Genetic Polymorphisms in Human Proton-Dependent Dipeptide Transporter PEPT1: Implications for the Functional Role of Pro\textsuperscript{586}

Eric Y. Zhang, Dong-Jing Fu, Youngeen A. Pak, Trent Stewart, Nitai Mukhopadhyay, Steven A. Wrighton, and Kathleen M. Hillgren

Discovery Drug Disposition and New Technology (E.Y.Z., Y.A.P., S.A.W., K.M.H.), Functional Genomics (D.-J.F., T.S.), and Diagnostics and Experimental Medicine (N.M.), Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Indiana

ABSTRACT

The human proton-dependent dipeptide transporter (PEPT1, gene SLC15A1) is important for intestinal absorption of di- and tripeptides and a variety of peptidomimetic compounds. Using a DNA polymorphism discovery panel of 44 ethnically diverse individuals, nine nonsynonymous and four synonymous coding-region single-nucleotide polymorphisms (SNPs) were identified in PEPT1. HeLa cells were transiently transfected with plasmids constructed by site-directed mutagenesis for each of the nine nonsynonymous variants. Quantitative polymerase chain reaction showed that the mRNA transcription level of all of the mutants was comparable with the mRNA transcription level of the reference sequence in transfected HeLa cells. Functional analysis in transiently transfected HeLa cells revealed that all nonsynonymous variants retained similar pH-dependent activity and $K_t$ values for [glycyl-1,2-$^{14}$C]glycylsarcosine (Gly-Sar) uptake as the reference PEPT1. In addition, a group of seven peptide-like drugs showed inhibitory effect on Gly-Sar uptake by these variants comparable with the reference, suggesting conserved drug recognition. Of the nine nonsynonymous SNPs, a single SNP (P586L) demonstrated significantly reduced transport capacity as evidenced by a much lower $V_{\text{max}}$ value. This was consistent with lower immunoactive protein level (Western analysis) and lower plasma membrane expression (immunocytochemical analysis). Therefore, Pro\textsuperscript{586} may have profound effect on PEPT1 translation, degradation, and/or membrane insertion.

In recent years, molecular characterization of numerous drug transporters has greatly expanded our understanding of cellular mechanisms of drug disposition. By controlling the cellular entry and exit of compounds, drug transporters have been recognized as important determinants of drug absorption, distribution, and elimination. In the small intestine, nutrient transporters, such as the intestinal peptide transporter, are involved in the active absorptive influx of xenobiotics (i.e., $\beta$-lactam antibiotics) from the intestinal lumen to the portal blood (Dantzig, 1997). In opposition to compound influx, P-glycoprotein is responsible for the active efflux of drugs (i.e., digoxin) from intestinal epithelial cells back to the lumen (Sababi et al., 2001). In the liver, there is a large set of influx and efflux transporters that play important roles in the clearance and excretion of drugs. For example, pravastatin (Tokui et al., 1999) is extracted efficiently from portal vein by the liver through the organic anion transporting polypeptide C (OATP-C). In the kidney, antiviral reagents such as adefovir and cidofovir can undergo active secretion by the organic anion transporter (Ho et al., 2000).

Genetic variations in the enzymes responsible for phase I and II metabolism have been shown to be a source of interindividual variability in drug effect and disposition (Meyer and Zanger, 1997; Hayes and Strange, 2000; Mackenzie et al., 2000; Rodrigues and Rushmore, 2002). Therefore, it is not surprising that genetic variations in drug transporter genes have been also implicated in changes of transporter expression level and function, which could affect drug disposition (Hoffmeyer et al., 2000; Ito et al., 2001; Tirona et al., 2001; Leabman et al., 2002). For example, alterations in the disposition of fexofenadine (Kim et al., 2001) and digoxin (Hoffmeyer et al., 2000) were found linked to polymorphisms in P-glycoprotein. Most recently, it has been shown that one...
commonly occurring SNP (T521C) in the OATP-C is likely to be related to altered pharmacokinetics of pravastatin in a Japanese population (Nishizato et al., 2003).

The intestinal proton-dependent dipeptide transporter PEPT1 was first cloned from rabbit (Fei et al., 1994) and subsequently from human, mouse, and rat (Liang et al., 1995; Saito et al., 1995; Fei et al., 2000). The nucleotide and primary amino acid sequence of PEPT1 is highly homologous across species and has a high transport capacity in small intestine. In addition to its naturally occurring substrates, di- and tripeptides, PEPT1 is capable of actively transporting a variety of chemically diverse compounds, including β-lactam antibiotics, renin inhibitors, and angiotensin-convverting enzyme inhibitors (Rubio-Aliaga and Daniel, 2002). Because of the broad substrate specificity of PEPT1, a prodrug approach, by which a poorly bioavailable drug is modified to be transported by PEPT1, has been intensively investigated as a promising strategy to improve oral absorption of certain molecules (Balimane et al., 1998; Steffansen et al., 1999; Friedrichsen et al., 2001; Thomsen et al., 2003). It is anticipated that with the unfolding of substrate structural requirements of PEPT1, more rationally designed peptidomimetic molecules and prodrugs targeting to PEPT1 will be generated in the future.

Despite increasing efforts in investigating PEPT1 as a possible drug delivery system for small peptides and peptide-like compounds, little information is available regarding potential functional consequence of genetic variations of human PEPT1. In the present study, we identified 13 cSNPs in PEPT1 using a DNA polymorphism discovery panel and studied their function in vitro. We show that with the unfolding of substrate structural requirements of PEPT1, more rationally designed peptidomimetic molecules and prodrugs targeting to PEPT1 will be generated in the future.

Materials and Methods

Materials. [glycyl-1,2-14C]Glycylsarcosine (Gly-Sar) (110 mCi/μmol) was purchased from Moravek Biochemicals (Brea, CA). Bicinchoninic acid protein assay kit was purchased from Pierce Chemical (Rockford, IL). Rabbit anti-human PEPT1 polyclonal antiserum was custom-made by Zymed Laboratories (South San Francisco, CA) and prepared from animals injected with a peptide corresponding to the last 16 amino acid residues of human PEPT1 (KSNPYFMSGANSQKQM). Rabbit anti-calnexin polyclonal antibody was purchased from Stressgen (Victoria, BC, Canada). Unlabeled Gly-Sar, glycine, cefadroxil, captopril, cephalaxin, enalapril, 1,3,4-dihydroxyphenylalanine (l-DOPA), besanin, and 5-aminolevulinic acid hydrochloride (lAminolevulinic acid) were obtained from Sigma-Aldrich (St. Louis, MO). HeLa cells (CCL-2) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 4.5 g/l glucose, 110 mg/l sodium pyruvate, 584 mg/l l-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humified atmosphere with 5% CO2.

SNP Identification. PEPT1 mRNA sequence was obtained from GenBank (accession no. NM_005073). This sequence was designated as the reference PEPT1 in the study. Determination of coding sequence, untranslated regions and intronic regions was based on annotation in the GenBank databases. Samples used for polymorphism discovery were obtained from a DNA polymorphism discovery panel (M44PRD) from Coriell Cell Repositories (Camden, NJ). The panel consists of genomic DNA isolated from 44 unrelated individuals of different ethnicity, including 11 European-American, 11 African-American, 11 Asian-American, six Mexican-American, and five native Americans.

PCR assays were designed to obtain a product of ~500 bp that includes 50–80 bp of flanking intronic sequence. The genomic PCR reaction was carried out with DNA polymerase AmpliTaq Gold (Applied Biosystems, Foster City, CA). A 25-μl PCR reaction was performed containing 100 ng of genomic DNA, 1 unit of AmpliTaq Gold, 2.5 pmol of forward primer, 2.5 pmol of reverse primer, 0.2 mM dNTPs, 1× AmpliTaq Gold buffer, and 4% dimethyl sulfoxide. The reaction mixture was denatured at 94°C for 3 min, followed by 12 touchdown cycles [94°C, 30 s; 60°C (0.5°C decrease for each cycle until 55°C), 30 s; 72°C, 2 min], 35 regular cycles [94°C, 30 s; 55°C, 30 s; 72°C, 2 min), and a final incubation at 72°C for another 5 min. Aliquots (1–2 μl) of unpurified PCR product were used for each sequencing reaction. The sequencing reaction was carried out from both directions using ABI Big Dye Terminator Sequencing kits on an ABI 3700 capillary analyzer (Applied Biosystems).

SNP identification was completed using the ABI sequence software for base calling. Chromatograms were transferred to a sequence assembly software-SequenceChainer (Gene Codes, Ann Arbor, MI). Each base call was compared with the consensus sequence, and the variants were confirmed by visual inspection of the chromatograms.

Haplotype Analysis. Genotype results of the nine cSNPs from the panel of 44 individuals were used to estimate the haplotypes constructed out of these cSNPs. Haplotypes were determined using the “HaploTyper” algorithm (Niu et al., 2002). Specifically, the algorithm partitions the set of SNPs into smaller sets, estimating the phase for each of the smaller sets by a Gibbs sampler and then combining the adjacent sets using Gibbs sampler again to construct one level bigger haplotypes and thus continuing until the whole haplotype was estimated.

Construction of Expression Vectors for the Reference and Variant PEPT1s. The open reading frame of the human reference PEPT1 was first amplified from human small intestine QUICK-CLONE cDNA (BD Biosciences Clontech, Palo Alto, CA) by pfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and subcloned into pcDNA3.1 (Invitrogen). Then, the cDNA fragment of human PEPT1 was constructed to contain BamH1 site at 5′ side and XbaI site at 3′ side. Finally, the fragment was introduced into BamH1/XbaI sites of pcDNA3.1 expression vector (Invitrogen).

Point mutations were introduced into the constructed vector containing the reference PEPT1 using the QuikChange site-directed mutagenesis kit (Stratagene). Each variant PEPT1 in the vector was fully sequenced to ensure that only desired mutation was introduced.

Transfection, Uptake Study, and Western Analysis of the Reference and Variant PEPT1s. HeLa cells were seeded onto 24-well plates. When the cells reached 70 to 80% confluence, transfection was performed with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to manufacturer’s protocol. PEPT1-mediated [14C]Gly-Sar uptake activity was measured in the cells cultured in 24-well plates 24 h post-transfection. The uptake medium was maintained at either pH 6.0 or 7.4 as described previously (Liang et al., 1995). Nonspecific uptake due to passive diffusion was determined in parallel experiments in HeLa cells transfected with the empty pcDNA3.1 vector. The cells were incubated for [14C]Gly-Sar uptake for 3 min at room temperature. At the end of incubation, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and lysed in 0.3 ml/well of PBS with 1% Triton X-100 for 30 min. The aliquots were subjected to both liquid scintillation counting and protein quantification by the bicinchoninic acid protein assay kit. Uptake activity was determined as the number of picomoles of Gly-Sar per milligram of protein per 3 min.

To measure Gly-Sar transport kinetics, [14C]Gly-Sar uptake in a concentration range of 0.094 to 3 mM was assessed with incubation time of 3 min, which is within the linear phase of the uptake process (the first 5 min). Passive diffusion (Kp, diffusion coefficient) was determined in parallel experiments in HeLa cells transfected with an
empty pcDNA3.1 vector. Experimental data were fitted by KaleidaGraph (Synergy Software, Reading, PA), in which a model describing the uptake as a process combining diffusion and single site carrier-mediated transport (Liang et al., 1998) was used. The fitted parameters were presented as the maximal uptake velocity, \( V_{\text{max}} \), and the concentration, \( K_{\text{m}} \), when uptake rate reaches one-half of \( V_{\text{max}} \). All experiments were carried out in triplicate on two to three different experimental days.

For immunoblotting studies, the transfected HeLa cells were washed with PBS and lysed in lysing buffer as described previously (Wong et al., 1995). Lysates were diluted with Laemmli sample loading buffer and separated by a 4 to 20% SDS-polyacrylamide gel. After transfer onto Immun-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), blots were probed with anti-human PEPT1 (1:2000) or anti-calnexin (1:2000) antibodies and visualized using a biotinylated anti-rabbit IgG and a chromogenic detection system (Vector Laboratories, Burlingame, CA).

**Immunocytochemical Analysis.** HeLa cells were grown on the four-well Lab-Tek chambered coverglass (Nalge Nunc International, Rochester, NY) and transfected with expression vectors containing the reference or P586L variant as described above. Cells were fixed in 1% formaldehyde in PBS for 10 min, permeabilized in PBS containing 0.025% Nonidet P-40 (Sigma-Aldrich), and 1% bovine serum albumin for 15 min, and blocked with Protein Block (DakoCytomation California Inc., Carpinteria, CA) for 30 min. Cells were then incubated with the rabbit anti-human PEPT1 antibody for 1 h at room temperature, washed three times with PBS containing 1% bovine serum albumin, and incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at a dilution of 1:500 in the antibody diluent (DakoCytomation California Inc.) for 1 h. Finally, cells were counterstained by 0.075 mM propidium iodide (Molecular Probes, Eugene, OR) in PBS for 5 min before visualization and analysis by MRC1024-UV confocal system (Bio-Rad, Hemel Hemstead, UK).

**Quantitative Real-Time PCR.** The mRNA expression level of each PEPT1 variant in comparison with the reference in transiently transfected HeLa cells was measured using SYBR Green-based quantitative real-time PCR. Specifically, transfected HeLa cells were washed with PBS 24 h post-transfection, trypsinized, and collected by centrifugation. Total RNA was extracted from cells by RNeasy Mini kit (QIAGEN, Valencia, CA) according to manufacturer’s protocol. Purified total RNA (8 \( \mu \)g) was digested by BamH1 and XbaI restriction enzymes (Invitrogen) to linearize the residue plasmids. The rest of haplotypes include some variants with double or triple mutations. Of the 44 individuals is available online as a data supplement to this article. According to a putative PEPT1 membrane topology model (Fig. 1), most mutations occur on the extracellular loops, and two of nonsynonymous SNPs are in the putative transmembrane regions. Many of these amino acids are highly conserved among PEPT1 orthologs and human PEPT2 (Fig. 2).

Haplotype analysis was conducted on the nine nonsynonymous cSNPs identified in this study. The estimated haplotypes are summarized in Fig. 3. Ten haplotypes were deduced. The dominant (>62%) haplotype is the one corresponding to the reference PEPT1 gene. The other haplotypes with relatively high estimated incidence are S117N (22.7%) and G419A (6.8%). The rest of haplotypes include some variants with double or triple mutations.

### TABLE 1

Summary of cSNPs in the human PEPT1 gene

<table>
<thead>
<tr>
<th>SNP ID*</th>
<th>Nucleotide Sequence</th>
<th>Effect</th>
</tr>
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<tbody>
<tr>
<td>5.1</td>
<td>5’ TGGCA [CT] CCCCC 3’</td>
<td>T1141</td>
</tr>
<tr>
<td>5.2</td>
<td>CGACA [GA] CTTTC</td>
<td>S117N</td>
</tr>
<tr>
<td>5.3</td>
<td>TGCA [GC] TGGTA</td>
<td>V122M</td>
</tr>
<tr>
<td>16.2</td>
<td>GGAAA [CT] GAAGT</td>
<td>N393</td>
</tr>
<tr>
<td>16.3</td>
<td>AGATG [GC] TGACA</td>
<td>V416L</td>
</tr>
<tr>
<td>17.1</td>
<td>ACTTG [GC] CCAAA</td>
<td>G419A</td>
</tr>
<tr>
<td>17.2</td>
<td>AACTG [GA] TGAAC</td>
<td>A449</td>
</tr>
<tr>
<td>17.5</td>
<td>CTTG [CT] AAATC</td>
<td>V450L</td>
</tr>
<tr>
<td>17.4</td>
<td>GCCAA [CT] GCCAC</td>
<td>E482</td>
</tr>
<tr>
<td>18.2</td>
<td>CCAGA [AG] AAAGG</td>
<td>E482</td>
</tr>
<tr>
<td>19.1</td>
<td>GGCAA [CT] ATCAC</td>
<td>N509</td>
</tr>
<tr>
<td>21.4</td>
<td>AATCC [GC] GCAGT</td>
<td>P586L</td>
</tr>
</tbody>
</table>

* SNP identification numbers are designated according to the corresponding exon number.
triple mutations, and all of them are estimated to occur at relatively low frequency (≤3.4%).

mRNA Expression of the Reference and PEPT1 Variants in Transfected HeLa Cells. To determine the level of mRNA for each variant in comparison with the reference gene when transiently expressed in HeLa cells, quantitative RT-polymerase chain reaction was performed using PEPT1-specific primers and 18s rRNA as an endogenous control. The amplification efficiency of PEPT1 gene and 18S rRNA were higher than 90%, assessed by the slope and the linear regres-
sion ($R^2 \geq 0.99$) of their standard curves. Each PEPT1-related $C_v$ value from the no-RT-control RNA sample was sufficiently higher ($> 9$) than values obtained from each sample and thus was not considered interfering with RNA analysis (data not shown). 18S rRNA expression level was invariable among different samples ($C_t$ average was $9.17 \pm 0.22$). As shown in Fig. 4, the mRNA level of each variant ranged from 1.6- to 2.2-fold of the reference level.

Functional Analyses of PEPT1 Variants in Transfected HeLa Cells. Using site-directed mutagenesis, expression vectors containing each PEPT1 variant were constructed. When transiently expressed in HeLa cells, the reference PEPT1 transported Gly-Sar in a pH-dependent manner (Fig. 5A). This pH-dependent uptake was seen with the other variants, all of which transported Gly-Sar at a higher rate at pH 6.0 than at pH 7.4. Under the conditions used in this experiment, uptake rate of R459C was similar to the reference value, whereas uptake rates of the other variants (except for P586L) ranged from 1.3- to 1.6-fold of the reference value. Most distinctively, P586L showed a greatly reduced uptake rate ($p < 0.01$; Fig. 5A).

Both anti-PEPT1 and anti-calnexin antibodies were used in Western blot studies. The anti-PEPT1 antibody detected PEPT1-specific bands (~75 to ~100 kDa) in the cell extract from PEPT1 reference and variants (Fig. 5B), but not from the mock-transfected cells. In addition, it is evident that the immunoreactive protein level of P586L was lower than those of the reference and the other variants, compared with the loading baseline provided by calnexin (Fig. 5C), which has been shown to be consistently expressed in transfected HeLa cells (Alvarez et al., 2003). This reduction in protein level seems consistent with the considerably diminished uptake rate of P586L (Fig. 5A).

As shown in Fig. 6, the same anti-PEPT1 antibody was used to detect PEPT1 expression in immunocytochemical analysis. The amplified green fluorescent signal was observed mostly on the cell membrane of transfected HeLa cells (Fig. 6, B and C), whereas some nonspecific cytoplasmic staining could be observed with mock-transfected cells (Fig. 6A). Moreover, the level of plasma membrane expression of the reference PEPT1 (Fig. 6B) seemed much higher than that of the P586L variant (Fig. 6C). These data, again, are in good agreement with the lower immunoreactive protein level of P586L (Fig. 5B) as well as its diminished uptake rate (Fig. 5A).

For a more comprehensive functional characterization, the concentration-dependent kinetics of Gly-Sar uptake was assessed for each PEPT1 variant. Determined by nonlinear curve fitting, the kinetic parameters are summarized in Table 2. All variants possessed $K_t$ and $V_{max}$ values similar to the reference, except for P586L, which retained a comparable $K_t$ value, but had an approximately 10-fold lower $V_{max}$ value ($p < 0.01$) than the reference and other variants.

For a further characterization of substrate recognition of each variant, seven previously reported PEPT1 drug substrates (Rubio-Aliaga and Daniel, 2002) were used to test relative inhibitory effects on Gly-Sar uptake by each variant (Table 3). Glycine (negative control, 10 mM) showed minimal inhibition on [14C]Gly-Sar uptake by PEPT1 reference and variants, whereas unlabeled Gly-Sar (positive control, 10 mM) achieved nearly complete inhibition. The inhibition on Gly-Sar uptake by the reference and variants was similar for most drug substrates as demonstrated by the percentage of inhibition. To be specific, for bestatin, 5-aminolevulinic acid hydrochloride, and enalapril, each compound inhibited Gly-Sar uptake by all variants and the reference to a relatively high extent; for cefadroxil, captopril, and cephalaxin, each showed medium inhibitory effect on all variants and the reference; L-DOPA was the weakest inhibitor for all variants and the reference. Therefore, based on this information, we conclude that the selected seven drug substrates for human PEPT1 (reference) are likely to be substrates of all the PEPT1 variants, and the recognition of these drug substrates seems to be conserved among PEPT1 reference and variants.

To examine the phenotype of PEPT1 haplotypes, we also constructed the expression plasmids corresponding to the estimated haplotypes containing double or triple mutations (Fig. 3), i.e., S117N/T451N, S117N/G419A/R459C, S117N/V416L, and S117N/V122M/G419A. These double- or triple-altered variants retained similar pH-dependent Gly-Sar uptake as the reference and those of the variants with the single amino acid changes (data not shown). These data suggest
that variants associated with Ser\textsuperscript{117}, Val\textsuperscript{122}, Val\textsuperscript{416}, Gly\textsuperscript{419}, and Arg\textsuperscript{459} did not alter PEPT1 function.

**Discussion**

Recently, a set of 24 membrane transporter genes was selected by the Pharmacogenetics of Membrane Transporters (PMT) project to be examined for genetic polymorphisms (Leabman et al., 2003). As a result, preliminary sequence information on some genetic variations of PEPT1 became public (www.pharmgkb.org); however, no functional data are available, and little can be surmised regarding whether these PEPT1 polymorphisms could lead to interindividual variabi-

by in oral absorption of PEPT1 drug substrates. In the current study, we screened a DNA panel composing 44 ethnically diverse individuals and found nine nonsynonymous and four synonymous coding-region SNPs in human PEPT1. Then, we studied the functional relevance of the nonsynonymous cSNPs by using a heterologous expression system.

Our sequencing data are compared with preliminary data on PEPT1 from PMT project (Table 4). Although both DNA panels studied in our study and PMT project were from Coriell Institute, our panel, which is smaller in sample size, is not a subset of the DNA samples in PMT project. The two

**TABLE 2**

Kinetic parameters for \textsuperscript{[14]}C Gly-Sar uptake by PEPT1 variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_d$</th>
<th>$V_{max}$</th>
</tr>
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<tbody>
<tr>
<td>Reference</td>
<td>0.33 ± 0.10</td>
<td>12.0 ± 2.7</td>
</tr>
<tr>
<td>T114I</td>
<td>0.32 ± 0.08</td>
<td>14.9 ± 2.0</td>
</tr>
<tr>
<td>S117N</td>
<td>0.43 ± 0.10</td>
<td>16.1 ± 1.8</td>
</tr>
<tr>
<td>V122M</td>
<td>0.65 ± 0.17</td>
<td>19.5 ± 2.6</td>
</tr>
<tr>
<td>V416L</td>
<td>0.38 ± 0.05</td>
<td>16.1 ± 1.8</td>
</tr>
<tr>
<td>G419A</td>
<td>0.45 ± 0.08</td>
<td>18.5 ± 2.6</td>
</tr>
<tr>
<td>V450I</td>
<td>0.35 ± 0.06</td>
<td>12.9 ± 1.6</td>
</tr>
<tr>
<td>T451N</td>
<td>0.35 ± 0.05</td>
<td>14.1 ± 0.6</td>
</tr>
<tr>
<td>R459C</td>
<td>0.32 ± 0.07</td>
<td>16.4 ± 1.1</td>
</tr>
<tr>
<td>P586L</td>
<td>0.25 ± 0.09</td>
<td>1.34 ± 0.3*</td>
</tr>
</tbody>
</table>

$p < 0.01$. 

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**Fig. 5.** Expression of the reference and PEPT1 variants in HeLa cells. A, pH-dependent Gly-Sar uptake. Transfection was conducted by using FuGENE 6. PEPT1-mediated \textsuperscript{[14]}C Gly-Sar (50 μM) uptake activity was measured in the cells in 24-well plates under two pH conditions (6.0 and 7.4). Cells transfected with the empty pcDNA3.1 vector was served as control. Data are shown as mean ± S.E. (B) and immunoblot analyses (C). Electrophoreses were run in parallel. The blots were probed with a rabbit anti-human PEPT1 (B) or anti-calnexin (C) polyclonal antibodies and visualized using a biotinylated anti-rabbit IgG and a chromogenic detection system.

**Fig. 6.** Immunocytochemical detection of PEPT1 in transfected HeLa cells. HeLa cells were transfected with empty vector (A), reference sequence (B), and P586L (C). Expressed proteins were visualized by Alexa Fluor 488 shown in green. Nuclei were stained by propidium iodide shown in red. Green fluorescent molecules, Alexa 488, were excited with a Krypton/Argon laser at 488 ± 10 nm, and the emission was detected at 522 ± 17 nm. Red fluorescent molecules, propidium iodide, were excited with a 568 nm Krypton/Argon line, and the emission was detected at 585 nm.

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most common nonsynonymous cSNPs, S117N and G419A, were identified in both studies with similar allelic frequency. In addition, three other nonsynonymous cSNPs (V122M, V450I, and T451N) and all four synonymous cSNPs identified in our study were also found in the PMT project, although with different frequencies. However, all low-frequency cSNPs (≤0.4%, occurring in one or two chromosomes in the total of 247 subjects) in PMT dataset were not found in our study. The converse is true for three low-frequency cSNPs, including T114I, R459C, and P586L (≥1.1%), identified in our study, each of which occurred once in the total of 88 chromosomes. It seems that the smaller sample size of 44 individuals screened in our study is sufficient to identify major PEPT1 variants, because all of the relatively high-frequency cSNPs found in our study matched well with the results from the PMT project. However, surprisingly, three low-frequency nonsynonymous cSNPs (T114I, R459C, and P586L) were found in our subjects, but not in PMT project. Furthermore, a resequencing effort on the individual DNA samples with these PEPT1 variations has ruled out the possibility of sequencing error.

When the transport function of the identified nine nonsynonymous variants was assessed in vitro in comparison with the reference PEPT1 gene, all variants, including P586L, similar to the reference, possessed the higher uptake rate at the lower pH value (Fig. 5A). This retained pH dependence on Gly-Sar uptake indicates that these codon changes seem not to occur in PEPT1 regions essential for proton cotransport. Kinetic studies revealed comparable Kₘ values for Gly-Sar uptake by all variants and the reference (Table 2). To further assess whether this conserved substrate recognition can be seen with drug substrates, seven chemically diverse compounds, including β-lactams antibiotics (cefadroxil and cephalexin), angiotensin-converting enzyme inhibitors (captopril and enalapril), peptidomimetic drugs (bestatin), and nonpeptidic substrates (l-DOPA and 5-aminolevulinic acid), were selected. A single concentration (10 mM) for all drug substrates was chosen in the uptake inhibition study. At this tested concentration, the inhibitory effect of each compound could be roughly categorized as relatively high, medium, and low (see Results). Table 3 demonstrates that the inhibition ranking of each drug substrate observed in the reference PEPT1 was, by and large, similarly seen among all the variants. Therefore, collectively, these results suggest that all variants likely retain a substrate-recognition/binding pocket similar to the reference PEPT1.

One interesting finding in the study was the reduced uptake capacity of variant P586L. This variant retains pH-dependent uptake, but with significantly reduced capacity. Kinetic studies revealed that P586L has similar Kₘ to Gly-Sar as the reference and other variants, but with a transport capacity 10-fold less as measured by Vₘ₉. In addition, the inhibitory effect by the selected drug substrates on Gly-Sar uptake by P586L was similar to that observed with the other variants. Therefore, collectively, these results suggest that all variants likely retain a substrate-recognition/binding pocket similar to the reference PEPT1.

When the transport function of the identified nine nonsynonymous variants was assessed in vitro in comparison with the reference PEPT1 gene, all variants, including P586L, similar to the reference, possessed the higher uptake rate at the lower pH value (Fig. 5A). This retained pH dependence on Gly-Sar uptake indicates that these codon changes seem not to occur in PEPT1 regions essential for proton cotransport. Kinetic studies revealed comparable Kₘ values for Gly-Sar uptake by all variants and the reference (Table 2). To further assess whether this conserved substrate recognition can be seen with drug substrates, seven chemically diverse compounds, including β-lactams antibiotics (cefadroxil and cephalexin), angiotensin-converting enzyme inhibitors (captopril and enalapril), peptidomimetic drugs (bestatin), and nonpeptidic substrates (l-DOPA and 5-aminolevulinic acid), were selected. A single concentration (10 mM) for all drug substrates was chosen in the uptake inhibition study. At this tested concentration, the inhibitory effect of each compound could be roughly categorized as relatively high, medium, and low (see Results). Table 3 demonstrates that the inhibition ranking of each drug substrate observed in the reference PEPT1 was, by and large, similarly seen among all the variants. Therefore, collectively, these results suggest that all variants likely retain a substrate-recognition/binding pocket similar to the reference PEPT1.

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changes on protein expression level (especially for P586L). Results (Fig. 4) suggested that all variants (including P586L) showed slightly higher mRNA level than the reference gene in the transient transfected cell system. Therefore, the significant reduction of P586L protein expression level seems not to be attributed to variations on gene transcription, but mainly due to alterations occurring post-transcriptionally. Given that Pro586 is located on the putative membrane boundary of the transmembrane (TM) domain and could be an essential residue for α-helix packing of TM domain (Brandl and Deber, 1986), we propose that substitution of proline with leucine at position 586 may have profound effect on protein stability or translational control, which leads to low protein expression level on the cell membrane. Experiments to determine the effect of P586L on post-transcriptional events are beyond the scope of this study and are the subject of future work.

When using site-directed mutagenesis to study protein structure-function relationships, it is assumed that important amino acids are conserved in the protein family and changes in conserved residues should affect protein function. Similarly, this reasoning should stand when it comes to determining whether a nonsynonymous SNP of a given transporter can alter transport function and lead to a functional consequence. As shown in Fig. 3, we created sequence alignments for partial regions in hPEPT1 containing the nine cSNPs and sequences from six animal species and human PEPT2. Some of the SNPs resulted in replacement of a conserved amino acid by a different amino acid present in another species in the same position (i.e., S117N and T451N). Gly419 was not conserved across species, whereas the substituted alanine is observed in rat and mouse PEPT1. Based on this alignment, we expected that these three cSNPs (S117N, T451N, and G419A) should be functionally neutral, whereas the other cSNPs occurring at conserved positions would have a greater probability of resulting in a functional consequence, depending on the type of substituting residue. For example, P586L may have altered function, because Pro586 is in an evolutionarily conserved region, and the substituting Leu is structurally different from Pro. Recently, a computational method was proposed to predict the cSNPs that might have a functional consequence. This program, Sorting Intolerant from Tolerant (Ng and Henikoff, 2002), is actually a sequence homology based tool that evaluates whether amino acid changes are conserved within the protein family to predict which cSNPs would affect protein function. The Sorting Intolerant from Tolerant program predicted that most PEPT1 cSNPs identified were tolerated mutations, except for P586L (data not shown). Not surprisingly, our experimental results on P586L concurred with the prediction from this sequence homology based analysis.

Despite the efforts to elucidate the structure-function relationship of PEPT1, the exact PEPT1 protein domains important for proton coupling and substrate translocation remain elusive in the absence of a high-resolution three-dimensional X-ray structure (Zhang et al., 2002). To date, some amino acid residues or domains in PEPT1 have been revealed functionally important, and the results in the present study are largely compatible with those earlier findings: 1) Previous studies on PEPT1 and PEPT2 chimeras (Doring et al., 1996) suggested that the large extracellular loop between TM9 and TM10 is not essential for phenotypical characteristics of transporter function. This observation is in good agreement with our results: five cSNPs located on this loop have little influence on transporter function in terms of pH dependence, substrate uptake kinetics, and inhibition specificity. 2) Previous site-directed mutagenesis studies on human PEPT1 have revealed five functionally important conserved residues, which are all located in putative TM domains. Specifically, two histidine residues, i.e., His57 and His122 (Chen et al., 2000), are on TM2 and TM4, respectively; Tyr167 is on TM5 (Bolger et al., 1998; Yeung et al., 1998); Try294 is on TM7, and Glu595 is on TM10 (Bolger et al., 1998). When human PEPT1 was mutated at each of these sites, transport function was lost or significantly diminished. In addition to identification of those functionally important residues in PEPT1, it has been proposed that the N-terminal one-half including TM7, TM8, and TM9 is the region of PEPT1 responsible for proton and substrate recognition (Doring et al., 1996; Fei et al., 1998; Terada et al., 2000), and a recent study with cysteine mutagenesis on PEPT1 revealed that TM5 may be part of the substrate translocation pathway (Kulkarni et al., 2003). Additionally, it has been speculated that the C-terminal region, although not directly involved in substrate recognition and binding, may be important for either protein trafficking or functional regulation (Doring et al., 1996). Located on the membrane boundary of TM10 in C-terminal portion of PEPT1, Pro586 was shown not to be important for protein function in terms of pH-dependent uptake, Gly-Sar affinity, and inhibitor specificity, because P586L behaved similarly to the reference gene with respect to these parameters. However, Pro586 seemed important in determining PEPT1 expression level in the membrane. Coincidentally, the Ala-substituted mutant on another conserved residue in the TM10, Glu595, retained the Kt value similar to the wild-type PEPT1, but it had significantly reduced transport capacity (Vmax) in transfected human embryonic kidney cells (Bolger et al., 1998). Although no experiment was carried out to assess the expression level of E595A, we speculate, in the context of the present study, that the low transport capacity (Vmax) of E595A may be associated with lower incorporation of the protein in the membrane. Together, these results provide supporting evidence for the concept (Doring et al., 1996) regarding the potential role of C-terminal to PEPT1 function, i.e., regulating transporter expression level. Further analyses are required to determine how these residues in the C-terminal region of PEPT1 modulate the expression level of PEPT1.

Although P586L seems to be associated with a decrease in Vmax in transfected HeLa cells, we speculate that the reduction of uptake capacity in vitro may not be consequential in vivo, given that PEPT1 is expressed at a high level along the gastrointestinal tract (Ganapathy and Leibach, 1996). The fact that no mutation identified in the studied subjects that resulted in complete loss of hPEPT1 function may reflect the strong selection and evolutionary pressure on this important mammalian peptide transporter, which has counterparts in bacteria, yeast, fungi, plants, and invertebrate and vertebrate animals. Therefore, the maintenance of appropriate di/tripeptide cellular uptake seems essential for the well being of all living creatures.

In this study, we reported the identification and in vitro functional characterization of human PEPT1 variants. Analyses of these cSNPs revealed that the mutations seem to be
functionally neutral except for P586L, which maintained a similar affinity for Gly-Sar and drug substrates as the other variants but had reduced protein expression in transfected HeLa cells. These data suggest that the C-terminal region of PEPT1 (where Pro residues) seems to be involved in regulation on PEPT1 expression, which is consistent with previous studies. However, the P586L allele seems to be a rare variant (found in one chromosome of 44 subjects in our study, but not found in another larger group of subjects, i.e., 247 individuals in PMT study) and therefore would have minimal impact on oral absorption of PEPT1 drug substrates. In conclusion, human PEPT1 transport functionality seems to be highly conserved, and PEPT1 polymorphisms are not expected to be a significant factor with respect to intersubject variability in oral absorption of its drug substrates.

Acknowledgments

We thank Anne Dantzic for critical reading of the manuscript, Sandra Kirkwood and Michael Flagella for discussion, and Mike Esterman and Xiaoling Xie for technical support.

References


