Direct Evidence for Efficient Transport and Minimal Metabolism of L-Cephalexin by Oligopeptide Transporter 1 in Budded Baculovirus Fraction

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The oligopeptide transporter PEPT1 (*SLC15A1*) is responsible for absorption of peptidic nutrients in the small intestine. Although the L-diastereomer of the β-lactam antibiotic cephalexin (L-cephalexin) is likely to be transported by PEPT1, there has been no direct demonstration of PEPT1-mediated L-cephalexin transport. Indeed, after the incubation with L-cephalexin, the intact form of L-cephalexin has not been identified inside vesicles/proteoliposomes prepared from brush border membrane of intestinal epithelial cells or cultured cell lines exogenously transfected with PEPT1 gene. Thus, it appears that L-cephalexin is rapidly metabolized by PEPT1 or PEPT1-associated proteins. Here, we attempted to verify whether L-cephalexin is transported by PEPT1 and whether it is hydrolyzed by PEPT1 itself, by using budded baculovirus expressing PEPT1 protein. Marked uptake of L-cephalexin in PEPT1-expressing budded baculovirus, compared with wild-type virus, indicated that L-cephalexin is a substrate for PEPT1. The uptake was found to be pH sensitive, and was strongly inhibited by the D-diastereomer of cephalexin and glycylsarcosine, but not by glycine. Thus, L-cephalexin is transported by PEPT1 itself. Upon the transport of both L- and D-cephalexin by PEPT1, dose-dependent membrane depolarization was observed; the EC_{50} values of 0.18 and 2.9 mM, respectively, indicate that the affinity of L-cephalexin for PEPT1-mediated transport is much higher than that of the D-diastereomer. On the other hand, the L-cephalexin metabolite 7-aminodesacetoxycephalosporanic acid was not detected in PEPT1-expressing or wild-type virus at either pH 6.0 or 7.4. We conclude that L-cephalexin is transported by PEPT1 with high affinity, but is not metabolized by PEPT1 itself.

Key words oligopeptide transporter; cephalexin; budded baculovirus

The oligopeptide transporter PEPT1 (*SLC15A1*) accepts di- and tripeptides as endogenous substrates[1,2] and also transports various peptide-mimetic therapeutic agents (*e.g.*, cephalexin).[3] PEPT1 is primarily expressed in apical membrane of small intestinal epithelial cells.[4] PEPT1 is thought to be involved in gastrointestinal absorption of its substrates. Involvement of PEPT1 may explain the difference in bioavailability between valacyclovir (PEPT1 substrate) and acyclovir (non-substrate) in humans.[5,6] We have recently reported that *rab8*-null mice, which exhibit minimal expression of PEPT1 on apical membranes of small intestine, lack saturable uptake of a prototypical PEPT1 substrate glycylsarcosine (Gly-Sar).[7] This may indicate a predominant role of PEPT1 in the peptide absorption. In addition to small intestine, PEPT1 is functionally expressed in some human cancer cell lines[8—10] and therefore would be a promising target for tumor detection. Indeed, a positron emitting probe targeted to PEPT1, ^{11}C-glycylsarcosine, was recently demonstrated to be able to distinguish between tumor and inflamed tissues.[11]

The β-lactam antibiotic cephalexin ((6*R*,7*R*)-7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; D-cephalexin) is a typical substrate of PEPT1.[12—14] This D-cephalexin is now clinically used for chemotherapy. PEPT1 is presumably responsible for membrane permeation of D-cephalexin in small intestine, because small intestinal uptake of D-cephalexin assessed with an everted sac method was greatly reduced in *rab8*-null mice.[15] The L-diastereomer of cephalexin (L-cephalexin) is also probably transported by PEPT1 in Caco-2 cells.[16] However, intact L-cephalexin was not found in vesicles or proteoliposomes prepared from brush border membrane of intestinal epithelial cells.[17] Although, L-cephalexin was not detected, and the hydrolysis product, 7-aminodesacetoxycephalosporanic acid (7-ADCA), appeared very rapidly in serum and urine after oral administration in rats.[18] This observation suggests that L-cephalexin can be absorbed from gastrointestinal tract. However, there has been no direct demonstration that L-cephalexin is transported by PEPT1 protein.

We recently proposed that orally administered L-cephalexin might be transported and metabolized by PEPT1.[13] Indeed, hydrolysis of L-cephalexin was markedly increased by exogenous transfection of PEPT1 gene into cultured cell lines, and metabolism of cephalexin was observed even in membrane-permeabilized cells expressing PEPT1, in which intracellular accumulation of L-cephalexin was negligible.[13] This observation may imply that L-cephalexin hydrolysis is mediated by PEPT1 itself, although possible involvement of endogenous peptidases cannot be excluded, because these experimental systems utilized mammalian cells.

The purpose of the present study was to clarify whether or not PEPT1 transports and/or metabolizes L-cephalexin. We performed L-cephalexin metabolism and uptake experiments using a highly purified PETP1 protein-expressing system, budded baculovirus[19] which lacks endogenous mammalian metabolic enzymes.

MATERIALS AND METHODS

Materials The L-diastereoisomer of cephalexin was ob-

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tained from Nippon Bulk Yakuhin Co., Ltd. (Osaka, Japan). The D-stereoisomer of cephalexin, (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, and 7-ADCA, 7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Budded Baculovirus The budded baculovirus fraction was prepared as reported previously[19] from the supernatant after 72-h or 96-h infection at MOI 5 or 1 for the PEPT1-expressing or wild-type system, respectively. Protein concentration was determined by DC Protein Assay Reagent (Bio-Rad, Hercules, CA, U.S.A.) using bovine serum albumin as a standard.

Metabolism Assay The reaction (37 °C) was initiated by adding L-cephalexin to baculovirus (50 μg protein) suspended in assay buffer to give a final concentration of 1 mM. The assay buffer was 25 mM MES/Tris (≤pH 6.5) or HEPES/Tris (>pH 6.5) containing (mM) NaCl (140), KCl (5.4), $CaCl_2$ (1.8), $MgCl_2$ (0.8), and D-glucose (5). The reaction was terminated by adding ice-cold acetonitrile containing 10 μM cefslodine (Sigma-Aldrich) as an internal standard.

Uptake Assay Uptake was assessed by a rapid filtration technique as described previously[19] in the assay buffer containing budded baculovirus (50 μg protein) and 1 mM L-cephalexin at 37 °C.

Quantification of 7-ADCA and L-Cephalexin The HPLC system used for quantification of 7-ADCA and L-cephalexin was previously described.[13] Hydrophilic Interaction Chromatography (HILIC) packed column, COSMOSIL HILIC 4.6 mm I.D.×250 mm (Nacalai Tesque, Inc., Kyoto Japan) was used as an analytical column, and the UV detector was set at 260 nm. The mobile phase was 10 mM ammonium acetate/acetonitril (30 : 70, v/v). The flow rate was 1.0 ml/min. The limits of quantification for 7-ADCA and L-cephalexin were 0.3 and 0.2 ng/ml, and the calibration curves were linear from 0.3 to 20 (R^2=0.995) and from 0.2 to 30 ng/ml (R^2=0.997), respectively.

Membrane Potential Assay Measurements of membrane potentials were performed as previously described[20] using a FlexStation II (Nihon Molecular Devices, Osaka, Japan). Membrane potential changes were tracked with a voltage-sensitive fluorescent indicator, membrane potential assay kit, blue (Nihon Molecular Devices). The dye solution reconstituted in HBSS (60 μl) was first added to wells, and mixed with the cells and incubated for 30—60 min at 37 °C. Assays were then carried out at 37 °C and were initiated by the addition of 60 μl of test compound. Fluorescence was measured every 2 s for 120 s, using wavelengths of 530 (excitation) and 565 nm (emission). Raw fluorescence data were analyzed with SoftMax Pro 5.0.1 (Nihon Molecular Devices). The EC_{50} values (concentration at half-maximum fluorescence response) were obtained using KaleidaGraph (Synergy Software, Reading, PA, U.S.A.) by fitting the data to a four-parameter logistic, in which the baseline response (Fl_{base}), the maximum response (Fl_{max}), the slope (S), and EC_{50} are related by the equation $y=(Fl_{base}-Fl_{max})/[1+(x/EC_{50})^S]+Fl_{max}$.

Statistical Analysis Statistical comparisons were performed by means of ANOVA with Dunnett *post hoc* tests using KaleidaGraph or by use of an unpaired 2-tailed Student's *t*-test.

RESULTS AND DISCUSSION

To verify whether L-cephalexin is transported and hydrolyzed by PEPT1, we used budded baculovirus expressing only PEPT1 protein and viral envelope protein gp64, the major coat protein of the budded virion.[19] Whether or not L-cephalexin is transported by PEPT1 has been controversial. One group reported that L-cephalexin was not transported by peptide transporter(s),[17] whereas another group observed substrate-induced intracellular acidification caused by transport of L-cephalexin *via* a peptide transporter, possibly PEPT1, though they did not detect L-cephalexin itself.[16] L-Cephalexin was not detected in membrane vesicles, proteoliposomes or intact cell lines endogenously or exogenously expressing PEPT1.[13,16,17] These observations and the fact that a metabolite of L-cephalexin, 7-ADCA, was detected immediately after oral dosing[18] imply that PEPT1 itself may mediate L-cephalexin hydrolysis, although to date, only we[13] have suggested that hydrolytic enzyme activity may be associated with PEPT1. In the present study using budded baculovirus, which expresses PEPT1, but not any endogenous mammalian enzyme, PEPT1-mediated transport of L-cephalexin was clearly observed, since substantial amount of intact L-cephalexin was detected in PEPT1-expressing budded baculovirus (Fig. 1A). The uptake of L-cephalexin by wild-type virus was much smaller (Fig. 1A). The formation of 7-ADCA in wild-type virus was below the detection limit, suggesting that hydrolase contamination in the virus was minimal. The uptake of L-cephalexin in PEPT1-expressing budded baculovirus was higher at pH 6.0 than at pH 7.4 (Fig. 1B), suggesting that L-cephalexin transport may be proton-driven. The uptake of L-cephalexin in the presence of D-cephalexin (5 mM), or the prototypical PEPT1 substrate glycylsarcosine (20 mM) or the non-substrate amino acid glycine (20 mM), assessed at 37 °C and pH of 6.0, amounted to 3.02±0.24, 1.09±0.13% and 86.0±7.3% of the control, respectively (mean±S.E., n=3). Thus, the uptake of L-cephalexin was markedly inhibited by D-cephalexin and gly-

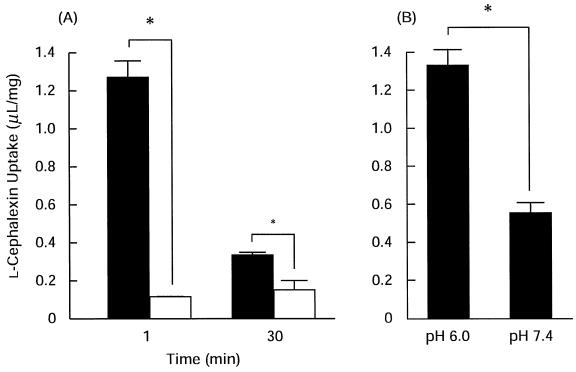

Fig. 1. Transport of L-Cephalexin in PEPT1-Expressing Budded Baculovirus

(A) Time-dependent uptake of L-cephalexin in PEPT1-expressing (●) or wild-type (○) budded baculovirus. (B) pH dependence of L-cephalexin uptake in PEPT1-expressing virus. In each panel, 50 μg protein of baculovirus was incubated with 1 mM L-cephalexin. Each point represents the mean±S.E. (n=3). *p<0.05 *vs.* wild-type (A) or pH 7.4 (B).

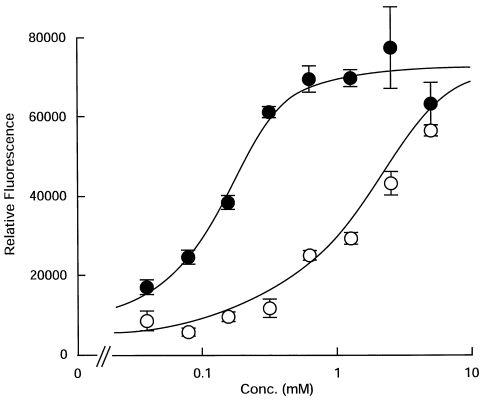

Fig. 2. Membrane Potential Assay Indicating High-Affinity Transport of L-Cephalexin

The peak fluorescence intensity normalized to the baseline was plotted against concentration of L-(●) or D-cephalexin (○). Data were fitted to a four-parameter nonlinear dose–response curve with four parameters. Each point represents the mean±S.E. (n=3).

cylsarcosine, but not glycine. These findings indicate that L-cephalexin is indeed transported by PEPT1. To examine the affinity of L-cephalexin for PEPT1, we carried out membrane potential assay using FlexStation II in HeLa cells stably expressing PEPT1, because this methodology is more sensitive than the detection of uptake by means of HPLC-UV. Upon PEPT1 activation with L-cephalexin, dose-dependent membrane depolarization was observed with an EC_{50} of 178±32 μM (Fig. 2). Because the EC_{50} of D-cephalexin (2.87±3.15 mM) was much larger than that of L-cephalexin, it is clear that L-cephalexin, which resembles natural dipeptides (L-isoform), has much higher affinity for PEPT1 than dose D-cephalexin.

We next examined the metabolic activity of PEPT1 using the same budded virus. Unexpectedly, 7-ADCA, a metabolite of L-cephalexin, was not detected in PEPT1-expressing budded virus or wild-type virus at pH 6.0 or 7.4 during incubation for up to 120 min (≤0.015 pmol/μg protein/min). The limit of quantification for 7-ADCA was similar to that for L-cephalexin (see Materials and Methods). Nevertheless, strong L-cephalexin uptake was detected (Fig. 1), whereas formation of 7-ADCA was below detection limit. Thus, it can be concluded that L-cephalexin is not hydrolyzed by PEPT1 alone. However, the present finding does not exclude the possibility that PEPT1 itself can hydrolyze L-cephalexin, but requires additional cofactor(s). In addition, it has recently been demonstrated in mouse small intestine that PEPT1 physically interacts with PDZ (PSD95/Dlg/ZO1) domain-containing scaffold protein PDZK1, which increases the transport activity of PEPT1 and other solute carrier transporters.[21,22] PDZK1 interacts with various intracellular and membranous proteins which have the so-called PDZ interacting motif at their extreme carboxyl terminus.[21] Therefore, it is considered that in *in vivo* and *in vitro* cultured cell lines, PEPT1 protein may be strongly linked with endogenous hydrolase(s) *via* such a scaffold protein like PDZK1, and di- and tripeptides taken up by PEPT1 would be efficiently hydrolyzed by this enzyme.

In conclusion, L-cephalexin is transported more effectively than D-cephalexin by PEPT1, but is not metabolized by PEPT1 alone. The present findings imply that drug design to improve gastrointestinal absorption *via* PEPT1 may also be applicable with a prodrug strategy to deliver active metabolites into the systemic circulation.

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