Recognition of β-lactam antibiotics by rat peptide transporters, PEPT1 and PEPT2, in LLC-PK₁ cells

TOMOHIRO TERADA, HIDEYUKI SAITO, MAYUMI MUKAI, AND KEN-ICHI INUI
Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan

Recognition of β-lactam antibiotics by rat peptide transporters, PEPT1 and PEPT2, in LLC-PK₁ cells. Am. J. Physiol. 273 (Renal Physiol. 42): F706–F711, 1997.—PEPT1 and PEPT2 are H⁺-coupled peptide transporters expressed preferentially in the intestine and kidney, respectively, which mediate uphill transport of oligopeptides and peptide-like drugs such as β-lactam antibiotics. In the present study, we have compared the recognition of β-lactam antibiotics by LLC-PK₁ cells stably transfected with PEPT1 or PEPT2 cDNA. Cephalosporin (anionic cephalosporin without an α-amino group) showed potent inhibitory effects on the glycylsarcosine uptake in the PEPT1-expressing cells. Other β-lactams, such as cephalaxin, cefadroxil, and cephradine (aminocephalosporins), inhibited modestly the PEPT1-mediated glycylsarcosine uptake. Except for cephalaxin, these β-lactams showed much more potent inhibitions on the glycylsarcosine uptake via PEPT2 than via PEPT1. Comparison of the inhibition constant (Kᵢ) values between cefadroxil and cephalaxin suggested that the hydroxyl group at the NH₂-terminal phenyl ring increased affinity for both PEPT1 and PEPT2. It is concluded that PEPT2 has a much higher affinity for β-lactam antibiotics having an α-amino group than PEPT1 and that substituents at the NH₂-terminal side chain of these drugs are involved in the recognition by both peptide transporters.

PEPTIDE TRANSPORTERS contribute to the maintenance of protein nutrition, mediating the efficient absorption of protein digestive products in the small intestine and kidney (8, 9). Intestinal peptide transporter has also been indicated to recognize a broad range of peptide-like drugs, such as orally active β-lactam antibiotics (19, 20, 28), anti-cancer agent, Bestatin (10, 22), and angiotensin converting enzyme inhibitors (26), thereby having an affinity for both PEPT1 and PEPT2. It is concluded that historical residues at position 57 and 121 of rat PEPT1 for its activity (29). By using chemical modification and site-directed point mutation techniques, we demonstrated that histidine residues at position 57 and 121 of rat PEPT1 mediate H⁺-gradient-dependent transport of differently charged β-lactam antibiotics by measuring uptake in Xenopus oocytes (23) and in the stably transfected LLC-PK₁ cells (30). When expressed in Xenopus oocytes, both rat PEPT1 and PEPT2 stimulated the uptake of Bestatin, a dipeptide-like antineoplastic drug (24). More recently, we have identified essential residues of rat PEPT1 for its activity (29). To understand the structure-function relationships and multispecificities of the peptide transporters, we have established the LLC-PK₁ cells stably expressing rat PEPT2 and compared recognition of β-lactam antibiotics by using rat PEPT1- and PEPT2-expressing transfectants.

MATERIALS AND METHODS

Cell culture and transfection. The parental LLC-PK₁ cells obtained from the American Type Culture Collection (CRL-1392) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (GIBCO Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD) without antibiotics in an atmosphere of 5% CO₂-95% air at 37°C (25). The clonal LLC-rPEPT1 cells were used as described previously (29, 30). In the case of LLC-rPEPT2 cells, a cDNA encoding rat PEPT2 was subcloned into the Sal I- and Not I-cut mammalian expression vector pBK-CMV (Stratagene, La Jolla, CA) and transfected into LLC-PK₁ cells by the CaPO₄ coprecipitation method (29, 30). G-418 (1 mg/ml)-resistant cells were picked up, and PEPT2-expressing cells (LLC-rPEPT2) were selected by measuring [¹⁴C]glycylsarcosine transport activity.

Uptake studies by cell monolayers. Uptake of [¹⁴C]glycylsarcosine was measured in cells grown on 60-mm plastic dishes as described previously (29). The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 CaCl₂, 0.5 Mglycine, 5 glucose, and 5.2-N-(methylamino)ethanesulfonic acid (pH 6.0) or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4). The pH of the medium was adjusted with a solution of NaOH or HCl. The cells were preincubated for 10 min at 37°C with 2 ml of the incubation medium. After the medium was removed, the cells were incubated with 2 ml of incubation medium (pH 6.0) containing [¹⁴C]glycylsarcosine (20 µM, 37 kBq/ml) for 15 min at
After incubation, the medium was aspirated, and the cells were rapidly rinsed twice with 5 ml of ice-cold incubation medium. The cells were scraped with a rubber policeman into 1 ml of 1 N NaOH, and the cell-associated radioactivity was determined in ACS II (Amersham International, Buckinghamshire, UK) by liquid scintillation counting. The protein content of cell monolayers solubilized in 1 N NaOH was determined by the method of Bradford (2), using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with bovine $\gamma$-globulin as the standard.

Materials. Amoxicillin, cefdinir, and cefixime (Fujiwasa Pharmaceutical, Osaka, Japan), cefditoren and cephalixin (Shionogi, Osaka), cefadroxil (Bristol Meyers, Tokyo, Japan), cephalexin (Sanko, Tokyo), cyclacillin (Takeda Chemical Industries, Osaka), and Bestatin [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine; Nippon Kayaku, Tokyo] were gifts from the respective suppliers. [14C]glycylsarcosine (1.78 GBq/mmol) was obtained from Daiichi Pure Chemicals (Ibaraki, Japan). Glycysarcosine and ampicillin were obtained from Sigma Chemical (St. Louis, MO). All other chemicals used were of the highest purity available. Figure 1 shows the general molecular structures of penicillin and cephalosporin antibiotics.

RESULTS

Characteristics of glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. The pBK-CMV expression vector with the rat PEPT2 cDNA inserted was transfected into LLC-PK1 cells, and approximately 50 G-418-resistant cells were picked up and examined for [14C]glycylsarcosine transport activity. A clone with the highest transport activity was selected (LLC-rPEPT2 cells) and used for further characterization of rat PEPT2. First, we examined the time course of [14C]glycylsarcosine uptake at pH 6.0 by LLC-rPEPT1 (rat PEPT1-expressing LLC-PK1 cells) and LLC-rPEPT2 cells. As shown in Fig. 2, the rate of glycylsarcosine uptake in LLC-rPEPT1 cells was much greater than that in LLC-rPEPT2 cells. On the other hand, the uptake of glycylsarcosine by the parental LLC-PK1 cells transfected with control vector (LLC-pBK cells) was negligible compared with that by LLC-rPEPT1 and LLC-rPEPT2 cells.

Figure 3 shows effects of the medium pH on glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. In LLC-rPEPT1 cells, glycylsarcosine uptake was maximal at pH 5.5–6.0, whereas it was maximal at pH 6.5–7.0 in LLC-rPEPT2 cells, suggesting that LLC-rPEPT1 cells prefer a more acidic pH region to transport glycylsarcosine than LLC-rPEPT2 cells.

Inhibition of glycylsarcosine uptake by various $\beta$-lactam antibiotics. To compare substrate specificity of PEPT1 and PEPT2, the ability of several $\beta$-lactam antibiotics to inhibit the uptake of glycylsarcosine was examined. Figure 4 shows dose-dependent inhibition of glycylsarcosine uptake by several $\beta$-lactam antibiotics in the LLC-rPEPT1 and LLC-rPEPT2 cells. The PEPT1-mediated glycylsarcosine uptake was inhibited by cephadroxil (aminopenicillin) > ceftibuten (anionic cephalosporin without an $\alpha$-amino group) > cefadroxil (aminopenicillin) in the order of inhibitory potency. Ampicillin (aminopenicillin) had much less effect on the uptake. In contrast, the PEPT2-mediated glycylsarcosine uptake was suppressed by cefadroxil > cephadroxil > ampicillin = ceftibuten, in the order of inhibition. The inhibition patterns of these antibiotics were apparently different between LLC-rPEPT1 and LLC-rPEPT2 cells, suggesting that PEPT2 had a much higher affinity for $\beta$-lactams except for ceftibuten.

Therefore, we further examined the inhibitory effects of other oral $\beta$-lactam antibiotics on the glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells.
uptake via these transporters, and the inhibition constant (\(K_i\)) values for the competitors were estimated by nonlinear regression analysis of the competition curves with one component. The chemical structures and \(K_i\) values for the antibiotics to compete with glycylsarcosine uptake are summarized in Table 1. The \(K_i\) values suggested that the affinities of PEPT1 for the antibiotics were in the following order: cefdinir > cefradin > cefixime > cephalexin > amoxicillin > ampicillin. On the other hand, the affinities of PEPT2 were in the following order: cefradin > cefixime > cephalexin > amoxicillin > ampicillin > cefditabten > cefdinir. The \(K_i\) values of cefdinir (monocarboxylic cephalosporin) for PEPT1 and PEPT2 suggested that cefdinir had low affinities for both transporters compared with other cephalosporins examined. Except for ceftibuten and cefdinir, these aminopenicillins and aminoccephalosporins showed much more potent inhibitions on the glycylsarcosine uptake via PEPT2 than via PEPT1 (\(P < 0.05\) by the Mann-Whitney U test). Notably, the \(K_i\) value of cefadroxil, an aminoccephalosporin with a hydroxyl group at the phenyl ring of cephalexin, on the glycylsarcosine uptake via both PEPT1 and PEPT2 was much smaller than that of cephalexin. Another peptide-like drug, Bestatin, an antineoplastic drug possessing a \(\beta\)-amino group, had potent inhibitory effects on the dipeptide uptake via both PEPT1 and PEPT2. The \(K_i\) values of Bestatin were 505 \(\mu\)M for PEPT1 and 20 \(\mu\)M for PEPT2.

**DISCUSSION**

Studies using isolated membrane vesicles have shown that the uptake of oligopeptides and \(\beta\)-lactam antibiotics in renal brush-border membranes is mediated by at least two distinct transport systems, i.e., the high-affinity/low-capacity and the low-affinity/high-capacity...
peptide transporters (4, 19). In contrast, the peptide transport studies with intestinal brush-border membrane vesicles have suggested that a single peptide transporter contributes to translocate the oligopeptides (15). Most recently, two distinct but homologous oligopeptide transporters, PEPT1 of human (12), rabbit (5), and rat (23) and PEPT2 of human (13), rabbit (1), and rat (24), have been identified by a cDNA cloning technique. The rat PEPT1 mRNA was expressed in both the small intestine and kidney (23), whereas rat PEPT2 mRNA was expressed abundantly in the kidney but not in the small intestine (24). Therefore, both PEPT1 and PEPT2 are coexpressed in the kidney, suggesting that the two transporters contribute to tubular reabsorption of oligopeptides. However, substrate specificities of the two peptide transporters have not yet been fully characterized.

In the present study, we compared the apparent $K_m$ values of glycylsarcosine uptake by the rat PEPT1- and PEPT2-expressing transfectants and affirmed that PEPT2 is the high-affinity type transporter, whereas PEPT1 is the low-affinity type transporter in the kidney. Daniel et al. (4) reported the existence of two types of oligopeptide transport systems in the brush-border membranes isolated from rat kidney. Therefore, it seems reasonable to consider that the two distinct peptide transport systems in the renal brush-border membranes are mediated via the PEPT1 and PEPT2. The differences in the substrate affinity and in the pH dependence (Fig. 3) of PEPT1 and PEPT2 for glycylsarcosine transport may suggest that these transporters have the distinct roles in tubular reabsorption of oligopeptides. The physiological and pharmacological roles of both transporters should be clarified by determining their localizations along the nephron.

Table 1. Configurations and inhibition constants for β-lactam antibiotics

<table>
<thead>
<tr>
<th>Drug</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>PEPT1</th>
<th>PEPT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
<td>47,800</td>
<td>669</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td></td>
<td></td>
<td>13,000</td>
<td>179</td>
</tr>
<tr>
<td>Cydacidil</td>
<td></td>
<td></td>
<td>168</td>
<td>27</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>CH₃</td>
<td></td>
<td>4,500</td>
<td>49</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>CH</td>
<td></td>
<td>2,170</td>
<td>3</td>
</tr>
<tr>
<td>Cephradine</td>
<td>CH₃</td>
<td></td>
<td>8,540</td>
<td>47</td>
</tr>
<tr>
<td>Cefdinir</td>
<td>CH=CH₂</td>
<td></td>
<td>11,900</td>
<td>20,100</td>
</tr>
<tr>
<td>Cefitubutin</td>
<td>H</td>
<td></td>
<td>597</td>
<td>1,340</td>
</tr>
<tr>
<td>Cefixime</td>
<td>CH=CH₂</td>
<td></td>
<td>6,920</td>
<td>11,900</td>
</tr>
</tbody>
</table>

Each value is mean of 2 experiments. Chemical structures illustrated as $R_1$ and $R_2$ represent substituents in penicillin or cephalosporin shown in Fig. 1. LLC-rPEPT1 and LLC-rPEPT2 cells were incubated for 15 min at 37°C with incubation medium containing $[^{14}C]$glycylsarcosine (20 µM, 37 kBq/ml) in the presence of each competitor. Thereafter, the radioactivity of solubilized cells was determined. The apparent inhibition constant ($K_i$) values were calculated from the inhibition plots based on the transformed Michaelis-Menten equation using nonlinear least square regression analysis.
Importantly, the present findings revealed that rat PEPT1 and PEPT2 had different characteristics not only in substrate affinity for native dipeptide but also in recognition of a variety of peptide-like drugs. Various β-lactam antibiotics showed the different inhibitory potencies against the glycylsarcosine uptake between rat PEPT1 and PEPT2. Ganapathy et al. (6) evaluated the K_i values of cefadroxil (aminocephalosporin) and cyclacillin (aminopenicillin) for glycylsarcosine uptake by Caco-2 cells (human adenocarcinoma cell line expressing human PEPT1), SKPT cells (rat kidney-derived cell line expressing PEPT2), and HeLa cells transfected transiently with human PEPT1 or PEPT2 cDNA. They suggested the differential recognition of these antibiotics by the two peptide transporters; PEPT1 had a much higher affinity for cyclacillin than for cefadroxil, whereas PEPT2 preferred cefadroxil to cyclacillin. The inhibition patterns for these antibiotics observed by Ganapathy et al. (6) agreed with our findings for rat PEPT1 and PEPT2. In addition, it should be emphasized that affinities of aminopenicillins and aminocephalosporins for rat PEPT1 in this study closely correlated with the absorption of these antibiotics from in situ rat small intestinal loops (31).

Among oral β-lactam antibiotics, there are some cephalosporins without an α-amino group, such as ceftibuten, cefixime, and cefdinir. Boll et al. (1) reported that β-lactam antibiotics without an α-amino group appeared not to be transported by rabbit PEPT2; in other words, the α-amino group of substrates was required to be recognized by rabbit PEPT2. In contrast, it was demonstrated that ceftibuten uptake by the rat renal brush-border membrane vesicles was mediated via two peptide transport systems (19), probably by PEPT1 and PEPT2. We also reported previously that ceftibuten and cefixime were transported by human PEPT1 in Caco-2 cells (14) and by rat PEPT1 expressed in oocytes (23) and in transfected cells (30) with relatively high affinities. Therefore, it has been controversial as to how PEPT1 and PEPT2 are involved in the renal transport of the cephalosporins without an α-amino group. Interestingly, we found in the present study that ceftibuten and cefixime inhibited the glycylsarcosine uptake both via rat PEPT1 and via rat PEPT2, suggesting that both antibiotics were recognized not only by PEPT1 but also by PEPT2. In addition, those drugs were twofold more potent in inhibiting the glycylsarcosine uptake via rat PEPT1 than via rat PEPT2, suggesting that β-lactam antibiotics without an α-amino group had low affinity for the rat PEPT2.

Daniel et al. (3) previously reported that increasing the hydrophobicity of the NH2-terminal side chain increased the affinities of aminocephalosporins and aminopenicillins to the renal H+/oligopeptide cotransporter. Because cyclacillin with a very hydrophobic NH2-terminal side chain showed relatively higher affinities to the PEPT1 and PEPT2 than other β-lactams used, the hydrophobicity of the NH2-terminal side chain was suggested to contribute to the interaction of β-lactams to the peptide transporters (Fig. 5; Table 1).

In comparing the K_i values for cefadroxil and cephalixin or for amoxicillin and ampicillin, we found that cefadroxil and amoxicillin had much higher affinities for both PEPT1 and PEPT2 than did cephalixin and ampicillin, respectively. Cefadroxil and cephalixin (aminocephalosporins) or amoxicillin and ampicillin (aminopenicillins) have very similar structures; the former two have a p-hydroxyphenyl group, and the latter two have a phenyl group at the side chain in the position 7 or 6 of cephalosporin and penicillin structure, respectively. Because the p-hydroxyphenyl group is much less hydrophobic than the phenyl group, the hydroxyphenyl group at the NH2-terminal side chain of some β-lactams would make a stronger interaction of the peptide transporter than hydrophobic interaction.

In conclusion, the present findings suggest that rat PEPT2 has a much higher affinity for β-lactam antibiotics with an α-amino group than rat PEPT1 and that not only hydrophobicity but also the hydroxyphenyl group at the side chain of these antibiotics is involved in substrate recognition by both rat PEPT1 and PEPT2. Further studies on mechanisms of drug recognition by stably transfected cells expressing rat PEPT1 and PEPT2 will provide useful information for drug design and delivery system to improve the efficiency of chemotherapy.

This work was supported in part by a Grant-in-Aid for Scientific Research (B) and a Grant-in-Aid for Scientific Research on Priority Areas of “Channel-Transporter Correlation” from the Ministry of Education, Science, and Culture of Japan, and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research. Address for reprint requests: K. Inui, Dept. of Pharmacy, Kyoto Univ. Hospital, Sakyo-ku, Kyoto 606-01, Japan.

Received 24 February 1997; accepted in final form 19 June 1997.

REFERENCES


