Cloning of Human PEX cDNA

EXPRESSION, SUBCELLULAR LOCALIZATION, AND ENDOPEPTIDASE ACTIVITY*

(Received for publication, February 9, 1998, and in revised form, March 24, 1998)

Mark L. Lipman,∗,b,c Dibyendu Panda,∗,b,d,e Hugh P. J. Bennett,† Janet E. Henderson,∗,g Elizabeth Shane,∗ Yingnian Shen,∗ David Goltzman,∗ and Andrew C. Karaplis∗,h

From the Divisions of ∗Nephrology and Endocrinology, Department of Medicine, and Lady Davis Institute for Medical Research, McGill University, 3755 Côte Ste-Catherine Road, Montréal H3T 1E2, Canada, the †Calcium Research Laboratory and Endocrine Laboratories, Royal Victoria Hospital, Department of Medicine, and Sheldon Biotechnology Centre, McGill University, Montréal H3A 1A1, Canada, and the hDepartment of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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 nephrilysin 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 prominent 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.

X-Linked hypophosphatemic rickets (HYP)1 is the most common 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

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

* This work was supported in part by grants from the Medical Research Council of Canada (to M. L. L., J. E. H., D. G., and A. C. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Chercheuse Boursier du Fonds de la Recherche en Santé du Québec.

‡ Scholar of the Kidney Foundation of Canada.

§ Contributed equally to the results of this work.

¶ Scholar of the Medical Research Council of Canada. To whom correspondence should be addressed: Div. of Endocrinology, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Lady Davis Institute for Medical Research, 626, 3755 Côte-St-Catherine Rd., Montréal, Québec H3T 1E2, Canada. Tel.: 514-340-8260 (ext. 4907); Fax: 514-340-7573; E-mail: akarapli@ldi.jgh.mcgill.ca.

1 The abbreviations used are: HYP, X-linked hypophosphatemia;
Cloning, Expression, and Peptidase Activity of PEX

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 lumbar spinal spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal (10.8 mg/dl), 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 D3 and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was 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 62-year-old woman with classic features of OHO (15). Resection of a benign extraskelatal 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.

PAX Expression in OHO-associated Tumors—RNA was extracted from tumor tissue using the RNaseasy Total RNA kit (Qiagen, Chat- sworth, 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 PAX-specific oligonucleotide primers PAX-1 (5’-GGAGGAATTGGTTGAGGGCG-3’) and PAX-2 (5’-GTAGACCAGAGATCAGCAG-3’), designed from the published cDNA sequence (1295 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 PAX cDNA—Cloning of the 5’ end of PAX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumors and mRNA 900 ng of an internal PAX-specific antisense oligomer (PAX-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 then amplified using human PAX-specific oligonucleotide primers PAX-1 (5’-GGAGGAATTGGTTGAGGGCG-3’) and PAX-2 (5’-GTAGACCAC-CAAGGATCCAG-3’), designed from the published cDNA sequence (1295 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 PAX cDNA—Cloning of the 5’ end of PAX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumors and mRNA 900 ng of an internal PAX-specific antisense oligomer (PAX-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 then amplified using human PAX-specific oligonucleotide primers PAX-1 (5’-GGAGGAATTGGTTGAGGGCG-3’) and PAX-2 (5’-GTAGACCAC-CAAGGATCCAG-3’), designed from the published cDNA sequence (1295 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.

For expression studies, an EcoRV (in the polylinker of pPCRII/AccI) fragment containing the 5’ end of PAX cDNA was ligated into the pPCRII vector containing the 3’ end of PAX cDNA following digestion with AccI and EcoRV. The resulting plasmid was restricted with KpnI and NotI excising the full-length PAX cDNA that was then inserted into pcDNA3 vector digested at the KpnI/NotI sites in the polylinker region, resulting in plasmid pPAX. The full-length PAX cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

Northern Blot Analysis—Total RNA was obtained from tumor I and human Saos-2 osteosarcoma cells using the RNaseasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)+ 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)+ RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N+, Amersham). Hybridization was performed with 32P-labeled full-length human PAX cDNA (3.1 kb) in 7× 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 pPAX 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 [3H]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 ENHANCE (NEN Life Science Products, as described previously (17). Generation of myc-tagged PAX, Transfection in COS-7 Cells, and Triton X-114 Extraction—Plasmid pPAX-myc was generated by PCR amplification of the 5’ end of PAX cDNA using 1.5 μl of 10× PCR buffer, 0.2 μg of PEX-1 sense primer (5’-TTGGATGTCAACGCCTCG-3’), 519 is the nucleotide position of the 5’ end of this primer designed from the cloned human PAX cDNA) and PexMyc2 as the antisense (5’-CTACCCACAATCTGATTTGTCAGGCCTCTCTCTGTTATCCCATGAGTCGCCTCTG-3’) primer. The latter encodes the human c-myc tag sequences (underlined) and PEX sequences corresponding to the carboxyl-terminal extension (underlined) of the polylinker region (hydration (In the polylinker of pcDNA3) and then 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 μg/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 3×106 cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed twice with PBS and incubated with 2 μg of pPAX-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 NIH 3T3 Cells with pPEX-myc—2×104 cells were cultured in DMEM with 10% fetal calf serum and were transfected with the pPAX-myc plasmid by electroporation and selection initiated with 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-
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 Glu642 and His710 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 NH2-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 NH2-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 746-CRLW 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

---

**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
FIG. 2. Human PEX cDNA cloned from OHO tumors. A, nucleotide and deduced amino acid sequence of tumor-derived human PEX cDNA. The numbering begins at the 5' end nucleotide as determined by anchored PCR. Amino acids are given below each codon using the single letter code.
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)⁺ RNA extraction) and poly(A)⁺ 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 μg 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)⁺ 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 transcript 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. 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 nepilysin, 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
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 membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

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-Ala2,Leu5]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 Glu646 and His711), it lacks a residue equivalent to Arg102 shown to be crucial for the dipeptidylcarboxypeptidase activity of neprilysin. Therefore,
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 constitutive 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 DVHN 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 Glu446 and His711 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 recombiant PEX indeed functions as an endopeptidase. Unlike neprilysin, however, the protein does not possess dipeptidylcarboxypeptidase activity since it lacks a residue equivalent to Arg102 of neprilysin. Our unexpected observation that PEX indeed functions as an endopeptidase. Unlike neprilysin, no peptidase activity had been postulated to exist in 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.

Acknowledgments—We gratefully acknowledge G. Hendry for providing the human fetal tissues, B. He for technical assistance with the Northern blot analysis, and S. James for help with the HPLC and mass spectrometry.

REFERENCES

1. Scrivener, C. R., and Tenenhouse, H. S. (1992) J. Inherited Metab. Dis. 15, 610–624
2. Eicher, E. M., Southard, J. L., Scrivener, C. R., and Glorieux, F. H. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4667–4671
3. Lyon, M. F., Scrivener, C. R., Baker, L. R. I., Tenenhouse, H. S., Kronick, J., and Mandula, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4989–4903
4. The HYP Consortium. (1995) Nat. Genet. 11, 130–136
5. Du, L., Desbarats, M., Viel, J., Glorieux, F. H., Cawthorn, C., and Ecarot, B. (1996) Genomics 36, 22–28
6. Ryan, E. A., and Reiss, E. (1984) Am. J. Med. 77, 501–512
7. Weidner, N., Bar, R. S., Weiss, D., and Strottman, M. P. (1985) Cancer 55, 1691–1705
8. Aschinger, L. C., Solomon, L. M., Zeis, P. M., Justice, P., and Rosenthal, I. M. (1977) J. Pediatr. 91, 56–60
9. Siris, E. S., Clemens, T. L., Dempster, D. W., Shane, E., Segre, G. V., Lindsay, R., and Bilezikian, J. P. (1987) Am. J. Med. 82, 307–312
10. Miyaschi, A., Fukuze, M., Tsutsumi, M., and Fujita, T. (1988) Clin. Endocrinol. Metab. 61, 46–53
11. Segre, G. V., and Dickerson, G. R. (1989) N. Engl. J. Med. 321, 1812–1821
12. Cai, Q., Hodgson, S. F., Kao, P. C., Lennson, V. A., Klee, G. G., Zimmieister, A. R., and Kumar, R. (1994) N. Engl. J. Med. 330, 1645–1649
13. Wilkins, G. E., Granleese, S., Hegele, R. G., Holden, J., Anderson, D. W., and Bendy, G. P. (1995) J. Clin. Endocrinol. Metab. 80, 1628–1634
14. Econs, M. J., and Dreznzer, M. K. (1994) N. Engl. J. Med. 330, 1679–1681
15. Shane, E., Parisien, M., Henderson, J. E., Dempster, D. W., Feldman, F., Hardy, M. A., Tohme, J. F., Karapisl, A. C., and Clemens, T. L. (1997) J. Bone Miner. Res. 12, 1502–1511
16. Amazuzka, N., Karapisl, A. C., Henderson, J. E., Warsawsky, H., Lipman, M. L., Matsuks, Y., Ejiri, S., Tanaka, M., Izumi, N., Ozawa, H., and Goltzman, D. (1996) Dev. Biol. 173, 166–176
17. Karapisl, A. C., Lim, S.-K., Baba, H., Arnold, A., and Kronenberg, H. M. (1995) J. Biol. Chem. 270, 1629–1635
18. Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
19. Ervan, E. G., Lewis, J. C., and Buss, J. E. (1992) Curr. Biol. 4, 626–636
20. Cox, A. D., and Der, C. J. (1992) Curr. Opin. Cell Biol. 4, 1008–1016
21. Beck, L., Soumouyou, Y., Martel, J., Krishnamurthy, G., Gauthier, C., Godfrey, C. G., and Tenenhouse, H. S. (1997) J. Clin. Invest. 99, 1290–1299
22. Grief, M., Mumm, S., Waecht, P., Mazzarella, R., Whyte, M. P., Thakker, R. V., and Schlessinger, D. (1997) Biochem. Biophys. Res. Commun. 231, 635–640
23. Guo, R., and Orlans, L. D. (1997) J. Bone Miner. Res. 12, 1099–1107
24. Dixon, P. H., Wooding, C., Christie, P., Grief, M., Schlessinger, D., Whyte, M. P., and Thakker, R. V. (1997) J. Bone Miner. Res. 12, Suppl. 1, S128
25. Xu, D., Emoto, N., Gaid, A., Slaughter, C., Kaw, S., deWit, D., and Yanagisawa, M. (1994) J. Clin. Invest. 93, 473–485
26. Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, M., Koike, H., Yamamoto, Y., and Tanazawa, K. (1995) Biochem. J. 311, 657–665
27. Neshlitt, T., Coffman, T. M., Grithiths, R., and Dreznzer, M. K. (1992) J. Clin. Invest. 89, 1455–1459
28. Glorieux, F. H., Marie, P. J., Petitfier, J., and Delvin, E. E. (1980) N. Engl. J. Med. 303, 1023–1031
29. Bar-Errot-Charrier, B., Glorieux, F. H., Travers, R., Desbarats, M., Bouchard, F., and Hinek, A. (1988) Endocrinology 123, 768–773
30. Econs, M. J., Oudet, C. L., Buss, J. E. (1992) J. Clin. Invest. 89, 1455–1459