Human Dipeptide Transporter, hPEPT1, Stably Transfected into Chinese Hamster Ovary Cells

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Purpose. A cDNA encoding the H^+-coupled peptide transporter, hPEPT1, has previously been cloned from human ileum (8). The objective of this study was to establish a stably transfected cell line expressing hPEPT1 in mammalian cell culture.

Methods. The hPEPT1 cDNA was subcloned into an expression vector carrying the CMV promoter and a neomycin resistance gene. This vector, pCDNA3-PEPT1, was transiently transfected into several cell lines to identify those capable of expressing PEPT1 transport function. CHO cells were selected and stably transfected with PEPT1 (CHO-PEPT1). Dipeptide transport activity was measured with ^{3}H-Gly-Sar, in the presence and absence of inhibitors.

Results. The clonal cell line, CHO-PEPT1, displayed high transport activity. Dipeptide transport was sensitive to pH and specific for dipeptides and other small peptides. Peptidomimetic antibiotics, such as cephalexin, were competitors for peptide transport.

Conclusions. The stably transfected cell line, CHO-PEPT1 exhibits enhanced transport over that of cell lines with native expression of PEPT1, and therefore, represents a useful tool for rapid screening of drugs that utilize the peptide transporter in the human intestine for absorption.

KEY WORDS: PEPT1; H^+-coupled transporter; peptides; β-lactam antibiotics; cephalexin; intestinal absorption.

INTRODUCTION

Intestinal membrane transporters play important roles in the utilization of essential nutrients and in drug absorption (1). Small peptides and β-lactam antibiotics were shown to be transported via an H^+-coupled transporter system in small intestines (2–4) and in the human colon adenocarcinoma cell line, Caco-2 (5–6). Recently, a dipeptide transporter, PEPT1, with 12 putative transmembrane domains (TMDs), has been cloned from rabbit and human intestines (7–8). However, a different gene, HPT1, with a single predicted TMD, has been cloned from Caco-2 cells and characterized in stably transfected CHO cells as a putative small peptide transporter (9). Transfection of HPT1 in CHO cells yielded increased uptake of cephalexin and bestatin (9). On the other hand, PEPT1 also induced active transport of small peptides when transiently expressed in HeLa cells using a viral transfection system or when its cRNA was injected to Xenopus laevis oocytes (8). In our experiments, we found detectable levels of dipeptide uptake expressed natively in HeLa and MDCK cells. Because of the different cellular background in which these two genes were expressed, it is difficult to compare transport functions, and the physiological roles of PEPT1 and HPT1 still need to be clarified.

The human colon adenocarcinoma cell, Caco-2, serves as a model system to study intestinal drug absorption in vitro. However, Caco-2 cells express both PEPT1 and HPT1, as well as other transporters such as MCT1 (monocarboxylic acid transporter) and GLUT5 (glucose-fructose transporter) (10–11). It is therefore difficult to distinguish which transporter is responsible for uptake of a test compound. Furthermore, dipeptide transport in these cells is rather low. Moreover, transient transfection of PEPT1 in several mammalian cell lines has yielded only moderate functional expression without the aid of a viral expression system (see results in this study), and a cell line stably expressing PEPT1 had not yet been developed. In the present study, we have established and characterized a clonal CHO cell line stably expressing the human homolog, hPEPT1. This cell line exhibited enhanced transport activities and represents a useful model system for studying peptoid drug transport.

MATERIALS AND METHODS

Materials

Glycyl-[^{3}H]-sarcosine (specific radioactivity, 39 Ci/mmol) was synthesized by Amershan (Arlington Heights, IL). Cephalexin, glycylsarcosine, the dipeptide Ala-Ala, and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Bacterial strains, Top10F′ and DH5α were purchased from Invitrogen (San Diego, CA).

Cell Culture

Chinese hamster ovary cells (CHO) were obtained from American Type Culture Collection and grown in DMEM containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 μg/ml) mixture at 37°C in a humidified atmosphere with 5% CO2. Other cells used in this study included human embryonic kidney cell HEK293, HeLa, monkey Cos-7 and Madin-Darby canine kidney cells MDCK, were also obtained from the American Type Culture Collection and grown in proper media under similar conditions.

Construction of Expression Plasmid Containing the hPEPT1 Gene

The hPEPT1 gene in pBluscript (8) (provided by Dr. F. H. Leibach) was subcloned into an expression plasmid, pCDNA3 carrying the CMV promoter and a neomycin resistance gene (Invitrogen, CA). The EcoRV-NotI fragment containing the hPEPT1 cDNA was excised from pBluscript and ligated into pCDNA3 cut with EcoRV-NotI. In this expression plasmid, pCDNA3-PEPT1, the hPEPT1 cDNA is under the CMV promoter.

Transfection and Selection of Stable Cell Lines

Cells were transfected with pCDNA3-PEPT1 or pCDNA3 vector alone by electroporation. Briefly, 5×10^6 CHO cells were mixed with plasmid DNA (7 μg) and subsequently pulsed at 960 μF capacitance and 0.3 kV. For transient transfection,

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cells were incubated in growth medium for 2 days before analysis of transport. For stable transfection, cells were recovered in growth media containing 300 μg/ml G418. Individual colonies were selected after growth under selection for two weeks. Transport assays were used to select clonal lines with functional transport activity. The cell line displaying the highest transport of ^{3}H-Gly-Sar was selected for further study (CHO-PEPT1). These cells were harvested, and aliquots were frozen for future use. Upon thawing and replating, the CHO-PEPT1 cells can be maintained in culture for up to two months, after which time transport begins to diminish. Depending on the time the cells were kept in culture, and the cell density at which the transport assay was performed, some fluctuation in maximum uptake was observed. The ratio of selective dipeptide transport over nonspecific uptake ranged from 2.5- to 5-fold.

Transport Assay of ^{3}H-Gly-Sar in CHO-PEPT1 Cells

Transport assays were performed as described previously (8). After incubation of 30 minutes, uptake of ^{3}H-Gly-Sar was measured with approximately 10^6 cells in triplicate for each experiment, and at least two independent experiments were performed for each assay. The concentration of ^{3}H-Gly-Sar was 20 μM, 0.1 μCi/ml in 3 mM Hepes/Mes/Tris (pH 6.0) containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The incubation time for uptake was 30 min. In competition assays, the uptake of ^{3}H-Gly-Sar was measured in the presence of unlabeled compounds (10 mM). For inhibition studies, the tracer was incubated together with increasing concentrations of test compounds (0.010, 0.033, 0.1, 0.33, 1.0, 3.3, 10, and 100 mM). The IC_{50} values (*i.e.* concentration of unlabeled compounds to inhibit 50% of the uptake of radiolabeled substrate) were determined from the dose-response inhibition curves. These values were calculated with the Sigma plot program, as described previously (15), using the equation $f = (a - d)/(1 + (x/c)^*b) + d$, where a and d represents the maximum and minimum uptake (in dpm), respectively, x the concentration of inhibitors, and b the slope factor. The slope factors did not significantly deviate from unity and are not reported separately.

RESULTS

Transient Expression of PEPT1 in Mammalian Cells

To select a suitable cell line for analyzing hPEPT1 function, transient expression of PEPT1 was tested in several mammalian cell lines. There is no detectable uptake of tracer in transfected or untransfected HEK293 cells. The uptake of the dipeptide tracer in transfected MDCK and HeLa cells was only marginally higher than that of untransfected cells possibly because of a higher background resulting from endogenous transport activities. Measurable uptake of ^{3}H-Gly-Sar was found in transfected CHO and Cos-7 cells. There was a nearly 2 fold higher uptake of ^{3}H-Gly-Sar in CHO cells transfected with hPEPT1 than in cells transfected with vector DNA alone, and an even better signal to noise ration in Cos-7 cells (data not shown). These results suggest that the acquired ability to accumulate dipeptide tracer in CHO-PEPT1 cells is due to the transfected gene, hPEPT1. Even though transient transfection into Cos-7 and CHO cells was variable, both of these cell lines are nevertheless useful for rapid testing of functional expression of hPEPT1 mutants carrying epitope tags (Covitz et al., unpublished). However, for extensive analysis of peptoid drug transport, a stably transfected cell line is required. Since Cos-7 cells are unsuitable for stable transfection, a stable clone was selected from transfected CHO cells, named CHO-PEPT1.

Peptide Transport Activities in the CHO-PEPT1 Cells

The time course for the accumulation of ^{3}H-Gly-Sar in the newly developed cell line (CHO-PEPT1) stably expressing the hPEPT1 gene is shown in Figure 1. Chinese Hamster Ovary (CHO) cells exhibited a low background level of transport activity, while CHO-PEPT1 cells showed a marked capacity for uptake. Accumulation was linear for the first 30 min. In the presence of unlabeled Gly-Sar (25 mM), this uptake was reduced to that of untransfected CHO cells. The latter was unaffected by the presence of 25 mM Gly-Sar, indicating the absence of saturable uptake in non-transfected CHO cells (not shown). Specific transport activity was 3–5 fold higher than nonspecific transport (Figure 1).

The concentration dependence of the accumulation of ^{3}H-Gly-Sar was measured by adding varying concentrations of unlabeled Gly-Sar. The calculated IC_{50} for Gly-Sar is 0.6 ± 0.3 mM (Table I.).

Effect of pH on Peptide Transport

The dipeptide transport activity in intestine (3, 14) and in Caco-2 cells (5) requires a proton gradient. In order to determine whether PEPT1 mediated transport is proton dependent, the uptake of ^{3}H-Gly-Sar into CHO-PEPT1 cells was measured at pH 5.5, 6.0 or 7.5 (Figure 2). Nonspecific uptake was determined in the presence of unlabeled Gly-Sar (25 mM). Maximal

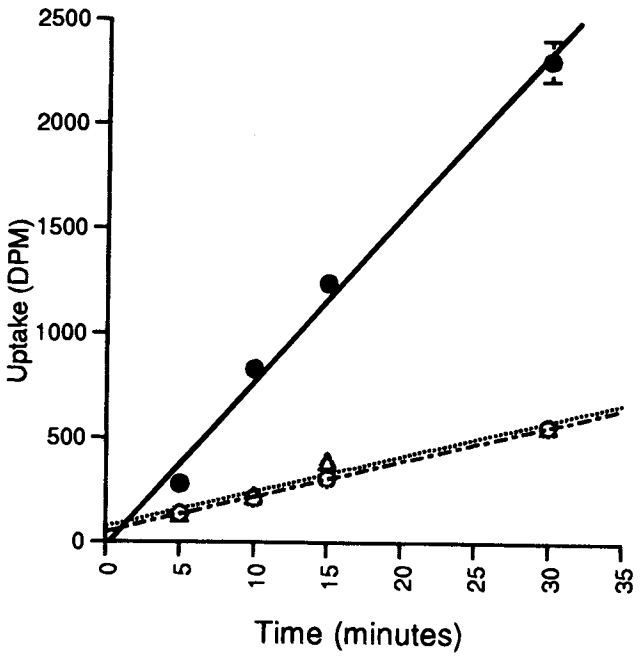

Fig. 1. Transport of glycylsarcosine (Gly-Sar) in CHO-PEPT1 cells. Uptake of ^{3}H-Gly-Sar was measured at pH 6.0 in the presence (○) or absence (●) of 25 mM Gly-Sar. The uptake in untransfected CHO cells at pH 6.0 is also shown in the absence of 25 mM Gly-Sar (△). The lines are fitted linear regression curves.

Table I. Competitive Inhibitors of ^{3}H-Gly-Sar Uptake

Inhibitors	IC_{50}(mean ± SD)
Gly-Sar	0.6 ± 0.3 mM
Ala-Ala	0.063 ± 0.005 mM
Gly-Gly-Gly	1.4 ± 0.7 mM
Cephalexin	5.2 ± 1.1 mM
Ampicillin	10.0 ± 1.5 mM

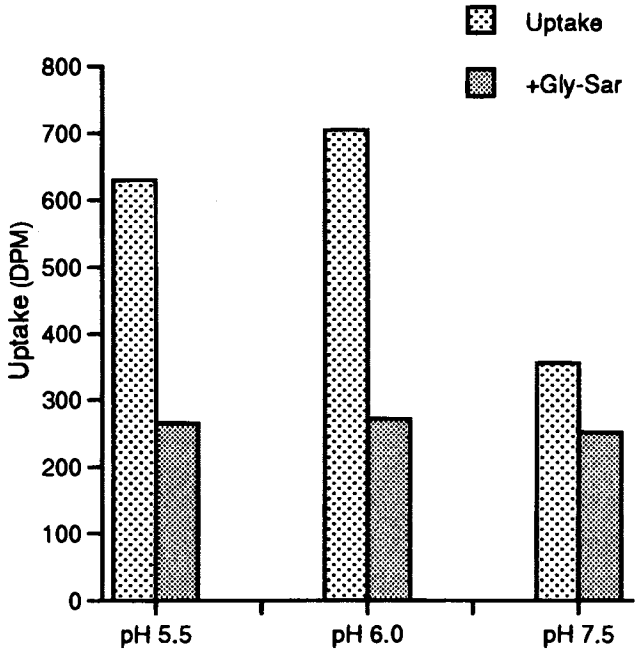

Fig. 2. The pH dependence of the peptide transport in CHO-PEPT1 cells. Uptake of ^{3}H-Gly-Sar was measured after 30 minutes incubation at pH 5.5, pH 6.0 and pH 7.5. Nonspecific accumulation was defined as the level of uptake in the presence of 25 mM Gly-Sar.

uptake occurred at pH 6, consistent with the reported requirement for a proton gradient in PEPT1 mediated transport (3, 8).

Specificity of the Peptide Transport in CHO-PEPT1 Cells

The H^+-coupled dipeptide transport system is selective for dipeptides, tripeptides and some peptide-like compounds, but not single amino acids (5,8). In order to determine if this specificity is maintained in CHO-PEPT1, competition assays were carried out. The uptake of ^{3}H-Gly-Sar in the presence of 10 mM unlabeled compounds was measured (Figure 3). The transport activities were reduced to background level in the presence of the unlabeled dipeptides, Gly-Sar and Ala-Ala, and the tripeptide, Gly-Gly-Gly (Gly3), while transport was only slightly affected by the presence of glycine, sarcosine, or a longer peptide, Gly-Gly-Gly-Gly (Gly4). Inhibition studies were performed with varying concentrations of the dipeptide Ala-Ala, and with the tripeptide Gly-Gly-Gly. Their IC_{50} values are 0.063 ± 0.005 mM and 1.4 ± 0.7 mM, respectively (Table I). These results suggest that the substrate specificity of the PEPT1 transporter is maintained in CHO-PEPT1 cells.

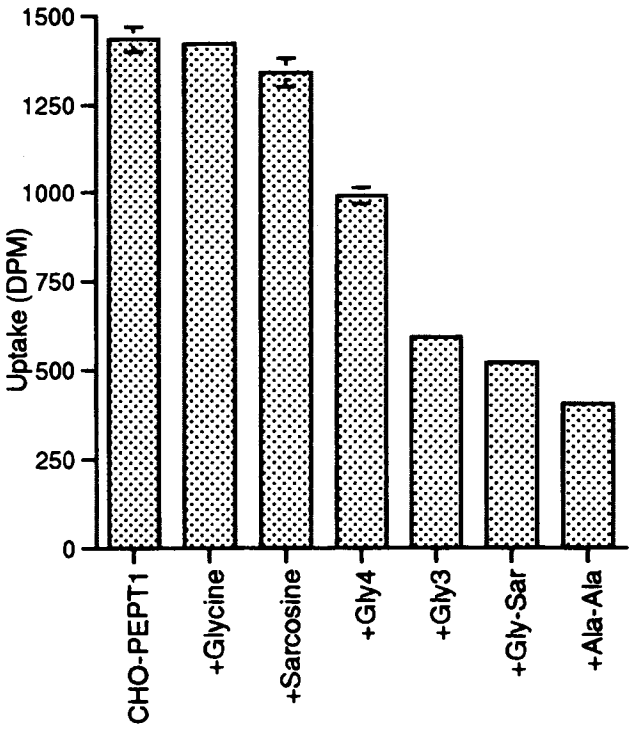

Fig. 3. Selectivity of peptide transport in CHO-PEPT1 cells. Uptake of ^{3}H-Gly-Sar was measured at pH 6.0. Competition assays were performed with different compounds at 10 mM.

Effect of Antibiotic Drugs on Peptide Transport

Peptidomimetic antibiotics, such as cephalexin and β-lactam antibiotics, have been shown to be taken up into Caco-2 cells (6). Also, cephalexin (10 mM) inhibits the uptake of ^{3}H-Gly-Sar in HeLa cells transiently transfected with hPEPT1 using a viral transfection system (8). In order to compare to these previous studies, the uptake of ^{3}H-Gly-Sar in CHO-PEPT1 cells in the presence of cephalexin or ampicillin at various concentrations was measured (Figure 4). The uptake decreased as the concentration of unlabeled cephalexin (Figure 4A) or ampicillin (Figure 4B) increased. The IC_{50} values for cephalexin and ampicillin are 5.2 ± 1.1 mM and 10.0 ± 1.5 mM, respectively (Table I). Because of the low potency of these agents in inhibiting ^{3}H-Gly-Sar uptake, and the additional osmotic pressure at the highest concentration tested (100 mM), nonspecific tracer uptake may have been affected in these experiments; therefore, the IC_{50} estimates should be considered approximations only.

DISCUSSION

We have established a cell line stably transfected with the human PEPT1 cDNA expressing a proposed H^+-coupled dipeptide transporter (8). The level of functional expression permits routine screening of compounds for their ability to interact with the hPEPT1 transporter. This type of stably transfected cell line thus represent a new paradigm for studying drug absorption or targeting, in vitro. In the field of drug metabolism, a similar approach is already being implemented using cell lines stably transfected with the gene encoding enzymes responsible for drug metabolism (12). Hence, the use of a large panel of stably

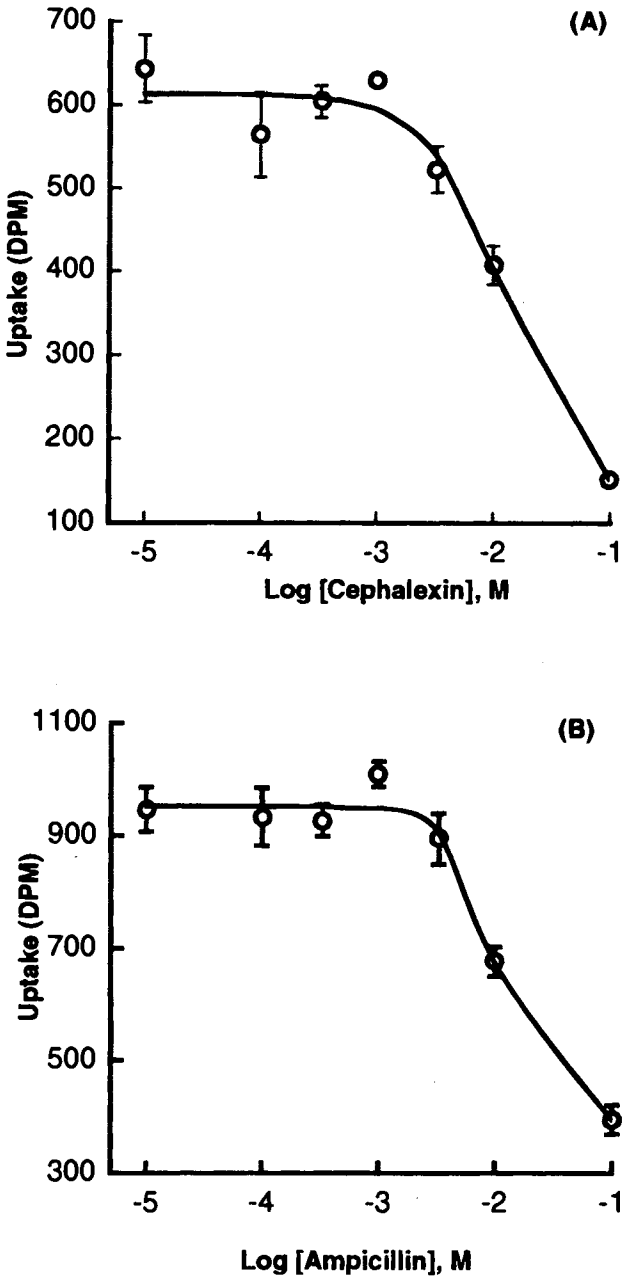

Fig. 4. Inhibition of cephalexin and ampicillin in CHO-PEPT1 cells. Uptake of ^{3}H-Gly-Sar was measured at pH 6.0, in the presence of cephalexin (A) or ampicillin (B), at various concentrations.

transfected cell lines for the study of drug transport and metabolism may become standard in drug design and development.

Our results further help to clarify which dipeptide transporter, PEPT1 or HPT1, is likely to contribute to previously observed uptake results in Caco-2 cells. The substrate specificity in CHO-PEPT1 cells (Figure 3) is consistent with that observed in Caco-2 cells (5). The IC_{50} values of dipeptides and cephalexin, listed in Table I, are in good agreement with their K_m values measured with ^{14}C-cephalexin uptake in Caco-2 cells (6). This finding suggests that the main transporter for dipeptides and peptoid drugs in Caco-2 cells may be encoded by PEPT1 rather than HPT1. However, there are at least two dipeptide carrier system in Caco-2 cells, *i. e.*, a basolateral dipeptide transporter and an apical H^+-coupled dipeptide transporter (13). Direct comparison of hPEPT1 and HPT1 transfected into CHO cells would be required to resolve their roles in peptoid drug absorption. However, additional proteins may also contribute to dipeptide transport in vivo.

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