Human recombinant endopeptidase PHEX has a strict S'_1 specificity for acidic residues and cleaves peptides derived from fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein

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The *PHEX* gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) encodes a protein (PHEX) with structural homologies to members of the M13 family of zinc metallo-endopeptidases. Mutations in the *PHEX* gene are responsible for X-linked hypophosphataemia in humans. However, the mechanism by which loss of PHEX function results in the disease phenotype, and the endogenous PHEX substrate(s) remain unknown. In order to study PHEX substrate specificity, combinatorial fluorescent-quenched peptide libraries containing *o*-aminobenzoic acid (Abz) and 2,4-dinitrophenyl (Dnp) as the donor–acceptor pair were synthesized and tested as PHEX substrates. PHEX showed a strict requirement for acidic amino acid residues (aspartate or glutamate) in S'_1 subsite, with a strong preference for aspartate. Subsites S'_2, S_1 and S_2 exhibited less defined specificity requirements, but the presence of leucine, proline or glycine in P'_2, or valine, isoleucine or histidine in P_1 precluded hydrolysis of the substrate by the enzyme. The peptide Abz-GFSDYK(Dnp)-OH, which contains the most favourable residues in the P_2 to P'_2 positions, was hydrolysed by PHEX at the N-terminus of aspartate with a k_{cat}/K_m of 167 $mM^{-1} \cdot s^{-1}$. In addition, using quenched fluorescence peptides derived from fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein sequences flanked by Abz and *N*-(2,4-dinitrophenyl)ethylenediamine, we showed that these physiologically relevant proteins are potential PHEX substrates. Finally, our results clearly indicate that PHEX does not have neprilysin-like substrate specificity.

Key words: combinatorial library, internally quenched fluorogenic substrate, M13 endopeptidase, PHEX substrate specificity.

INTRODUCTION

PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) was first identified by positional cloning as the gene that is mutated in patients with X-linked hypophosphataemia (XLH) [1]. XLH is the most prevalent form of inherited rickets in humans and is characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphataemia and renal defects in phosphate reabsorption and vitamin D metabolism [2]. Much of our knowledge about XLH has been obtained from studies of the *Hyp* mouse, which has a large deletion of the *Phex* gene [3] and has been used as an animal model for the human disease [4].

The product of the *PHEX* gene (PHEX) shows striking homologies to members of the M13 family of zinc metallo-endopeptidase. These enzymes are type-II integral membrane glycoproteins [5] and include neprilysin (NEP; neutral endopeptidase 24.11) [6], endothelin-converting enzymes (ECE-1 and ECE-2) [7], Kell blood group protein [8], ECE-like enzyme/distress-induced neuronal endopeptidase ('ECEL1/DINE') [9,10], soluble endopeptidase/NEP-like enzyme 1/neprilysin 2 ('SEP/NL1/NEP2') [11–13] and membrane metallo-endopeptidase-like 2 ('MMEL2') [14], although the latter might be the human homologue of SEP/NL1/NEP2. The use of specific inhibitors has demonstrated the involvement of NEP, ECE-1 and ECE-2 in regulating the amount and hence activity of several bioactive peptides [15,16]. Sequence similarity of PHEX to other members of M13 family suggests a similar role for PHEX. The demonstration that a purified recombinant soluble and secreted form of PHEX can hydrolyse parathyroid hormone-related peptide residues ($PTHrP_{107-139}$) is consistent with this hypothesis [17]. However, the physiological relevance of $PTHrP_{107-139}$ as a PHEX substrate and its role in the pathogenesis of XLH are still unknown.

The major sites of PHEX expression are bone [3,18–22] and teeth [22], but PHEX mRNA was also detected in ovary [23], fetal lung [3,24] and in the parathyroid gland [25]. PHEX expression is notably absent from kidney [3], suggesting that it may regulate renal phosphate reabsorption by controlling the activity of a circulating factor. Consistent with this hypothesis was the demonstration that inhibition of Na^+-dependent phosphate transport in cultured renal cells can be achieved by a factor in conditioned medium from cultured osteoblasts derived from *Hyp* mice [26,27].

Phosphaturic activity(ies) have also been found in tumours from patients with tumour-induced osteomalacia (TIO; also known as oncogenic hypophosphataemic osteomalacia), an acquired renal phosphate wasting disorder with the phenotypic features of XLH [2]. The term 'phosphatonin' was designated to depict the

Abbreviations: Abz, *o*-aminobenzoic acid; ADHR, autosomal dominant hypophosphataemic rickets; DMP1, dentin matrix protein 1; Dnp, 2,4-dinitrophenyl; DSPP, dentin sialophosphoprotein; ECE, endothelin-converting enzyme; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; FGF-23, fibroblast growth factor-23; IQFP, internally quenched fluorogenic peptide; LLC-PK_1 cells, porcine renal epithelial proximal tubule cells; MALDI–TOF, matrix-assisted-laser-desorption ionization–time-of-flight; MEPE, matrix extracellular phosphoglycoprotein; NEP, neprilysin; OPN, osteopontin; *PHEX*, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; $PTHrP_{107-139}$, parathyroid hormone-related peptide residues 107–139; secPHEX, soluble secreted PHEX; TIO, tumour-induced osteomalacia; XLH, X-linked hypophosphataemia in humans.

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phosphaturic tumours factor(s) [28], and although the exact nature of phosphatonin remains to be determined, several candidates have been proposed (for review, see [29]). It has been shown that mutations in the *FGF23* gene that encodes a novel growth factor, fibroblast growth factor-23 (FGF-23) is responsible for autosomal dominant hypophosphatemic rickets (ADHR), an inherited disorder that resembles XLH and TIO [30]. Moreover, it was demonstrated that over-expression of FGF-23 in animal models elicits renal phosphate wasting, a reduction in serum phosphate levels and osteomalacia [31]. Of interest was the finding that FGF-23 is over-expressed in tumours from patients with TIO [32]. In addition to FGF-23, other proteins such as Frizzled-related protein 4 [32] and matrix extracellular phosphoglycoprotein (MEPE) [33] are over-expressed in TIO tumours. However, the phosphaturic activity of Frizzled-related protein-4 and MEPE in animal models remains to be determined.

To better understand the relationship between PHEX and phosphatonin, and to clarify conflicting data in the literature about the hydrolytic activity of PHEX [17,20,34,35], we have designed experiments to determine its substrate specificity using internally quenched fluorogenic peptides (IQFPs) containing the groups *o*-aminobenzoic acid (Abz), as the fluorescent donor, and *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) attached to a glutamine residue or 2,4-dinitrophenyl (Dnp) attached to the ε-amino group of lysine, as the fluorescent acceptor. Our results indicate that PHEX has a strong requirement for an acidic amino acid residue in the P_1' position of its substrates, and that the enzyme prefers substrates with free C-terminal groups. Furthermore, we demonstrated that several peptides derived from biologically relevant proteins, such as FGF-23 and MEPE, are PHEX substrates.

EXPERIMENTAL

Expression and purification of recombinant PHEX

The human recombinant soluble secreted form of PHEX (secPHEX) was obtained as described previously [17]. The concentration of purified secPHEX was determined using the Bradford method (DC protein assay kit; Bio-Rad, Mississauga, Canada). Membrane-bound recombinant human PHEX was expressed in porcine renal epithelial proximal tubule (LLC-PK_1) cells [17], and membranes were prepared essentially as described by Guo et al. [34]. Briefly, transfected cells resuspended in lysis buffer (150 mM NaCl/20 mM Tris/HCl, pH 7.6) were disrupted by sonication. Cell debris was removed by centrifugation at 300 ***g*** for 15 min at 4 °C. Membranes were collected by centrifugation at 30 000 ***g*** for 50 min and solubilized for 2 h with 1 % *n*-dodecyl-β-D-maltoside in lysis buffer. Insoluble material was pelleted by centrifugation at 10 000 ***g*** for 15 min, and protein concentrations of the supernatant were determined using the Bradford method (DC protein assay kit). PHEX amounts were evaluated by immunoblotting as described previously [17]. NEP expression and purification were as described previously [36].

N-terminal sequence determination

Purified secPHEX was analysed by SDS/PAGE on a 10.0 % gel [37]. Proteins were transferred to a PVDF membrane (Immobilon O, Millipore), stained with Coomassie Blue, destained and washed extensively with distilled water. The bands were excised and the N-terminal sequence determined in a PPSQ-23 protein sequencer (Shimadzu, Tokyo, Japan).

Synthesis of peptide libraries

Positional scanning IQFP combinatorial libraries were synthesized as described previously [38,39], with the exception that Abz/Dnp was used as fluorescence donor–acceptor pair. For preliminary experiments we prepared a library with the general structure Abz-GXXZXK(Dnp)-OH, where the Z position was successively filled with one of 19 amino acids (cysteine was omitted to avoid dimerization) and X contained randomly incorporated residues. To ensure equal coupling of the randomized residues, a balanced mixture of 19 amino acids was used following the optimum composition described previously [40]. Three other libraries were prepared with structures Abz-GXXDZK(Dnp)-OH, Abz-GXZDXK(Dnp)-OH and Abz-GZXDXK(Dnp)-OH, in which the P_1' position was pre-fixed as aspartate, a second fixed position (Z = P_2', P_1 and P_2) included 19 naturally occurring amino acids and the other positions were randomized (X = one of 17 amino acids; cysteine was excluded to avoid dimerization; aspartate and glutamate were excluded of the mixture in order to force the hydrolysis only at the fixed residue). Stock solutions were prepared in DMSO and the concentrations were measured using the Dnp molar absorption coefficient, $\varepsilon_{365} = 17\,300\ M^{-1} \cdot cm^{-1}$.

Synthesis and purification of peptides

IQFPs containing the group EDDnp attached to a glutamine residue were synthesized by the solid-phase synthesis method described previously [41] in a Shimadzu model PSSM-8 automated solid-phase peptide synthesizer. The IQFPs containing the Dnp group incorporated to the ε-NH_2 of a lysine residue were synthesized by the solid-phase methodology, using fluoren-9-ylmethoxycarbonyl-Lys(Dnp)-OH to introduce the quencher group. All the peptides obtained were purified by semi-preparative HPLC. The molecular mass and purity of synthesized peptides were checked by amino acid analysis and by molecular mass determination with matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF MS), using a TofSpec E (Micromass, Manchester, U.K.). Stock solutions of Dnp or EDDnp-peptides were prepared in DMSO and the concentrations were measured using the Dnp molar absorption coefficient, $\varepsilon_{365} = 17\,300\ M^{-1} \cdot cm^{-1}$.

Peptide library screen

Hydrolysis of peptides in the library was monitored under optimum pH and salt concentrations previously established for recombinant secPHEX using $PTHrP_{107-139}$ as substrate [17], namely 0.1 M Hepes, pH 6.5, containing 0.15 M NaCl. The number of Zn^{2+} ions present in the enzyme after purification has not been determined. However, in these conditions, addition of 10^{-6} M $ZnCl_2$ did not increase activity, suggesting that the enzyme has retained its full complement of zinc during purification. Enzymic activity in a final volume of 0.35 ml was continuously followed at 37 °C in a Hitachi F-2000 fluorimeter by measuring the fluorescence at $\lambda_{emission} = 420$ nm and $\lambda_{excitation} = 320$ nm. The assays were performed at low substrate concentrations where the reactions followed first-order conditions ($[S] \ll K_m$), and the rate constant (k_{obs}) was determined by the non-linear regression data analysis Grafit program version 3.0 [42]. These determinations were done at two different substrate concentrations and the apparent catalytic efficiencies, designated $^*k_{cat}/K_m$, were obtained by dividing the k_{obs} by the enzyme

concentration. The error was less than 5 % for any obtained value.

Optimum pH determination

The pH dependence was studied using 0.2 μM Abz-GFSDYK (Dnp)-OH over a pH range 3.5–8.5. The buffers used were as follows: 0.01 M sodium acetate ($3.5 < pH < 5.1$), 0.01 M Bis/Tris ($5.1 < pH < 6.5$), 0.01 M Hepes ($6.5 < pH < 7.4$) and 0.01 M Tris/HCl ($7.4 < pH < 8.4$) containing 0.14 M NaCl. Enzymic activity was followed at 37 °C using the continuous fluorimetric assay. For each pH, the apparent second-order rate constant ($^*k_{cat}/K_m$) was determined at low substrate concentrations where the reactions followed first-order conditions ($[S] \ll K_m$).

NaCl influence on catalytic activity

The influence of salt on secPHEX catalytic activity was investigated using 0.2 μM Abz-GFSDYK(Dnp)-OH as substrate at 37 °C in 0.01 M Bis/Tris buffer, pH 5.5, over a NaCl range 0–500 mM. The increase in fluorescence was continuously measured at 37 °C and the apparent second-order rate constant ($^*k_{cat}/K_m$) for the different NaCl concentrations was calculated as described above.

Determination of kinetic parameters for synthetic peptides derived from the peptide libraries and from putative natural substrates

The hydrolysis of the IQFPs at 37 °C in 10 mM Bis/Tris buffer, pH 5.5, (0.35 to 1.0 ml final volume) containing 150 mM NaCl, was continuously followed measuring the fluorescence at $\lambda_{emission} = 420$ nm and $\lambda_{excitation} = 320$ nm in a Hitachi F-2000 spectrofluorimeter. The cuvette containing the buffer and the substrate was placed in a thermostatically controlled cell compartment for 5 min before the addition of the enzyme, and the increase in fluorescence with time was continuously recorded for 5–10 min. The enzyme concentration for initial rate determination was chosen at a level intended to hydrolyse less than 5 % of the substrate present. The slope was converted into μmol of substrate hydrolysed per min based on a calibration curve obtained from complete hydrolysis of each peptide. The inner-filter effect was corrected using an empirical equation as described previously [43]. The kinetic parameters K_m and k_{cat} were calculated by the non-linear regression data analysis Grafit program. The k_{cat}/K_m values were calculated as the ratio of these two determined parameters. The apparent second-order rate constant k_{cat}/K_m ($^*k_{cat}/K_m$) was determined under pseudo-first-order conditions, where $[S] \ll K_m$. These determinations were performed in two different substrate concentrations and the errors were less than 5 % for any obtained value.

Determination of secPHEX cleavage sites

To determine the position of cleavage in peptides of the IQFPs combinatorial libraries, the products resulting from total hydrolysis by secPHEX were submitted to N-terminal amino acid sequencing in a PPSQ-23 protein sequencer (Shimadzu). The scissile bonds in the IQFPs derived from $PTHrP_{107-139}$, FGF-23 and MEPE were determined by amino acid sequencing and by MALDI-TOF MS (TofSpec-E) after isolation of the fragments resulting from the hydrolysis by secPHEX by analytical HPLC.

RESULTS

secPHEX specificity determined by peptide libraries

Positional scanning synthetic combinatorial libraries were used to identify the P_2 to P'_2 substrate specificity of secPHEX. P_2 to P'_2 are defined according to the nomenclature of Schechter and Berger [44]. We found that secPHEX requires an acidic residue in P'_1 position, as demonstrated using the library with the general sequence Abz-GXXZXK(Dnp), where only the peptides Abz-GXXDXK(Dnp) and Abz-GXXEXK(Dnp) were hydrolysed (Figure 1A). The products resulting from cleavage were submitted to N-terminal amino acid sequencing, and the presence of an aspartate or a glutamate as the first residue of the fragments DXK(Dnp) and EXK(Dnp) respectively was confirmed. However, a clear preference was observed for an aspartate residue in this position, as substitution with a glutamate residue resulted in almost 5-fold reduction on the catalytic efficiency ($^*k_{cat}/K_m$) (Figure 1A).

Based on the results from the first library, three other libraries with general structures Abz-GXXDZK(Dnp), Abz-GXZDXK(Dnp) and Abz-GZXDXK(Dnp), in which P'_1 was fixed as aspartate, were synthesized and tested with recombinant secPHEX to determine the S'_2, S_1 and S_2 specificities respectively. We observed that the P'_2 position showed a moderate preference for amino acids with aromatic side chains such as phenylalanine and tyrosine, as well as for polar residues such as serine and threonine (Figure 1B). In contrast, substrates containing leucine, proline and glycine in this position were resistant to hydrolysis by secPHEX. The S_1 subsite accepted a broad range of amino acids, although substrates containing isoleucine, valine and histidine in this position were resistant to hydrolysis (Figure 1C). No impeditive residues were detected in P_2 position, since secPHEX was able to hydrolyse all substrates of the Abz-GZXDXK(Dnp) series. However, in this library, peptides containing the amino acids arginine, lysine, asparagine and glutamine in P_2 were the least preferred substrates (Figure 1D).

A model peptide containing the most favourable amino acid residue in each position screened by the libraries was next synthesized. The resulting sequence, Abz-GFSDYK(Dnp)-OH, was used to better characterize the enzyme and to establish the optimum assay conditions for the kinetic studies.

pH activity profiles and NaCl dependence

The effect of pH on the hydrolysis of Abz-GFSDYK(Dnp)-OH by secPHEX was determined over a pH range 3.5–8.5. A bell-shaped curve was obtained, with maximum k_{cat}/K_m values occurring around pH 5.5 (Figure 2). A significant influence of NaCl concentration on the catalytic efficiency of recombinant secPHEX was also detected, as shown in the inset of Figure 2. k_{cat}/K_m values in presence of 10 mM Bis/Tris, containing 150 mM NaCl, pH 5.5, were more than 2-fold lower than in the absence of added salt. However, the presence of the salt is essential for the K_m and k_{cat} determinations, since in absence of NaCl the enzyme is gradually inactivated (results not shown).

Effects of substrate C-terminus modification

We next examined the effect of amidating the C-terminus of substrates on cleavage efficiency by secPHEX. Two substrates, Abz-GFSDYK(Dnp)-OH and Abz-GFSEYK(Dnp)-OH (peptides I and IV, Table 1), and their C-terminus amidated analogues (peptides II and V, Table 1), were used to compare the activity of secPHEX. The kinetic parameters presented in Table 1 show that

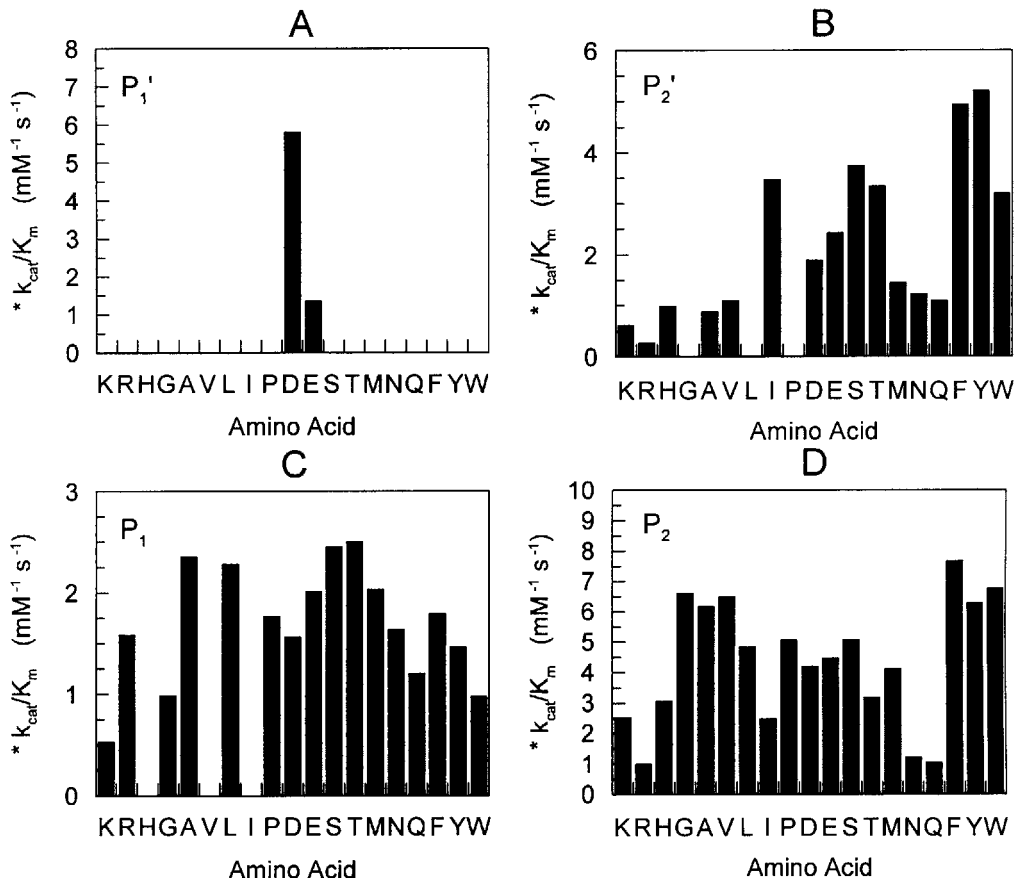

Figure 1 Scanning for amino acid residues preferred by secPHEX in substrate subsites P_2 to P'_2

Positional scanning fluorimetric combinatorial libraries with general sequences Abz-GXXZXK(Dnp)-OH (**A**), Abz-GXXDZK(Dnp)-OH (**B**), Abz-GXZDXK(Dnp)-OH (**C**) and Abz-GZXDXK(Dnp)-OH (**D**) were incubated with secPHEX (0.2–0.45 μM) in 0.1 M Hepes/150 mM NaCl, pH 6.5, at 37 °C. The assays were performed at a low substrate concentration where the reactions followed first-order conditions ([S] $\ll K_m$). The *y*-axis represents the apparent catalytic efficiency values ($*k_{cat}/K_m$) obtained as described in the Experimental section. The *x*-axis shows the specific amino acid represented by the one-letter code. The errors were less than 5 % for any obtained value.

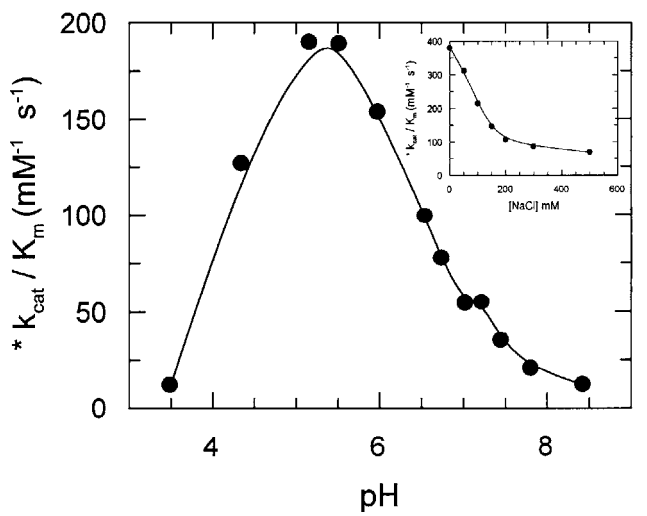

Figure 2 pH and NaCl (inset) dependence of Abz-GFSDYK(Dnp)-OH hydrolysis by secPHEX

The pH activity profile for the hydrolysis of Abz-GFSDYK(Dnp)-OH by PHEX in presence of different buffers and pH conditions is as described in the Experimental section. Inset: the effect of NaCl is shown; Abz-GFSDYK(Dnp)-OH was incubated with secPHEX in 0.01 M Bis/Tris buffer, pH 5.5, with NaCl (0–0.5 M).

Table 1 Kinetic constants for the hydrolysis by PHEX of IQFPs designed by screening combinatorial libraries

The assays were performed at 37 °C in 10 mM Bis/Tris containing 150 mM NaCl, pH 5.5. Measurements were made as described in the Experimental section. Cleavage sites are indicated as arrows (↓). The S.D. of the kinetic constants were less than 5 %.

Number	Peptides	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I	Abz-GFS ↓ DYK(Dnp)-OH	3	0.5	167
II	Abz-GFS ↓ DYK(Dnp)-NH_2	53	1.3	25
III	Abz-GFS ↓ DYQ-EDDnp	13	0.6	46
IV	Abz-GFS ↓ EYK(Dnp)-OH			9*
V	Abz-GFSEYK(Dnp)-NH_2	resistant		

* k_{cat}/K_m values were determined under pseudo-first-order conditions.

the model peptide Abz-GFSDYK(Dnp)-OH (peptide I, Table 1) was the best substrate of the series ($k_{cat}/K_m = 167\ mM^{-1} \cdot s^{-1}$), due to its high affinity for PHEX. On the other hand, its amidated analogue Abz-GFSDYK(Dnp)-NH_2 (peptide II, Table 1) was hydrolysed by the enzyme with a high k_{cat} value, but with a 7-fold decrease in catalytic efficiency due to its low affinity for the enzyme. As expected from the results of the peptide library studies described above, secPHEX catalytic efficiency

Table 2 Kinetic constants for hydrolysis by PHEX of IQFPs based on $PTHrP_{107-139}$ sequence

Conditions of hydrolysis are as described in Table 1. Cleavage sites are indicated by arrows (↓). The S.D. of the kinetic constants were less than 5 %.

Number	Peptides*	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I	Abz- D^{124}-HLS ↓ DTSTQ-EDDnp	1.3	0.1	77
II	Abz-L^{134}-EL ↓ DSRQ-EDDnp	0.9	0.05	55
III	Abz-A^{110}-WL ↓ DSGVQ-EDDnp	1.5	0.04	27

* The numbers in the peptides sequences identify the position of the residues in $PTHrP_{107-139}$.

was lower with Abz-GFSEYK(Dnp)-OH (peptide IV, Table 1), containing glutamate in P_1' and a free carboxy group, than that observed with the Asp-containing analogue (peptide I, Table 1). Abz-GFSEYK(Dnp)-NH_2 (peptide V, Table 1) was resistant to hydrolysis, confirming that substrates with blocked C-terminal groups are less susceptible to hydrolysis by secPHEX. Replacing K(Dnp) by Q-EDDnp at the C-terminal end of the model peptide resulted in an decrease of the k_{cat}/K_m value for peptide Abz-GFSDYQ-EDDnp (compare peptides I and III, Table 1). Q-EDDnp is present in peptides prepared by the solid-phase synthesis method.

Hydrolysis of peptides based on $PTHrP_{107-139}$

The secPHEX was reported to cleave $PTHrP_{107-139}$ at three positions [17]. To determine cleavage efficiency of this substrate, peptides with sequences encompassing the identified hydrolysed peptide bonds were synthesized and incubated with secPHEX. The enzyme had a high affinity for these peptides (low K_m values), but low k_{cat} values (Table 2). Despite the low catalytic constant, peptide Abz-D_{124}-HLSDTSTQ-EDDnp (peptide I, Table 2) was hydrolysed with k_{cat}/K_m value of 77 $mM^{-1} \cdot s^{-1}$, and was a good substrate for secPHEX. In all cases, hydrolysis of the substrates occurred at N-terminus of an aspartate residue.

Hydrolysis of peptides based on human FGF-23 sequence

FGF-23 has been proposed as a PHEX substrate [45]. Using the information gathered from the combinatorial libraries about the most favourable amino acids in the P_2 to P_2' positions, the FGF-23 sequence [31] was scanned for putative cleavage sites and IQFPs analogues containing aspartate or glutamate were synthesized. Cysteine residues found in human FGF-23 sequence were substituted with methionine to avoid synthesis problems. In spite of the presence of more than one putative scissile bond in some peptides, all substrates of this series were hydrolysed at a single cleavage site at the N-terminus of aspartate residues, as determined by N-terminal sequencing. The kinetic parameters presented in Table 3 reveal that Abz-R^{175}-RHTRSAEDDSERQ-EDDnp and Abz-R^{175}-RHTQSAEDDSERQ-EDDnp (peptides III and IV), the longest substrates tested, showed the highest k_{cat}/K_m values of this series, due to their high affinities for the enzyme. The peptide Abz-L^{94}-MMDFRGQ-EDDnp (peptide I, Table 3) had a low affinity for the enzyme, but the highest k_{cat} value. Peptides Abz-S^{212}-AEDNSPQ-EDDnp and Abz-R^{76}-SEDAGFQ-EDDnp (peptides V and VI, Table 3) presented poor catalytic efficiencies, and Abz-N^{122}-GYDVYHQ-EDDnp (peptide II, Table 3) was the poorest substrate of this series. The Arg^{179} to Gln mutation in FGF-23 was shown to result in ADHR in humans [30]. To test

Table 3 Kinetic constants for hydrolysis by PHEX of IQFPs based on FGF-23 sequence

Conditions of hydrolysis are as described in Table 1. Cleavage sites are indicated as arrows (↓). The S.D. of the kinetic constants were less than 5 %.

Number	Peptides*	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I	Abz- L^{94}-MM ↓ DFRGQ-EDDnp	17	0.8	47
II	Abz- N^{122}-GY ↓ DVYHQ-EDDnp	9	0.01	1.1
III	Abz-R^{175}-RHTRSAED ↓ DSERQ-EDDnp	3	0.2	67
IV	Abz-R^{175}-RHTQSAED ↓ DSERQ-EDDnp	1.7	0.15	88
V	Abz- S^{212}-AE ↓ DNSPQ-EDDnp	22	0.3	14
VI	Abz- R^{76}-SE ↓ DAGFQ-EDDnp	4	0.06	15

* The numbers in the peptide sequences identify the position of the residues as in FGF-23.

Table 4 Kinetic constants for hydrolysis by PHEX of IQFPs based on MEPE sequence

Conditions of hydrolysis are as described in Table 1. Cleavage sites are indicated as arrows (↓). The standard deviations of the kinetic constants were less than 5 %.

Number	Peptides*	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I	Abz-GP^{197}-QR ↓ DSQAQ-EDDnp	47	0.2	4
II	Abz-I^{232}-PS ↓ DFEGQ-EDDnp			80†
III	Abz-T^{294}-HL ↓ DTKKQ-EDDnp	15	0.02	1.3
IV	Abz-G^{337}-SN ↓ DIMGQ-EDDnp	13	0.01	0.8
V	Abz-G^{386}-SS ↓ DAAEQ-EDDnp	2.4	0.3	125
VI	Abz-R^{441}-GL ↓ DNEIQ-EDDnp	7	0.03	4
VII	Abz-N^{449}-EM ↓ DSFNQ-EDDnp	37	0.3	8
VIII	Abz-R^{506}-RD ↓ DSSEQ-EDDnp	5	0.5	100
IX	Abz-S^{513}-S ↓ DSGSQ-EDDnp	32	0.2	6

* The numbers in the peptide sequences identify the residues as in MEPE.
† k_{cat}/K_m value determined under pseudo-first-order conditions.

whether this mutation interferes with secPHEX cleavage of FGF-23, peptide Abz-R^{175}-RHTQSAEDDSERQ-EDDnp (peptide IV, Table 3) was synthesized and incubated with secPHEX. The mutation did not alter secPHEX cleavage of the peptide (compare peptides III and IV, Table 3).

Hydrolysis of peptides based on human MEPE sequence

As was done for FGF-23, the human MEPE sequence [33] was mapped, and IQFPs derivatives containing aspartate or glutamate were synthesized and tested as secPHEX substrates. Again, all peptides were hydrolysed at a single cleavage site in the N-terminus of an aspartate residue as presented in Table 4. The majority of the substrates of this series were hydrolysed with poor catalytic efficiency due to low k_{cat} and high K_m values. However, peptides Abz-G^{386}-SSDAAEQ-EDDnp and Abz-R^{506}-RDDSSEQ-EDDnp (peptides V and VIII, Table 4) showed the best k_{cat}/K_m values, which was due mainly to their high affinity for the enzyme.

Comparison of membrane-bound PHEX and secPHEX activity

It can be argued that engineering a soluble form of PHEX may change the specificity of the enzyme. To rule out this possibility, we first performed N-terminal sequencing of purified secPHEX

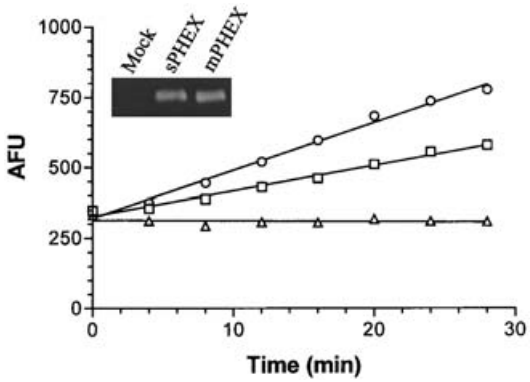

Figure 3 Hydrolysis of Abz-GFSDYK(Dnp)-OH by secPHEX and membrane-bound PHEX

Abz-GFSDYK(Dnp)-OH at a concentration of 10 μM was incubated at 37 °C in 0.01 M Bis/Tris buffer, pH 5.5, with 150 mM NaCl, containing 2.1 μg of total proteins from membranes extracted from LLC-PK_1 cells transfected with vector alone (△), 2.1 μg of total proteins from membranes extracted from LLC-PK_1 cells transfected with the membrane-bound form of human PHEX (□) or 2.1 μg of total proteins from membranes extracted from LLC-PK_1 cells transfected with vector alone to which 500 ng of purified secPHEX was added (○). Fluorescence was monitored every 4 min for a period of 30 min. Amounts of secPHEX (sPHEX) or membrane-bound PHEX (mPHEX) in the enzymic reactions were evaluated by immunoblotting (inset). Mock, cells transfected with vector alone. AFU, arbitrary fluorescence units.

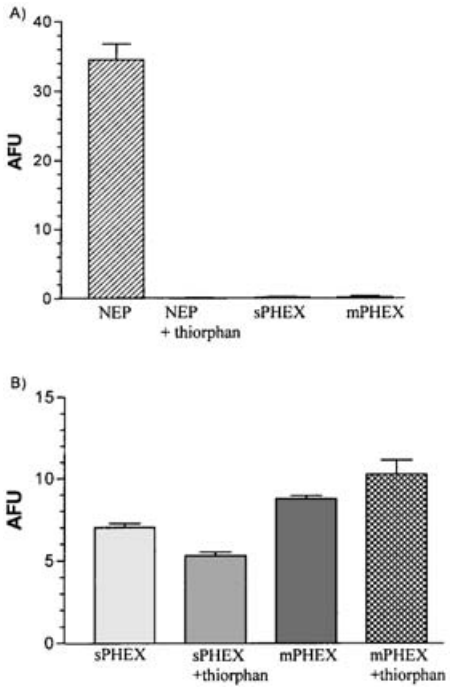

Figure 4 Activity of PHEX in the presence of NEP-specific substrate and inhibitor

NEP, secPHEX (sPHEX) and membrane-bound PHEX (mPHEX) activities were measured after 30 min of incubation in presence of either 10 μM Abz-rRL-EDDnp (upper panel) or 10 μM Abz-GFSDYK(Dnp)-OH (lower panel). Fluorescence detection (AFU, arbitrary fluorescence units) at $\lambda_{emission} = 420$ nm and $\lambda_{excitation} = 320$ nm was determined as described in the Experimental section. When present, thiorphan, a NEP-specific inhibitor, was at a concentration of 10^{-6} M. Results are the means ± S.D. for three different experiments.

to determine the number of amino acid residues lacking in the enzyme ectodomain. Residue Ser^{46} (of human PHEX sequence as reported in [3]) was found as the first secPHEX residue.

LLC-PK_1 cells were then transfected with membrane-bound PHEX cDNA, and PHEX activity in the membrane fraction was compared with secPHEX, using Abz-GFSDYK(Dnp)-OH as substrate. To obtain a better comparison between secPHEX and membrane-bound PHEX in this series of experiments, secPHEX was added to membranes purified from cells transfected with vector alone. When similar amounts of secPHEX and membrane-bound PHEX, as evaluated by immunoblotting (Figure 3, inset), were added to the enzymic assay, degradation of the substrate was observed (Figure 3). No substrate hydrolysis was evident with membranes from cells transfected with vector alone. A higher rate of hydrolysis was observed with secPHEX, which also showed a slightly lower K_m value (5×10^{-6} M and 10×10^{-6} M, for secPHEX and membrane-bound PHEX respectively). N-terminal sequencing of the hydrolysis products showed that both enzymes cleaved substrate Abz-GFSDYK(Dnp)-OH at the N-terminus of the aspartate residue.

We also compared the activities of membrane-bound PHEX and secPHEX with the NEP-specific substrate Abz-rRL-EDDnp [46]. Neither enzyme hydrolysed this substrate (Figure 4, upper panel). In addition, the NEP-specific inhibitor thiorphan did not prevent cleavage of substrate Abz-GFSDYK (Dnp)-OH by membrane-bound or secPHEX (Figure 4, lower panel). In contrast, NEP activity was completely inhibited by the same concentration of thiorphan (Figure 4, upper panel). These results demonstrated that engineering human PHEX into a soluble enzyme did not affect its activity and specificity, and that PHEX does not display a NEP-like activity.

DISCUSSION

Data collected from the Hyp mouse model and human XLH patients [2] suggest that PHEX plays an important role in the control of phosphate homoeostasis and skeletal mineralization. However, the mechanism(s) by which PHEX regulates these physiological processes is still unknown. Homologies between PHEX and endopeptidases of the M13 family suggest that PHEX may hydrolyse biologically active peptides and in this way control their activity. Although several candidates, such as parathyroid hormone [20], $PTHrP_{107-139}$ [17], FGF-23 [45] and MEPE [47], have been proposed, no physiologically relevant PHEX substrate has yet been identified, and the substrate specificity of the enzyme is not known. In the present study, we used a soluble secreted form of recombinant human PHEX (secPHEX) that can be easily purified to probe the enzyme substrate specificity. We showed that the secPHEX protein lacks 45 amino acid residues at the N-terminal, starting at residue Ser^{46} of the native PHEX sequence. Considering that PHEX has a 20-amino-acid-long cytoplasmic domain (from Met^1 to Arg^{20}) and a transmembrane segment of at least 20 residues, our results indicate that secPHEX is lacking, at the most, five residues from its extracellular domain. The three-dimensional structure of NEP indicated that this segment of the protein probably forms the stalk that maintains the enzyme away from the surface of the cell, and does not contain amino acid residues important for substrate binding or catalytic activity [48]. It is thus unlikely that the absence of these five residues would change secPHEX specificity. This hypothesis was confirmed by our demonstration that both the recombinant membrane-bound and soluble secreted forms of the enzyme cleaved the substrate Abz-GFSDYK(Dnp)-OH at the same position and that both forms of the enzyme have a similar affinity for the substrate.

The development of positional scanning combinatorial fluorogenic peptide libraries allowed us to test and evaluate

PHEX substrate specificity *in vitro*. The results clearly show an unequivocal preference of PHEX for cleavage at the N-terminus of acidic amino acid residues (aspartate or glutamate), with a strong bias for aspartate residues. These results are in accordance with the previously reported observation that PHEX hydrolysed $PTHrP_{107-139}$ at three positions all located at the N-terminus of an aspartate residues, and consistent with our observation that a positively charged arginyl side chain is possibly present in the PHEX S'_1 pocket and could form an ionic bond with the aspartate side chain of the substrate [17]. Indeed, whereas the NEP three-dimensional structure showed that the S'_1 pocket is lined with seven hydrophobic amino acids residues, namely Phe^{106}, Ile^{558}, Phe^{563}, Met^{579}, Val^{580}, Val^{692} and Trp^{693}, sequence comparisons have identified Tyr^{109}, Glu^{554}, Phe^{559}, Val^{576}, Ile^{577}, Val^{691} and Arg^{692} as the homologous residues in PHEX [17]. It is interesting to underline that neither secPHEX nor membrane-bound PHEX could hydrolyse the NEP substrate Abz-rRL-EDDnp, and that neither enzyme was inhibited by the NEP inhibitor thiorphan. These results support our previous observation [17] that PHEX could not cleave several well-known NEP substrates, including enkephalins and substance P. Thus our work clearly shows that PHEX and NEP have different substrate specificities, and is not in agreement with reports that PHEX is able to hydrolyse NEP substrates, such as Leu-enkephalin [34] and the chromogenic peptide benzyloxycarbonyl-Ala-Ala-Leu-*p*-nitroanilide [35], as these peptides do not have acidic residues in their amino acid sequences.

Libraries with a fixed asparate residue in the P'_1 position were next synthesized in order to define extended substrate specificity. We found that the S'_2, S_1 and S_2 subsites are much less restrictive than the S'_1 subsite. However, we observed that specific residues in the P'_2 (leucine, proline and glycine) and P_1 (isoleucine, valine and histidine) positions precluded hydrolysis by secPHEX. From these results, a model peptide with the most favourable amino acid residue in each position was synthesized. This peptide, Abz-GFSDYK(Dnp)-OH, was the best PHEX substrate among all the peptides tested in the present study ($k_{cat}/K_m = 167\ mM^{-1} \cdot s^{-1}$), and was used to determine the optimum conditions for a PHEX enzymic assay.

We also showed that, similar to NEP, PHEX prefers substrates with a free C-terminus. Amidating the C-terminus of substrate Abz-GFSDYK(Dnp)-OH resulted in an almost 7-fold decrease in PHEX catalytic efficiency. Similar results were also obtained with substrate Abz-GFSEYK(Dnp)-OH and its amidated counterpart. In NEP, Arg^{102} has been proposed to form an ionic interaction with the free C-terminus of some substrates [49]. By sequence homology, Lipman et al. [20] could not identify an equivalent residue in PHEX. Our results suggest that the residue responsible for the ionic interaction with the free C-terminus of substrates might also be present in the active site of PHEX. Clearly, a more systematic study, using substrates modified at their C-terminus and PHEX proteins in which critical amino-acid substitutions have been introduced by site-directed mutagenesis of the cDNA, is necessary to identify this residue.

Based on biochemical and genetic evidence, several proteins/peptides have been proposed as PHEX substrates. These include $PTHrP_{107-139}$ [17], FGF-23 [45] and MEPE [47]. To determine whether PHEX cleavage sites were present, the sequences of these proteins/peptides were scanned for regions containing aspartate or glutamate residues in a favourable context for PHEX cleavage, as identified above, and IQFPs derivatives were synthesized accordingly. Our results show that these three proteins/peptides contain several PHEX cleavage sites that are hydrolysed more or less efficiently by the enzyme. In particular, the enzyme had a high affinity for the FGF-23-derived substrate

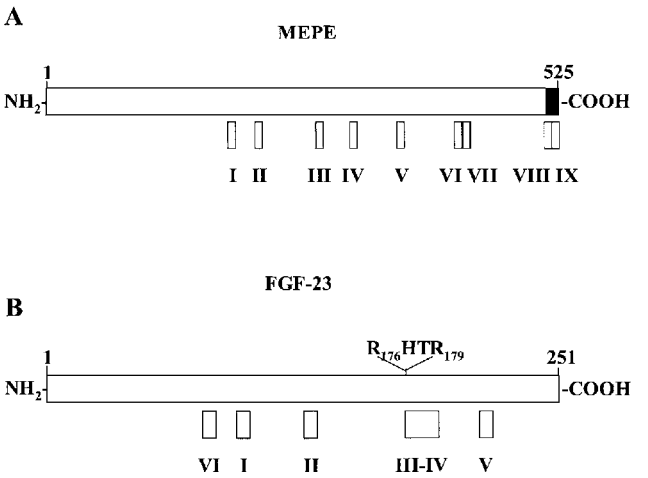

Figure 5 Representation of the positions of the synthetic peptides in MEPE and FGF-23 structure

(**A**) MEPE structure: numbers correspond to the first and last amino acid residues of MEPE, including the signal sequence. The black box represents MEPE sequence homologous to sequences found in DMP1, DSPP and OPN. The boxes under MEPE structure correspond to the position in MEPE sequence of the peptides presented in Table 4. Numbering is as in Table 4. (**B**) FGF-23 structure: numbers correspond to the first and last amino acid residues of FGF-23, including the signal sequence. $R_{176}HTR_{179}$ (Arg^{176}-His-Thr-Arg^{179}) indicate the position of the convertase cleavage site found in FGF-23 sequence. The boxes under FGF-23 structure correspond to the position in FGF-23 sequence of the peptides presented in Table 3. Numbering is as in Table 3.

Abz-R^{175}-RHTRSAEDDSERQ-EDDnp (peptide III, Table 3) and for MEPE-derived peptide Abz-R^{506}-RDDSSEQ-EDDnp (peptide VIII, Table 4), and in both cases cleaved substrates between two aspartate residues. The latter peptide is located in the C-terminal domain of MEPE (see Figure 5A), a region with strong homologies with several bone and teeth proteins including dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and osteopontin (OPN) [33]. These homologies suggest that DMP1, DSPP and OPN are also putative PHEX substrates. This region also contains a protein kinase CK2 (casein kinase II) phosphorylation site that suggests a regulatory role.

Of particular interest is the peptide Abz-R^{175}-RHTRSAEDDSERQ-EDDnp. This sequence contains FGF-23 furin cleavage site that is mutated in ADHR (Arg^{176} and Arg^{179}; see Figure 5B) [30]. Bowe et al. [45] have observed that *in vitro*-translated wild-type FGF-23 was hydrolysed by *in vitro*-translated PHEX, whereas mutant FGF-23 with a $Arg^{179} \rightarrow Gln$ substitution (R179Q) was resistant to hydrolysis by the enzyme, suggesting that the mutation could interfere with PHEX cleavage of this protein. However, we could not reproduce this result using short peptides. The FGF-23-derived peptide substrate carrying the R179Q mutation was cleaved as efficiently by PHEX as the wild-type FGF-23-derived peptide. This result is consistent with results generated from the combinatorial libraries, demonstrating that only subsites close to the cleavage site have an impeditive influence. Guo et al. [34] have reported that FGF-23 peptide P^{172}-IPRRHTRSAEDDSE was resistant to hydrolysis by PHEX. This peptide has the PHEX cleavage site identified in Abz-R^{175}-RHTRSAEDDSERQ-EDDnp (in between the two aspartate residues), and incubation with PHEX should release the tripeptide Asp-Ser-Glu. It is, however, possible that the release of this very hydrophilic tripeptide was missed in the HPLC system used to monitor PHEX activity.

The discrepancy in the results raises the question of whether the PHEX substrate specificity observed with small synthetic peptides

can be extended to the hydrolysis of large natural proteins. The crystal structure of NEP indicates that the presence of a two-domain structure restricts the access of the active site to small peptides [48]. Although the three-dimensional structure of PHEX is still not known, its homology with NEP would suggest that both enzymes function as oligopeptidases, as these proteins present a common origin and a similar fold [48]. If this hypothesis is correct, full-length FGF-23 and MEPE could not be cleaved by PHEX. However, it has been shown that FGF-23 is processed to smaller fragments during its biosynthesis, and that mutations in FGF-23 resulting in ADHR in human patients precluded this processing [50]. Similarly, Argiro et al. [47] postulated that MEPE is first cleaved by an uncharacterized metallopeptidase, and that one of the fragments generated is the phosphaturic factor processed by PHEX. In addition, Quarles and Drezner [51] have reported phosphaturic activity of truncated MEPE fragments. Thus, despite functioning as an oligopeptidase, PHEX may be involved in controlling the activity of FGF-23 and/or MEPE by cleaving their processed peptide products. Further studies are necessary to characterize the size selectivity of PHEX substrates.

In conclusion, we have developed an enzymic assay for PHEX using IQFPs that has the advantage of high sensitivity and allows the possibility to monitor the reaction on a continuous basis. This enzymic assay can be helpful to develop inhibitors, better characterize the enzyme and understand its physiological role. We have also provided evidence that peptides derived from physiologically relevant proteins, such as FGF-23 and MEPE, are PHEX substrates.

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