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RESEARCH PAPER

Functional Expression of PEPT2 in the Human Distal Lung Epithelial Cell Line NCI-H441

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ABSTRACT

Purpose The peptide transporter PEPT2 is expressed in alveolar type II epithelial cells. So far, however, no appropriate alveolar epithelial cell line for studying PEPT2 function has been known. In this study, we examined the functional expression of PEPT2 in the human distal lung epithelial cell line NCI-H441 (H441).

Methods Expression of PEPT2 mRNA and protein was examined in H441 cells. Transport function of PEPT2 was studied using glycylsarcosine (Gly-Sar) as a substrate.

Results Lamellar bodies were well developed in H441 cells and mRNA expression of type II cell markers and PEPT2 increased during time in culture. PEPT2 protein expression was confirmed in H441 cells, but not in A549 cells, by immunostaining and Western blotting. The uptake of Gly-Sar in H441 cells was inhibited by cefadroxil, and the cefadroxil-sensitive uptake was pH-dependent and peaked at pH 6.5. Gly-Sar uptake in H441 cells showed saturation kinetics with a K_m value of 112.5 μM. In addition, apical-to-basal, but not basal-to-apical, transport of cephalexin across H441 cell monolayers was sensitive to cefadroxil.

Conclusions PEPT2 is functionally expressed in H441 cells, making the cell line a good *in vitro* model to study PEPT2 function and its regulation in human distal lung.

KEY WORDS alveolar epithelial cells · Gly-Sar uptake · proton-coupled oligopeptide transporter · pulmonary drug disposition · transcellular transport

ABBREVIATIONS

3OMG	3-*O*-Methyl-D-glucose
ABCA3	ATP-binding cassette A3
CLSM	Confocal laser scanning microscopy
DEX	Dexamethasone
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gly-Sar	Glycylsarcosine
H441	NCI-H441
HPLC	High performance liquid chromatography
ITS	Insulin-transferrin-sodium selenite
LysoTracker Red	Lyso-Tracker® Red DND-99
NOD1	Nucleotide-binding oligomerization domain protein 1
PBS	Phosphate-buffered saline
rBBM	Renal brush-border membrane
SP-C	Surfactant protein C
TEER	Transepithelial electrical resistance
γ-iE-DAP	γ-D-Glutamyl-meso-diaminopimelic acid

INTRODUCTION

Water and solute transport is fundamental to maintain fluid homoeostasis along the extensive surface area of the human lungs (about 100 m^2) and to constantly remove serum components (including proteins and peptides) from the airspace (1,2). Moreover, transporter involvement has been speculated to contribute to pulmonary drug disposition. Therefore, improving our understanding of expression, function and regulation

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of drug transporters at the air-blood barrier - in health and disease - is an important challenge in physiology and pharmacology alike (3,4).

In this context, the absorption of therapeutic and serum proteins has extensively been studied (5,6). Far less investigated is the fate of oligopeptides, which occur as fragments of larger proteins and peptides broken down by extracellular peptidases, and which need to be removed from the airspace in order to prevent microbial growth. In this context, di- and tripeptide transporters PEPT1 (SLC15A1) and PEPT2 (SLC15A2) are important facilitators for the cellular uptake and/or absorption of oligopeptides. Also, these transporters are pharmacologically important, because they transport various peptidomimetic drugs such as β-lactam antibiotics, antivirals and anticancer agents, and therefore, control the pharmacokinetics and pharmacodynamics of these drugs (7,8).

PEPT2 is widely distributed in various tissues including the kidneys, brain, and lungs (9,10). PEPT1 and PEPT2 are members of the proton-coupled oligopeptide transporter superfamily and transport their substrates through secondary active transport coupled with an electrochemical proton gradient (7,11). Generally, PEPT2 is a high-affinity type transporter compared with PEPT1 (12,13).

In the lungs, PEPT2 was shown to be functionally expressed in bronchial epithelial cells and alveolar type II epithelial cells, as evidenced by the uptake of fluorophore-conjugated dipeptide derivatives (14,15). Recently, we examined the expression of PEPT2 and characteristics of glycylsarcosine (Gly-Sar) uptake in freshly isolated rat alveolar epithelial cells in primary culture, and showed that PEPT2 was functionally expressed in type II cells, but the expression decreased along with transdifferentiation, and PEPT2 function was almost completely lost in cells with type I-like phenotype (16). On the other hand, the expression of PEPT1 was hardly detected or ambiguous in the lungs (4). Thus, PEPT2 in type II cells may be a potential target for the pulmonary delivery of oligopeptides and peptidomimetics.

In order to investigate the expression and function of PEPT2 and its regulation in type II cells, a robust *in vitro* cell culture systems would be a powerful tool. Unfortunately, primary cultured type II cells rapidly transdifferentiate into type I-like cells, and thus lose PEPT2 activity as described above (16,17). The A549 cell line is an *in vitro* model which is supposed to have type II cell phenotype. A549 cells, however, have not yet been reported to express PEPT2 and moreover, lack the ability to form confluent monolayers of polarized cells (18).

The human distal lung epithelial cell line NCI-H441 (H441) was originally isolated from the pericardial fluid of a patient with papillary adenocarcinoma of the lung. H441 cells have alveolar type II and/or club cell-like phenotype confirmed by the production of surfactant proteins and the formation of lamellar bodies (19,20). In addition, H441 cells have the ability to form confluent, electrically tight monolayers with transepithelial electrical resistance values up to ~ 1000 $\Omega \cdot cm^2$; which closely resembles alveolar epithelial cells *in vitro* and renders them useful for transepithelial transport studies (21). In this study, the expression and transport function of PEPT2 was studied in H441 cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was purchased from MP Biomedicals (Solon, OH, USA). Insulin-transferrin-sodium selenite (ITS) supplement was purchased from Roche AG (Basel, Switzerland). RPMI-1640 medium, penicillin-streptomycin, trypsin (1:250, powder), and Lyso-Tracker® Red DND-99 (LysoTracker Red), a fluorescent marker for lamellar bodies, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Nile red, a selective fluorescent stain for intracellular lipid droplets in lamellar bodies, was purchased from COSMO BIO Co. Ltd. (Tokyo, Japan). Hoechst 33342 solution, a fluorescent nuclear marker, cephalexin and dexamethasone (DEX) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Cefadroxil and sodium pyruvate solution (1%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]-Gly-Sar was obtained from Moravek Biochemicals (Brea, CA, USA), and [^{3}H]-3-*O*-methyl-D-glucose (3OMG) from American Radiolabeled Chemicals (St. Louis, MO, USA). Antibodies against PEPT2 (SC-19918) and secondary antibodies (SC-3855, SC-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ReverTra Ace® and THUNDERBIRD ® SYBER® qPCR Mix were from TOYOBO (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Cell Culture

H441 cells were obtained from American Type Culture Collection (ATCC, HTB-174) and cultured in RPMI-1640 medium supplemented with 5% FBS, 1% sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2-95% air at 37°C. Twenty four hours after seeding, the medium was replaced with RPMI-1640 medium containing in addition to the supplements mentioned above, DEX (200 nM) and ITS. For the transepithelial transport studies, cells were grown on Transwell Clear inserts (12 mm in diameter, pore size 0.4 μm; Corning, NY, USA), and apical and basolateral fluid volumes were 0.5 and 1.5 ml, respectively. A549 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, and were cultured as described previously (22).

Observation of Lamellar Bodies by Confocal Laser Scanning Microscopy (CLSM)

H441 cells grown on 35-mm glass-bottom culture dishes for 9 days were incubated with LysoTracker Red (70 nM) and Hoechst 33342 (1 mM) for 30 min, or Nile red (2 μM) for 60 min at 37°C. For comparison, A549 cells cultured for 4 days were also stained with LysoTracker Red and Hoechst 33342. After washing with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4), fluorescence signals were visualized by CLSM using a LSM5 Pascal microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) equipped with a 63x oil immersion objective lens.

Messenger RNA Expression Analysis of H441 Cells

H441 cells were cultured for 7, 9, 11 and 13 days, and total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). The total RNA was reverse transcribed into cDNA by using ReverTra Ace. Real-time PCR was performed on a CFX Connect Realtime-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using THUNDERBIRD SYBR qPCR Mix. The PCR conditions were: initial denaturation for one cycle of 1 min at 95°C, followed by specified cycles of 10 s at 95°C (denaturation), 15 s at 60°C (annealing), and 15 s at 72°C (extension). After the reaction, a melting curve was obtained to confirm the single product. The primers used for real-time PCR were shown in Table I. The expression level of each mRNA was normalized as to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The mRNA expression of PEPT1 was examined by conventional reverse-transcription-polymerase chain reaction (RT-PCR) analysis as described recently (23), for the comparison with that of PEPT2. The PCR conditions consisted of an initial denaturation at 95°C for 30 s, followed by amplification for 22 cycles of 10 s at 95°C (denaturation), 15 s at 63°C (annealing), and 15 s at 72°C (extension). The primer sequences for PEPT1 were: sense, 5′- TTGCTTCT GGTCGTCTGTGTA-3′ and antisense, 5′- ATCCTCCA CTTGCCTCCTGA-3′ (expected size, 188 bp).

Table I Primer Sequences for Real-Time PCR

Gene name	Sequence (F: forward R: reverse)	Product size (bp)
SP-C	F: 5′-GTCTCCACATGAGCCAGAAACA-3′ R: 5′-GCGCCCCAATGCTCATC-3′	57
ABCA3	F: 5′-CGGGAAGACCACGACTTT-3′ R: 5′-GCTGCCGCACCTTTC-3′	110
PEPT2	F: 5′-AGGAAAATGGCTGTTGGTATGATC-3′ R: 5′-CGCAACTGCAAATGCCAG-3′	51
GAPDH	F: 5′-CCACCCATGGCAAATTCC-3′ R: 5′-TGGGATTTCCATTGACAA-3′	69

Detection of PEPT2 Protein by Immunostaining and Western Blotting in H441 and A549 Cells

H441 and A549 cells used for the immunostaining of PEPT2 were grown on 35-mm glass-bottom culture dishes for 13 and 7 days, respectively. The expression of PEPT2 was examined by immunostaining as described previously, using goat polyclonal anti-PEPT2 antibodies (SC-19918; 1:50 dilution) and rhodamine-conjugated donkey anti-goat IgG secondary antibodies (SC-3855; 1:100 dilution) (16). For Western blotting, the crude membrane fraction was prepared from H441 and A549 cells as described previously (24). Renal brush-border membrane (rBBM) was isolated from the renal cortex of male SD rats as described previously (25), and used as a positive control. Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" of Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University. The expression of PEPT2 was examined by Western blotting as described previously, using goat polyclonal anti-PEPT2 antibodies (SC-19918; 1:50 dilution) and a horseradish peroxidase-coupled donkey anti-goat IgG (SC-2020; 1:2000 dilution) (16).

Uptake of [^{3}H]-Gly-Sar in H441 Cells

To evaluate the transport activity of PEPT2 in H441 cells, cells were seeded at a density of 3×10^4 cells per well in 24-well plates and cultured for 13 days. Cells were preincubated with HEPES buffer (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) for 10 min at 37°C. Then, MES buffer (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM MES, pH 6.5) containing D-glucose (5 mM) and [^{3}H]-Gly-Sar (50 μM) was added, with or without 1 mM cefadroxil, a potent inhibitor of PEPT2, and the cells were incubated for the given times at 37°C or 4°C. To examine the effect of extracellular pH on [^{3}H]-Gly-Sar uptake, H441 cells were incubated in MES buffer (pH 5.0–6.5) or HEPES buffer (pH 7.0–8.5). After incubation, the cells were solubilized with 0.1 M NaOH, and each cell lysate was used for radioactivity counting and protein assay. For the measurement of radioactivity, Ultima GOLD (PerkinElmer, MA, USA) was added to the lysate and the radioactivity was determined by liquid scintillation counting on a LSC5100 (Hitachi Aloka Medical, Ltd., Tokyo, Japan). Protein concentrations were measured by the Lowry method with bovine serum albumin as a standard.

Transepithelial Transport Studies

Transepithelial electrical resistance (TEER) of H441 cell monolayers was measured at days 3 to 13 after seeding, using a Millicell® ERS voltohmmeter (Merck KGaA, Darmstadt,

Germany). Transport studies with cell monolayers were conducted at day 13 in culture. The cell monolayers were preincubated for 10 min at 37°C with RPMI-1640 medium. After preincubation, RPMI-1640 medium containing cephalexin (100 μM) was added either to the apical (0.5 ml) or basolateral (1.5 ml) compartment. At the specified time points, a 100-μl sample from the apical side or a 300-μl sample from the basolateral side was removed from the acceptor compartment. Cephalexin in each sample was analyzed by High Performance Liquid Chromatography (HPLC) method (JASCO Corporation, Tokyo, Japan) with elution through a reverse phase TSKgel ODS-80TM column (5 um particle size, 100×4.6 mm; Tosoh Bioscience, Tokyo, Japan) using a mixture of methanol and 1% acetic acid (20:80 *v/v*) as the mobile phase. A chromatogram was recorded by UV detection at 260 nm.

Statistical Analysis

Data were expressed as means ± standard error (SE). Statistical analysis was performed by Student's *t*-test or by one-way ANOVA followed by Tukey's test for multiple comparisons. The level of significance was set at $*p<0.05$ or $**p<0.01$.

RESULTS

Morphological Features of H441 Cells in Culture

When H441 cells were plated at a density of 1.0×10^4 cells per 35-mm culture dish, the cells grew with a doubling time of about 40 h and reached confluence at around 13 days after seeding (data not shown). At day 13, H441 cells formed monolayers with epithelial-like morphology and dome-like structures were observed (Fig. 1a). Lamellar bodies are characteristic intracellular structures typically observed in alveolar type II but not in type I epithelial cells (2,26). Using Nile red (a selective fluorescent stain for lipid droplets) and LysoTracker Red (an acidic organelle-selective cell-permeant fluorescence probe), lamellar bodies were clearly observed by CLSM in H441 cells (Fig. 1b, c). In comparison, the number of lamellar bodies in A549 cells was considerably lower (Fig. 1d).

Expression of Surfactant Protein C (SP-C), ATP-Binding Cassette A3 (ABCA3), PEPT2 and PEPT1 mRNAs in H441 Cells

The expression of SP-C, ABCA3 and PEPT2 mRNAs was measured in H441 cells at day 7, 9, 11 and 13 after seeding. The expression of these mRNAs increased along with the time in culture (Fig. 2a, b and c). Especially, the expression of SP-C

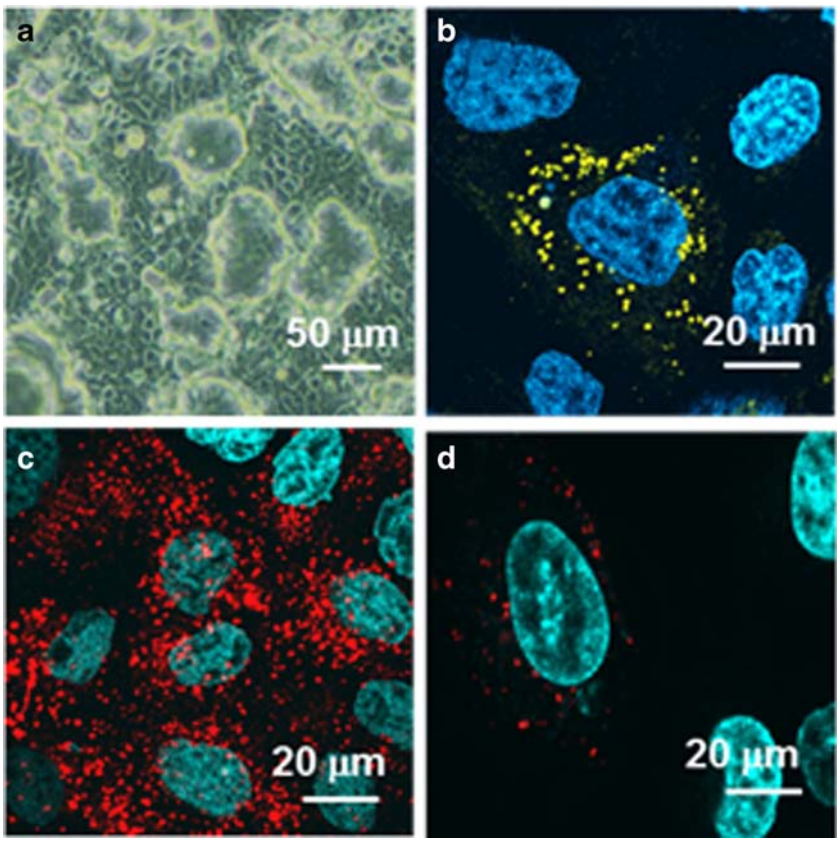


Fig. 1 Morphology of H441 cell monolayers (**a**), and lamellar body formation in H441 (**b**, **c**) and A549 (**d**) cells. (**a**) Phase contrast micrograph of H441 cells. (**b**, **c**, **d**) In confocal laser scanning micrographs, cells were stained with Nile red (*yellow*; **b**) or LysoTracker red (*red*; **c**, **d**) for lamellar bodies. Hoechst 33342 (*blue*; **b**, **c**, **d**) was used to stain cell nuclei.

mRNA was remarkably increased, and was about 25-fold higher at day 13 compared with that at day 7. For the comparison, the expression of PEPT1 mRNA in H441 cells at day 13 was examined by RT-PCR. However, the expression of PEPT1 mRNA was hardly detected in contrast to that of PEPT2 (Fig. 2d). In the following experiments, H441 cells cultured for 13 days were used.

Expression of PEPT2 Protein in H441 and A549 Cells

The expression of PEPT2 protein was examined by immunostaining observed by CLSM in H441 and A549 cells (Fig. 3a, b). H441 cells were stained with PEPT2 antibodies, while A549 cells were not. In H441 cells, PEPT2 seemed to be expressed on the apical membrane (Fig. 3a inset). The expression of PEPT2 protein in H441 was also confirmed by Western blotting, while no expression was observed in A549 cells (Fig. 3c).

Effect of Cefadroxil on Gly-Sar Uptake in H441 Cells

The uptake of Gly-Sar, a *bona fide* substrate for PEPT2, into H441 cells and the effect of cefadroxil, a potent inhibitor of PEPT2, on this uptake were examined at extracellular pH 6.5. As shown in Fig. 4a, Gly-Sar was taken up by H441 cells in a time-dependent fashion up to 120 min. This uptake was inhibited by cefadroxil at 37°C. In contrast, cefadroxil-sensitive uptake of Gly-Sar was not observed at 4°C (Fig. 4b). Gly-Sar uptake at 37°C was blocked by cefadroxil in a concentration-dependent manner (Fig. 4c). However, the inhibition of Gly-Sar uptake by cefadroxil was not complete. The nature of the remaining uptake of Gly-Sar in the presence of high concentrations of cefadroxil is not clear, but it may be diffusion into the cells or the paracellular diffusion into the basal side of the domes, because H441 cells formed many dome-like structures (Fig. 1a).

Effect of Extracellular pH on Gly-Sar Uptake in H441 and A549 Cells

The uptake of Gly-Sar was measured at various extracellular pH values in H441 and A549 cells (Fig. 5). Cefadroxil-sensitive uptake of Gly-Sar exhibited a bell-shaped pH-dependence with a maximal uptake at pH 6.5 in H441 cells. In

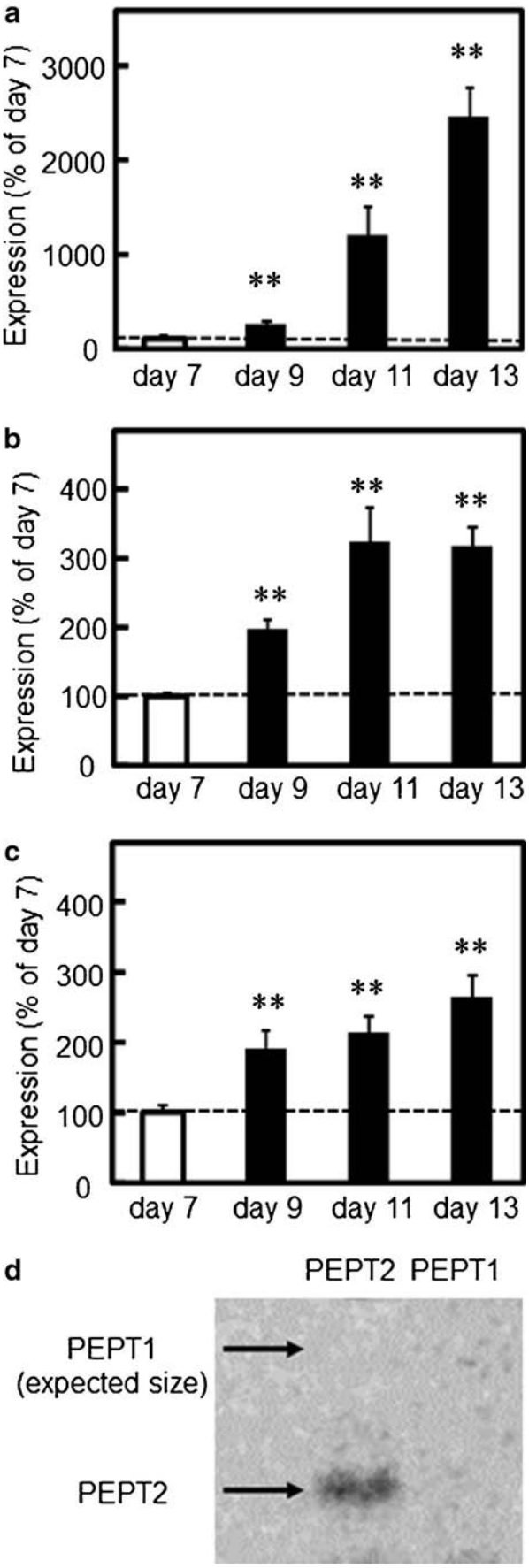


Fig. 2 The mRNA expression of the type II markers (SP-C and ABCA3), ▶ PEPT2 and PEPT1 in H441 cells. Total RNA was extracted from H441 cells at different days in culture, and real-time PCR analysis was performed to evaluate the expression level of SP-C (**a**), ABCA3 (**b**) and PEPT2 (**c**) mRNAs which was normalized to GAPDH. Each value represents the mean ± SE of three RNA samples. $^{**}p < 0.01$, significantly different from the expression level at day 7. The *dotted line* indicates the mRNA expression level in the cells cultured for 7 days (100%). (**d**) RT-PCR analysis of PEPT2 and PEPT1 mRNA expression in H441 cells at day 13.

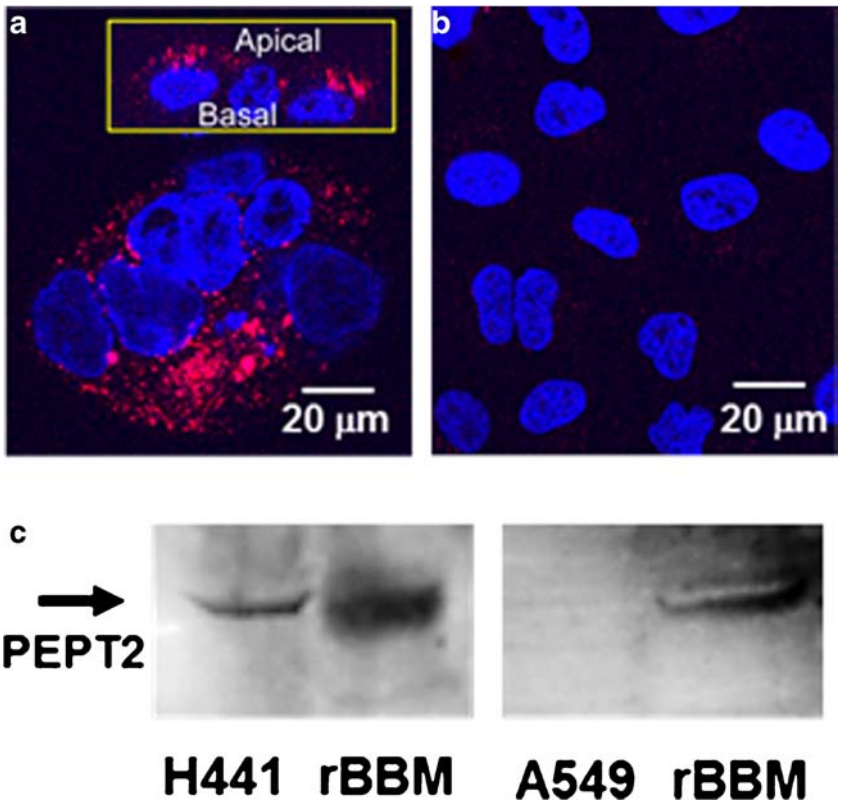


Fig. 3 The expression of PEPT2 protein in H441 and A549 cells. (**a**, **b**) Expression of PEPT2 protein was examined by immunostaining in H441 (**a**) and A549 (**b**) cells. The *inset* in (**a**) showed polarized expression of PEPT2 on the apical membrane. *Red*: PEPT2, *Blue*: nuclei. (**c**) Expression of PEPT2 protein was examined by Western blotting. rBBM was used as a positive control.

contrast, pH-dependent uptake of Gly-Sar was absent in A549 cells. Moreover, uptake activity in A549 was only a fraction of what was observed in H441 cells.

Concentration-dependence of Gly-Sar Uptake in H441 Cells

The concentration-dependence of Gly-Sar uptake was examined at a concentration range of 10–1000 μM. Cefadroxil-sensitive Gly-Sar uptake into H441 cells showed saturation kinetics, and the Eadie-Hofstee plot indicated the involvement of a single transport system, most likely PEPT2 (Fig. 6). The Michaelis-Menten constant (K_m) and maximum uptake rate (Vmax) for Gly-Sar uptake estimated from the Eadie-Hofstee plot were 112.5±14.3 μM and 2.2±0.15 pmol/mg protein/min, respectively.

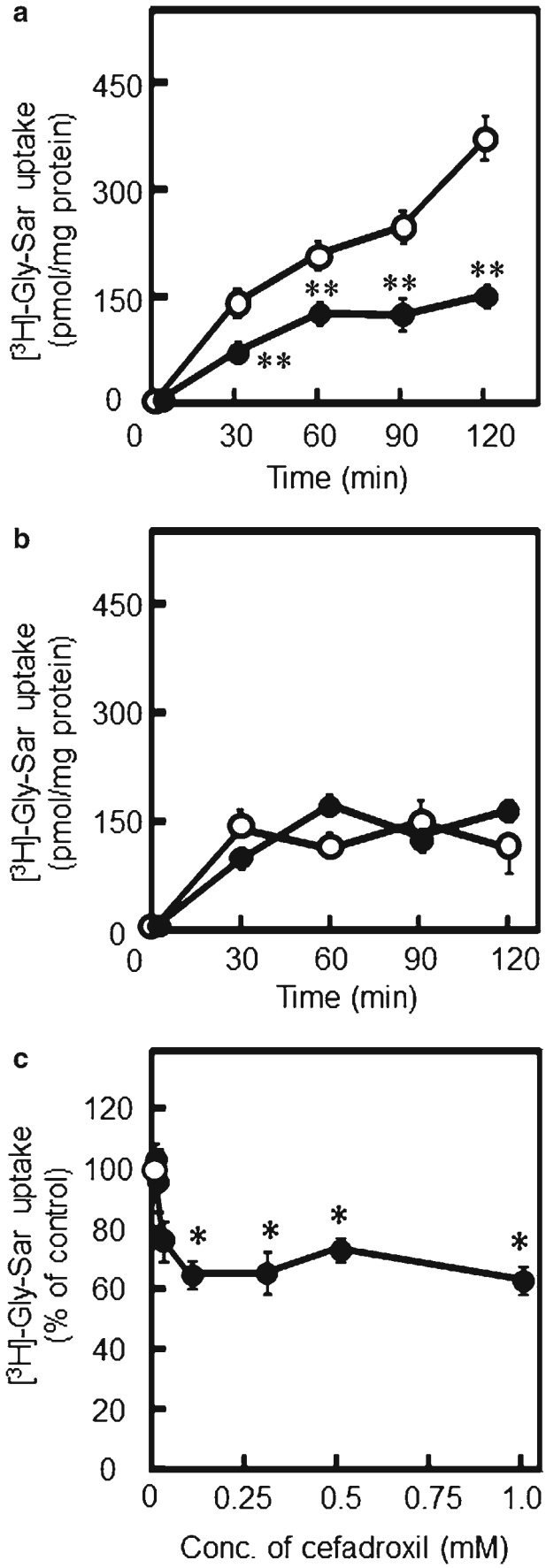
Transcellular Transport of Cephalexin in H441 Cells

Transport of cephalexin across H441 cell monolayers grown on the permeable inserts was examined. Under the present experimental conditions, TEER of H441 cell monolayers increased with time in culture and reached a peak of 454±36 $\Omega \cdot cm^2$ at day 13 (Fig. 7a). Cefadroxil-sensitive transport of cephalexin across H441 cell monolayers was observed in apical-to-basal, but not in basal-to-apical, direction (Fig. 7b).

DISCUSSION

In the present study, H441 cells formed epithelial-like monolayers with dome-like structures (Fig. 1a), suggesting ion, and subsequent water, transport from the apical to the basal surface. Formation of dome-like structures was already reported in H441 cells by Shlyonsky *et al.* (20), and is a hallmark of alveolar epithelial cells in primary culture (27,28). After staining with Nile red or LysoTracker Red, strong signals indicative of lamellar bodies were observed by CLSM in H441 cells (Fig. 1b, c), whereas lamellar body formation was much less pronounced in A549 cells (Fig. 1d). Although commonly used as surrogate alveolar type II cells, A549 (a cell line derived from human lung carcinoma) and RLE-6TN (a cell line derived from rat normal lung) (29,30), lamellar bodies are generally not well developed in these cell lines (31,32). In this aspect, H441 may be more representative of alveolar type II epithelial cells.

Fig. 4 Effect of cefadroxil on Gly-Sar uptake in H441 cells. Time-dependent uptake of [^{3}H]-Gly-Sar (50 μM) at extracellular pH 6.5 was measured at 37°C (**a**) or 4°C (**b**) in the absence (*open circles*) or presence (*solid circles*) of cefadroxil (1 mM). (**c**) Concentration-dependent effect of cefadroxil on [^{3}H]-Gly-Sar uptake. The uptake of [^{3}H]-Gly-Sar (50 μM) was measured at pH 6.5 in the absence (*open circle*) or presence (*solid circles*) of various concentrations of cefadroxil. Each point represents the mean ± SE of three independent cultures. *$p < 0.05$ and **$p < 0.01$, significantly different from each value in the absence of cefadroxil.



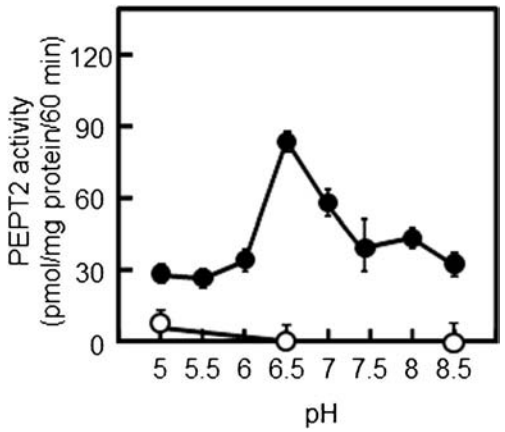


Fig. 5 pH-dependence of Gly-Sar uptake in H441 and A549 cells. The uptake of [^{3}H]-Gly-Sar (50 μM) was measured at various extracellular pH values between 5.0 and 8.5 in H441 cells (*solid circles*) and in A549 cells (*open circles*). PEPT2-mediated uptake of [^{3}H]-Gly-Sar (PEPT2 activity) was calculated by subtracting the uptake value in the presence of cefadroxil (1 mM) from that in the absence of cefadroxil.

The expression of alveolar type II cell markers, SP-C and ABCA3, was confirmed in H441 cells on mRNA level (Fig. 2a, b). The expression of these mRNAs increased significantly with time in culture. Gazdar *et al.* (33), who established H441 cell line, examined mRNA expression of SP-A, SP-B, and SP-C in various human lung cancer cell lines by Northern blot analysis. They reported that SP-A and SP-B mRNAs were detected in H441 cells, while SP-C mRNA was not detected even after DEX stimulation. In contrast, the expression

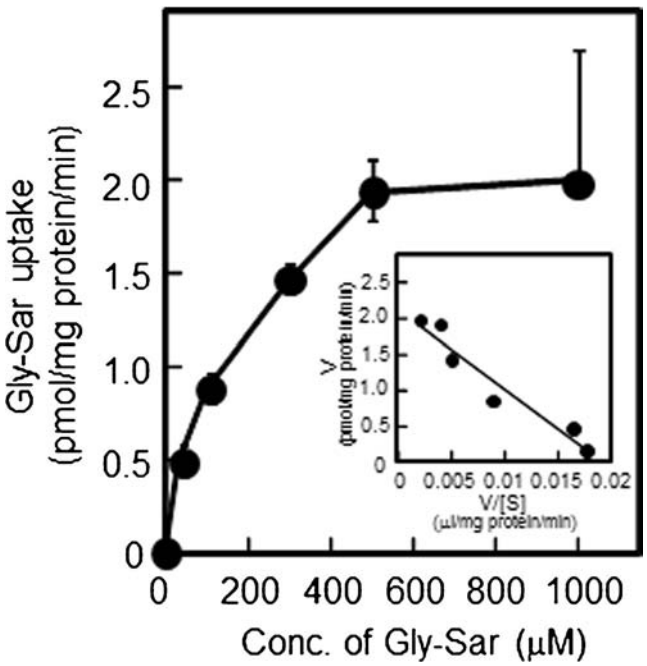


Fig. 6 Concentration-dependence of Gly-Sar uptake in H441 cells. The uptake of various concentrations of Gly-Sar was measured at pH 6.5. The *inset* shows an Eadie-Hofstee plot. Each point represents the mean ± SE of three independent cultures.

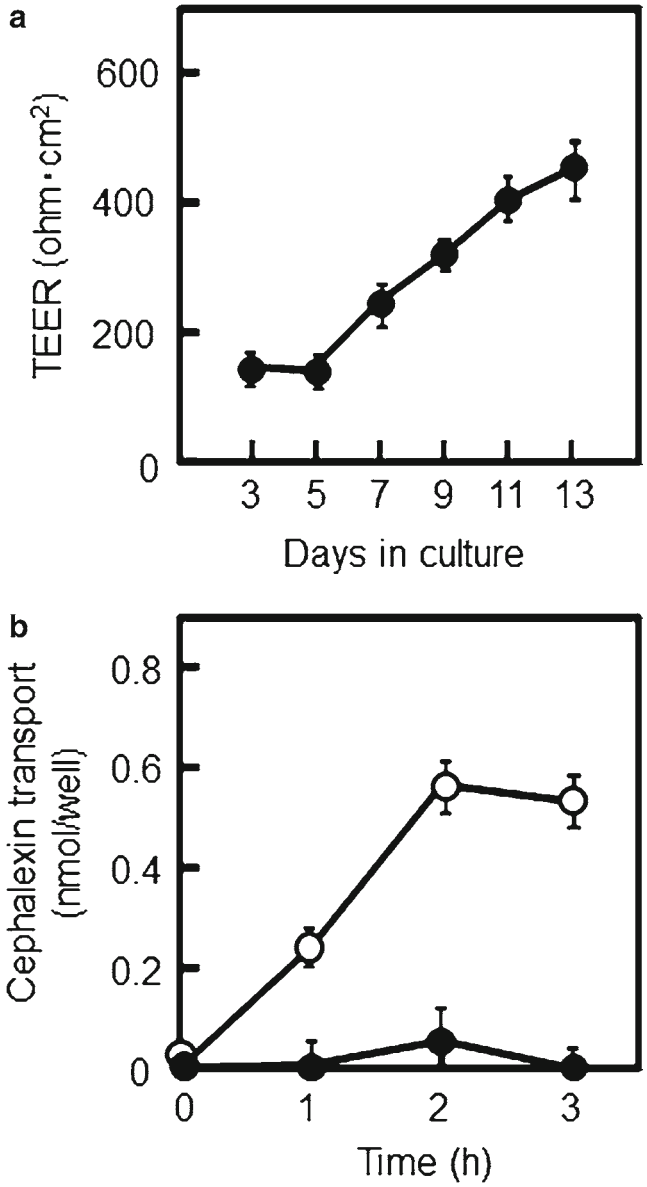


Fig. 7 Transepithelial transport of cephalexin across H441 cell monolayers. (**a**) Time course of TEER development in H441 cell monolayers. Cells were seeded at a density of 2.23×10^5 cells/cm^2 on Transwell Clear inserts and TEER values were measured at the days indicated. Each point represents the mean ± SE of four independent cultures. (**b**) Transepithelial transport of cephalexin (100 μM) in the apical-to-basal (*open circles*) and the basal-to-apical (*closed circles*) directions across H441 cell monolayers. PEPT2-mediated transcellular transport of cephalexin was calculated by subtracting the value in the presence of cefadroxil (2 mM) from that in the absence of cefadroxil. Each point represents the mean ± SE of three independent cultures.

of SP-C mRNA was clearly observed in H441 cells in this study, which may be due to the differences in culture conditions.

Human alveolar epithelial cells in primary culture formed electrically tight monolayers with peak TEER values of 1000–1500 Ω·cm^2 and 2000–2500 Ω·cm^2, when they were cultured on their own and co-cultured with pulmonary microvascular endothelial cells, respectively (34). Reports on maximal TEER values of H441 cell monolayers are quite variable, and range from 200 Ω·cm^2 for cells in monoculture (19) and 440 Ω·cm^2 in the presence of 10 nM DEX (35) to 1000 Ω·cm^2 in liquid-covered conditions (21), probably, depending on the culture conditions. In this study, H441 cell monolayers grown on the permeable support showed TEER values of approximately 450 Ω·cm^2 at day 13 (Fig. 7a), which was profoundly higher than TEER values observed in A549 and RLE-6TN cells in our hands (data not shown). DEX appears to be a key molecule in the formation of electrically tight H441 cell monolayers (35) and it can be speculated that this is due to an up-regulation of members of the claudin family of tight junctional proteins (Dr. Winfried Neuhaus (University of Vienna), personal communication).

The expression of PEPT2 mRNA and protein was confirmed in H441 cells (Figs. 2c, d and 3). In contrast, the expression of PEPT1 mRNA was hardly detected in H441 cells (Fig. 2d). Gly-Sar is a *bona fide* substrate for PEPT2, with an apparent affinity (K_m value) of approximately 50–100 μM (12,13,36), which is very similar to the K_m value determined in H441 cells in our study (*i.e.*, 112.5 μM; Fig. 6). The uptake of Gly-Sar was inhibited by cefadroxil (Fig. 4), a potent inhibitor for PEPT2-mediated transport (12,13). In addition, Gly-Sar uptake in H441 cells was pH-dependent and was peaked at pH 6.5 (Fig. 5), as reported for Gly-Sar uptake in PEPT2 over-expressing LLC-PK$_1$ cells (12). Taken together, these results strongly suggest that PEPT2 is functionally expressed on the apical membrane of H441 cells. On the other hand, PEPT2-mediated Gly-Sar uptake in A549 cells was found to be marginal (Fig. 5). In accordance with this functional assay, the expression of PEPT2 protein was not detected in A549 cells (Fig. 3). Recently, Sakamoto *et al.* (37) examined PEPT2 protein expression in H441 and A549 cells by liquid chromatography-tandem mass spectrometry. However, PEPT2 protein expression was not detected not only in A549 cells but also in H441 cells. The reason for the apparent discrepancy between this and their studies on PEPT2 expression in H441 cells is not clear, but may be due to a detection limit in mass-based assays or the differences in cell culture conditions. In their study, H441 cells cultured for a shorter period (7 days) in the RPMI-1640 medium without DEX and ITS supplements were employed.

In order to speculate whether Gly-Sar uptake process is concentrative or not in this culture model, we estimated the intracellular volume of H441 cells using 3OMG. 3OMG is a non-metabolized analogue of D-glucose and is a good substrate of facilitative glucose transporter. The intracellular volume of H441 cells estimated from the equilibrium uptake value of 3OMG (uptake clearance) was 30.9 μl/mg protein (the mean of three independent cultures). On the other hand, the clearance of cefadroxil-sensitive Gly-Sar uptake at extracellular pH 6.5 was 4.5 μl/mg protein at 120 min (Fig. 4a), indicating that the uptake was not concentrative even in the presence of an inwardly directed proton gradient. This may be due to the low expression of PEPT2 in H441 cell line or

due to the fact that PEPT2-mediated uptake represents only a small fraction of total uptake, the diffusion being the primary mode of entry. Further studies are needed to understand the regulation factors affecting the expression and functional activity of PEPT2 in H441 cells.

Søndergaard *et al.* (38) examined the expression and function of PEPT1 and PEPT2 in the human bronchial airway epithelial cell line, Calu-3. Surprisingly, they showed that Gly-Sar transport in Calu-3 cells was mediated by PEPT1 rather than PEPT2, although both mRNAs were expressed in the cells. On the other hand, we have previously shown PEPT2 to be functionally expressed in freshly isolated rat alveolar type II epithelial cells in primary culture (16). In that study, a K_m value of 72 μM was measured for PEPT2-mediated Gly-Sar uptake, which is very close to what is found in H441 cells. Thus, although the expression and function of PEPT1 and PEPT2 may be different in different sites in the lungs - or indeed, different tumor cell lines - the H441 cell line can be recommended as an *in vitro* model to study PEPT2 function in the human distal lung.

Besides di- and tripeptides, PEPT2 transports a number of pharmacologically important compounds including some β-lactam antibiotics, angiotensin-converting enzyme inhibitors, antiviral prodrugs and δ-aminolevulinic acid (13,39). Hence, PEPT2 is one of the key factors determining the absorption, distribution and excretion of these drugs. In the lungs, PEPT2 may play a role in the pulmonary absorption of peptidomimetics into the systemic circulation. In this study, cefadroxil-sensitive transcellular transport of cephalexin, another PEPT2 substrate, from the apical to the basal side was observed (Fig. 7b). For the transcellular transport of cephalexin, a highly hydrophilic antibiotic, not only the apical transporter (*i.e.*, PEPT2) but also the basolateral transporter would be needed. Terada *et al.* (40) studied the basolateral peptide transporter in human intestinal cell line Caco-2. They reported that basolateral Gly-Sar uptake was mediated by a single facilitative peptide transporter, which was sensitive to cefadroxil but was different from PEPT1. They also examined Gly-Sar uptake across the basolateral membrane of Madin-Darby canine kidney (MDCK) cells, and suggested that the renal basolateral peptide transporter was distinct from PEPT1/PEPT2 and the basolateral peptide transporter in Caco-2 cells (41). In addition, Putnam *et al.* (42) examined the transepithelial transport of cephalexin across MDCK cell monolayers, and found that apical-to-basal transport was much higher than basal-to-apical transport, and apical-to-basal transport, but not basal-to-apical transport, was inhibited by Gly-Sar. These findings suggest that putative peptide transporters are involved in the transport of oligopeptides and peptidomimetics across the basolateral membrane of the intestinal and renal epithelial cells, though the transporters are not yet identified. On the other hand, there is little information concerning the transport characteristics of oligopeptides and peptidomimetics in the basolateral membrane of alveolar epithelial cells. In this context, H441 cells would be a good *in-vitro* model to further study the oligopeptide transport in the basolateral membrane of alveolar epithelial cells.

At this stage, the contribution of alveolar type II epithelial cells to the total pulmonary absorption of peptidomimetic drugs is not known. On one hand, it was reported that PEPT2 was involved in bacterial peptide recognition and immune activation. Using primary cultures of human upper airway epithelial cells, Swaan *et al.* (43) showed that γ-D-glutamyl-meso-diaminopimelic acid (γ-iE-DAP), a bacterial dipeptide, was taken up by PEPT2. In the cells, γ-iE-DAP activated the innate immune response in a nucleotide-binding oligomerization domain protein 1(NOD1)-dependent manner. Consequently, it was proposed that PEPT2 plays a vital role in microbial recognition and participates in host defense in the lungs. Because the expression of NOD1 was also observed in alveolar type II epithelial cells in murine and human lungs (44), it could be interesting to examine whether such an immune response also occurs in H441 cells *via* a PEPT2-mediated mechanism.

CONCLUSION

We could demonstrate, for the first time, the functional expression of PEPT2 in the human distal lung epithelial cell line H441. This cell line is useful to study the regulation of the expression and function of PEPT2, transport of oligopeptides and peptidomimetics, and the role of PEPT2 in the immune response; all important issues which are not yet well understood in alveolar type II epithelial cells.

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