Characterization of PHEX endopeptidase catalytic activity: identification of parathyroid-hormone-related peptide$_{107–139}$ as a substrate and osteocalcin, PP_i and phosphate as inhibitors

Guy BOILEAU*†[1], Harriet S. TENENHOUSE‡, Luc DESGROSEILLERS* and Philippe CRINE*†

*Département de biochimie, Faculté de médecine, Université de Montréal, C.P. 6128, Succ. Centre-Ville, Montréal, QC, Canada H3C 3J7, †BioMep Inc., 4631 Cumberland Ave., Montreal, QC, Canada H4B 2L5, and ‡Department of Pediatrics and Human Genetics, McGill University–Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal, QC, Canada H3H 1P3

Mutations in the *PHEX* gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) are responsible for X-linked hypophosphataemia, and studies in the *Hyp* mouse model of the human disease implicate the gene product in the regulation of renal phosphate (P_i) reabsorption and bone mineralization. Although the mechanism for PHEX action is unknown, structural homologies with members of the M13 family of endopeptidases suggest a function for PHEX protein in the activation or degradation of peptide factors involved in the control of renal P_i transport and matrix mineralization. To determine whether PHEX has endopeptidase activity, we generated a recombinant soluble, secreted form of human PHEX (secPHEX) and tested the activity of the purified protein with several peptide substrates, including a variety of bone-related peptides. We found that parathyroid-hormone-related peptide$_{107–139}$ is a substrate for secPHEX and that the enzyme cleaves at three positions within the peptide, all located at the N-terminus of aspartate residues. Furthermore, we show that osteocalcin, PP_i and P_i, all of which are abundant in bone, are inhibitors of secPHEX activity. Inhibition of secPHEX activity by osteocalcin was abolished in the presence of Ca^{2+}. We suggest that PHEX activity and mineralization may be controlled *in vivo* by PP_i/P_i and Ca^{2+} and, in the latter case, the regulation requires the participation of osteocalcin.

Key words: bone mineralization, PHEX expression and purification, PHEX substrate specificity.

INTRODUCTION

The *PHEX* gene (formerly *PEX*; a phosphate regulating gene with homologies to endopeptidases on the X chromosome) was identified by positional cloning as the gene responsible for X-chromosome-linked hypophosphatemia (XLH) in humans [1]. XLH is a Mendelian disorder of P_i homoeostasis characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphataemia and renal defects in P_i reabsorption and vitamin D metabolism [2]. Several groups have cloned and sequenced the human and mouse PHEX/Phex cDNAs [3–6] (*PHEX* and *Phex* refer to the human and mouse genes respectively). Amino acid sequence comparisons have demonstrated homologies between PHEX/Phex protein and members of the M13 endopeptidase family, as previously observed in the partial sequence of the candidate gene [1]. The M13 endopeptidases are zinc-containing type II integral membrane glycoproteins with a relatively short cytoplasmic N-terminal region, a single transmembrane domain, and a long extracytoplasmic domain, which contains the active site of the enzyme [7]. In addition to PHEX, this family includes neprilysin (NEP, neutral endopeptidase 24.11), a widely distributed endopeptidase involved in the degradation of several bioactive peptides [8], the endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2), responsible for the processing of inactive big-endothelins into active endothelins [9], the Kell blood-group protein, a protein of the erythrocyte membrane with unknown function [10], and ECE-like enzyme/distress-induced neuronal endopeptidase (ECEL/DINE) [11,12] and soluble endopeptidase/NEP-like enzyme 1 (SEP/NL1) [13,14], two recently reported peptidases with sequence similarity to the family.

PHEX and *Phex* are expressed predominantly in bone [3–6,15,16] and teeth [16]. However, the precise physiological role of the PHEX protein is unknown, and the mechanisms whereby loss of PHEX function causes renal P_i wasting and impaired bone mineralization are not understood. Sequence similarity of PHEX to members of the M13 family of zinc metallopeptidases suggests a role in regulating the activity of bioactive peptide(s). In support of this hypothesis, Lajeunesse et al. [17] and Nesbitt et al. [18] provided evidence for the presence of a renal P_i transport inhibitory factor in conditioned medium of cultured osteoblasts isolated from X-chromosome-linked *Hyp* mice, an animal model for human XLH [19]. Although it has been postulated that PHEX may be involved in the inactivation of this phosphaturic factor (designated phosphatonin, [18,20]), the molecular identity of this factor remains to be determined.

As a first step in obtaining a better understanding of the physiological role of PHEX, we engineered a soluble, secreted form of the human protein (secPHEX), purified it to homogeneity and tested its activity with several peptide substrates. We show

Abbreviations used: CGRP, calcitonin gene-related peptide; ECE, endothelin-converting enzyme; ECEL/DINE, ECE-like enzyme/distress-induced neuronal endopeptidase; endo H, endoglycosidase H; LLC-PK_1 cells, porcine kidney cells; NEP, neprilysin; *PHEX*, **ph**osphate-regulating gene with homologies to **e**ndopeptidases on the **X** chromosome (PHEX is the endonuclease itself); *Phex* is the equivalent mouse gene; PNGaseF, peptide:N-glycosidase F; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide ($PTHrP_{107–139}$ means residues 107–139 of PTHrP). RER, rough endoplasmic reticulum; RP-HPLC, reverse-phase HPLC; SA domain, signal peptide/membrane anchor domain; secPHEX, soluble, secreted form of PHEX; SEP/NL1, soluble endopeptidase/NEP-like enzyme 1; XLH, X-chromosome-linked hypophosphataemia; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; SP-, sulphopropyl; Gla, γ-carboxyglutamic acid.

[1] To whom correspondence should be addressed (e-mail boileaug@bch.umontreal.ca).

that the 107–139-residue fragment of parathyroid-hormone (PTH)-related peptide ($PTHrP_{107–139}$) was the only peptide degraded by secPHEX and that metallopeptidase inhibitors, physiological concentrations of PP_i and P_i, and osteocalcin inhibited enzyme activity.

MATERIALS AND METHODS

DNA manipulations

All DNA manipulations were performed according to standard protocols [21]. Site-directed mutagenesis was performed using a PCR-based strategy, as described previously [22].

Construction of expression vectors

Human PHEX cDNA was cloned previously in one of our laboratories [4]. For expression of the *PHEX* gene in cultured mammalian cells, a restriction fragment (*Spe*I–*Eco*RV), which contained the entire *PHEX* coding sequence, was digested, blunted, and subcloned into the mammalian expression vector pCDNA3/RSV [23] (RSV refers to rous sarcoma virus). This vector also contains the bacterial *neo* gene that confers resistance to the antibiotic neomycin (G418) to cells that express it. The resulting vector was called pCDNA3/RSV/PHEX.

To generate a soluble, secreted form of PHEX, the signal peptide/membrane anchor domain (SA domain) of the protein was transformed into a cleavage-competent signal sequence using a strategy similar to that previously described for NEP [24]. However, in the case of PHEX, further genetic manipulations were required. In addition to the introduction of hydrophilic amino acid residues in the SA domain as described for NEP [24], it was necessary to delete four codons in PHEX (Figure 1A below). These modifications were achieved by introducing site-directed mutations into the pCDNA3/RSV/*PHEX* vector by PCR mutagenesis (eight codons) and deletions (four codons), using appropriate oligonucleotide primers, as described previously [22] (Figure 1A below).

To generate a catalytically inactive form of secPHEX, vector pCDNA3/RSV/sec*PHEX*E581V, harbouring a Glu^{581}-to-Val substitution, was produced by site-directed mutagenesis using the same PCR-based strategy referred to above and appropriate oligonucleotide primers.

Expression of recombinant proteins

Expression of human *PHEX*, sec*PHEX* and sec*PHEXE581V* was induced by transfection of LLC-PK_1 cells (porcine pidney cells; A.T.C.C. No. CRL-1392) with appropriate vectors, and transfected cells were selected with G418 (Life Technologies, Burlington, ON, Canada) and cultured as described previously [25].

Immunoblot analysis of cell extracts and culture media were performed essentially as described previously [26] using a monoclonal antibody raised against a recombinant human PHEX fragment (K^{121}–E^{294}) [15].

To determine the glycosylation state of the proteins, samples were incubated prior to electrophoresis with endoglycosidase H (endo H) or peptide:N-glycosidase F (PNGaseF) as suggested by the distributor (New England Biolabs Inc., Mississauga, ON, Canada).

Production and purification of secPHEX or secPHEXE581V

To produce large amounts of secPHEX or secPHEXE581V, confluent cells (in thirty 200-mm-diameter Petri dishes) were incubated for 4 days in 199 medium (Life Technologies) supplemented with 2.5 μg/ml insulin, 17.5 μg/ml transferrin, 2 μg/ml ethanolamine, 100 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin. Sodium butyrate was present at a concentration of 10 mM. After 4 days, the media were recovered, centrifuged and concentrated on Centriprep-50 columns. Typically, 600 ml of crude spent medium from transfected LLC-PK_1 cells were concentrated to 30 ml and loaded, at a flow rate of 2 ml/min, on an 8 ml sulphopropyl (SP)-Sepharose cation-exchange column (Amersham Pharmacia Biotech Inc. Baie d'Urfée, QC, Canada)

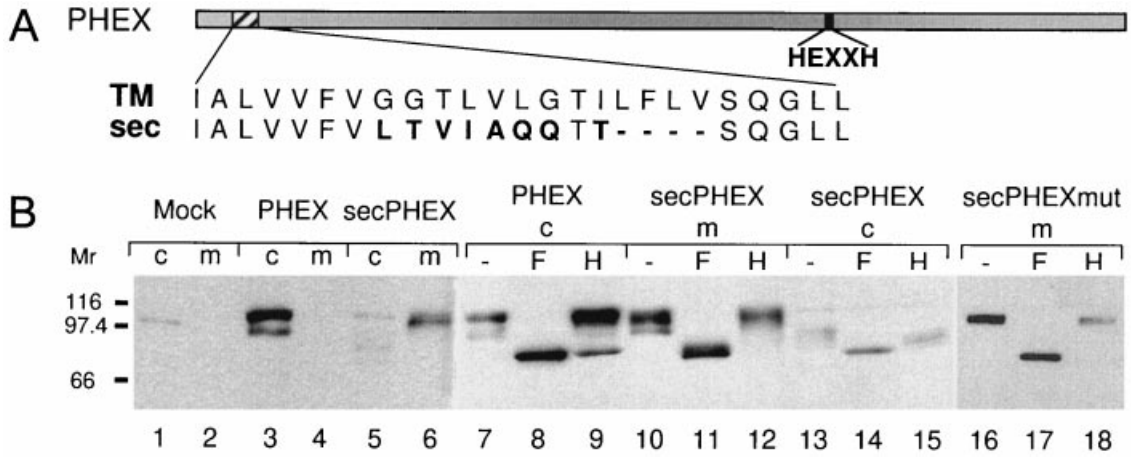

Figure 1 Structure and expression of PHEX and secPHEX

(**A**) Schematic representation of the PHEX protein ('PHEX'), and the amino acid sequence of the wild-type ('**TM**') and mutated ('**sec**') transmembrane domains. The hatched box indicates the position of the transmembrane domain and the black box indicates the position of the zinc-binding amino acids ('**HEXXH**'). Amino acid sequences are presented in the one-letter code. In the **sec** sequence, **bold** letters show the position of mutated amino acid, whereas hyphens (-) depict deleted residues. (**B**) Immunoblot analysis of PHEX and secPHEX expression. Proteins from cellular extracts ('c') corresponding to 1/50th (approx. 50 μg) of one Petri dish or the same proportion from corresponding culture media ('m') of mock-transfected LLC-PK_1 cells ('Mock') or cells transfected with either PHEX, secPHEX or secPHEXE581V were resolved on an SDS/7.5%-PAGE gel and revealed with a PHEX-specific antibody as described in the Materials and methods section. Some samples were treated with PNGase F ('F') or endo H ('H') before electrophoresis. '-' Refers to untreated samples. The positions of M_r ('Mr') markers are indicated on the left.

previously equilibrated with 50 mM sodium phosphate, pH 6.6, containing 50 mM NaCl. The column was washed at the same flow rate with 10 column vol. of the same buffer and the proteins were eluted with a 50 mM–1 M NaCl gradient. Fractions were analysed by SDS/PAGE and immunoblotting as described above, and fractions containing secPHEX or secPHEXE581V were revealed by silver staining.

Fractions containing secPHEX or secPHEXE581V were pooled and concentrated to approx. 1.5 mg/ml using Centriprep-50 columns. The protein solution was then diluted to 0.1 mg/ml with buffer A [50 mM P_i (pH 7.0)/1 M ammonium sulphate], centrifuged at 9000 ***g*** for 15 min and the supernatant loaded on a 1 ml butyl-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) at a flow rate of 1 ml/min. The column was washed, at the same flow rate, with buffer A to a stable baseline and the proteins were eluted with a 40 ml gradient from 100% buffer A/0% buffer B (50 mM P_i, pH 7.0) to 0% buffer A/100% buffer B. Fractions were analysed as described above, and those containing secPHEX or secPHEXE581V were revealed by silver staining. Fractions containing pure secPHEX or secPHEXE581V were pooled, concentrated and dialysed against 50 mM Mes (pH 6.5)/150 mM NaCl. Protein concentrations were determined using the Bradford method (DC protein assay kit; Bio-Rad, Mississauga, ON, Canada).

Enzymic assay

A 1 μg portion of purified secPHEX or secPHEXE581V was incubated with 5 μg of peptide substrate for 30 min at 37 °C in 200 μl of 50 mM Mes (pH 6.5)/150 mM NaCl. The reaction mixture also contained 1 μg/μl of the tripeptide Tyr-Gly-Gly, which is not a PHEX substrate and was used as an internal standard. The peptides tested for degradation by secPHEX were: [Leu]enkephalin, α-endorphin, substance P, bradykinin, big-endothelin-1, endothelin-1, α-calcitonin gene-related peptide (α-CGRP), calcitonin, osteocalcin, $PTH_{1–84}$, $PTH_{1–34}$, $PTHrP_{1–34}$, $PTHrP_{107–139}$ and osteogenic growth peptide. All peptides were of human origin and obtained from Bachem, Philadelphia, PA, U.S.A. or Peninsula Laboratories, Belmont, CA, U.S.A., except for human osteocalcin, which was purchased from Peptide Research Institute, Osaka, Japan. After the incubation period, the reaction was stopped by the addition of EDTA to a final concentration of 5 mM. Identification of peptide products was performed by reverse-phase (RP-)HPLC on a C_{18} μBondapak analytical column (Waters, Mississauga, ON, Canada) as described previously [14]. In some cases, inhibitors were added under conditions described in the legends to the Figures. Results were quantified by comparing the area under the peaks of undigested and digested $PTHrP_{107–139}$, after normalization for the amount of Tyr-Gly-Gly present in the sample. secPHEX digestion products were characterized by matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF-MS) at the McGill University Mass Spectrometry Center.

RESULTS

Construction and expression of a soluble, secreted form of PHEX

To obtain a soluble, secreted form of recombinant human PHEX protein, we first attempted to transform the PHEX SA domain into a cleavage-competent signal peptide [24]. This strategy resulted in the production of a misfolded PHEX protein that remained trapped in the rough endoplasmic reticulum (RER) of transfected cells (results not shown). Therefore an alternate strategy was developed which involved the deletion of selected amino acids in the SA domain of PHEX in addition to the substitution of others as was done for NEP (Figure 1A).

The membrane or soluble, secreted forms of PHEX were expressed in LLC-PK_1 cells and permanent cell lines established as described in the Materials and methods section (LLC-PK_1/PHEX and LLC-PK_1/secPHEX cells, respectively, for the membrane-bound and soluble, secreted forms). Immunoblotting of extracts of LLC-PK_1/PHEX cells with a PHEX-specific monoclonal antibody [15] revealed a major band of 105 kDa and a minor band of 95 kDa (Figure 1B, lane 3). No protein was detected in the culture medium, as expected for an integral membrane protein (Figure 1B, lane 4). In contrast, secPHEX appeared in the culture medium as a 100 kDa species (Figure 1B, lane 6), with very little enzyme detected in the cell extract (Figure 1B, lane 5).

To characterize the glycosylation state of PHEX and secPHEX, we next submitted the recombinant proteins to deglycosylation by PNGase F and endo H. PNGase F treatment revealed that all PHEX and secPHEX species were N-glycosylated, since their electrophoretic mobility was increased following digestion (Figure 1B, compare lanes 7 and 8, 10 and 11, 13 and 14). Treatment of PHEX with endo H transformed the minor 95 kDa band into a faster-migrating band (Figure 1B, lane 9), indicating that this species is likely to be an underglycosylated RER-associated form. However, the major 105 kDa band was resistant to endo H digestion (Figure 1B, lane 9), consistent with a cell-surface expression of the enzyme as shown previously [6]. secPHEX present in the culture medium was also resistant to endo H digestion (Figure 1B, lane 12), suggesting true secretion of the enzyme. In contrast, residual secPHEX in the cell extract was sensitive to endo H treatment (Figure 1B, lane 15). The differences between the glycosylation state of secPHEX in the culture medium with that in the cellular extract suggest that the cell-associated form of secPHEX is an intracellular species that has not travelled through the Golgi complex.

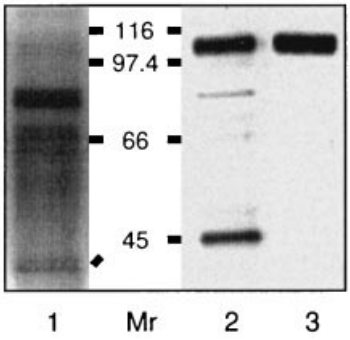

Figure 2 Purification of secPHEX

Proteins present in the culture medium of secPHEX-producing LLC-PK_1 cells (lane 1; 1 μg of protein) or in fractions pooled after SP-Sepharose (lane 2; 150 ng of protein) or butyl-Sepharose 4 (lane 3; 150 ng of protein) chromatography were separated on 7.5% acrylamide gels and silver-stained. The positions of M_r ('Mr') markers are indicated.

Purification of secPHEX

SecPHEX could be purified to homogeneity using a two-step procedure (Figure 2). First, the concentrated culture medium (Figure 2, lane 1) was loaded on an SP-Sepharose column and the proteins were eluted with a 0.05–1 M NaCl gradient. secPHEX was eluted at 150–200 mM NaCl. We estimated that the amount of secPHEX recovered after this first step was

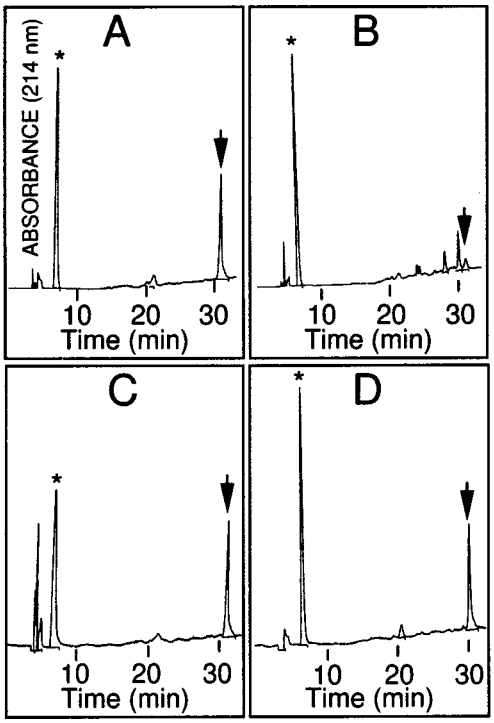

Figure 3 HPLC analysis of $PTHrP_{107–139}$ degradation products

Degradation of $PTHrP_{107–139}$ by purified secPHEX was determined as described in the Materials and methods section. All reaction mixtures contained the tripeptide Tyr-Gly-Gly (1 μg/μl) as an internal standard. (**A**) $PTHrP_{107–139}$ in the absence of secPHEX; (**B**) $PTHrP_{107–139}$ in presence of secPHEX; (**C**) $PTHrP_{107–139}$ in the presence of secPHEX and 1 mM EDTA; (**D**) $PTHrP_{107–139}$ in the presence of secPHEXE581V. Arrows indicate the elution positions of $PTHrP_{107–139}$ and asterisks the elution position of Tyr-Gly-Gly.

$\approx$ 2 mg/litre of culture medium. The two contaminant proteins visible after this first chromatographic step (Figure 2, lane 2) were separated from secPHEX on the butyl-Sepharose 4 column (Figure 2, lane 3). The final yield of purified secPHEX was estimated to be $\approx$ 1 mg/litre of culture medium.

Activity of secPHEX

SecPHEX activity was assayed in 50 mM Mes, pH 6.5, containing 150 mM NaCl, the buffer routinely used to assess NEP activity [26]. NaCl was added to the reaction mixture because we observed that secPHEX precipitated out in solutions containing less than 50 mM salt.

Of the peptide substrates tested (see the Material and methods section), only $PTHrP_{107–139}$ was degraded by secPHEX. In the absence of secPHEX, no digestion of $PTHrP_{107–139}$ (elution time 31.5 min) was evident (Figure 3A). In the presence of secPHEX, however, approx. 75–80% degradation of the peptide was observed (Figure 3B). (The peak that was eluted at 7 min corresponds to the Tyr-Gly-Gly used as internal standard). Digestion of $PTHrP_{107–139}$ by secPHEX resulted in the production of four degradation products eluted at 23.5, 24.2, 27.0 and 29.4 min (Figure 3B). As expected for a zinc metallopeptidase, secPHEX activity was fully inhibited by the addition of 1 mM EDTA (Figure 3C) or 1 mM 1,10-phenanthroline (results not shown) to the reaction mixture.

To confirm that the activity of secPHEX was not due to a contaminant protease co-purifying with it, a mutant of secPHEX, secPHEXE581V, in which the critical catalytic Glu^{581} was replaced by a valine residue, was constructed. A similar mutation introduced in NEP [27] or ECE-1 [28] resulted in total loss of catalytic activity. secPHEXE581V was produced in LLC-PK_1 cells and showed an expression pattern essentially identical with that of secPHEX (Figure 1B, compare lanes 10, 11 and 12 with lanes 16, 17 and 18 respectively). However, in contrast with the wild-type form of the secreted enzyme, purified secPHEXE581V failed to degrade $PTHrP_{107–139}$ under similar conditions (Figure 3D).

To determine the cleavage site specificity of secPHEX, RP-HPLC peaks corresponding to the degradation products of $PTHrP_{107–139}$ were collected and analysed by MALDI–TOF-MS. Figure 4 depicts the $PTHrP_{107–139}$ fragments identified. As can be seen from the cleavage sites identified, hydrolysis of the peptide by secPHEX occurred at the N-terminus of aspartate residues.

The pH optimum for the reaction was determined by progressively increasing the pH of the Mes buffer from 5.0 to 7.0 or of a Tris buffer (50 mM Tris/HCl/150 mM NaCl) from 7.0 to 9.0. Maximum activity was observed at pH 6.5 (Figure 5). secPHEX activity rapidly decreased at more basic pH values.

Inhibition of secPHEX activity

In previous studies we noticed that NEP activity was sensitive to the presence of P_i in the incubation medium (C. Vezina and G. Boileau, unpublished work). To determine whether secPHEX had the same sensitivity to P_i, we examined the effect of P_i, from 0.1 to 50 mM, on secPHEX activity and found that 50% inhibition was achieved by 3.5 mM P_i. Since P_i proved to be an effective inhibitor of secPHEX and since pyrophosphate, an alkaline phosphatase substrate, is abundant in bone [29], we also examined its effect on secPHEX activity. Increasing the PP_i concentration from 0.1 to 50 mM demonstrated that 50% inhibition of enzyme activity was achieved at 2.5 mM (Figure 6A).

secPHEX specificity for aspartate residues (Figure 4) suggested that the S_1' pocket can accommodate negatively charged side chains of amino acid residues. Although osteocalcin, which contains three negatively-charged γ-carboxyglutamic acid residues (Gla), was not degraded by secPHEX (results not shown), it was a potent inhibitor of secPHEX-mediated $PTHrP_{107–139}$ hydrolysis. A 50% inhibition of secPHEX activity was achieved at 3.6 μM osteocalcin (Figure 6A).

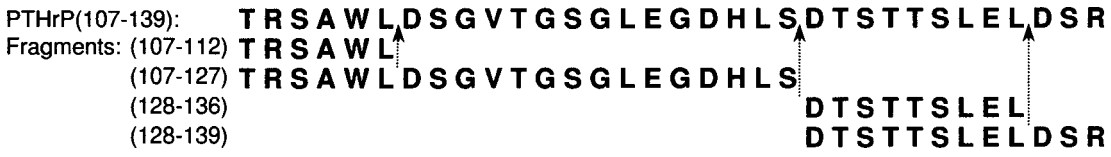

Figure 4 Identification of secPHEX cleavage sites in $PTHrP_{107–139}$

The sequences of $PTHrP_{107–139}$ and fragments identified by MS are presented. Cleavage sites are indicated by arrows. The one-letter code is used to represent the amino acid residues.

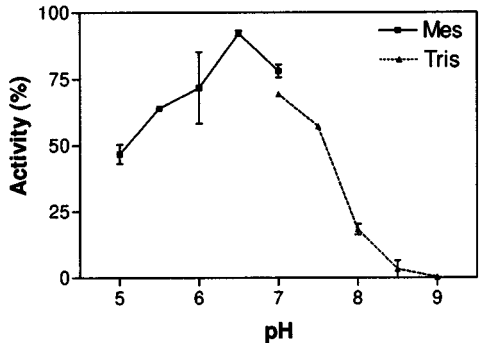

Figure 5 pH-dependency of secPHEX activity

secPHEX and $PTHrP_{107–139}$ were incubated under different pH conditions as described in the Materials and methods section and the extent of substrate hydrolysis was determined by HPLC. Conditions yielding the highest activity were arbitrarily set to 100%. Assays performed in Mes buffer (■) or Tris (▲) buffer.

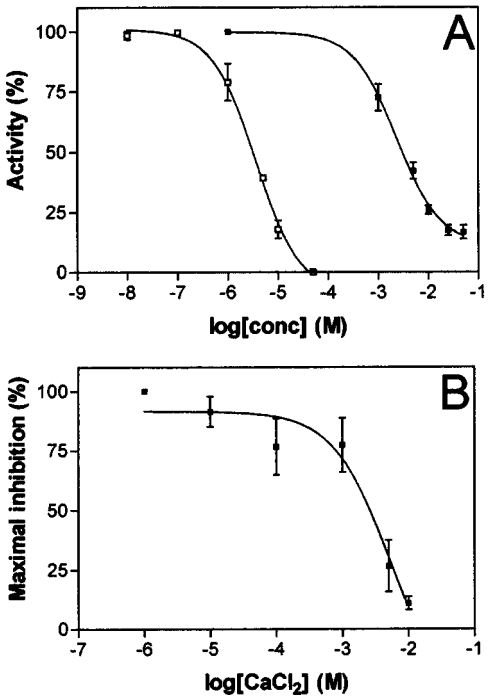

Figure 6 Effects of increasing concentrations of PP_i, osteocalcin and Ca^{2+} in the presence of osteocalcin on $PTHrP_{107–139}$ degradation by purified secPHEX

secPHEX activity was measured in the presence of increasing concentrations of (**A**) pyrophosphate (closed symbols) and osteocalcin (open symbols), and (**B**) $CaCl_2$ (with a constant osteocalcin concentration of 2 μM). In (**A**), 100% corresponds to the activity of secPHEX in the absence of inhibitors. In (**B**), osteocalcin inhibitory potency is measured, and 100% corresponds to the inhibition observed in the presence of 2 μM osteocalcin and the absence of $CaCl_2$.

The Gla residues of osteocalcin are known to bind Ca^{2+} [30]. We thus examined the effect of Ca^{2+} on the inhibitory action of osteocalcin. By varying the $CaCl_2$ concentration in the assay from 10 μM to 100 mM, we showed that 5 mM Ca^{2+} was necessary to reduce the inhibitory potency of osteocalcin by 50% (Figure 6B). Ca^{2+} had no effect on secPHEX activity in the absence of osteocalcin (results not shown).

DISCUSSION

In the present study we engineered a novel soluble, secreted form of human PHEX (secPHEX), expressed it in mammalian cells, and used the purified recombinant enzyme to identify, for the first time, substrates and inhibitors of enzyme activity.

Enzymes of the M13 endopeptidase family, including PHEX, are type II transmembrane glycoproteins. These proteins have, near their N-termini, a unique hydrophobic peptide (SA domain) acting both as a signal peptide to direct the translocation of the protein through the membrane of the RER and as a transmembrane domain for anchoring the protein in the plasma membrane of the cell [31]. These proteins, unlike type I transmembrane proteins, cannot be easily transformed into soluble forms by deletion of the SA domain, since such a manipulation would also prevent translocation into the RER and transport to the cell surface. We have used site-directed mutagenesis to transform the SA domain of PHEX into a cleavage-competent signal peptide. The presence of secPHEX in the culture medium of cells transfected with vector pCDNA3/RSV/secPHEX and its resistance to digestion by endo H indicate that the strategy used was successful and that the enzyme travelled through the Golgi apparatus where complex sugars have been added.

The main advantage of a soluble, secreted form of PHEX is that it can be easily purified from the spent culture medium without the use of detergents. The enzyme was purified using a two-step purification procedure that yielded $\approx$ 1 mg of secPHEX/litre of culture medium. It can be argued that cleavage of the cytosolic and transmembrane domains from the extracellular domain of PHEX results in a protein with enzymic properties different from those of the wild-type enzyme. However, several lines of evidence point to the contrary. First, recombinant soluble forms of NEP [24,32] and ECE-1 [33] were obtained using similar strategies and, in all cases, the soluble enzymes showed catalytic parameters that were identical with those of their membrane-bound counterparts. These data suggest that the ectodomain, which harbours the catalytic site, can fold into an active enzyme without the contribution of the cytosolic and transmembrane domains. Secondly, a soluble form of NEP, generated by treating solubilized renal brush-border membranes with trypsin, retained the catalytic features of the native enzyme [34]. Finally, Oefner et al. [35] determined the three-dimensional structure of a soluble and secreted form of human NEP complexed to the specific inhibitor phosphoramidon and found that the inhibitor was well positioned in the active site. Taken together, these results indicate that the soluble, secreted forms of enzymes of the M13 family can be engineered without affecting their catalytic parameters. Thus the features of secPHEX reported in the present study are likely to apply to the native enzyme.

Members of the M13 family, such as NEP [7], SEP/NL1 [13,14] and ECE-1 [36], generally have a wide substrate specificity. In this respect, PHEX is different from other members of the M13 family. Of the 14 peptides tested, only $PTHrP_{107–139}$ was cleaved by the enzyme. Cleavage occurred at three positions within the peptide, all at the N-terminus of an aspartate residue. It is noteworthy that several of the peptides tested, including osteocalcin and $PTH_{1–34}$, have aspartate residues in their structure but are not cleaved by secPHEX. This observation is consistent with a restricted specificity of PHEX and suggests that other structural motifs are required for substrate binding. Interestingly, all aspartate residues found at cleavage sites in $PTHrP_{107–139}$ are followed by a hydroxylated amino acid (Ser or Thr). Thus additional studies with synthetic peptides are necessary to define the substrate specificity of PHEX.

$PTH_{1–34}$ and $PTH_{1–38}$ were reported previously as PHEX substrates [6]. We could not confirm this result with our purified enzyme. Although the reason for this discrepancy is not clear, it may be that the crude membrane preparation used contained contaminant protease(s) [6].

PHEX appears unique among the members of the M13 family in that its S_1' pocket can accommodate negatively charged amino acid side chains. All other members of the family accommodate hydrophobic amino acid residues in the S_1' pocket [14,34,37]. The three-dimensional structure of NEP showed that the S_1' pocket is lined with seven hydrophobic amino acid residues, namely Phe^{106}, Ile^{558}, Phe^{563}, Met^{579}, Val^{580}, Val^{692} and Trp^{693} [35]. Sequence comparisons between NEP and PHEX, using PSI-BLAST program [38], identified Tyr^{109}, Glu^{554}, Phe^{559}, Val^{576}, Ile^{577}, Val^{691} and Arg^{692} as the homologous residues in PHEX. The S_1' pocket of PHEX thus contains charged residues, including a positively charged arginine side chain that could form an ionic bond with the aspartate side chain of the substrate.

The identification of $PTHrP_{107–139}$ as a PHEX substrate *in vitro* raises the question of whether it is a physiologically relevant substrate. PHEX is expressed in osteoblasts [3,5,15,16], where PTHrP is produced [39]. Presumably, $PTHrP_{107–139}$ is generated by intracellular processing of the PTHrP precursor by furin or a furin-like enzyme [40]. However, since the PHEX cleavage sites, which we identified in human $PTHrP_{107–139}$, are not conserved in rat, mouse and chicken PTHrP [41], our data suggest that any biological role for PHEX-mediated degradation of $PTHrP_{107–139}$ would be confined to humans. It should be noted that the latter also produces a 173-amino-acid PTHrP isoform derived from exon 5 which is not present in either the rodent or chicken PTHrP genes [41]. The function of $PTHrP_{107–139}$ in bone is still obscure, with both inhibitory [42,43] and stimulatory [44,45] effects on osteoclast [42,44] and osteoblast [43,45] function reported. It would, however, appear that $PTHrP_{107–139}$ is not phosphatonin, the putative phosphaturic factor found in culture media of osteoblasts derived from *Hyp* mice [17,18] and secreted by mixed mesenchymal tumours derived from patients with oncogenic hypophosphateic osteomalacia [20,46,47]. This conclusion is based on studies in which we failed to show any effect of $PTHrP_{107–139}$ on Na/P_i co-transport in opossum kidney cells, the same renal proximal tubular cell line used to demonstrate the inhibitory effects of phosphatonin on Na/P_i co-transport [18,46] (results not shown).

Given that PHEX is expressed predominantly in bone [3–6,15,16], it is likely that loss of PHEX function in XLH, and in the *Hyp* mouse model of the human disorder, contributes to the skeletal defect, in concert with the hypophosphataemia arising from the renal P_i leak. However, the precise mechanism for the skeletal phenotype is not understood. *Hyp* mice present with an enlarged osteoid area [15,48] that is not the result of abnormal matrix deposition [49] but rather is due to impaired mineralization [48]. A relationship between PHEX and the mineralization process is further supported by our observations that the onset of *Phex* expression and mineralization coincide at embryonic day 15 in the mouse [16]. In this regard, our findings that osteocalcin and pyrophosphate are inhibitors of PHEX activity are of interest. Osteocalcin, the most abundant of the non-collagenous proteins produced by osteoblasts, inhibits growth of hydroxyapatite crystals *in vitro* [50], and disruption of the osteocalcin genes in mice is associated with an increase in bone mass [51]. These results indicate that osteocalcin is an inhibitor of bone formation. Similarly, pyrophosphate, a substrate of alkaline phosphatase, inhibits hydroxyapatite crystallization *in vitro* (for a review, see [52]). Moreover, pyrophosphate accumulation in patients with alkaline phosphatase deficiency is associated with defective skeletal mineralization, possibly by inhibiting mineral crystal growth. Our results suggest that osteocalcin and pyrophosphate may also control mineralization by modulating the activity of PHEX.

The mechanisms by which osteocalcin, pyrophosphate and P_i function as PHEX inhibitors are still unknown. Both molecules have negatively charged groups (Gla residues in osteocalcin) that may interact with the S_1' pocket of the enzyme. The observation that Ca^{2+} can prevent PHEX inhibition by osteocalcin supports the hypothesis that Gla residues are involved in osteocalcin/PHEX interactions. Indeed, it has been shown that vitamin K-dependent γ-carboxylation of osteocalcin glutamic acid residues 17, 21 and 24 is required for Ca^{2+} binding [30]. However, we cannot rule out that Ca^{2+} binding to Gla residues induces a conformational change in other regions of the molecule important for PHEX interaction. Structural studies have been initiated to examine the molecular interactions between PHEX and osteocalcin.

In conclusion, we established novel systems to produce and purify recombinant PHEX protein and developed an assay to measure PHEX catalytic activity and to identify substrates and inhibitors of the enzyme. Although we demonstrated that $PTHrP_{107–139}$ is a substrate for PHEX, the physiological significance of PHEX-mediated $PTHrP_{107–139}$ degradation in humans remains to be determined. Our *in vitro* assay will provide a mechanism to investigate the impact of disease-causing *PHEX* mutations [53] on catalytic activity and, in doing so, will improve our understanding of PHEX structure–function relationships and provide an insight into genotype/phenotype correlations. Finally, we demonstrated that osteocalcin, pyrophosphate and P_i are inhibitors of PHEX activity. We propose that PHEX activity and mineralization can be modulated physiologically by pyrophosphate, P_i and Ca^{2+}, in the latter case by a mechanism involving osteocalcin.

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