Drug Inhibition of Gly-Sar Uptake and hPepT1 Localization using hPepT1-GFP Fusion Protein

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ABSTRACT An hPepT1-GFP fusion construct was made to study drug inhibition of dipeptide uptake and apical, basolateral, or subcellular hPepT1 localization. The hPepT1 stop codon was mutated by polymerase chain reaction and was subsequently cloned into the pEGFP-N1 vector. The hPepT1-GFP fusion construct was then transfected into Caco-2 and HeLa cells, and drug inhibition was studied by inhibiting ^{3}H-Gly-Sar uptake. Western blot analysis was used to determine hPepT1-GFP expression levels and confocal microscopy was used to examine the localization. Both anti-hPepT1 antibody and anti-GFP antibody recognized a 120-kd hPepT1-GFP fusion protein in the transfected cells. The ^{3}H-Gly-Sar uptake in transfected HeLa cells was enhanced more than 20 times compared with the control. Valacyclovir (5 mmol/L) was able to completely inhibit ^{3}H-Gly-Sar uptake in these transfected cells. Confocal microscopy showed that the hPepT1-GFP mainly localized in the Caco-2 cell apical membrane, but was present throughout the entire HeLa cell membranes. The hPepT1-GFP fusion protein was not found in either early endosome or lysosome of Caco-2 cells under normal conditions; however, it was detected in some subsets of lysosomes and early endosomes in phorbol 12-myristate 13-acetate (PMA)-treated Caco-2 cells.

KeyWords: hPepT1, Localization, Apical Membrane, Lysosome, Valacyclovir

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INTRODUCTION

Drugs taken orally are absorbed by passive diffusion, through the paracellular pathway, or by carrier-mediated drug transport. Since modern molecular biology techniques have developed, more and more transporters have been found to be involved in the oral absorption processes. There are limited passive diffusion and paracellular pathways available for the absorption of large, polar molecules; therefore, intestinal transporters are great potential carriers for the uptake of these drugs (1, 2).

The human dipeptide transporter (hPepT1) was cloned by screening a human intestinal complementary DNA (cDNA) library with a rabbit cDNA probe. When this cDNA was expressed in HeLa cells and *Xenopus laevis* oocytes, it displayed a proton-dependent peptide uptake activity (3). Human PepT1 displays 81% identity with and 91% similarity to the rabbit dipeptide transporter. The hPepT1 transporter is highly expressed in duodenum and jejunum, while lower expression levels are also detected in ileum, colon, cecum, kidney, and liver (4-6). In situ hybridization detects hPepT1 in the tip of mucosal cell villi but not in the crypts. The predicted protein has 708 amino acids with 12 transmembrane domains (7, 8). This peptide transporter accepts dipeptides and tripeptides as its substrates, but it cannot transport free amino acids (9). It is believed that the following drugs are absorbed by hPepT1: β-lactams such as cephalosporins (cephalexin, cepharadine, cefadroxil), cyclacillin, ampicillin, and various angiotensin-converting enzyme (ACE) inhibitors, as well as antiviral prodrugs such as valacyclovir (9-11).

Cells that overexpress hPepT1 are useful models for screening the permeability of drugs that are transported by hPepT1 (12). Caco-2 cells and

MDCK cells, which are highly polarized cells, are good cell lines for in vitro oral drug absorption evaluation. Other cell lines such as HeLa cells and CHO cells are also commonly used in transporter overexpression studies. However, the apical or basolateral hPepT1 localization in these overexpressed cell lines is unknown. In this article, we constructed an hPepT1-GFP fusion plasmid and transfected it into polarized and nonpolarized cells. The hPepT1-GFP fusion protein expression level, its functional ^{3}H-Gly-Sar uptake, and drug inhibition were monitored in these transfected cells. In addition, confocal microscopy studies determined the apical or basolateral and subcellular hPepT1-GFP localization.

MATERIALS AND METHODS

Materials

The antilysosome-associated membrane protein (anti-LAMP) monoclonal antibody (H4A3) developed by Drs J. Thomas August and E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute ofChild Health and Development (NICHD) and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). All culture mediums and reagents were purchased from Gibco (Grand Island, NY). The culture dishes and plates were from Falcon (Lincoln Park, NJ). Chamber slides were purchased from Nalge Nunc International (Rochester, NY). ^{3}H-Glycyl-sarcosine (Gly-Sar) (17 Ci/mmol) was from Moravek Biochemicals (Brea, CA). Rhodamine-labeled rabbit-antimouse immunoglobulin (Ig)G, fluorescein isothiocyanate (FITC)-labeled goat-antirabbit IgG, and FITC-labeled rabbit-antigoat IgG were from Chemicon International (Temecula, CA). Antitransferrin receptor monoclonal antibody was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Anti-rab7 and anti-rab5A antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The pEGFP-N1 vector was from Clontech (Palo Alto, CA). Restriction enzymes were purchased from Promega and New England Biolabs (Beverly, MA). The QIAquick gel extraction kit and QIAprep miniprep and Maxprep kits were from Qiagen (Valencia, CA). Polymerase chain reaction (PCR) reagents and Taq DNA polymerase were from Perkin Elmer Biosystem (Norwalk, CT). Lipofectamine transfection reagent was from Gibco, and Fugene transfection reagent was from Roche Molecular Biochemicals. Precast sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) gels were purchased from Bio-Rad (Hercules, CA).

Construction of hPepT1-GFP fusion plasmid

The hPepT1 cDNA (in pBluscript II SK) was digested with XhoI/ApaI at 37°C for 2 hours, then the 5' end 1.4-kilobase (kb) fragment was separated on 0.7% agarose gel and purified using a QIAquick gel extraction kit according to the manufacturer's manual. Two primers were designed to perform PCR for the amplification of the 3' end 0.7-kb fragment to mutate the stop codon, as well as to introduce ApaI/BamHI cloning sites. The 2 primer sequences are

Primer 1:
5'-CTAGTGTGGGCCCCCAATCAC-3'
Primer 2:
5'-CCTGAGGATCCGGCATCTGTTTCTGTG-3'

The hPepT1 stop codon (TGA) was mutated to GGA using primer 2, which also included a BamHI site (GGATCC). To keep the GFP fusion in-frame, two extra bases (GG) were added to primer 2 after the BamHI site.

The PCR conditions used are as follows: DNA template 20 ng, dNTP 0.25 mmol/L, Primers 1 pmol, 1x PCR buffer (Perkin Elmer), Taq DNA polymerase 1.5 U (Perkin Elmer), and pfu DNA polymerase 0.3 U (Promega). The amplification temperatures were 94°C for 5 minutes, 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After 20 cycles, a final extension was done for 7 minutes at 72°C. The 0.7-kb PCR product was digested with ApaI/BamHI. Then the DNA fragments were separated on 1% agarose gel and purified using a QIAquick gel extraction kit.

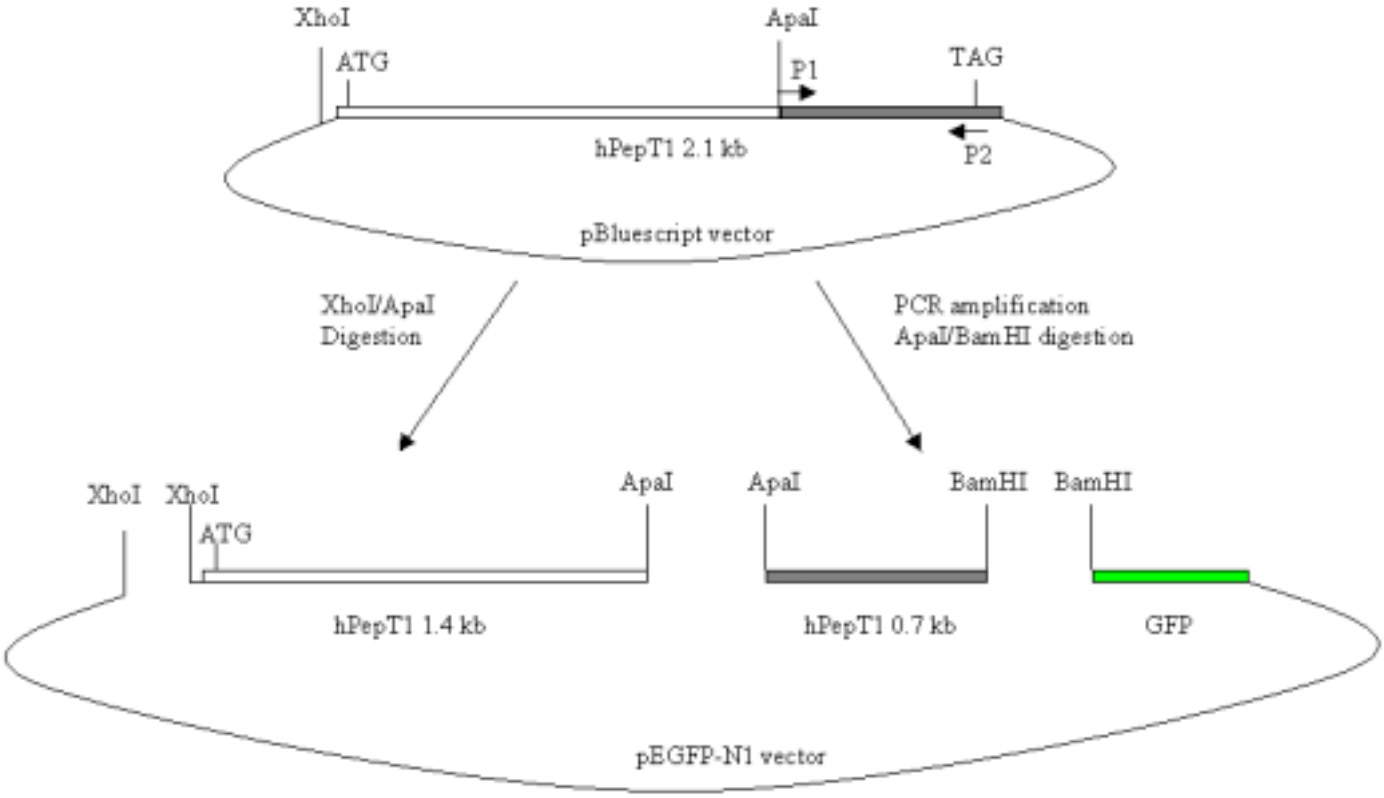

Fig. 1. Construct map of hPepT1-GFP. P1 and P2 indicate two primers for PCR amplification. Gray bar indicates C-terminal 0.7 kb hPepT1 fragment amplified by PCR. Green bar indicates green fluorescent protein. Restriction enzymes are labeled.

These two pieces of hPepT1 DNA fragment were ligated into the pEGFP-N1 vector XhoI/BamHI site using a rapid DNA ligation kit (Boehringer Mannheim, Indianapolis, IN) at room temperature for 10 minutes (Figure 1). Three microliters of ligation mixture was transformed into DH5α by heat shock. The transformed bacteria were plated onto a kanamycin agar plate and incubated at 37°C for 16 hours. Bacterial colonies were grown up for DNA purification using a QIAprep miniprep kit. The clone with the correct insert and the correct orientation was checked by enzyme digestion. Plasmid preparations of positive clones were purified with a Qiagen plasmid Maxiprep kit and sequenced at the University of Michigan Core Facility.

Transfection of hPepT1-GFP to Caco-2 and HeLa cells

Cells were cultured in Dulbecco modified Eagle medium (DMEM; high glucose [Gibco]) supplement with 1% nonessential amino acid, 1% L-glutamine, 1% sodium pyruvate, and 10% fetal bovine serum (FBS). Cells were plated onto a 6-well plate (Falcon) or a chamber slide (Nalge Nunc International) for 24 hours (for HeLa cells) or 48 hours (for Caco-2 cells) before transfection. Transfection was performed after cells reached 50%-70% confluence. When Lipofectamine (Gibco) was used for transfection, the cells were washed twice with Dullbecco's phosphate buffered saline (D-PBS) (Gibco), and incubated with Lipofectamine/DNA complex (5:1) in Opti-MEM I (Gibco)without serum for 12 hours, and then changed to DMEM medium with 10% FBS. When Fugene (Boehringer Mannheim) was used for transfection, the cells were directly incubated with Fugene/DNA complex (3:1) in DMEM medium with 10% FBS for 24 hours, with 1 change of medium after 24 hours. Cells were incubated for 48 hours after transfection before the functional assay.

Uptake studies of ^{3}H-Gly-Sar in hPepT1-GFP-transfected cells

After a 48-hour transfection, cells were washed twice with transporter buffer (pH 6, 1 mmol/L $CaCl_2$, 1 mol/L $MgCl_2$, 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L NaH_2PO_4, 5 mmol/L D-Glucose, 5 mmol/L MES) and incubated with 10 µmol/L Gly-Sar (9.94 µmol/L Gly-Sar and 0.06 µmol/L ^{3}H-Gly-Sar) in 1 mL transporter buffer for 30 minutes at room temperature. In the drug inhibition study, cephalexin (10 mmol/L), acyclovir (5 mmol/L), or valacyclovir (5 mmol/L) was also included in the transporter buffer. After 30 minutes, the uptake was stopped by the addition of 0.5 mL ice-cold transporter buffer. Cells were washed 3 times with ice-cold transporter buffer, collected in 0.5 mL 1.5% Triton X-100, and sonicated 3 times for 10 seconds. Two hundred microliters of sonicated cell suspension was used for scintillation counting, and the remaining sample was saved for protein assay.

Western blot assay for hPepT1-GFP expression

Forty-eight hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS) and collected in 0.5 mL PBS with 1 mmol/L PMSF (phenylmethysulfonyl fluoride) and 60 µg/mL of soybean trypsin inhibitor, and sonicated 3 times for 10 seconds. The suspension was incubated with 2x loading buffer for 30 minutes at room temperature, and then 10 µg of protein was subjected to electrophoresis in 4%-20% SDS-polyacrylamide minigels (Bio-Rad). The protein was transferred to Hybond-P PVDF transfer membrane (Amersham, Piscataway, NJ) for 1.5 hours under 250 mA. The membrane was incubated with an anti-hPepT1 polyclonal antibody (1:1000 in 2.5% milk Tris buffered saline tween-20 (TBS-T), provided by Dr. Wolfgang Sadee at the University of California, San Francisco) or anti-GFP antibody (1:2000 in 2.5% milk TBS-T [Roche Molecular Biochemicals]) at room temperature for 1 hour, washed 5 times with PBS for 5 minutes, and then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham) for 1 hour at room temperature. An enhanced chemiluminescence system ECL+Plus (Amersham) was used to detect the expression level.

Confocal Microscopy and Immunostaining

Transfected cells were washed twice with ice-cold PBS 48 hours posttransfection and fixed with 4% paraformaldehyde for 15 minutes at 4°C, washed 4 times again with PBS, and stored at 4°C in PBS. The slides were mounted in Fluoromount-G medium and the apical or basolateral hPepT1-GFP fusion protein localization was examined using confocal microscopy (Noran O2 confocal laser scanning system with Nikon TE 200 inverted microscope and SGI workstation (Thermo NORAN, Middleton, WI). The X-Y picture of the transfected cells was taken at 490 nm and Z-series pictures were taken at 0.5 µm intervals at the same wavelength.

For immunostaining, the cells were transfected with hPepT1-GFP fusion plasmid or hPepT1 in pcDNA3

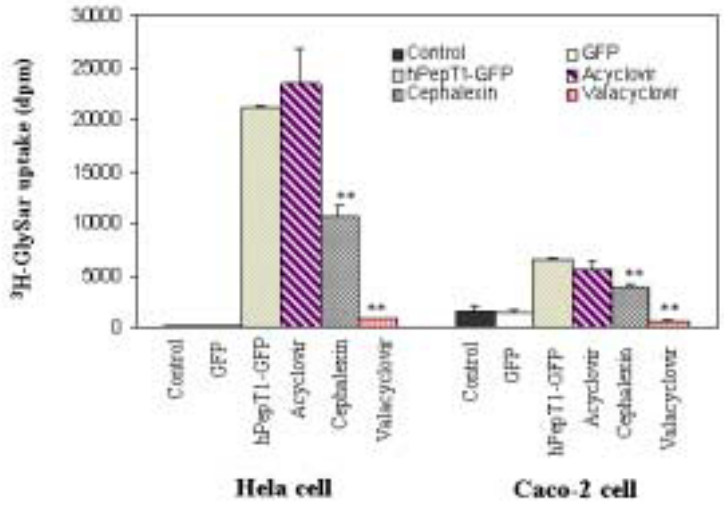

Fig 3. Uptake of ^{3}H-Gly-Sar in hPepT1-GFP transfected cells and drug inhibition. Uptake activity was measured during 30 min incubation. Acyclovir (5 mM), valacyclovir (5 mM), and cephalexin (10 mM) were used to inhibit Gly-Sar uptake in hPepT1-GFP transfected cells. **p<0.01 vs. hPepT1-GFP transfected cells.

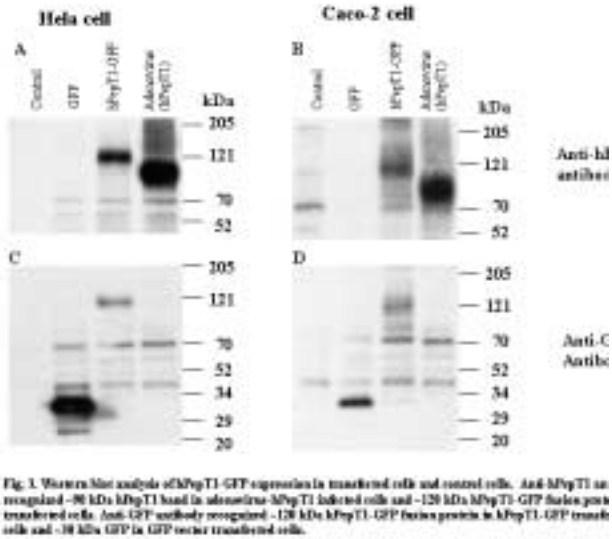

Figure 3. Western blot analysis of hPepT1-GFP expression in transfected cells and control cells. Anti-hPepT1 antibody recognized ~90 kDa hPepT1 band in adenovirus-hPepT1 infected cells and ~120 kDa hPepT1-GFP fusion protein in transfected cells. Anti-GFP antibody recognized ~120 kDa hPepT1-GFP fusion protein in hPepT1-GFP transfected cells and ~30 kDa GFP in GFP vector transfected cells.

vector, and fixed with 4% paraformaldehyde for 15 minutes at room temperature 48 hours posttransfection. For the phorbol 12-myristate 13-acetate (PMA) treatment experiment, cells were incubated with 50 nmol/L PMA in PBS for 1 hour at 37°C 80 hours after transfection, and then fixed with 4% paraformaldehyde, as described above. Cells were permeablized with 0.2% saponin and 0.1% BSA in PBS for 30 minutes at room temperature and washed 2 times with PBS (0.1% saponin, 0.1% BSA). Then cells were incubated with primary antibody diluted in PBS with 0.1% saponin and 0.1% BSA for 1 hour at room temperature (anti-LAMP antibody 1:100, antitransferrin receptor antibody 1:100, anti-Rab7 antibody 1:100, anti-Rab5A antibody 1:100, anti-hPepT1 antibody 1:100), washed 5 times for 5 minutes each time with PBS (0.1% saponin, 0.1% BSA), and incubated with rhodamine or FITC-labeled secondary antibody (diluted 1:100 in PBS with 0.1% saponin and 0.1% BSA) for 1 hour at room temperature. The cells were washed with PBS 5 times for 5 minutes each time and mounted. The colocalization of lysosome or early endosome and hPepT1-GFP was examined under a confocal microscope at wavelengths of 490 nm and 570 nm.

RESULTS

Uptake of ^{3}H-Gly-Sar and drug inhibition in hPepT1-GFP-transfected cells

The ^{3}H-Gly-Sar uptake was measured, after a 30-minute incubation, by counting the radioactivity in the cell lysates. In the control and the GFP-transfected cells, the ^{3}H-Gly-Sar uptake was very low. However, the hPepT1-GFP-transfected HeLa and Caco-2 cells showed 20 and 4 times, respectively, more uptake than the control cells. Three commercial drugs were included in the transporter buffer to test their ^{3}H-Gly-Sar uptake inhibition. Cephalexin (Sigma, St. Louis, MO) (10 mmol/L) inhibited 50% of the ^{3}H-Gly-Sar uptake in hPepT1-GFP-transfected HeLa cells and 40% of ^{3}H-Gly-Sar uptake in hPepT1-GFP-transfected Caco-2 cells. The second drug used, valacyclovir (Sigma) (5 mmol/L), inhibited more than 90% of ^{3}H-Gly-Sar uptake in both hPepT1-GFP-transfected HeLa and Caco-2 cells. However, the third drug, Vacyclovir (University of Michigan, Ann Arbor, MI) (5 mmol/L), was unable to inhibit the ^{3}H-Gly-Sar uptake in hPepT1-GFP-transfected cells (Figure 2).

Expression of hPepT1-GFP in transfected cells

The hPepT1-GFP expression was determined through Western blot analysis using a polyclonal anti-hPepT1 antibody and an anti-GFP antibody as primary antibodies. As shown in Figure 3, the anti-hPepT1 polyclonal antibody recognized a 120-kd hPepT1-GFP fusion protein band in the hPepT1-GFP-transfected HeLa and Caco-2 cells. It also detected a 90-kd band in the adenovirus-hPepT1-infected HeLa and Caco-2 cells (Figure 3A,B). However, hPepT1 expression was undetectable in the control Caco-2 cells (passage 35) using the hPepT1 polyclonal antibody after 4 to 5 days of cultivation, which is the result of the low expression levels during short cultivation time. The anti-GFP antibody recognized the GFP tag in hPepT1-GFP fusion protein and showed a 120-kd band in hPepT1-GFP-transfected HeLa and Caco-2 cells, and it recognized a 30-kd band in the control GFP vector-transfected cells (Figure 3C,D).

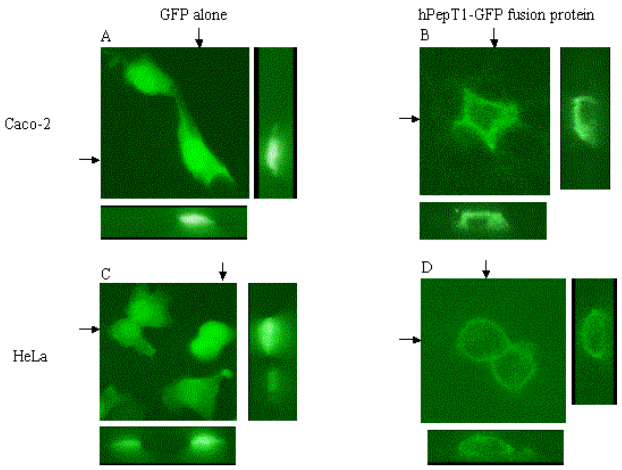

Fig. 4. hPepT1-GFP and GFP localization in transfected Caco-2 cells (A, B) and HeLa cells (C, D). To the right and below each picture are Z-series of confocal pictures. Arrows indicate the points where Z-series pictures are taken. A and C are GFP transfected cells; B and D are hPepT1-GFP transfected cells.

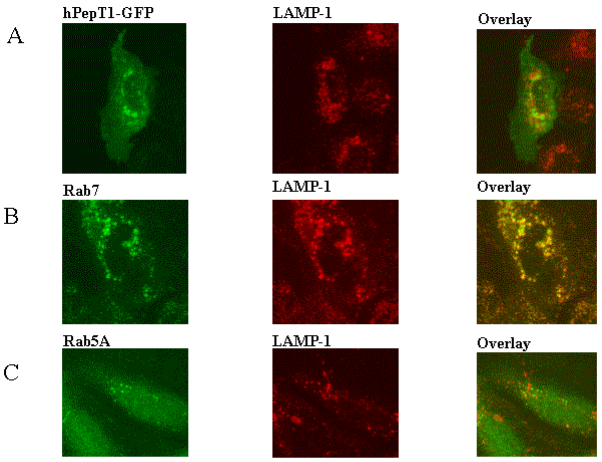

Fig. 5. Co-localization of hPepT1-GFP with LAMP-1 in Caco-2 cells (A). LAMP-1 was stained with its monoclonal antibody H4A3 and rhodamine-labeled secondary antibody (red). Rab7 was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (B). Rab5A was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (C). Yellow color in (B) indicates the overlay of Rab7 and LAMP-1.

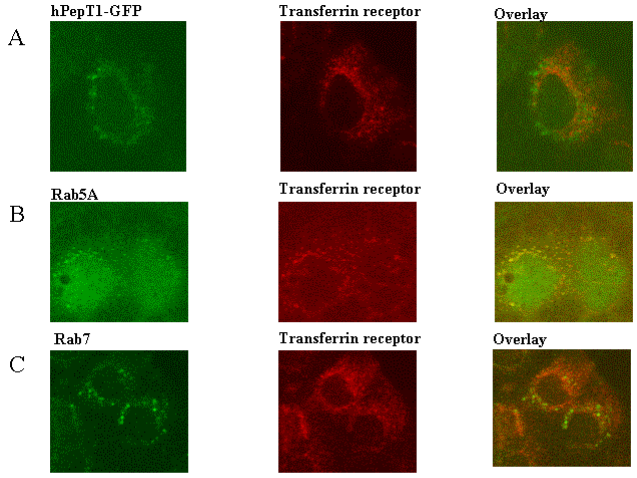

Fig. 6. Co-localization of hPepT1-GFP with transferrin receptor in Caco-2 cells (A). Transferrin receptor was stained with its monoclonal antibody and rhodamine-labeled secondary antibody (red). Rab5A was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (B). Rab7 was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (C). Yellow color in (B) indicates the overlay of Rab5A and transferrin receptor.

Figure 4. hPepT1-GFP and GFP localization in transfected Caco-2 cells (A, B) and HeLa cells (C, D). To the right and below each picture are Z-series of confocal pictures. Arrows indicate the points where Z-series pictures are taken. A and C are GFP transfected cells; B and D are hPepT1-GFP tranfected cells.

Figure 5. Colocalization of hPepT1-GFP with LAMP-1 was stained with its monoclonal antibody h4A3 and rhodamine-labeled secondary antibody (red). Rab 7 was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (B). Rab5A was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (C). Yellow color in (B) indicates the overlay of Rab7 and LAMP-1.

Figure 6. Co-localization of hPepT1-GFP with tranferrin receptor in Caco-2 cells (A). Transferrin receptor was stained with its monclonal antibody and FITC-labeled secondary antibody (green) (B). Rab7 was stained with its polyclonal antibody and FITC-labeled secondary antibody (green) (C). Yellow color in (B) indicates the overlay of Fab5A and transferrin receptor.

Membrane localization of hPepT1-GFP fusion protein

The confocal microscopy X-Y picture showed that hPepT1-GFP is mainly localized in the Caco-2 and HeLa cell plasma membranes. From the Z-series pictures, hPepT1-GFP was mainly found to be localized in the Caco-2 apical membrane (Figure 4B). It seems that there was some hPepT1-GFP in the basolateral membrane, but much less than in the apical membrane, whereas the GFP control cells showed an even GFP protein distribution throughout the transfected cells (Figure 4A,C). However, since HeLa cells are not polarized, hPepT1-GFP was found throughout the entire HeLa cell membrane, and it was unable to distinguish between apical and basolateral membranes (Figure 4D).

Subcellular localization of hPepT1-GFP fusion protein

The subcellular hPepT1-GFP localization in transfected Caco-2 cells was also determined using confocal microscopy. The lysosome and late endosome were stained with LAMP-1 monoclonal antibody and rhodamine-labeled secondary

antibody. We observed clear lysosomal staining using this lysosomal marker. However, we did not find colocalization of hPepT1-GFP and LAMP-1 in transfected Caco-2 cells after a 48-hour transfection (Figure 5A). In the positive control cells, the protein Rab7, which is known to localize in lysosome and late endosome, was stained with its primary antibody and FITC-labeled secondary antibody. The results showed that Rab7 completely overlaid with LAMP-1 (Figure 5B). In the negative control experiment, the protein Rab5A, which is known to localize in early endosome, was stained with its primary antibody and FITC-labeled secondary antibody. The double staining showed no overlay of Rab5A and LAMP-1 (Figure 5C). The early endosome was also stained with a transferrin receptor antibody and a rhodamine-labeled secondary antibody. The hPepT1-GFP was not found colocalized with transferrin-receptor staining (Figure 6A). In contrast, positive-control Rab5A staining was overlaid with transferrin receptor (Figure 6B), while the negative-control Rab7 was not colocalized with transferring receptor (Figure 6C). Daniels and Amara (13) reported that PMA could stimulate cell membrane turn over and membrane transporter internalization more rapidly. Therefore, PMA was also used in our experiment. When Caco-2 cells were transfected with hPepT1-GFP for 80 hours and treated with PMA (50 nmol/L) for 1 hour and then stained with anti-LAMP-1 antibody or antitransferrin receptor antibody, the hPepT1-GFP was found overlaid with LAMP-1 and transferrin receptor in some lysosomes and early endosomes (Figure 7A,B). However, hPepT1-GFP was not found in all lysosomes or early endosomes. In hPepT1-pcDNA3-transfected Caco-2 cells that were double stained with hPepT1 polyclonal antibody and anti-LAMP-1 antibody, we did not find the hPepT1 and LAMP-1 colocalization after a 48-hour transfection (Figure 8A). However, when the transfected cells were treated with PMA for 1 hour after 80 hours of transfection, hPepT1 was found colocalized in some lysosomes (Figure 8B). Similar to the hPepT1-GFP results, hPepT1 was not found in all lysosomes.

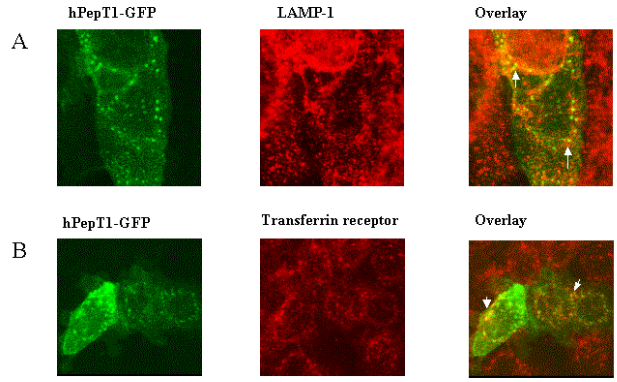

Fig. 7. Co-localization of hPepT1-GFP with LAMP-1 (A) or with transferrin receptor (B) in Caco-2 cells after 1 hr treatment of PMA. LAMP-1 was stained with its monoclonal antibody H4A3 and rhodamine labeled secondary antibody. Transferrin receptor was stained with its monoclonal antibody and rhodamine labeled secondary antibody. Yellow color (arrows) indicates the overlay spots.

Figure 7. Co-localization of hPepT1-GFP with LAMP-1(A) or with transferrin receptor (B) in Caco-2 cells after 1 hr treatment of PMA. LAMP-1 was stained with its monclonal antibody and rhodamine labeled secondary antibody. Transferrin receptor was stained with its monoclonal antibody and rhodamine labeled secondary antibody. Yellow color (arrows) indicates the overlay spots.

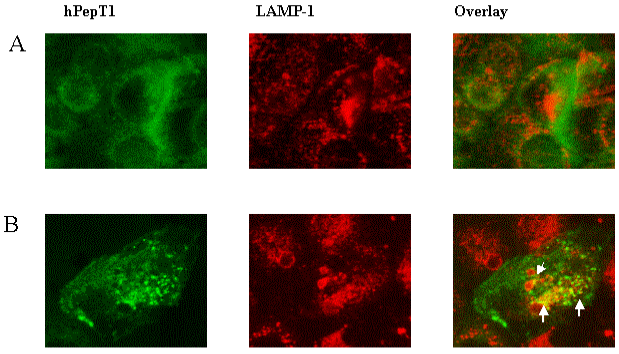

Fig. 8. Co-localization of hPepT1 with LAMP-1 in Caco-2 cells 48 hrs post-transfection (A) and after 1 hr PMA treatment in 80 hrs post-transfected Caco-2 cells (B). hPepT1 was stained with its polycolonal antibody and FITC-labeled secondary antibody. LAMP-1 was stained with its monoclonal antibody H4A3 and rhodamine labeled secondary antibody. Yellow color (arrows) indicates the overlay spots.

Figure 8. Co-localization of hPepT1 with LAMP-1 in Caco-2 cells 48 hrs post transfection (A) and after 1 hr PMA treatment in 80 hrs post-transfected Caco-2 cells (B). hPepT1 was stained with its polyclonal antibody and FITC-labeled secondary antibody. LAMP-1 was stained with its monoclonal antibody H4A3 and rhodamine labeled secondary antibody. Yellow color (arrows) indicates the overlay spots.

DISCUSSION

When hPepT1-GFP plasmid was introduced into Caco-2 and HeLa cells, hPepT1-GFP fusion protein expression in these cell lines was detected by Western blot analysis. The results show a 120-kd hPepT1-GFP fusion protein in the transfected cells, which is 30 kd larger than the hPepT1 protein alone. The function of this fusion protein was confirmed by ^{3}H-Gly-Sar uptake studies in transfected HeLa and Caco-2 cells.

Various drugs have been reported to be the substrates of hPepT1 such as β-lactam antibiotics and ACE inhibitors, and they are absorbed via this transporter in the intestine. Interestingly, some antiviral drugs such as acyclovir are not hPepT1 substrates, however, when it is converted to amino acid ester prodrug valacyclovir, it can be transported by the hPepT1 transporter (11). In the ^{3}H-Gly-Sar uptake and drug inhibition studies, the β-lactam antibiotic cephalexin (10 mmol/L) showed only moderate ^{3}H-Gly-Sar uptake inhibition in hPepT1-GFP-transfected cells (40%-50% inhibition). The antiviral drug acyclovir (5 mmol/L) did not inhibit ^{3}H-Gly-Sar uptake, although its prodrug valacyclovir (5 mmol/L) strongly inhibited ^{3}H-Gly-Sar uptake in hPepT1-GFP-transfected cells. These results indicate that valacyclovir inhibits Gly-Sar uptake better than cephalexin.

Confocal microscopy was used to determine hPepT1-GFP apical or basolateral localization by detecting its green fluorescence. The hPepT1-GFP fusion protein localized in the plasma membrane, while the GFP vector alone showed an even GFP protein distribution in the cells. In polarized Caco-2 cells, the hPepT1-GFP fusion protein was mainly detected in apical membrane with a lesser amount found in the basolateral membrane. In contrast, the hPepT1-GFP fusion protein was found throughout the entire cell membrane in nonpolarized HeLa cells. These results provide important information for in vitro drug absorption screening, because hPepT1 localization depends on whether the hPepT1 overexpressed cells are polarized or nonpolarized.

On the topic of subcellular hPepT1 localization, there is still some debate over whether it is present in the lysosome and exhibits functional peptide transport in this organelle. The assumption that hPepT1 exists in the lysosomal membrane is based on the fact that the acidic lysosome environment might provide a great proton gradient (high inside of lysosome) for the hPepT1 transporter. There are some reports that indicate the existence of an oligopeptide transporter in the lysosome. Thamotharan et al showed that when lysosomal membrane vesicles were incubated with the dipeptide Gly-Gln, the uptake was saturable and proton-dependent (14). The lysosomal membrane vesicles were also able to take up Gly-Gly, Gly-Ala, or the tripeptides MMK, AYK, and RCK (15, 16). In 3H-ribonuclease-loaded lysosome preparations, 8 to 18 amino acid peptides were detected in the release medium (15).

Yet there has been no direct evidence to suggest which transporter is responsible for the peptide uptake activity in the lysosome. hPepT1 was thought to be a candidate for this lysosomal peptide transporter (17). In our experiments, double staining was used to distinguish between early endosome and lysosome or late endosome. LAMP-1 is a lysosome marker that is present only in lysosome and late endosome, while transferrin receptor is an early endosome marker. The rhodamine-labeled secondary antibody and primary transferrin receptor antibody as well as LAMP-1 antibody were used to stain early endosome, late endosome, or lysosome, respectively (18). In the positive and negative control experiments, two known functional proteins Rab5A (in early endosome) and Rab7 (in late endosome and lysosome) were chosen to confirm the organelle staining (19) and compare their localization with that of hPepT1-GFP. Using double immunostaining, Rab7 was found colocalized with LAMP-1, which is in late endosome and lysosome, but not colocalized with transferrin receptor, which is in early endosome. Rab5A was detected overlaid with transferrin receptor, but not with LAMP-1. Surprisingly, when hPepT1-GFP was examined in lysosome or early endosome, the results did not show colocalization of hPepT1-GFP with LAMP-1, nor did hPepT1-GFP and transferrin receptor after a 48-hour transfection in Caco-2 cells. However, when hPepT1-GFP-transfected Caco-2 cells were treated with PMA to stimulate the membrane turnover rate and membrane transporter internalization rate (13), hPepT1-GFP

was found in some lysosomes and early endosomes, but not all. These results suggest that hPepT1-GFP does not seem to be functional in lysosome or early endosome; rather, it might be internalized into lysosome for degradation. However, the lysosomal localization and the possible function of hPepT1 in that organelle require further investigation.

In summary, GFP was cloned onto the C-terminus of hPepT1, and this hPepT1-GFP fusion protein can be expressed in transfected cells and can uptake dipeptides. Valacyclovir, a substrate of hPepT1-GFP, inhibits Gly-Sar uptake in hPepT1-GFP-transfected cells. Using confocal microscopy, the hPepT1-GFP fusion protein was found mainly in the apical membrane of overexpressed Caco-2 cells. In addition, hPepT1-GFP fusion protein was not found in the lysosome or early endosome of overexpressed Caco-2 cells under normal conditions, but it was detected in lysosome and early endosome in PMA-treated Caco-2 cells.

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