

# Cloning of Human *PEX* cDNA

EXPRESSION, SUBCELLULAR LOCALIZATION, AND ENDOPEPTIDASE ACTIVITY\*

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Mutations in the *PEX* gene are responsible for X-linked hypophosphatemic rickets. To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its tissue expression, subcellular localization, and peptidase activity. We show that the cDNA encodes a 749-amino acid protein structurally related to a family of neutral endopeptidases that include neprilysin as prototype. By Northern blot analysis, the size of the full-length *PEX* transcript is 6.5 kilobases. *PEX* expression, as determined by semi-quantitative polymerase chain reaction, is high in bone and in tumor tissue associated with the paraneoplastic syndrome of renal phosphate wasting. *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase from Triton X-114 extractions of transiently transfected COS cells. Immunofluorescence studies in A293 cells expressing *PEX* tagged with a c-myc epitope show a predominant cell-surface location for the protein with its COOH-terminal domain in the extracellular compartment, substantiating the assumption that *PEX*, like other members of the neutral endopeptidase family, is a type II integral membrane glycoprotein. Cell membranes from cultured COS cells transiently expressing *PEX* efficiently degrade exogenously added parathyroid hormone-derived peptides, demonstrating for the first time that recombinant *PEX* can function as an endopeptidase. *PEX* peptidase activity may provide a convenient target for pharmacological intervention in states of altered phosphate homeostasis and in metabolic bone diseases.

mon inherited disorder of renal phosphate wasting characterized by severe hypophosphatemia, renal phosphate wasting, reduced serum concentrations of 1,25-dihydroxyvitamin D levels, and defective bone mineralization (1). Until recently, much of our understanding of HYP has been facilitated by the availability of two murine homologues, the *Hyp* and *Gy* mice, which exhibit many of the phenotypic features of HYP (2, 3). Through positional cloning, however, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in non-deletion patients with the disorder, was identified (designated *PEX*) and its partial cDNA sequence reported (4). The predicted human *PEX* gene product, as well as its murine homologue (5), exhibit homology to a family of neutral endopeptidases involved in either activation or degradation of a number of peptide hormones. It has been postulated that *PEX* metabolizes a peptide hormone that modulates renal tubular phosphate handling. Such an activity could involve either the processing of a phosphate-reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. These speculations notwithstanding, the physiologic function of the *PEX* gene product and the mechanisms that lead to the renal and skeletal abnormalities of HYP remain to be defined.

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disorder of phosphate homeostasis with biochemical and physical abnormalities similar to HYP (reviewed in Ref. 6). This syndrome is associated with a variety of histologically distinct, usually benign, mesenchymal tumors (7) whose excision promptly reverses the metabolic abnormalities and results in cure of the bone disease. It is generally thought that a factor(s) produced by these tumors promotes phosphaturia and inhibits the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. The nature of the phosphaturic substance remains unknown and is likely distinct from both parathyroid hormone (PTH) and calcitonin, two polypeptide hormones known to inhibit the renal tubular reabsorption of phosphorus (8–13). Because of the striking similarity in the clinical presentation of patients with OHO and HYP, it is postulated that the factor causing phosphaturia in OHO is the active form of the *PEX* substrate. The identification and characterization of the putative *PEX* substrate, referred to as phosphatonin (14), however, will require first a better understanding of *PEX* function. Toward this objective, we have cloned a cDNA encoding

X-Linked hypophosphatemic rickets (HYP)<sup>1</sup> is the most com-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81970.

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<sup>1</sup> The abbreviations used are: HYP, X-linked hypophosphatemia;

*PEX*, phosphate regulating gene with homologies to endopeptidases on the X chromosome; OHO, oncogenous hypophosphatemic osteomalacia; PTH, parathyroid hormone; PCR, polymerase chain reaction; kb, kilobase(s); RT, reverse transcriptase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

the full-length human PEX protein, and determined the tissue distribution of PEX transcripts. In addition, we have examined the subcellular localization of recombinant PEX protein and demonstrated its peptidase activity.

#### EXPERIMENTAL PROCEDURES

**Tumor Tissues**—Patient I was a 55-year-old woman who presented with a 2-year history of progressively increasing bone pain and difficulty in walking. X-rays of the lumbosacral spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal while serum phosphorus was low (0.41 to 0.57 mmol/liter; normal, 0.8–1.6 mmol/liter). Alkaline phosphatase was 232 units/liter (normal, 30–105 units/liter) and tubular reabsorption of phosphate while the patient was hypophosphatemic was decreased to 63% (normal, >80%). A search for a tumor was negative and the patient was treated with 1,25-dihydroxyvitamin D<sub>3</sub> and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was a fibrous hemangioma. Postoperatively, the patient noted increasing strength in her lower extremities and marked decrease in her pain. The serum phosphorus normalized (0.96 mmol/liter) and the tubular reabsorption of phosphate improved but did not completely normalize (71–76%). No recurrence of the tumor has been found 10 years later.

Patient II was a 21-year-old man with classic features of OHO (15). Resection of a benign extraskeletal chondroma from the plantar surface of the foot resulted in complete reversal of the biochemical and clinical abnormalities associated with the syndrome. Tumor tissue obtained from these two patients at surgery was frozen immediately in liquid nitrogen and stored at –70 °C.

**PEX Expression in OHO-associated Tumors**—RNA was extracted from tumor tissue using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA) and reverse transcribed using oligo(dT) primer and Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C in a final reaction volume of 30 µl. The resulting cDNA was then amplified using human PEX-specific oligonucleotide primers PEX-1 (5'-GGAGGAATTGGTTGAGGGCG-3') and PEX-2 (5'-GTAGACCACCAAGGATCCAG-3'), designed from the published cDNA sequence (1298 and 1807 are the nucleotide positions of the 5' end of the sense and antisense primers, respectively) (4). Following amplification (35 cycles), an aliquot of the PCR reaction was fractionated on a 1% agarose gel and visualized following staining with ethidium bromide.

**Cloning of Full-length PEX cDNA**—Cloning of the 5' end of PEX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumor II and mRNA was prepared. 1.5 µg of mRNA was reverse transcribed into cDNA using 100 ng of a PEX-specific antisense oligomer (PEX-2) and 200 units of Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C in a final reaction volume of 30 µl. The resulting cDNA was size fractionated on a 1% agarose gel and fragments corresponding to >600 base pairs were purified and resuspended in H<sub>2</sub>O. The 3' end of the first strand cDNA was homopolymer tailed with dGTP using 1 µl of terminal deoxynucleotidyl transferase at 37 °C for 30 min in a volume of 50 µl. Following heat inactivation of the enzyme, the RNA template was removed by incubation with RNase H and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H<sub>2</sub>O and an aliquot was used for anchored PCR analysis along with 200 ng of an internal PEX specific antisense primer (PEX-3, 5'-CGTGCCAGAACTAGGGTGCCACC-3'; nucleotide 98 of the published human cDNA sequence is the 5' end of the primer) and 200 ng of oligo(dC) as the sense primer. Forty cycles of PCR were performed using 0.5 µl of *Taq* polymerase (Promega Biotech, Madison, WI) in a reaction volume of 50 µl. Cycling parameters were: 1 min of denaturation at 94 °C, 2 min of annealing at 55 °C, and 2 min of extension at 72 °C. The PCR products were fractionated on a 1% agarose gel and a band of 700 base pairs was isolated, purified, and ligated into pPCRII vector (Invitrogen). Following transformation into *INVαF'* bacteria, clones containing the appropriate size insert were sequenced.

To clone the 3' end of PEX cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from mRNA obtained from tumor I was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 µg) was subjected to PCR using a PEX-specific sense oligomer (PEX-1) and an antisense oligomer corresponding to the SP6 RNA polymerase-binding site sequences present in the pCDNA3 vector. Thirty-five cycles of amplification were performed in a 50-µl reaction volume with each cycle consisting of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C. Amplified products were fractionated on a 1% agarose gel and a 1.2-kb

fragment corresponding to the 3' end of PEX cDNA was subcloned and sequenced.

For expression studies, an *EcoRV* (in the polylinker of pPCRII)/*AccI* (in the PEX sequence) fragment containing the 5' end of PEX cDNA was ligated into the pPCRII vector containing the 3' end of PEX cDNA following digestion with *AccI* and *EcoRV*. The resulting plasmid was restricted with *KpnI* and *NotI* excising the full-length PEX cDNA that was then inserted into pCDNA3 vector digested at the *KpnI/NotI* sites in the polylinker region, resulting in plasmid pPEX. The full-length PEX cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

**Tissue Expression of PEX mRNA**—PEX expression was examined in normal human tissues and in the Saos-2 human osteoblastic osteosarcoma cell line, by RT-PCR using oligonucleotides PEX-4 (5'-CTGGATCCTTGGTGGTCTAC-3') and PEX-5 (5'-CACTGTGCAACTGTCTCAG-3') as sense and antisense primers (2398 and 2895 are the nucleotide positions of the 5' end of these primers designed from the full-length human PEX cDNA). Semiquantitative PCR analysis for PEX expression in human tissues was performed as described previously (16), following normalization for *glyceraldehyde-3-phosphate dehydrogenase* message in all samples containing PEX transcripts.

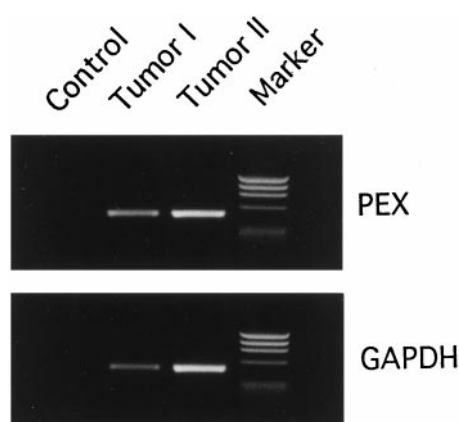
**Northern Blot Analysis**—Total RNA was obtained from tumor I and human Saos-2 osteosarcoma cells using the RNeasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)<sup>+</sup> RNA was isolated from Saos-2 total RNA using standard procedures. Twenty micrograms of tumor I total RNA and 20 µg of Saos-2 poly(A)<sup>+</sup> RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N<sup>+</sup>, Amersham). Hybridization was performed with <sup>32</sup>P-labeled full-length human PEX cDNA (3.1 kb) in 7 mM Tris-HCl, 50% formamide, 10% dextran sulfate, 4 × SSC, 2 × Denhardt's solution, and heat-denatured salmon sperm DNA (100 µg/ml). The blot was washed in 0.1 × SSC, 0.1% SDS for 20 min at 50 °C, and subjected to autoradiography for 4 days.

**In Vitro Transcription, Translation, and Analysis of Products**—Plasmid pPEX was linearized with *NotI* and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed in the presence of [<sup>3</sup>H]leucine according to the manufacturer's recommendations (Promega) with or without canine pancreas microsomal membranes. Products were analyzed by SDS-polyacrylamide gel electrophoresis (8%). Autoradiography was performed after treating the gel with EN<sup>3</sup>HANCE (NEN Life Science Products), as described previously (17).

**Generation of myc-tagged PEX, Transfection in COS-7 Cells, and Triton X-114 Extraction**—Plasmid pPEX-myc was generated by PCR amplification of PEX cDNA using oligonucleotide PexMyc1 as the sense primer (5'-TTGGATGTCACGCCTCG-3', 519 is the nucleotide position of the 5' end of this primer designed from the cloned human PEX cDNA) and PexMyc2 as the antisense (5'-CTACCACAATCTACAGTTGTTTCAGTCTCTTCGCTAATCAGCTTTTGTTCATAGAGTCCATGCCTCTG-3') primer. The latter encodes the human *c-myc* tag sequences (underlined) and PEX sequences corresponding to the carboxyl-terminal of the mature protein (<sup>742</sup>RGMDSM~~EQKLISEEDL~~NNCRLW). Following PCR, the amplified fragment was ligated to the pPCR II vector, excised by digestion with *KpnI/NotI*, and inserted into the corresponding sites in the polylinker region of pCDNA3. The in-frame fusion protein was verified by DNA sequencing.

COS-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/liter glucose with L-glutamine; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (Life Technologies, Inc.) and antibiotics (penicillin/streptomycin) were plated at a density of 3 × 10<sup>5</sup> cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed with twice with PBS and incubated with 2 µg of pPEX-myc plasmid DNA in 1 ml of DMEM containing 0.1% bovine serum albumin, and DEAE-dextran (Pharmacia) for 3.5 h at 37 °C. Following incubation, the transfection medium was aspirated, the cells were shocked with 10% dimethyl sulfoxide in PBS for 2 min, and then cultured in DMEM with 10% calf serum at 37 °C for 48 h. Triton X-114 extraction were performed on cultured cells expressing myc-tagged PEX as described (18). The samples were then analyzed by immunoblotting using the 9E10 anti-myc monoclonal antibody (19).

**Stable Transfection of A293 Cells and Immunofluorescence**—A293 cells maintained in DMEM with 10% fetal calf serum were transfected with the pPEX-myc plasmid by electroporation and selection initiated using G418 (600 mg/ml for 14 days and then decreased to 400 mg/ml). Populations of stably transfected cells were recovered at the end of the selection period. For myc-tagged PEX indirect immunofluorescence, stably transfected cells plated on gelatin-coated coverslips were washed twice with PBS, fixed in 4% paraformaldehyde, and in some experi-



**FIG. 1. PEX mRNA expression in OHO tumors.** Total RNA extracted from two tumors associated with OHO was reverse transcribed and amplified by PCR (35 cycles) using human PEX-specific primers, PEX-1 and PEX-2, designed from the published human sequence. The expected 509-base pair amplified fragment was obtained from both tumor samples. *Control*, no cDNA added to the amplification reaction, *i.e.* negative control; *Marker*,  $\Phi$ 174 DNA digested with *Hae*III restriction endonuclease. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

ments permeabilized with 0.5% Triton X-100. Cells were blocked with 10% fetal calf serum in DMEM for 30 min, washed, and incubated for 1 h at 37 °C with the 9E10 anti-*myc* monoclonal antibody (1:500 dilution). Cells were subsequently washed and incubated in turn with fluorescein-conjugated sheep anti-mouse secondary antibody (1:250 dilution). Coverslips were rinsed extensively with PBS, mounted in medium (glycerol:Tris; 1:1) containing 2.5% 1,4-diazabicyclo-(2,2,2)octane (Sigma) and examined with fluorescent microscopy using appropriate filters.

**Assay for Membrane-bound Endopeptidase Activity**—COS-7 cells transiently transfected with pCDNA3 vector alone, with vector containing human *NEP* cDNA (generous gift of P. Crine, Université de Montréal), or with pPEX plasmid, were washed and scraped in PBS. Following brief centrifugation, the cell pellets were resuspended in 50 mM Tris-HCl, pH 7.4, and disrupted by sonication. Homogenates were fractionated by sequential centrifugation at  $1,000 \times g$  for 10 min and then at  $100,000 \times g$  for 60 min. The final precipitate was washed with 50 mM Tris-HCl, pH 7.4, resuspended in the same buffer, and assayed for endopeptidase activity. The protein concentration in membrane fractions was determined by the method of Bradford (20) with bovine serum albumin as standard.

[D-Ala<sup>2</sup>,Leu<sup>3</sup>]Enkephalin (500  $\mu$ M) was incubated with COS cell membrane preparations (~60  $\mu$ g of protein) in 100 mM Tris-HCl, pH 7.0, at 37 °C for 30 min (final volume 30  $\mu$ l). The reaction was terminated by the addition of 100  $\mu$ l of 0.1% trifluoroacetic acid (*v/v*). Production of Tyr-D-Ala-Gly was monitored using reversed-phase HPLC (Bondpak C-18 reverse phase column, Waters) with a UV detector set at 214 nm. A linear solvent gradient of 0% B to 40% B in 60 min was used with a flow rate of 1.5 ml/min (mobile phase A = 0.1% trifluoroacetic acid (*v/v*); mobile phase B = 80% acetonitrile, 0.1% trifluoroacetic acid). Tyr-D-Ala-Gly was identified by co-chromatography with marker synthetic peptide. For assessing PEX endopeptidase activity, 10  $\mu$ g of PTH-(1–38) and PTH-(1–34) peptides (Peninsula Laboratories, Belmont, CA) were added to the membrane preparations. For HPLC analysis of hydrolysis products, a linear solvent gradient of 0% to 50% solution B was used at a rate of 1.5 ml/min. MALDI-TOF mass spectrometry was performed on specific peptide fragments.

## RESULTS

**Cloning of Human PEX cDNA**—At the initiation of these studies, *PEX* expression had been reported in minute amounts only in leukocytes and fetal brain. We postulated that in states of hypophosphatemia *PEX* expression may be increased and therefore opted to use the OHO tumor as a tissue source that may express considerably more *PEX*. Tissues obtained from two tumors associated with OHO were used to obtain total RNA and analysis for *PEX* mRNA expression was assessed by RT-PCR. As shown in Fig. 1, *PEX* transcripts were readily

amplified from both tumor samples demonstrating the expected 509-base pair fragment predicted from the published partial human *PEX* sequence (4).

The cloning of the 3' end of *PEX* transcript was performed by rapid amplification of the 3' end of the cDNA, while the 5' of the cDNA was amplified by anchored PCR, as described under "Experimental Procedures." Fig. 2A shows the nucleotide and predicted amino acid sequence of the full-length human *PEX* cDNA cloned from tumor tissues. The composite cDNA reveals a single open reading frame encoding a protein of 749 amino acids which displays homology (34.2% identity, 70% similarity) to human neprilysin (EC 3.4.24.11), and other members of the membrane-bound metalloendopeptidase family encompassing endothelin-converting enzyme-1 (66% similarity) and the Kell antigen (60% similarity), suggesting that *PEX* is a novel member of this family of neutral endopeptidases, as previously suggested (4). Like the other members, *PEX* is a likely a glycoprotein with eight potential *N*-glycosylation sites and 10 cysteine residues that may be important for the proper folding and hence native conformation of the protein.

The ATG codon at position 604 was assigned as the initiator methionine since it is preceded by two in-frame TGA termination codons 36 and 63 base pairs upstream and conforms favorably to the Kozak consensus for vertebrate initiation of translation (21). The cloned cDNA identifies the first 3 and the last 108 amino acids of the predicted *PEX* gene product in addition to the published partial sequence. These additional amino acids comprise residues such as Glu<sup>642</sup> and His<sup>710</sup> that are shared by neprilysin, and may be critical for the formation of the active site of the protein and hence its enzymatic activity. Three amino acid residues predicted from our cDNA clone differ from the published partial human *PEX* sequence, D363A (GAC to GCC), R403W (AGG to TGG), and A641G (GCG to GGA). To confirm that these alterations did not arise because of PCR errors, *PEX* sequences were amplified from Saos-2 human osteosarcoma cells (see below) and sequenced. In addition, the same alterations were subsequently described in the murine *Pex* cDNA (12), suggesting possible cloning artifacts in the published partial human *PEX* sequence. Our cloned sequences also encompass 603 nucleotides of the 5'-untranslated region, and 276 nucleotides of the 3'-untranslated region, including the canonical polyadenylation signal AATAAA, 19 nucleotides upstream of the poly(A) tract. The human and the published mouse *PEX* cDNA sequences share extensive homology within the protein coding region (96% identity) as well as in the 5' and 3' noncoding regions.

TMpred analysis of the human *PEX* sequence predicts that the protein has no apparent NH<sub>2</sub>-terminal signal sequence but has a single membrane-spanning helical domain comprising amino acid residues 21–39 (Fig. 2C). This predicts its transmembrane topology to be that of a type II integral membrane protein with a 20-residue NH<sub>2</sub>-terminal cytoplasmic tail and a COOH-terminal of 700 amino acid residues containing the catalytic domain in the extracellular compartment. Unexpectedly, a CXXX box motif comprising amino acid residues 746CRLW was also identified at the carboxyl terminus of *PEX*. This motif may serve as a site for prenylation, a post-translational lipid modification involved in a number of processes including facilitating membrane attachment, targeting of proteins to specific subcellular membrane compartments, promoting protein-protein interactions, and regulating protein function (22, 23).

**Tissue Expression of PEX mRNA**—We next examined *PEX* expression in a number of fetal and adult tissues and compared the level of expression to OHO tumor RNA using semi-quantitative RT-PCR (Fig. 3). *PEX* transcripts were expressed in



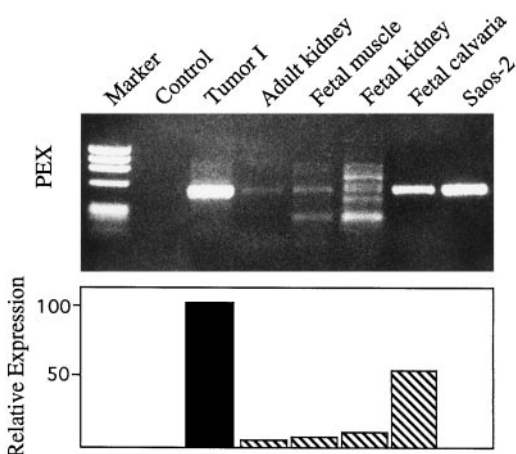


FIG. 3. **PEX expression in human tissues.** Quantitative RT-PCR amplification of the PEX transcripts from total RNA prepared from human tissues and OHO-associated tumor. Relative expression levels for the PEX transcript were measured by quantifying PEX product in reversed-transcribed RNA samples that have been previously normalized for glyceraldehyde-3-phosphate dehydrogenase levels. The specific primers used were as follows: for PEX, the forward primer was PEX-4 and the reverse primer PEX-5; for glyceraldehyde-3-phosphate dehydrogenase, the primers were as described previously (14). PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Control, negative control; Marker,  $\Phi$ 174 DNA digested with *Hae*III restriction endonuclease. Below, shown are the relative levels of PEX transcripts in various human tissues compared with those in the tumor.

human fetal calvarium and to a lesser degree in fetal kidney and skeletal muscle while no expression was apparent in fetal liver (data not shown). PEX expression was also observed in the human osteoblastic osteosarcoma cell line, Saos-2. In adult tissues, PEX mRNA was identified in kidney, but not in liver, or endomyocardium (not shown). Recent studies have also reported PEX expression in human fetal bone, skeletal muscle, and liver as well as fetal and adult ovary and lung (24, 25). Analysis following normalization for *glyceraldehyde-3-phosphate dehydrogenase* message in all tissues containing PEX transcript disclosed that bone PEX expression is 2–10-fold higher than in other normal tissues examined. In comparison, OHO tumor PEX expression was twice the levels observed in fetal calvarium, consistent with its relative “overabundance” in these tissues.

**Northern Blot Analysis**—To determine the size of the full-length PEX transcript, we isolated total RNA from tumor I (quantity of available tissue was insufficient for poly(A)<sup>+</sup> RNA extraction) and poly(A)<sup>+</sup> RNA from human Saos-2 osteosarcoma cells. This cell line was used since it is readily available and successful amplification of PEX sequences has been performed by RT-PCR (see above). Aliquots (20  $\mu$ g of each) were examined by Northern blot analysis using the cloned human PEX cDNA as probe. A single transcript of approximately 6.5 kb was readily detected only in the Saos-2-derived poly(A)<sup>+</sup> sample and contrasts with the predicted size of the cloned sequence of 3.1 kb (Fig. 4). This finding would therefore predict a ~4 kb 5'-untranslated region for PEX cDNA, consistent with published data from Northern blot analysis of PEX expression in mouse calvaria (5). A less well defined band was also detected in the Saos-2 sample corresponding to a potential tran-

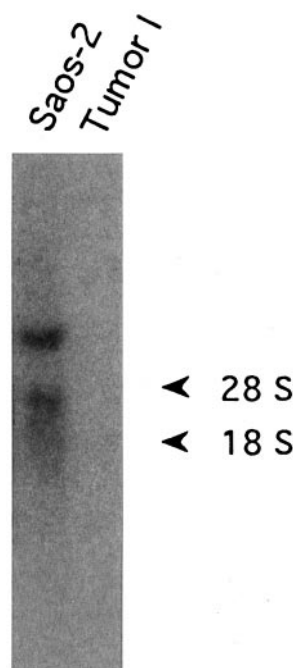


FIG. 4. **Northern blot analysis of PEX mRNA.** Approximately 20  $\mu$ g of poly(A)<sup>+</sup> RNA prepared from Saos-2 cells and 20  $\mu$ g of total RNA prepared from tumor I tissue were resolved on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. Following hybridization with radiolabeled PEX cDNA, the blot was washed and the signal detected by autoradiography. A transcript of ~6.5 kb was observed only in the lane containing Saos-2 poly(A)<sup>+</sup> RNA. There is suggestion of an additional band corresponding to a transcript of ~3.8 kb. Arrows indicate the position of the 28 S (approximately 4.8 kb) and 18 S (approximately 1.8 kb) ribosomal RNA.

script of ~3.8 kb, although the nature of this transcript remains unclear. Northern analysis of total RNA samples from tumor I and Saos-2 cells (results not shown) did not reveal any signal for PEX, consistent with the relatively low expression levels of the PEX transcript, previously described (4, 24, 25). This finding contrasts sharply with PEX expression levels demonstrated in murine calvaria and cultured osteoblasts (5) and may reflect tissue and species differences.

**In Vitro Translation of PEX cRNA**—*In vitro* translation studies using full-length human PEX cRNA were performed in the rabbit reticulocyte lysate cell-free system. In the absence of microsomal membranes, PEX cRNA was translated into an ~86-kDa protein, as predicted from the cloned cDNA sequence (Fig. 5). Following addition of canine microsomal membranes to the translation mixture, products of higher molecular mass (~100 kDa) became apparent, consistent with *N*-glycosylation of PEX at the eight potential glycosylation sites deduced from the predicted sequence.

**PEX is a Cell Membrane-associated Protein**—Previous studies have established that nephrilysin, endothelin-converting enzyme-1, and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent Triton X-114 and immunofluorescent localization to examine whether PEX is also a membrane-associated protein. For identification of PEX, we generated a construct in which the carboxyl terminus sequences of PEX are modified by a human

code. The putative start codon is indicated as /1 along with the deduced amino acid translation. Two stop codons preceding the predicted initiation ATG are in *bold type*. Large asterisk (\*) denotes the putative prenylation site. A potential polyadenylation signal in the 3'-untranslated region is *underlined*. Eight potential *N*-glycosylation sites are *boxed*. The sequence has been assigned GenBank accession number U82970. B, amino acid homology between PEX and human NEP cDNA. Sequence comparison was performed using the LALIGN program. C, TMpred analysis of the PEX sequence showing a single membrane-spanning domain encompassing amino acid residues 21–39 (*arrowhead*). Numbers on the *horizontal axis* refer to the amino acid sequence.

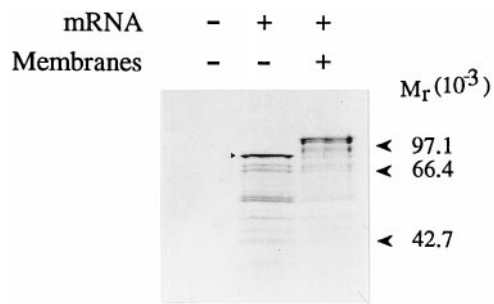


FIG. 5. *In vitro* translation of human PEX cRNA. Plasmid pPEX was linearized and sense RNA strand transcribed using T7 RNA polymerase. Translation of PEX cRNA was performed using rabbit reticulocyte lysate in the absence (*minus*) and presence (*plus*) of canine pancreas rough microsomes. Products were electrophoresed in a SDS-polyacrylamide gel (10%) and visualized by autoradiography. Arrowhead in lane 2 indicates full-length human PEX protein. The addition of microsomal membranes results in the appearance of higher molecular weight forms that likely represent glycosylated products.

*c-myc* tag. The epitope tag was inserted immediately upstream of the putative prenylation motif so that any potential lipid modification of the PEX protein may proceed uninterrupted.

Triton X-114 is a detergent that forms an aqueous solution at 4 °C but separates into hydrophobic and aqueous phases when the temperature is raised to 30–37 °C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. Triton X-114 extracts from COS-7 cells transiently expressing PEX tagged with the *c-myc* epitope showed that PEX partitions nearly exclusively into the detergent phase (Fig. 6A). This finding indicates that PEX is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of PEX, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, *myc*-tagged PEX immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus (Fig. 6B). If permeabilization was omitted, staining was localized exclusively to the plasma membrane (Fig. 6C), while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining (data not shown). Since the *myc*-tag was inserted in the carboxyl end of PEX, these findings further corroborate the sequence-based prediction that PEX is a type II integral membrane protein with its large COOH-terminal hydrophilic domain in the extracellular compartment.

**Recombinant PEX Protein Has Endopeptidase Activity**—The subcellular localization and sequence similarity between PEX and NEP strongly suggest that PEX functions as a membrane-bound metallopeptidase. However, no peptidase activity has been ascribed to PEX. As shown in Fig. 7A, when [D-Ala<sup>2</sup>,Leu<sup>5</sup>]enkephalin, used to assay for neprilysin activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human neprilysin or PEX proteins, as determined by Western blot analysis (data not shown), production of Tyr-D-Ala-Gly from the substrate was evident only in neprilysin-expressing membrane preparations. While the PEX sequence preserves two of the residues critical for catalytic activity of neprilysin (equivalent to Glu<sup>646</sup> and His<sup>711</sup>), it lacks a residue equivalent to Arg<sup>102</sup> shown to be crucial for the dipeptidylcarboxypeptidase activity of neprilysin. Therefore,

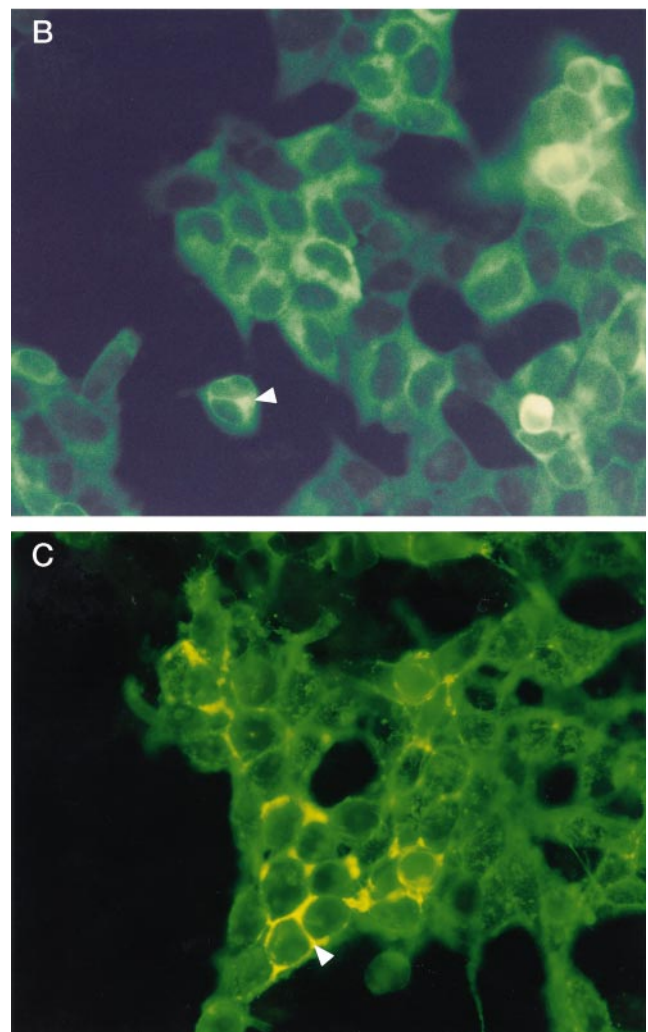
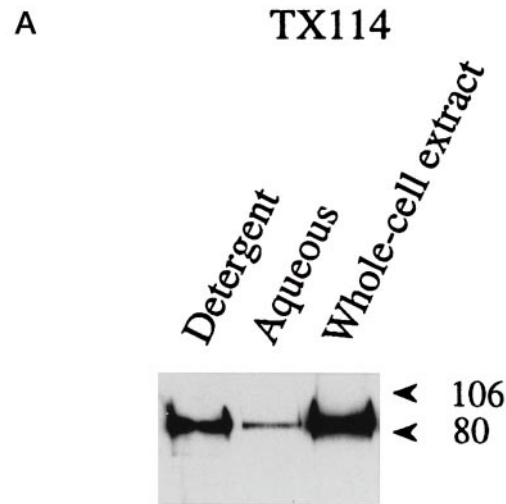


FIG. 6. Triton X-114 extraction and immunofluorescent localization of PEX. A, extraction and partitioning of PEX expressed in COS-7 cells with Triton X-114. Plasmid pPEX-*myc* was transiently transfected in COS-7 cells and 48 h later cells were extracted with Triton X-114. Whole cell extracts, as well as detergent and aqueous phases, were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-*myc* monoclonal antibody. Right margin indicates molecular mass  $\times 10^{-3}$ . B and C, localization of PEX using indirect immunofluorescence in stably transfected A293 cells with (B) and without (C) permeabilization with Triton X-100, respectively. Staining was carried out using the 9E10 anti-*myc* monoclonal antibody, followed by fluorescein-labeled secondary (sheep anti-mouse) antibody. Arrowheads indicate intracellular (B) and plasma membrane staining (C).

unlike neprilysin, PEX has no dipeptidylcarboxypeptidase activity, but likely functions as an endopeptidase.

To examine recombinant human PEX for endopeptidase activity, cell membrane preparations from COS cells transiently expressing the protein were incubated with human PTH-(1-38) or PTH-(1-34) and the cleavage products were analyzed by reverse-phase high pressure liquid chromatography (HPLC), as shown in Fig. 8. A parallel preparation from vector transfected COS cells did not appreciably cleave PTH-(1-38). However, in the presence of PEX, both PTH peptides were hydrolyzed in a highly reproducible pattern resulting in the formation of several peaks that absorb at 214 nm. Mass spectrometry of the peptide materials recovered from two product peaks gave  $m/z$  values of 861 and 630, respectively. While the former product was present in hydrolysates from both PTH-(1-38) and PTH-(1-34), the latter product was identified only in the PTH-(1-34) hydrolysate and likely corresponds to the carboxyl-terminal pentapeptide DVHNF of human PTH-(1-34). These findings provide the first direct evidence that recombinant PEX possesses endopeptidase activity and suggest that its substrate specificity may not be restricted to the putative phosphatonin but may include other circulating hormones or perhaps bone-derived autocrine/paracrine regulatory factors that regulate renal phosphate handling.

#### DISCUSSION

To gain insight into the role of PEX in normal physiology we have cloned the human full-length cDNA and studied its expression, subcellular localization, and peptidase activity. The cloned human PEX cDNA encodes a protein whose deduced amino acid sequence is identical to the published partial (4) and to the full-length sequences reported more recently (24-26). Its deduced topology is that of a type II integral membrane glycoprotein and in the present study we have provided experimental evidence to support this prediction. We have shown that PEX is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase following extraction with Triton X-114, consistent with the prediction from sequence analysis that it is an integral membrane glycoprotein. Nevertheless, the observed hydrophobic nature of PEX need not be attributed solely to it being an integral membrane protein. Lipophilic modification is known to cause cell membrane association, presumably through hydrophobic interaction of the modifying group with the lipid bilayer. Signaled by the COOH-terminal tetrapeptide CRLW motif, post-translational attachment of isoprenoids via a thioether linkage to the cysteine residue would be sufficient to promote effective membrane association. Further studies will be necessary to determine if such lipid modification of PEX does indeed take place. Of interest, however, is the observation that a nonsense mutation within this motif (R747Stop) has been reported to co-segregate with HYP and is likely to be associated with an inactive PEX gene product (27). Finally, the localization of PEX expressed in A293 cells is also consistent with the protein being membrane-associated and corroborates the sequence-based prediction that PEX is a type II integral membrane protein with its large COOH-terminal hydrophilic domain in the extracellular compartment. While protein expression was detected mostly on the cell surface, in some cells the signal was also localized intracellularly. This localization of the expressed protein would indicate that a portion of PEX activity is located in a membrane-bound compartment, possibly the Golgi membranes. The Golgi localization described for endothelin-converting enzyme-1 activity in cultured endothelial cells (28, 29) is proposed to promote the efficient conversion of big endothelin-1 because of the co-localization and concentration of enzyme and substrate through the constitu-

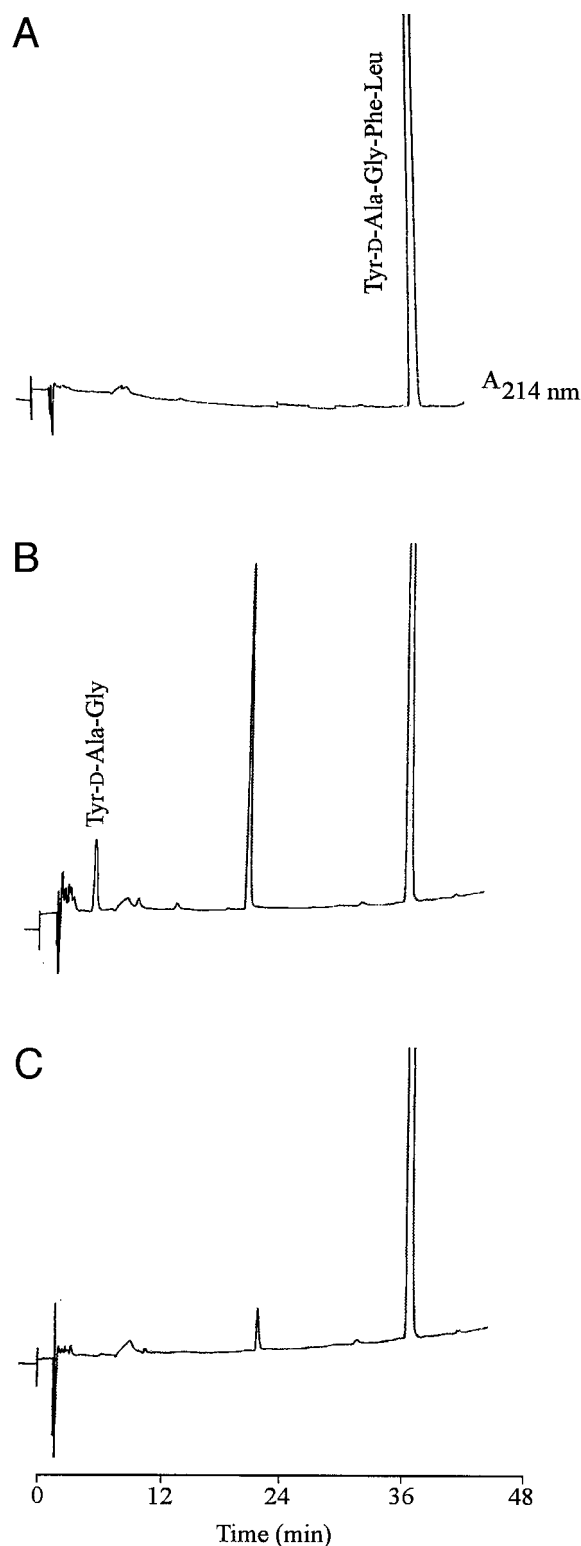
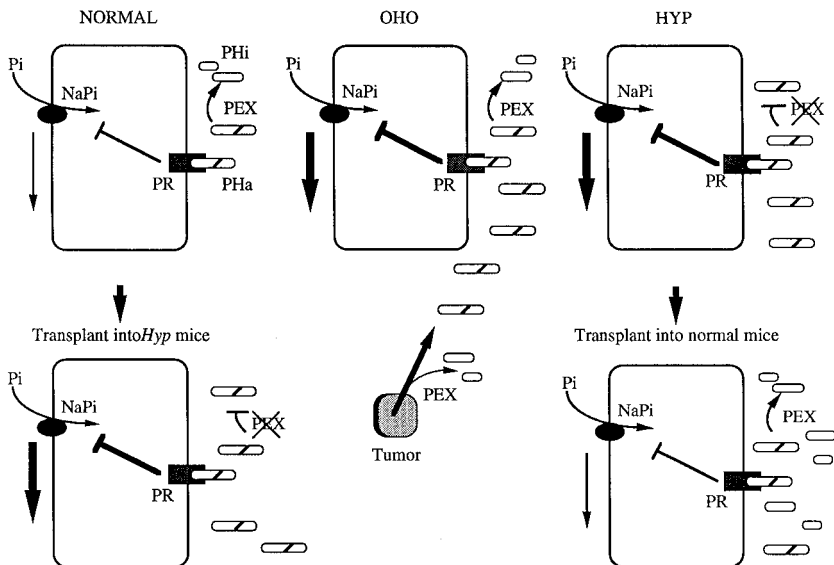
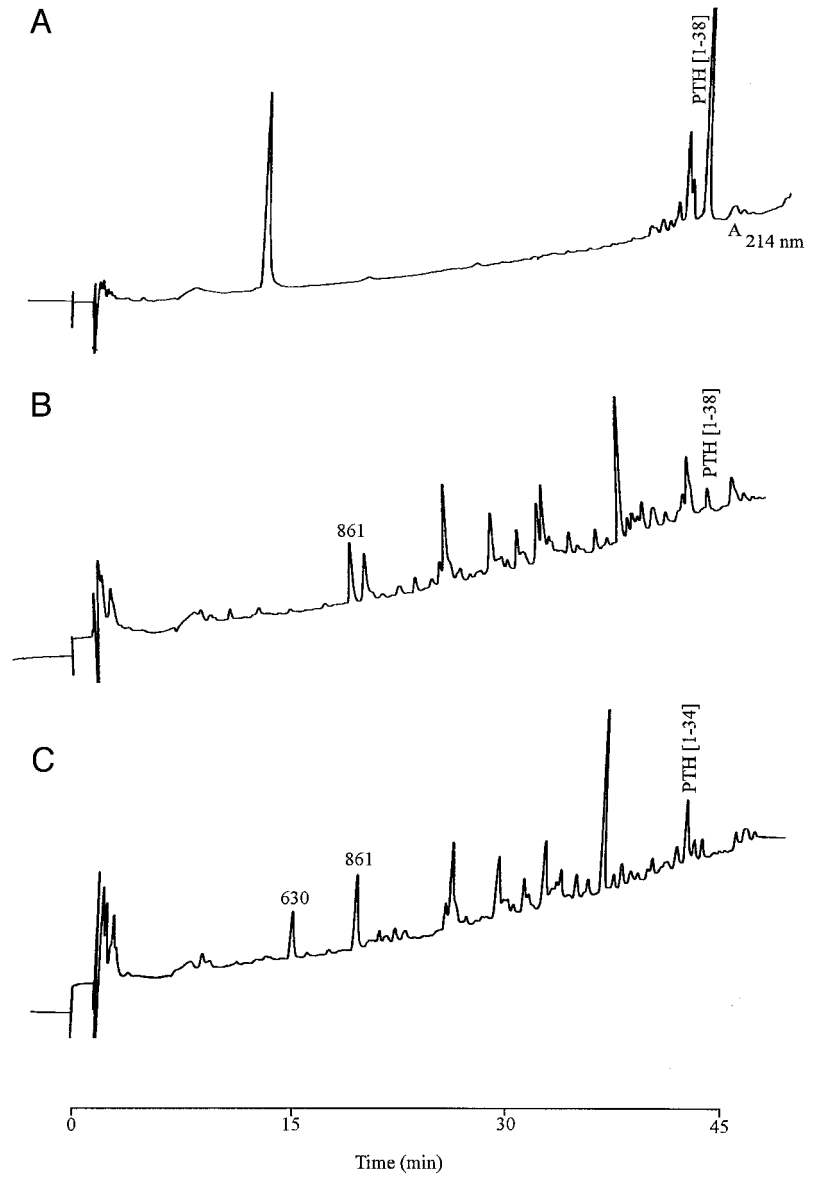


FIG. 7. HPLC analysis of the hydrolysis of [D-Ala<sup>2</sup>,Leu<sup>5</sup>]enkephalin. A, cell membrane preparations from vector transfected COS-7 cells; or B, from cells transiently expressing human NEP; or C, human PEX cDNAs were incubated in the presence of [D-Ala<sup>2</sup>,Leu<sup>5</sup>]enkephalin (500  $\mu$ M) and hydrolysis products were resolved by HPLC as described under "Experimental Procedures." Tyr-D-Ala-Gly was identified by chromatography of synthetic marker peptide.

tive secretory pathway. It is possible then, that in parallel fashion, the PEX enzyme mediates both intracellular and cell-surface conversions of its putative substrate.

The finding that wild-type PEX transcripts are expressed in

**FIG. 8. Hydrolysis of PTH-derived peptides by PEX endopeptidase activity.** *A*, human PTH-(1-38) was incubated with cell membrane preparations from vector transfected COS-7 cells; or *B*, from cells transiently expressing human PEX and hydrolysis products were resolved by HPLC. *C*, chromatographic profile of products arising from the hydrolysis of PTH-(1-34) when incubated with cell membranes from COS-7 cells transiently expressing PEX. The novel product with a molecular weight of 630 likely corresponds to the terminal pentapeptide DVHNF of human PTH-(1-34).



**FIG. 9. Schematic representation of phosphate handling in the proximal renal tubule in normal, OHO, and HYP states.** The diagrams indicate events proposed to occur at the level of the proximal renal tubule. A putative circulating phosphaturic hormone (*PHa*) interacts with its renal receptor (*PR*) and inhibits phosphate reabsorption across the renal brush-border membrane (-) by decreasing *NaPi* activity. Downward arrows indicate the degree of phosphate excretion. *PEX* expressed predominantly in extrarenal tissues modulates the levels of circulating *PHa* by converting it to its inactive form (*PHi*).

relative overabundance in OHO tumors poses a question in trying to understand the pathophysiology of these disorders. That is, how do we reconcile the apparently disparate observations that overexpression of PEX in OHO and loss of function in HYP patients, both lead to similar derangement in phosphate homeostasis? One of the physiological functions of PEX may well be the inactivation of a factor that normally promotes renal phosphate excretion (Fig. 9). In patients with OHO, the hyperphosphaturia that characterizes the syndrome would be the consequence of unregulated and excessive elaboration of the phosphaturic factor by the tumor. The modestly elevated PEX levels that we have documented in these tumors may arise either in response to the severe hypophosphatemia or to the abnormally high levels of the active phosphaturic factor. Yet, the increased PEX expression may not be sufficient to accommodate the increased substrate load, resulting in abnormally high circulating levels of the active phosphaturic hormone. The inactivation of PEX observed in HYP patients would similarly cause decreased turnover of this humoral phosphaturic factor and thereby lead to renal phosphate wasting.

This model is also consistent with the observation that the *Hyp* phenotype is neither corrected nor transferred following cross-transplantation of kidneys in normal and *Hyp* mice (30). Thus, when *Hyp* mice are engrafted with a normal kidney, phosphaturia ensues since circulating levels of the phosphaturic agent are excessive. On the other hand, engraftment of mutant kidneys in normal mice will not affect renal tubular phosphate handling of the recipients since circulating levels of the phosphaturic substance will be normally regulated by the enzymatic activity of extrarenal wild-type PEX. Indeed, analysis of the tissue distribution of PEX mRNA by RT-PCR has confirmed its expression in extrarenal tissues and particularly bone. Our present findings and those of others (5, 24–26) showing high levels of PEX expression in cells of the osteoblast lineage would be consistent with the intrinsic osteoblast defect postulated to exist in HYP patients (31) and in *Hyp* mice (32, 33).

Finally, although the deduced structure of PEX clearly suggests that it is a metalloprotease, no peptidase activity had been ascribed to the protein. The preservation of the catalytic glutamate and histidine residues (equivalent to Glu<sup>646</sup> and His<sup>711</sup> of neprilysin; Fig. 2B) would argue for such an activity. In addition, the wide range of PEX mutations in HYP patients that align with regions required for protease activity in neprilysin suggests that PEX also functions as a protease (34). Here, for the first time, we provide experimental evidence that recombinant PEX indeed functions as an endopeptidase. Unlike neprilysin, however, the protein does not possess dipeptidyl-carboxypeptidase activity since it lacks a residue equivalent to Arg<sup>102</sup> of neprilysin. Our unexpected observation that PEX effectively degrades PTH raises the question of whether circulating PTH is the putative phosphatonin. Although extracts from some OHO tumors have been reported to stimulate renal adenylate cyclase (15, 35) and this activity was inhibited by PTH antagonists (35), most studies have excluded PTH and PTH-related peptide (PTHrP) activity in OHO-associated tumors. Moreover, calcium homeostasis is generally preserved in patients with HYP. It is more likely, therefore, that the enzyme is rather promiscuous in its substrate specificity. PEX may indeed modulate PTH bioavailability and bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts

involved in regulating phosphate reabsorption and osteoblast maturation and mineralization. Although additional work will be required to clarify many of these issues, the availability of full-length human PEX cDNA now provides us with the opportunity to study the biology of PEX, identify its substrate(s), elucidate its role in pathological states characterized by dysregulated phosphate homeostasis, and determine its suitability as target for therapeutic intervention in the treatment of metabolic bone diseases.

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