 membranes cleaved MEPE. Pretreatment with leupeptin inhibited both cathepsin B and *Sf*9 mediated hydrolysis of rMEPE. Western blot analysis using anti-MEPE polyclonal antibody.

cathepsin B (Fig. 4B), similar to its effect to inhibit the activity in *Sf*9 membranes (Fig. 4A). An additional MEPE degradation product between 66 and 50 kDa was observed with *Sf*9 membranes, possibly indicating

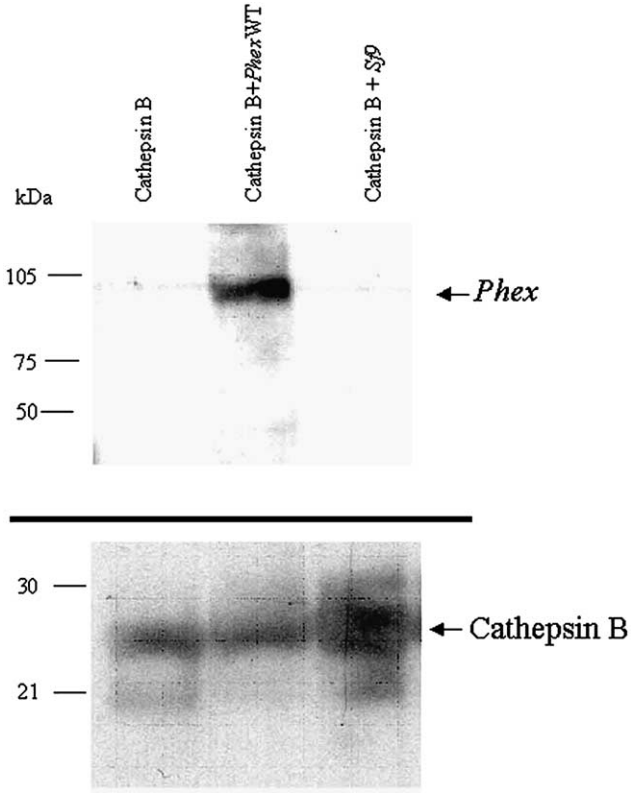

Fig. 5. rPhex and cathepsin B are not substrates for each other. Five μg of human liver cathepsin B was incubated with membrane preparation (30 μg) derived from *Sf*9 cells infected with vector alone or the *rPhex*WT construct as described in Materials and methods. Protein fractions were subjected to SDS–PAGE under reducing conditions and immunoblot analysis was performed with either an anti-V5 monoclonal antibody to detect epitope tagged *Phex* (upper panel) or a monoclonal antibody to cathepsin B (lower panel). The ~25 kDa band represents cathepsin B and the predominant band of ~100 kDa that corresponds to full-length *Phex*. *rPhex* did not cleave cathepsin B.

different cleavage site specificities of the enzymatic activity in the membranes.

The ability of *rPhex* to inhibit MEPE cleavage by endogenous enzymes in *Sf*9 membranes was not the result of *rPhex* degradation, since in previous studies we demonstrated that the *rPhex* produced in *Sf*9 cells was an enzymatically active protein of the predicted molecular weight [18]. To investigate if *Phex* metabolized cathepsin B or vice versa, we incubated purified *rPhex* with cathepsin B and evaluated potential degradation products by Western blot analysis. We found that *rPhex* and cathepsin B did not degrade each other (Fig. 5). These findings and the inability of EDTA to block the effects of *rPhex* on MEPE degradation (Fig. 3B) suggest that the C-terminal region of *Phex* somehow interferes with the enzymatic cleavage of MEPE, by either sequestering the MEPE or modifying the activity of cathepsin-like enzymes through non-enzymatic actions of *Phex*. To investigate potential interactions of *Phex* and MEPE, we used the extracellular domain if *Phex* in a yeast two-hybrid assay with MEPE. These studies

failed to identify any significant interactions between *Phex* and MEPE (data not shown).

Discussion

Since the discovery that inactivating mutations of PHEX cause XLH, efforts have focused on identifying the physiologically relevant substrates of this endopetidase that are responsible for renal phosphate wasting and impaired mineralization. Recent studies suggest that systemic consequences of *Phex* mutations, resulting from the failure to metabolize a phosphaturic factor, possibly FGF23 [17], may be distinct from the autocrine/paracrine effects, leading to impaired bone mineralization [18–20,22]. The presence of an intrinsic mineralization defect in *Hyp*-derived osteoblasts has led to the search for extracellular modulators of mineralization that are produced by osteoblasts and normally degraded by *Phex*.

The importance of the current study is that we show that MEPE (or OF45) is not a substrate for *Phex*. It is still possible, however, that MEPE is a candidate for a mineralization inhibitory factor, since this extracellular matrix protein is involved in the mineralization process [24,25] and its expression is increased in osteoblasts derived from the *Hyp*-mice [27]. Although we found that *Phex* does not directly hydrolyze MEPE, it does modify MEPE cleavage by cathepsin B and other undefined enzymes present in *Sf*9 membranes. In this regard, endogenous enzymatic activity in crude *Sf*9 membranes (Figs. 3 and 4A), as well as purified cathepsin B (Fig. 4B), resulted in the cleavage of rMEPE into two major fragments. Additional studies, using the inhibitor leupeptin, indicated that the activity in *Sf*9 membranes responsible for MEPE cleavage is probably a cysteine protease (Fig. 4). We did not identify the precise cleavage site in MEPE, but cathepsin B prefers a basic and hydrophobic amino acid at the P1 and P2 substrate sites [32] and there are several potential cleavage sites in MEPE that would generate the cleavage products of the observed molecular weights. The observation that the MEPE is degraded by cysteine proteases and Sf9 membranes is consistent with known role of enzymes to degrade organic matrix components present in bone.

Of potential greater importance is the observation that *Phex* indirectly regulates the metabolism of this extracellular matrix protein through the inhibition of matrix degrading enzymes (Figs. 3 and 4). The inhibition of MEPE cleavage required the C-terminal region of *rPhex*, since a C-terminal truncated form of (*Phex rPhex* 3′M; aa 537–749) failed to inhibit cleavage of MEPE by *Sf*9 membrane preparations (Fig. 3A). *Phex* inhibition of MEPE hydrolysis, however, does not appear to require enzymatic activity of *Phex*. In this regard, inhibition of r*Phex* activity by EDTA, which prevented *Phex* cleavage of the synthetic substrate ZAAL-pNA (Fig. 2), did not inhibit the actions of *Phex* to block degradation of MEPE (Fig. 3B). In addition, we failed to demonstrate *rPhex* cleavage of cathepsin B or visa versa (Fig. 5), indicating that the inhibition of MEPE cleavage is not due to an effect of the respective enzymes to degrade each other. Finally, we were unable to demonstrate interactions between *Phex* and MEPE using a yeast two-hybrid assay (data not shown). By exclusion of these possibilities, *rPhex* is likely interfering with the cysteine protease function, possibly acting as a pseudosubstrate to block cathepsin B activity.

There is a precedent for non-enzymatic actions of other metalloproteases that are mediated by protein–protein interactions. Other proteins, such as serpins, have amino acid residues that act as a pseudosubstrate to inhibit protease activity [33]. Other types of interactions are also possible. In this regard, the ADAM protease family possesses both proteolytic and adhesive properties that are RGD-dependent and-independent [34]. An RGD cell attachment sequence is present in MEPE [5], which might be involved in interactions. In addition, there is evidence to that members of the *Phex* family bind to other factors. For example, Kell, a related membrane zinc endopeptidase, interacts with XK, a protein with 10 membrane-spanning domains that shares structural similarities to membrane transporters [35,36]. Failure to demonstrate interactions between *Phex* and MEPE in the yeast-two hybrid assay, however, fails to support such mechanisms in explaining *Phex* inhibition of MEPE cleavage (Fig. 5). To date, the only studies investigating *Phex* binding to proteins involve its processing in the endoplasmic reticulum, which illustrate that *Phex* can be co-immunoprecipitated with the molecular chaperone calnexin [23]. The precise mechanism whereby *rPhex* interferes with MEPE metabolism remains to be established and examination of potential interactions between *Phex* and cathepsin-like enzyme or cell surface proteinases might provide additional insights into this mechanism.

The biological relevance of *Phex* inhibition of MEPE in vitro metabolism remains to be established. Although *Phex* is an ectoenzyme [4], *Phex* immunoreactivity has been identified in the Golgi apparatus and endoplasmic reticulum [37]; similarly, whereas cathepsin B is usually found in perinuclear lysosomes, it has recently been found in the plasma membranes of prostate tumor cells [38]. Moreover, cathepsin B has collagen-phagocytosing activity in osteoblast phagosomes [39] and has also been shown to cleave osteocalcin [40], another extracellular matrix protein synthesized by osteoblasts. Extracellular degradation of matrix proteins, however, does occur and MEPE may be processed after secretion [24]. Thus, it may not be necessary that *Phex*, cathepsin B-like enzymes, and MEPE, co-localize in cellular compartments, since these interactions may happen in the extracellular

milieu in vivo. On the other hand, cathepsin B itself may not be the physiological target for *Phex*, but other extracellular matrix degrading enzymes, of which *Phex* inhibition of cathepsin B activity represents a mechanistic paradigm, may be the physiological targets for *Phex*-mediated inhibition.

In summary, *Phex* may have effects that are distinct from its ability to hydrolyze substrates. These effects might be mediated by blocking the activity of other enzymes that process factors regulating mineralization of extracellular matrix. Given the role of MEPE deficiency to enhance mineralization, it is possible that MEPE degradation products are responsible for inhibiting mineralization in the setting of abnormal *Phex* rather than being a secondary event, resulting from impaired mineralization. If so, the observed elevated MEPE transcripts in calvaria (Fig. 1) and bone of *Hyp*-mice [27] could be a compensatory response to the increased degradation of MEPE occurring in the setting of abnormal *Phex*. We currently lack the tools to characterize MEPE protein expression and its potential fragments in bone, due to the lack of a suitable antibody. Our efforts using anti-MEPE antibodies have been unsuccessful in identifying MEPE in bone, possibly because of their low affinity. Additional investigations will be required to determine whether unprocessed MEPE accumulates in bone of XLH patients and/or *Hyp* mice, and contributes to the intrinsic mineralization defect caused by inactivating mutations of *Phex*.

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