

## Cloning of a lymphatic peptide/histidine transporter

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Although peptide transport across the plasma membrane has been characterized well in the kidney and the intestine, the functional relevance of this transport in other organs has not been addressed. Here we report the cloning of a cDNA for a novel peptide/histidine transporter found in the rat (rPHT2), whose mRNA is expressed mainly in the lymphatic system. *rPHT2* encodes a protein of 582 amino acids and showed 49% identity with the brain PHT (PHT1) [Yamashita, Shimada, Guo, Sato, Kohmura, Hayakawa, Takagi and Tohyama (1997) *J. Biol. Chem.* **272**, 10205–10211]. rPHT2 mRNA was abundant in lung, spleen and thymus, and detected faintly in brain, liver, adrenal gland and heart by Northern-blot analysis and reverse tran-

scriptase PCR (RT-PCR). Intense signals for the gene were found in immunocytes using *in situ* hybridization. Ectopic expression of rPHT2 protein in HEK-293T cells and BHK cells was not found on the cell surface, but was found on the lysosomal membrane using light- and electron-microscopic analysis. Recombinant rPHT2 protein reconstituted into liposomes showed proton-dependent transport activity with histidine and histidyl-leucine. These findings suggest that rPHT2 is involved in the protein catabolic pathway in the lymphatic system.

**Key words:** cellular localization, lysosome, macrophage, molecular cloning, subcellular localization.

### INTRODUCTION

Peptide transport is a specific biochemical process, in which small peptides are transported across a membrane by energy-dependent saturable carriers [1]. Cloning of the cDNAs encoding the mammalian oligopeptide transporters (PEPTs) has offered insight into the molecular mechanism for uptake of oligopeptides. They have been identified as proton-dependent electrogenic transporters, and their physiological roles are to absorb small peptides arising from the digestion of dietary protein in the small intestine for peptide transporter (PepT) 1 [2], as well as to absorb filtered peptide generated by luminal peptidases in the kidney for PepT2 [3]. We later isolated a third member, a neuro-specific peptide/histidine transporter (PHT1) [4], whose suggested role is uptake of degraded neuropeptides and neuro-modulators and to absorb small peptides for nutrition.

Bockman et al. [5], using immunofluorescence micrography, showed that PepT1, thought to be located in the plasma membrane, was localized in lysosomes and nuclei of the pancreas. This suggests an extended role of PEPT, where PEPT not only absorbs oligopeptides from outside of the cells, but is also involved in transporting them across the intracellular membrane in organs other than the intestine and the kidney.

In the mammalian lymphatic system, although proteins such as antigens are thought to be actively processed in intracellular organelles as lysosomes, the molecular mechanism by which the

generated small peptides and amino acids cross the intracellular membrane has not been well characterized.

Here we report the cloning of a novel PHT cDNA in the rat (*rPHT2*), which is a member of the growing PEPT family, whose mRNA is expressed mainly in the lymphatic system. Interestingly, ectopically expressed rPHT2 protein has been found in the lysosomal membrane.

### MATERIALS AND METHODS

#### Screening and sequencing

Two degenerate oligonucleotide primers (forward, 5'-ATGG-TSACCCTSGTSCCYTAYTGGATG-3', and reverse, 5'-SAG-RCARAARAARATGCCCAT-3') based on a human gene, clone 80149 (GenBank accession no. t64272) in the expressed sequence tag database, which is homologous to *PHT1* cDNA, were used to amplify cDNA prepared from rat lung mRNA by reverse transcriptase PCR (RT-PCR). The product was cloned into the pGEM-T vector (Promega). The cloned insert encoding part of a rat 80149 homologue was labelled with <sup>32</sup>P by random priming (5 × 10<sup>5</sup> c.p.m./ml) and used to screen a rat brain cDNA library constructed in the cloning vector ZAP II [primed with oligo(dT); Stratagene]. Hybridization and *in vivo* excision were carried out as described previously [4]. Both strands of the inserts

Abbreviations used: PHT, peptide/histidine transporter; r, rat; PepT, peptide transporter; PEPT, oligopeptide transporter; LAMP1, lysosome-associated membrane protein 1; RT-PCR, reverse transcriptase PCR.

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<sup>2</sup> The nucleotide sequence data reported for rat *PHT2* will appear in DDBJ, EMBL, GenBank® and GSDN Nucleotide Sequence Databases under the accession number AB026665.

of the positive clones were sequenced by the dideoxy chain-termination method [6,7].

### Northern-blot analysis

Total RNA (50 µg) was extracted from rat tissues using Isogen (Wako Pure Chemical Industries) as outlined by the manufacturer. The cDNA region for rPHT2 (corresponding to nucleotides 770–1187) was labelled with <sup>32</sup>P by random priming (5 × 10<sup>5</sup> cpm/ml). Hybridization was carried out as described previously [4].

### RT-PCR

The blood of rat tissues was removed by perfusion with saline solution and total RNAs (50 ng) were isolated from the rat tissues. The total RNAs were transcribed into single-stranded cDNAs with Moloney-murine-leukaemia virus reverse transcriptase (Life Technologies). PCR was performed using the cDNAs as templates, KOD Dash DNA polymerase (Toyobo Co.; 1 unit/20 µl of reaction mixture), primers (forward, 5'-CGCCACACCCGTCTTCATCACCAA-3', and reverse, 5'-GATGAGCAGGTATGGGGGATTTG-3'; fragment size, 670 bp; 0.5 µM) and dNTP (0.2 mM). The PCR cycles consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 4 s and extension at 74 °C for 30 s, for 50 cycles.

### In situ hybridization

The partial cDNA of rPHT2 (corresponding to nucleotides 770–1187) was used to synthesize hybridization riboprobes labelled with [<sup>35</sup>S]UTP using T7 RNA polymerase for sense and T3 RNA polymerase for antisense strands. *In situ*-hybridization techniques for rPHT2 mRNA (RNA probe) were performed as described previously [4].

### Transfections and immunofluorescence analysis

First, rPHT2 was subcloned into the mammalian expression vector pCAGGS [8] at the *Eco*RI site (rPHT2/pCAGGS). For FLAG (DYKDDDDK)-tagged rPHT2 vector, an *Xba*I site was added by PCR just before the initiation codon of rPHT2. Then rPHT2 was subcloned into the mammalian expression vector pCDNA (kindly provided by Professor G. Nunez, Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, U.S.A.) between the *Xba*I site and the *Eco*RI site that is just after the FLAG epitope tag (FLAG-tagged rPHT2/pCDNA). Sequencing confirmed the absence of unwanted substitutions in the FLAG-tagged rPHT2, due to the amplification processes. HEK-293T and BHK cells were transiently transfected with 0.25 µg of the control vector, FLAG-tagged rPHT2/pCDNA and rPHT2/pCAGGS as described previously [9].

Antibody to rPHT2 (anti-rPHT2) was raised in rabbits with a 20-amino-acid sequence at the C-terminus of rPHT2, RYER-TRQDPDSQNSTRVRG. A cysteine residue was added to the N-terminus of this peptide for coupling of keyhole limpet haemocyanin, before injection into a rabbit. Polyclonal antibodies were purified by passing them through a column of Sepharose 4B coupled with the 20-amino-acid peptide.

With the diluted anti-rPHT2 antibody (1:500) as first antibody and Cy3-conjugated anti-rabbit IgG (Amersham; 1:500) as second antibody, immunofluorescence analysis was performed with the transfected cells as described previously [9]. Double staining was performed with mouse lysosome-associated membrane protein 1 (LAMP1) monoclonal antibody (Stressgen; 1:200) as first antibody and FITC-conjugated anti-mouse IgG

(Jackson Laboratories; 1:500) as second antibody. Immunofluorescence was visualized with Zeiss Axiophot and Zeiss LSM510 laser confocal microscopy (Carl Zeiss).

### Pre-embedding immunogold method

FLAG-tagged rPHT2-transfected cells were pre-fixed in 0.1% glutaraldehyde/4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature. Specimens were incubated with 0.05 M lysine in PBS for 20 min to inactivate residual aldehyde. Blocking was done with 5% BSA/5% normal goat serum in incubation buffer (PBS supplemented with 0.8% BSA and 0.1% gelatin) for 30 min at room temperature. Then the sections were incubated in the anti-rPHT2 antibody (1:100) overnight at 4 °C. After washing in incubation buffer, the sections were incubated in goat anti-rabbit IgG conjugated to colloidal gold (1:30; 0.8 nm diameter; Auriongp-ultrasmall, Aurion, Wageningen, The Netherlands) for 2 h at room temperature. The sections were washed (four times in incubation buffer and twice in PBS), fixed with 2% glutaraldehyde in PBS for 10 min at room temperature and subsequently post-fixed in 1% osmium tetra-oxide in PBS for 1 h. After washing in distilled water, colloidal gold labelling was intensified using a silver enhancement kit mixed with arabic gum solution for 10 min at room temperature. Finally, the sections were washed thoroughly in distilled water, and dehydrated in an ascending series of ethanol dilutions. They were then treated with propylene oxide (twice for 10 min) and equilibrated in resin overnight (Durcupan ACM, Fluka, Neu-Ulm, Germany), mounted on glass slides and cured at 60 °C for 48 h. Serial thin sections were cut on a Reichert Ultracut E microtome and stained with lead citrate and uranyl acetate. Electron micrographs were taken at 100 kV on a JEM 1200EX.

### Protein production and reconstitution of glutathione S-transferase (GST)-rPHT2 protein into liposomes

Sf21 insect cells were infected with recombinant baculovirus carrying GST-rPHT2 established with the Baculovirus Expression Vector System (Pharmingen). Recombinant fusion proteins were extracted with 1% Triton X-100 and purified by affinity chromatography on glutathione-Sepharose 4B (Amersham).

GST-rPHT2 protein was reconstituted into liposomes as described previously [10]. Briefly, liposomes were prepared by sonication of 50 mg/ml soya bean phosphatidylcholine (type II-S, Sigma) in 5 mM dithiothreitol under argon for 15 min. GST-rPHT2 protein (1 µg) in 0.2% Triton X-100 and 1% octylglucoside was mixed with liposomes (250 µg) in buffer (0.1 M KCl/10 mM Hepes/Tris, pH 5.0). The mixture was frozen at -80 °C for 10 min and thawed rapidly at 37 °C, then diluted with 1 ml of the same buffer and centrifuged at 100000 g for 1 h. The pellet was suspended in 0.1 M KCl/10 mM Hepes/Tris (pH 5.0) buffer and used as proteoliposomes (GST-rPHT2 liposomes). Liposomes processed similarly but without the protein were used for controls.

### Immunoblotting

For detection of GST-rPHT2 protein, immunoblotting was carried out as described previously [6]. Briefly, the samples were mixed with reducing sample buffer containing 2% SDS and loaded on to SDS/polyacrylamide gel (10% gel). After electrophoresis, the gel was transferred to Immobilon P (Millipore). Blots were preblocked in PBS containing 5% non-fat milk, and washed with PBS containing 0.05% Triton X-100 (PBS-T).

Detection was performed with anti-rPHT2 antibody (1:500) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) in PBS-T with 5% non-fat milk by the alkaline phosphatase method.

### Transport assays

For the efflux assays of [<sup>14</sup>C]histidine, 15  $\mu$ M [<sup>14</sup>C]histidine (150 pmol; New England Nuclear) and 1 mM histidine were added during the freeze–thawing dilution procedure. About 20 pmol of [<sup>14</sup>C]histidine was estimated to be loaded into the liposomes by counting the [<sup>14</sup>C]histidine-treated liposomes before the reactions. The liposomes (5  $\mu$ l, 1  $\mu$ g of protein, 250  $\mu$ g of liposomes) were incubated for 30 s in 0.1 ml of washing buffer (0.1 M KCl/10 mM Hepes/Tris, pH 7.0 or pH 5.0) at room temperature and the loaded substrates were set to flow. Then the liposomes were filtered through 0.22  $\mu$ m membrane (type GS, Millipore) and washed with 4 ml of washing buffer. The remaining liposome-associated radioactivity was determined by liquid scintillation counting using a Beckman LS600011C scintillation counter.

For the counter-influx assays of [<sup>14</sup>C]histidine, 1 mM histidine, 1 mM histidyl-leucine (His-Leu) or 1 mM glycine was added during the freeze–thawing dilution procedure and loaded. The liposomes containing the substrates were incubated with 0.1 ml of washing buffer (pH 5) containing 15  $\mu$ M [<sup>14</sup>C]histidine for 5 min at room temperature, then washed with 4 ml of ice-cold washing buffer by filtration. The liposome-associated radioactivity was determined as described above. Statistical difference between experimental groups was tested using the Student's *t* test.

## RESULTS

### Isolation of full-length rPHT2 cDNA

To find novel oligopeptide transporters, we searched the ESTs of GenBank using the *PHT1* sequence. Some entries had scores that suggested significant homology with *PHT1*. According to the sequence of the longest clone, number 80149 in these entries, we designed degenerate primers and amplified a rat cDNA by RT-PCR. Using the cDNA as a probe in screening, we isolated seven positive clones from a rat brain cDNA library. Sequence analysis revealed that one of the clones was *PHT1* and that the other six clones were identical and designated *rPHT2*.

*rPHT2* was 1979 bp long with an open reading frame of 1748 bp (including the termination codon) encoding a protein of 582 amino acids (Figure 1A). The initiating ATG was proposed since there was a stop codon in the 5' region before it. A polyadenylation signal, ATAAA, was found in the 3'-non-coding region. The encoded protein was predicted to have a core molecular mass of 64.6 kDa. The Kyte–Doolittle hydrophobicity profile [11] suggested the presence of 12 stretches of hydrophobic residues that represent potential transmembrane domains. There was no N-terminal signal sequence. There was a di-leucine-based lysosome-sorting motif (Leu-Val) at positions 19 and 20 in the putative cytoplasmic N-terminal region [12] (Figure 1B). Potential sites for Asn-linked glycosylation were located at positions 223, 357 and 440 in the third, fourth and fifth extracellular loops, respectively. There were four potential sites for protein kinase C-dependent phosphorylation (174, 195, 283 and 577), and two sites for protein kinase A-dependent phosphorylation (304 and 567). The amino acid sequence of this clone showed 93% homology to a cAMP-inducible 1 protein (cI-1), which is predicted by *cI-1* [13]. *cI-1* is the clone recently isolated as a cAMP-inducible gene from a mouse macrophage cDNA, whose

function is unknown. Using database searches, Botka et al. [14] recently reported a human genomic P1-derived artificial chromosome (PAC) clone, pDJ606g6 (accession no. AC004126), containing the likely human orthologue of *cI-1*, which is termed *hPHT2* [14]. The amino acid sequence of rPHT2 showed 80% homology to the hPHT2 protein predicted by *hPHT2*. rPHT2 protein was similar to PHT1 (49% identity), to the *Arabidopsis thaliana* nitrate transporter CHL1 (27% identity) [15] and the *A. thaliana* PHT NTR1 (27% identity) [16,17]. There was a weaker similarity to other mammalian peptide transporters such as PepT1 (22% identity) [18,19] and PepT2 (24% identity) [20] (Figure 1C). The consensus sequence with rPHT2 and PHT1 proteins was most conserved in the regions corresponding to the putative fifth and tenth membrane-spanning portions (82% and 77% identities, respectively).

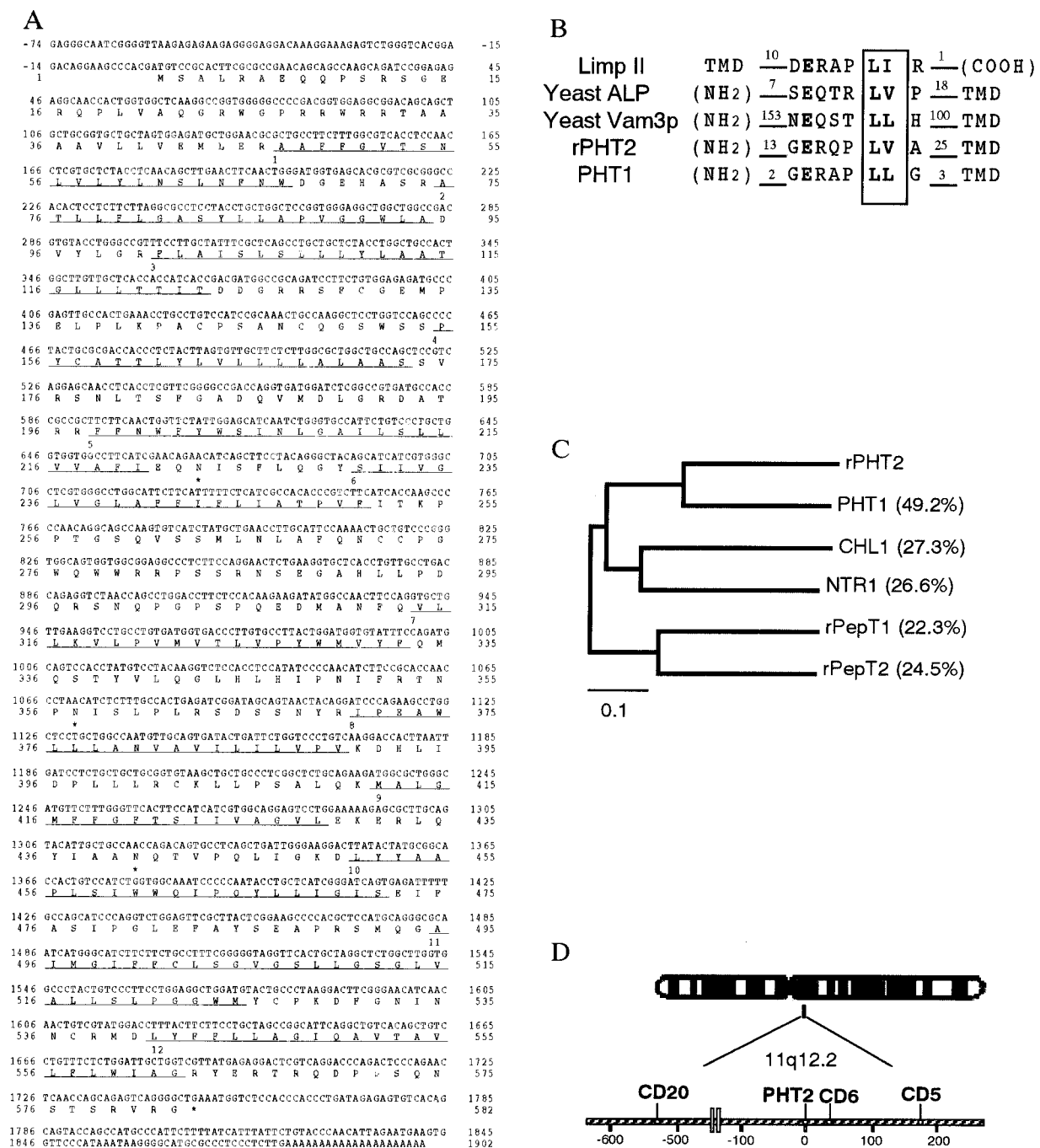
### Tissue expression of rPHT2 mRNA

We examined the tissue distribution of the mRNA corresponding to rPHT2 by Northern analysis (Figure 2). rPHT2 mRNA (2.3 kb) in rat tissues was expressed abundantly in the lung and spleen, and faintly expressed in the brain, liver and heart by Northern-blot analysis. We also performed RT-PCR using mRNAs prepared from the rat tissues without blood to exclude the effect of the lymphatic cells in blood. rPHT2 mRNA was detected in the lung, spleen, adrenal gland and thymus (Figure 2) by RT-PCR. The lower expression in brain and liver detected by RT-PCR may be caused by perfusion of the tissues, leading to removal of lymphatic cells, or by the use of a lower amount of cDNA than for the other samples. The distribution of rPHT2 mRNA was significantly different from that of PHT1 mRNA, which has been found to be expressed specifically in the brain and retina [4], and from mRNAs of the other mammalian oligopeptide transporters, PepT1 and PepT2, which have been found to be expressed abundantly in the small intestine, kidney and liver [2,3]. rPHT2 is the first member of a peptide-transporter family found to be predominantly expressed in the lymphatic system.

To determine the detailed localization of *rPHT2* gene expression, we performed *in situ* hybridization using a <sup>32</sup>S-labelled riboprobe (Figure 3). The specificity of hybridization signals for rPHT2 mRNA was confirmed with a control study using the sense probe (results not shown). Strong expression of rPHT2 mRNA was detected in the marginal zones (abundant with macrophages) and white pulp (abundant with B-cells), except around the central artery (periarterial lymphatic sheath, abundant with T-cells; Figures 3A a and 3B a). Eosinophils in the red pulp of the spleen were also positive (Figure 3B b). In lung, alveolar macrophages (Figures 3A b and 3B c) and lymphocyte infiltration (Figure 3B d) showed strong signals. In intestine, solitary follicles, Peyer's patches and lamina propria mucosae showed strong signals (Figure 3A c and 3B e). Some eosinophils in the lamina propria mucosae were positive (Figures 3B f and g). In thymus, there were ubiquitous signals (Figures 3A d and 3B h). Non-specific faint ubiquitous signals were seen in brain (results not shown).

### Subcellular localization

First, we examined uptake of histidine and peptide in *Xenopus laevis* oocytes injected with rPHT2 mRNA and water, as we did previously for PHT1 [4]. We found little or no difference in the uptake between these oocytes (uptake of [<sup>14</sup>C]histidine/histidine for 60 min at room temperature, rPHT2-injected oocytes, 2.9  $\pm$  1.2 pmol/oocyte; water-injected oocytes, 1.5  $\pm$  0.2 pmol/oocyte). This prompted us to examine its subcellular localization in mammalian cells. Since we could not detect the endogenous

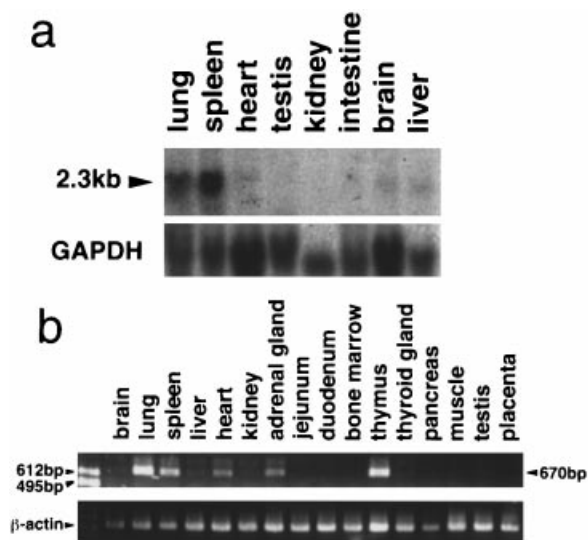


**Figure 1** The features of the *rPHT2* gene

(A) DNA sequence and predicted primary amino acids sequence of rPHT2. Three potential *M*-linked glycosylation sites are marked with asterisks. The predicted 12 transmembrane domains of rPHT2 are numbered and underlined. (B) The di-leucine-based lysosome-sorting signals of rPHT2. The di-leucine based sequences (boxed) and surrounding residues of membrane proteins targeted to the lysosome/vacuole are shown. Conserved glutamic acid residues located upstream are shown in bold. The number of additional amino acids in each cytoplasmic domain is indicated over a solid line. The positions of the N- or C-termini and the transmembrane domains (TMDs) are also shown. LIMP II, a type-II lysosomal integral membrane protein; ALP, an alkaline phosphatase; Vam3p, a vacuolar t-SNARE (target-membrane soluble *N*-ethylmaleimide-sensitive factor attachment-protein receptor; details in [12]). (C) Dendrogram of representative proteins of the PEPT family. The percentages show amino acid identities between rPHT2 and the other clones. (D) Chromosomal localization of the putative human PHT2 (*hPHT2*) gene at 11q12.2.

rPHT2 protein with the anti-rPHT2 antibody, presumably because of the low sensitivity of the antibody, we used transfected cells with high expression of rPHT2. With the anti-rPHT2 antibody, rPHT2 protein was detected in a pattern of intracellular punctate staining in BHK cells transiently transfected with the

rPHT2 expression vector by immunofluorescence microscopy (Figure 4b). The specificity of the anti-rPHT2 antibody was confirmed by the negative staining of the vector-transfected cells (Figure 4a) and by an absorption test with the antigenic peptide (results not shown). Double immunofluorescent staining with



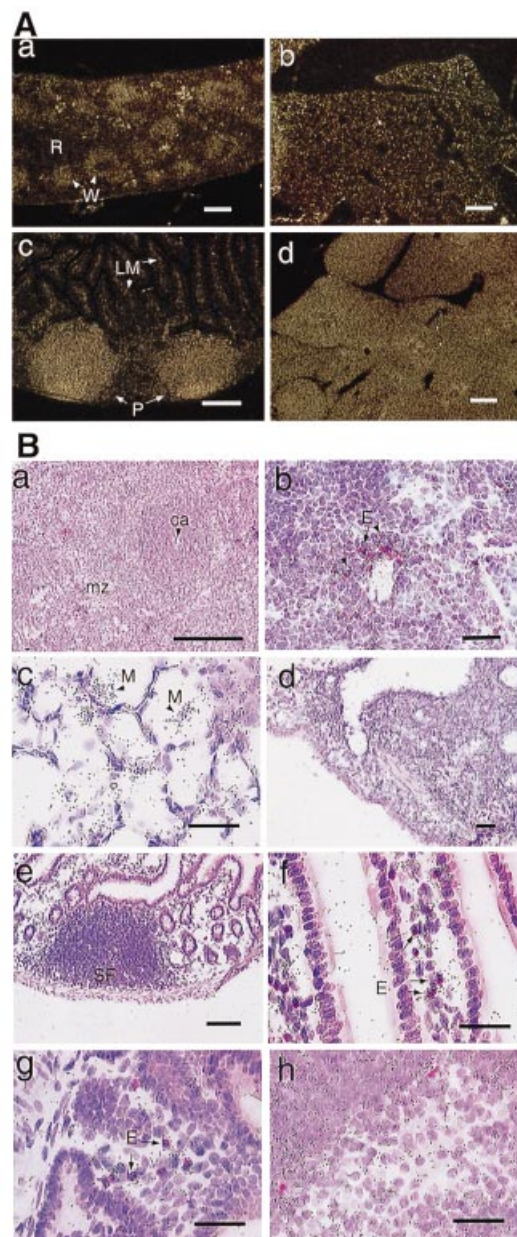
**Figure 2** Expression of rPHT2 mRNAs in rat tissues

(a) The expression of rPHT2 mRNAs (2.3 kb) was detected to be abundant in the lung and spleen, and detected faintly in brain, liver and heart by Northern-blot analysis. Control hybridization of each blot with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe is shown underneath. (b) rPHT2 mRNAs were amplified strongly in thymus, spleen and lung, moderately in heart and adrenal gland, and faintly in brain, liver, kidney and testis by RT-PCR.

monoclonal anti-LAMP1 antibody showed the co-localization of rPHT2 with LAMP1 in lysosomes, transported from the trans-Golgi network and endosomes [21,22] (Figures 4c and 4d). The precise localization of rPHT2 protein could be determined by electron microscopy with anti-rPHT2 antibody in HEK-293T and BHK cells transiently transfected with FLAG-tagged rPHT2. Tagging at the N-terminus of rPHT2 with FLAG did not affect its localization since the anti-rPHT2 antibody stained the same organelles co-localized with LAMP1 in FLAG-tagged rPHT2/pCDNA-transfected cells (Figure 4e). rPHT2 proteins were detected in the margins of lysosomes, autophagosomes and vacuoles, shown by silver-enhanced gold particles (Figures 4f, 4g and 4h), but not in the plasma membrane. Some cells showed the particle signals in the outer membrane of nuclei (Figure 4g).

### Transport activity of GST-rPHT2 protein reconstituted into liposomes

To determine the transport activity of rPHT2, we used liposomes reconstituted with recombinant rPHT2 protein at an interior pH of 5 as a model of lysosomes. Purified GST-rPHT2 protein produced by Sf21 cells was incorporated into liposomes by a freeze-thawing and dilution procedure. The incorporation of GST-rPHT2 protein into liposomes was confirmed by immunodetection (Figure 5a). Next, we examined the efflux of loaded [ $^{14}$ C]histidine from the GST-rPHT2 liposomes, measuring the remaining [ $^{14}$ C]histidine after the efflux. GST-rPHT2 liposomes had less remaining [ $^{14}$ C]histidine compared with the control liposomes (which were processed similarly but without the protein) and the reduction was greater with an outside pH of 7 than with an outside pH of 5 (Figure 5b). This indicates that rPHT2 transported the [ $^{14}$ C]histidine out of the liposome in a proton-dependent manner. To confirm that the efflux seen was

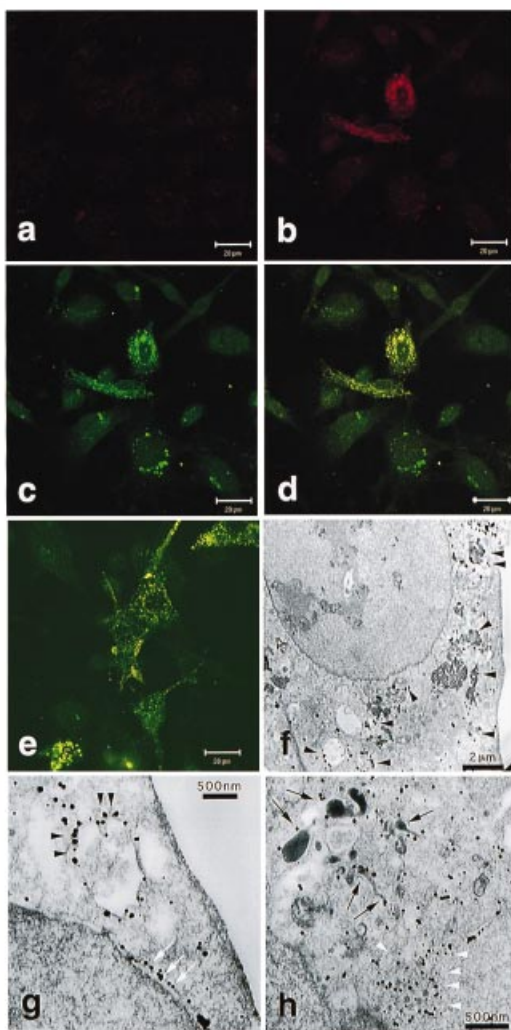


**Figure 3** *In situ* hybridization for rPHT2 mRNA

(A) Dark-field photomicrograph. Panel a, spleen; b, lung; c, intestine; d, thymus. (B) Bright-field micrograph. Panel a, white pulp in spleen; b, red pulp in spleen; c, alveolus in lung; d, lymphocyte infiltration in lung; e, solitary follicles in intestine; f, lamina propria mucosae in intestine; g, lymphocyte infiltration in intestine; h, thymus. W, white pulp; R, red pulp; P, Payer's patch; LM, lamina propria mucosae; ca, central artery; mz, marginal zone; E, eosinophil; M, alveolar macrophage; SF, solitary follicles. Scale bars indicate 250  $\mu$ m (A, a–d); B, a), 50  $\mu$ m (A, b–d, f–h) and 200  $\mu$ m (A, e).

not 'leakage' caused by the protein incorporation, we examined the counter-influx of [ $^{14}$ C]histidine outside into liposomes loaded with substrates. The influx of external [ $^{14}$ C]histidine by counter-flow was observed with GST-rPHT2 liposomes loaded with histidine or His-Leu, but not with glycine (Figure 5c). Similar counter-influx was observed with [ $^3$ H]carnosine as the external substrate for GST-rPHT2 liposomes loaded with histidine and

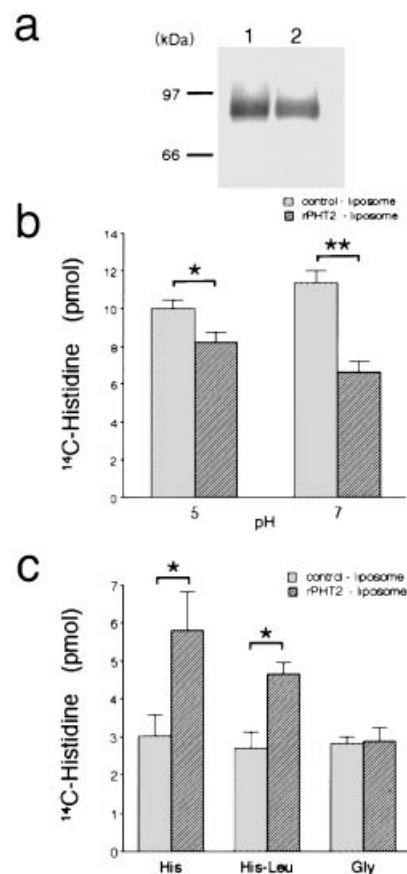




**Figure 4** Immunofluorescence (a–e) and electron (f–h) micrographs of BHK cells (a–f) and HEK-293T cells (g and h) transfected with the control vector (a), rPHT2/pCAGGS (b–d) and FLAG-tagged rPHT2/pCDNA (e–h)

(a–e) The cells were double-stained with the rabbit anti-rPHT2 antibody and a mouse anti-LAMP1 antibody as the first antibody, then with a Cy3-conjugated anti-rabbit IgG antibody and FITC-conjugated anti-mouse IgG antibody. The anti-rPHT2 antibody detected rPHT2 proteins in the intracellular membrane in rPHT2-transfected cells (b, red), but not in vector-transfected cells (a, red). The anti-LAMP1 antibody detected LAMP1 (c, green). The yellow staining indicates the co-localization of rPHT2 and LAMP1 (d, red for rPHT2 and green for LAMP1) and FLAG-tagged rPHT2 and LAMP1 (e, red for FLAG-tagged rPHT2 and green for LAMP1). (f–h) The silver-enhanced gold particles detecting FLAG-tagged rPHT2 protein were located around the lysosomes (black arrow) and the autophagosomes (black arrowhead). Some particles were located at the outer membranes of the nuclei (white arrow) and the Golgi-like apparatus (white arrowhead).

His-Leu. The counter-influx of  $0.5 \mu\text{M}$  [ $^3\text{H}$ ]carnosine for 5 min was as follows: control liposomes,  $0.58 \pm 0.17$  pmol/ $250 \mu\text{g}$  of liposomes, and GST-rPHT2 liposomes,  $1.02 \pm 0.06$  pmol/ $250 \mu\text{g}$  of liposomes when 1 mM unlabelled histidine was loaded; control liposomes,  $0.48 \pm 0.007$  pmol/ $250 \mu\text{g}$  of liposomes, and GST-rPHT2 liposomes,  $0.99 \pm 0.07$  pmol/ $250 \mu\text{g}$  of liposomes when 1 mM unlabelled His-Leu was loaded ( $n = 2$ , preliminary data). These results indicate that the substrates for rPHT2 are histidine and di-peptide, but not glycine. This is similar to PHT1, whose



**Figure 5** Recombinant rPHT2 protein reconstituted into liposomes transports [ $^{14}\text{C}$ ]histidine

(a) Immunodetection with GST-rPHT2 protein (1  $\mu\text{g}$ ; lane 1) and proteoliposome used with 1  $\mu\text{g}$  of GST-rPHT2 protein (lane 2). In lane 2, about 80% of the used GST-rPHT2 protein (lane 1) was detected by anti-rPHT2 antibody at 90 kDa (GST; 26 kDa + rPHT2). (b) Efflux of internal [ $^{14}\text{C}$ ]histidine by reconstituted liposomes. The loaded [ $^{14}\text{C}$ ]histidine was transported as described in the Materials and methods section, and the remaining radioactivity of [ $^{14}\text{C}$ ]histidine was determined. The loaded [ $^{14}\text{C}$ ]histidine was reduced more in GST-rPHT2 liposomes than in control liposomes (without GST-rPHT2 protein), and the reduction was greater when the external pH was 7 than when it was 5. The results shown are means  $\pm$  S.E.M. of six measurements from three independent experiments. (c) Counter-influx of external [ $^{14}\text{C}$ ]histidine by GST-rPHT2 liposomes loaded with 1 mM unlabelled substrates. The uptake of external [ $^{14}\text{C}$ ]histidine (15  $\mu\text{M}$ , at pH 5 for 5 min) was observed with GST-rPHT2 liposomes loaded with histidine (His) or His-Leu, but not with glycine. The results shown are means  $\pm$  S.E.M. of nine measurements from six independent experiments with histidine-loaded liposomes and of three measurements from three independent experiments with His-Leu- and glycine-loaded liposomes. (b and c) Significant differences (Student's paired  $t$  test), \* $P < 0.05$  and \*\* $P < 0.01$ .

substrates are histidine, di-peptide and tri-peptide, but not glycine [4].

## DISCUSSION

The amino acid sequence of rPHT2 was most similar to that of PHT1, and more similar to CHL1 and NTR1 (from the plant *A. thaliana*), than to the other mammalian peptide transporters, PepT1 and PepT2. The cloning of rPHT2 implicates a mammalian PHT subfamily with PHT1, of the growing PEPT family, corresponding to the nitrate-transporter family of plants.

The di-leucine-based motifs (LZ, where Z indicates L, V, I or A) and tyrosine-based motifs (YXXZ/NXXY, where Z indicates

one of the hydrophobic amino acids, L, I, V, M, C or A) in the cytoplasmic domain of membrane proteins are known to be important for the sorting to endosomes and lysosomes/vacuoles [12]. rPHT2 contains the LZ motif at the N-terminus and Tyr<sup>584</sup> in conjunction at the C-terminus, but not the complete tyrosine-based motif. Further, the amino acid sequence around the LZ motif is conserved with PHT1, which is also co-localized with rPHT2 and LAMP1 when expressed in culture cells (K. Sakata, T. Yamashita and M. Tohyama, unpublished work; Figure 1C). A type-II lysosomal integral membrane protein (LIMP II) also contains an LZ motif, but not a YXXZ/NXXY motif [23]. Further, the LZ motif of LIMP II has been demonstrated to be sufficient for the targeting of membrane proteins to lysosomes directly from the Golgi complex, without the tyrosine-based motif [24,25]. Furthermore, the LZ motif is known to be required for binding to AP-3 [26], an adaptor-protein complex that transports protein from late Golgi to the vacuole [27]. These data suggest that rPHT2 expressed in culture cells might be sorted directly from late Golgi to lysosomes/vacuoles in the same way as LIMP II, associated with AP-3.

Lysosomes are digestive organelles that contain enzymes which operate best at pH 5 to yield amino acids and small peptides. Previously, these small peptides were speculated to be transported through lysosomal membranes from the inside of the lysosome (a region of low di-peptidase activity) to the cytosol (a region of high di-peptidase activity) because of incomplete digestion [28]. Since then, the selective transport of di- and tri-peptides across the lysosomal membrane has been demonstrated [29]. Recently, the presence of an H<sup>+</sup>-coupled di- and tri-peptide transport system was demonstrated in lysosomal membranes in liver [30]. The character of the system is similar to that of the PEPT family members, except that the  $K_m$  of the transport system is much greater than that of the PEPT family, indicating the presence of a low-affinity proton-coupled PEPT in the lysosomal membrane. The finding that the rPHT2 protein is located on the lysosomal/autophagosomal membrane suggests that rPHT2 is one of the transport systems in the lysosomal membrane that transports digested histidine/peptides from inside the lysosome (a region of low pH) to the cytosol (a region of high pH), using protons as the driving force. The subcellular localization of endogenous rPHT2 and the precise mechanisms of the histidine/peptide transport activity of rPHT2 at the lysosomal membrane, accompanied with the substrate specificity and the transport kinetics, remain to be determined in future studies.

The functional role of the peptide transporter in nuclei has not yet been determined, although an intra-lysosomal enzyme, acid phosphatase, is known to change its localization between the perinuclear space, Golgi apparatus and lysosomes, depending on cell differentiation and the activity of the macrophages [31]. This evidence suggests that the membranes of these organelles are associated, and that the localization of rPHT2 in the nuclear membrane is one of the changeable distribution states.

High expression of rPHT2 mRNA was found in macrophages and eosinophils, the professional phagocytes, which actively digest antigenic organisms and dying or dead cells into amino acids and small peptides by phagocytosis [32]. Active transport of these across the phagosomal membrane to the cytosol has been suggested, and although no molecule responsible for this process has been reported, rPHT2 may contribute to the transport. rPHT2 gene expression in lymphocytes suggests that rPHT2 transports the small peptides made in autophagy and the processing of secretory proteins, such as antibodies and cytokines. In the small intestine, solitary follicles, Peyer's patches and lamina propria mucosae, which contain lymphocytes, macrophages and granular leucocytes, are known to play a protective

role against antigenic organisms entering from the lumen [32]. The high expression of rPHT2 mRNA suggests a role for these carriers in immune processes, whereas expression of PepT1 on the brush-border membranes of absorptive epithelial cells of villi is thought to facilitate the absorption of the peptides as nutrition resulting from protein digestion [33].

*hPHT2*, the homologous human gene of *cl-1* and *rPHT2*, is located on the human genomic clone pDJ606g6 [14]. This PAC clone comes from Best's disease (vitelliform macular dystrophy), with its region mapped between clones STS D11S461 and EST AHNAK [34]. However, the location of pDJ606g6 is centromeric outside of the Best's disease critical region, flanked by markers of D11S1765 and UGB [35,36]. Using a database search, we found that *hPHT2* was positioned around 20 kb and 130 kb centromeric to the adhesion molecules CD6 and CD5, respectively (Figure 1D). Deletions in chromosome bands 11q12.2–q12.3, one of the most frequent chromosome aberrations in B-cell chronic lymphocytic leukaemia (B-CLL), and B-CLL cells with 11q deletions were recently shown to reduce CD6 expression [37,38]. *hPHT2* might be involved in the pathogenesis of the subgroup of B-CLL characterized by the 11q deletion.

To summarize, this report concerns the cloning of *rPHT2*, a rat proton-coupled PHT expressed in the lymphatic system. The finding of rPHT2 expression at the lysosomal/autophagosomal membrane is expected to offer insights into the molecular mechanism of peptide transport across the lysosomal/autophagosomal membrane and the metabolic pathway that produces amino acids from proteins in lymphatic cells.

We thank Professor G. Nunez and Dr N. Inohara (Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, U.S.A.) for providing the expression vector FLAG-tagged pCDNA3. We thank Miss K. Nakano for DNA sequence, and Miss I. Jikihara for electron microscopy. K.S. is supported by a JSPS Research fellowship for Young Scientists and T.Y. by JSPS Postdoctoral Fellowships for Research.

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Received 31 July 2000/17 January 2001; accepted 6 March 2001