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Identification of the histidine residues involved in substrate recognition by a rat H^+/peptide cotransporter, PEPT1

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Abstract The LLC-PK_1 cells stably transfected with a rat PEPT1 cDNA transported ceftibuten (anion) and cephradine (zwitterion), both oral β-lactam antibiotics, in a H^+-gradient-dependent manner. Diethylpyrocarbonate, a histidine residue modifier, abolished ceftibuten uptake. This inhibition was prevented in the presence of glycylsarcosine or cephradine. When expressed in *Xenopus* oocytes, replacement of either histidine 57 or histidine 121 of the rat PEPT1 with glutamine by site-directed mutagenesis eliminated ceftibuten and [^{14}C]glycylsarcosine transport activities. Immunostaining of oocyte sections indicated that insertion of the mutant transporters in the plasma membranes was not impaired. These findings suggest that both histidine 57 and histidine 121, which are conserved in the rat, rabbit and human PEPT1, are involved in substrate recognition of this molecule.

Key words: Proton-coupled peptide transporter; Intestinal absorption; β-Lactam antibiotics; Mutagenesis; *Xenopus* oocyte; Rat intestine

1. Introduction

Absorption of oligopeptides in the intestinal epithelial cells are mediated by a peptide transport system localized in the brush-border membranes [1,2]. Using purified intestinal brush-border membrane vesicles, the peptide transport system was suggested to be an electrogenic and H^+-gradient-driven cotransporter [3,4]. Interestingly, the peptide transporter has been suggested to serve as a carrier protein for intestinal absorption of a broad range of pharmacologically active peptide-like drugs. For example, oral β-lactam antibiotics [5–12], the antineoplastic agent bestatin [13] and angiotensin-converting enzyme inhibitors [14,15] are recognized by the intestinal peptide transporter.

Recently, the H^+-coupled oligopeptide transporter PEPT1 has been cloned from rabbit [16] and human [17]. Ganapathy et al. [18] reported differential recognition of β-lactam antibiotics by human PEPT1 and PEPT2, another member of H^+/peptide transporter family specifically expressed in the kidney [19]. The biophysical and kinetic characterization of H^+-oligopeptide cotransport mediated by the human PEPT1 were reported in detail previously [20]. We also cloned and functionally expressed rat PEPT1 [21] and PEPT2 [22] in *Xenopus* oocytes, and found the localization of rat PEPT1 in the brush-border membranes of the small intestine [23]. When expressed in oocytes, the rat PEPT1 transported not only amino-β-lactams bearing structural similarity with native oligopeptides, but also anionic cephalosporin lacking an α-amino group such as ceftibuten [21]. The rat PEPT1 showed much higher affinity for ceftibuten than cephradine in the presence of an inward H^+ gradient [21]. To understand the molecular mechanisms involved in such multispecificity of PEPT1, it is necessary to define substrate binding and/or recognition sites located in the transporter protein. We report here the residues essential for substrate recognition by the PEPT1 transporter based on transfection and site-directed mutagenesis.

2. Materials and methods

2.1. Cell culture and transfection

The parental LLC-PK_1 cells obtained from the American Type Culture Collection (ATCC 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 Inc., Walkersville, MD) without antibiotics in an atmosphere of 5% CO_2–95% air at 37°C [24]. The LLC-PK_1 cells at 219th passage were used for transfection. The cDNA encoding rat PEPT1 was subcloned into the *Sal*I- and *Not*I-cut mammalian expression vector pBK-CMV (Stratagene, La Jolla, CA) [25]. LLC-PK_1 cells were plated at 3×10^6 cells per 100-mm plastic dishes 24 h before transfection. For transfection, DNA-calcium phosphate precipitate formed with 10 μg of pBK-CMV with or without the rat PEPT1 cDNA inserted was added to the cells and incubated at 37°C. Fifteen hours after transfection, cells were rinsed twice with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (pH 7.4) [PBS (−) buffer; (in mM) 137 NaCl, 3 KCl, 8 Na_2HPO_4 and 1.5 KH_2PO_4]. Then, 3 ml of PBS (−) containing 15% glycerol was added to the cells followed by incubation for 5 min at room temperature. After washing once with PBS (−), the cells were cultured under normal conditions. Forty-eight hours later, the cells were split at dilution of 1:75, 1:30 and 1:15. Twelve hours after splitting, G418 (1 mg/ml) was added to the culture medium. The medium was replaced with fresh medium containing G418 (1 mg/ml) every 3 days. Between 14 and 21 days, single colonies appeared and were picked up for subsequent screening. The G418-resistant clonal cells were grown to confluence as monolayers on 100-mm dishes. The cells were washed twice with PBS (−) and 1 ml of homogenization buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) was added. After centrifugation ($320\times g$, 5 min) of the scraped cells at 4°C in microfugetubes, the pellets were homogenized with 1 ml of homogenization buffer with a Polytron (Kinematica, Kriens-Luzern, Switzerland) for 1 min. The homogenates were centrifuged ($320\times g$, 10 min) at 4°C, and the supernatants were recentrifuged ($15\,000\times g$, 30 min) at 4°C. The pellets consisting of crude plasma membrane fractions were suspended in buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 0.5 mM PMSF). The membrane fractions obtained were analyzed by immunoblotting as described [21]. The brush-border membranes from rat duodenum were prepared as reported previously [6] and subjected to immunoblotting analysis.

2.2. Uptake studies by cell monolayers

Uptake of β-lactam antibiotics was measured in cells grown on 60-mm plastic dishes as described previously [12,13]. The protein content of cell monolayers solubilized in 1 N NaOH was determined by the

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Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DEPC, diethylpyrocarbonate

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method of Bradford [26], using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) with bovine γ-globulin as a standard. The protein content of the monolayers was 1.5–2.0 mg per dish.

2.3. Site-directed mutagenesis

The plasmid pSPORT1 (GIBCO) inserted with the rat PEPT1 cDNA at *Sal*I and *Not*I sites served as a template for PCR mutagenesis. PCR reactions was carried out under two sets of conditions: T7 promoter primer and mNOT (5′-GGATCCTCTAGAGCGTCCGC-3′), which eliminates the *Not*I site; and with mutagenic oligonucleotides and SP6 promoter primer. The mutagenic oligonucleotides used were as follows (base changes are in italics): 5′-GCCATCTAC-CA*A*ACGTTTGT-3′, which replaces a histidine residue at position 57 with glutamine (H57Q); 5′-CTTCCTTTGCA*G*GTAGCACT-3′, which replaces a histidine residue at position 121 with glutamine (H121Q). The two amplified products were combined and used as a template for second PCR with T7 and SP6 primers. Second PCR products containing the mutation possessed *Sal*I and *Not*I sites. Following digestion by *Sal*I and *Not*I, the fragments were directionally ligated into *Sal*I- and *Not*I-cut pSPORT1. Double mutation (H57Q and H121Q) was constructed under the same conditions as described above with the H121Q mutant as a template. All the mutations were confirmed by sequence analysis with Sequenase version 2.0 (United States Biochemical, Cleveland, OH) or Ampli Taq DNA polymerase (Perkin Elmer Applied Biosystems Division, Foster City, CA).

2.4. Expression in Xenopus oocytes

Twenty-ng aliquots of capped complementary RNA (cRNA) transcribed in vitro from *Not*I-linearized wild-type and mutant PEPT1 cDNA using T7 RNA polymerase were injected into *Xenopus* oocytes. Injected oocytes were maintained in modified Barth's medium at 18°C for 24–48 hours. Functional expression of the wild-type and mutant PEPT1 was assessed by measuring uptake of ceftibuten or [^{14}C]glycylsarcosine in groups of oocytes injected with 50 nl of water or cRNA as described previously [21]. Groups of 10 injected oocytes from the uptake experiments were prepared for immunostaining. Briefly, the oocytes were fixed in 3% paraformaldehyde at 4°C for 1 h and immersed in PBS (−) containing 30% sucrose at 4°C for 18 h. The fixed oocytes were embedded in O.C.T. compound (Miles Diagnostic Division, Elkhart, IN) and frozen at −20°C. Sections (8 μm thick) were cut, dried on glass slides, and washed with PBS (−). The sections were then covered with 10% normal goat serum (GIBCO) for 10 min, and incubated with anti-rat PEPT1 serum (1:100 dilution) at 4°C for 12 h. After washing with PBS (−), the sections were incubated with FITC-labeled goat anti-rabbit IgG (5 μg/ml, GIBCO) at 37°C for 30 min, and then washed again with PBS (−). The specimens were mounted in 11% glycerol in 56 mM Tris-HCl buffer (pH 9.0) containing 5% 1,4-diazabicyclo[2,2,2]octane (DABCO) as an anti-bleaching reagent. The sections were examined under an Olympus BX-50 microscope and photographed with an Olympus PM-30 microscope camera.

2.5. Materials

Ceftibuten (Shionogi and Co., Osaka, Japan) and cephradine (Sankyo Co., Tokyo, Japan) were gifts from the respective suppliers. [^{14}C]Glycylsarcosine (1.78 GBq/mmol) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). Glycylsarcosine was obtained from Sigma Chemical Co. (St. Louis, MO). Glycine and DEPC were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals used were of the highest purity available.

3. Results

The pBK-CMV vector with or without the rat PEPT1 cDNA inserted was transfected into LLC-PK$_1$ cells as described in Section 2. The membrane fractions isolated from G418-resistant cells were examined by immunoblotting using antiserum for rat PEPT1 [21,23]. LLC-PK$_1$ cells transfected with the rat PEPT1 cDNA (LLC-rPEPT1) expressed a novel protein with the apparent molecular mass of ~75 kDa, which was consistent with the protein found in the purified brush-border membranes of the rat duodenum (Fig. 1). On the other hand, LLC-PK$_1$ cells transfected with the control vector (LLC-pBK) showed no immunoreactive protein.

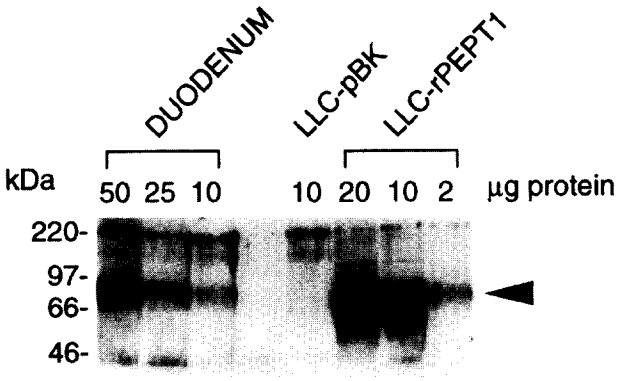

Fig. 1. Western blot analysis of crude membranes isolated from LLC-rPEPT1 and LLC-pBK, and of brush-border membranes purified from rat duodenum. Membrane proteins were separated by SDS-polyacrylamide gel (10%) electrophoresis and blotted to polyvinylidene difluoride membranes. The antiserum for rat PEPT1 [21] (1:1000 dilution) was used as primary antibody. A peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and strips of the blots were visualized by chemiluminescence on X-ray film. The arrow indicates the position of PEPT1.

Using the LLC-rPEPT1 cells, uptake of cephalosporins was examined. As illustrated in Fig. 2A, uptake of ceftibuten (an anionic cephalosporin lacking an α-amino group) was markedly stimulated by lowering pH of the medium, but uptake of cephradine (a zwitterionic aminocephalosporin) was maximal at pH 5.5–6.0 (Fig. 2B). The uptake rates of both drugs by LLC-pBK cells were markedly lower than that by LLC-rPEPT1 cells and remained unaffected by pH.

Previously, we reported that histidine residues are involved in the transport activity of the peptide transport system in purified brush-border membrane vesicles from the rabbit small intestine [27]. The inactivation of transport by DEPC, a histidine modifier, was abolished in the presence of cephradine or glycylsarcosine, suggesting that histidine residues in the transporter are essential for its substrate recognition. Therefore, we examined the effect of DEPC on ceftibuten uptake by LLC-rPEPT1 cells. As shown in Fig. 3, ceftibuten uptake was markedly inhibited by pretreatment with DEPC (1 mM) at 25°C. This inhibition was also found at 4°C (data not shown). Glycylsarcosine and cephradine, but not glycine, completely abolished the inhibitory effect of DEPC, suggesting that histidine residues are involved in the interaction of dipeptides or β-lactam antibiotics with the transporter. Pretreatment with either glycylsarcosine or cephradine in the absence of DEPC showed no inhibitory effect on ceftibuten uptake. In a separate experiment, [^{14}C]glycylsarcosine (20 μM) uptake by LLC-rPEPT1 cells was also completely inhibited by the DEPC pretreatment (control, 827 ± 38; DEPC, 49 ± 2, pmol/mg protein/15 min, mean ± S.E. of three monolayers). The inhibitory effect of DEPC on the [^{14}C]glycylsarcosine uptake was almost prevented by 10 mM cephradine but not by 10 mM ceftibuten (cephradine, 771; ceftibuten, 53, pmol/mg protein/15 min, mean of two monolayers).

The PEPT1 transporter is predicted to possess 12 transmembrane α-helices [16,17,21]. We assumed that histidine residues located in the putative transmembrane α-helices play an important role in substrate binding. Seven histidine

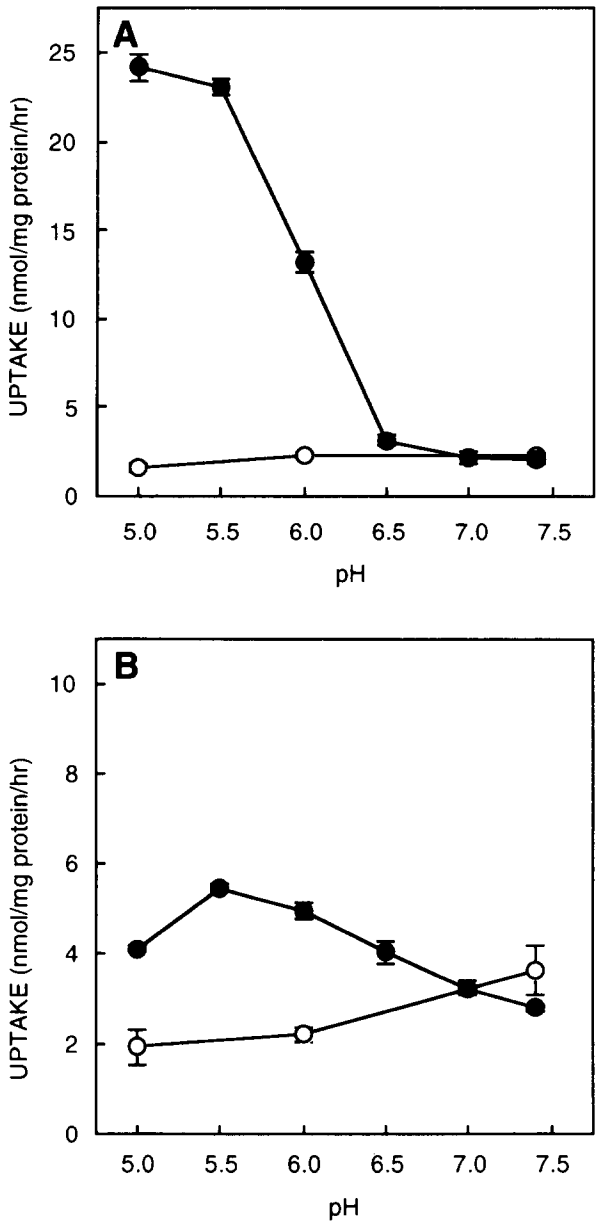

Fig. 2. The pH-dependence of ceftibuten (A) and cephradine (B) uptake by LLC-rPEPT1 and LLC-pBK cells. LLC-rPEPT1 cells (●) or LLC-pBK cells (○) were incubated for 60 min at 37°C with 1 mM ceftibuten or cephradine at various pH values. After incubation, cellular uptake of drugs was measured. Each point represents the mean ± S.E. of three independent monolayers. When the error bar is not shown, it is smaller than the symbol.

residues are conserved among rat [21], rabbit [16] and human [17] PEPT1. Among these residues, histidine 57 and histidine 121, which are located in the predicted transmembrane α-helices 2 and 4, respectively, were of special interest. Therefore, histidine residue 57 or 121, or both, were replaced by glutamine using site-directed mutagenesis. After the mutations were verified by sequencing, cRNA was synthesized for the wild-type and mutant PEPT1 and then injected into *Xenopus* oocytes. As shown in Fig. 4A, injection of wild-type PEPT1 cRNA stimulated [^{14}C]glycylsarcosine uptake about 3-fold above the uptake in water-injected oocytes. The uptake of [^{14}C]glycylsarcosine was completely abolished in the oocytes injected with cRNA of either histidine 57 mutant (H57Q) or histidine 121 mutant (H121Q), or histidine 57 and 121 double mutant (H57Q/H121Q). The wild-type PEPT1 also stimulated the uptake of ceftibuten about 5-fold above the water-injected control value as observed previously [21], and H^+-gradient-dependent transport activity of ceftibuten was not found in any of the oocytes injected with the mutant cRNA (Fig. 4B).

One possible reason for defective transport activity of these histidine mutants is decreased level of the mutant protein in the oocyte plasma membranes, which could be caused by reduced stability and/or impaired insertion to the membranes of the mutants. To examine this possibility, immunolocalization of the PEPT1 mutants in the oocytes was performed. As shown in Fig. 5, oocytes expressing the wild-type PEPT1 and all of the histidine mutants, H57Q, H121Q and double mutant, displayed a strong bright ring along the plasma membranes, whereas native oocytes (no injection) or those injected with water exhibited no labeling of the membranes.

4. Discussion

Recent molecular approaches have revealed that two structurally different H^+-coupled peptide transporters are localized in the intestine and kidney: PEPT1 is expressed primarily in the small intestine and to a lesser extent in the kidney [16,17,21], whereas the PEPT2 is expressed specifically in the kidney [19,22,28]. These transporters exhibit about 50% amino acid identity. Expression of the rabbit PEPT1 in *Xenopus* oocytes and of the human PEPT1 exhibited the electrogenic and H^+-gradient-driven glycylsarcosine transport [16,17]. The rabbit PEPT1 showed high apparent affinity for the anionic dipeptide alanyl-aspartate compared to other neutral or positively charged dipeptides [16]. When expressed in *Xenopus* oocytes, the rat PEPT1 accepted both ceftibuten, an anionic cephalosporin lacking an α-amino group, and cephradine, a neutral aminocephalosporin [21]. The PEPT1 appears to mediate translocation of these oral antibiotics regardless of the presence of an α-amino group in their molecules, and to prefer anionic species as substrates.

In the present study, we established a stable renal epithelial cell line transfected with the rat PEPT1 cDNA, LLC-rPEPT1, which expressed the ~75 kDa PEPT1 protein as detected by immunoblotting with anti-rat PEPT1 antiserum (Fig. 1). Most importantly, pH profiles of the uptake of ceftibuten and cephradine by LLC-rPEPT1 cells (Fig. 2) were consistent with those found in both brush-border membrane vesicles [8,29] and Caco-2 cells [11,12]. Ceftibuten bears two carboxyl groups ($pK_a = 2.3$ and 3.2) and one aminothiazole group ($pK_a = 4.5$), and cephradine has one carboxyl group ($pK_a = 2.6$) and one amino group ($pK_a = 7.4$). Therefore, ionic species of both drugs are different in the range of pH 5.0–7.4, namely ceftibuten and cephradine exist mostly in the form of an anion and zwitterion, respectively. Therefore, the marked differences in their pH profiles cannot be explained simply by the changes in ionic species of each drug over the pH range examined. If charged amino acid residues are present in the substrate binding sites of rat PEPT1, affinity of the transporter for charged substrates might be affected by environmental pH, thereby exhibiting such differences in dependence on pH.

To investigate involvement of charged amino acid residues in substrate recognition by rat PEPT1, site-directed mutagenesis was performed. Mutations at histidine 57 and histidine

121 were of special interest, because these residues are located in the predicted transmembrane α-helices and are highly conserved in all PEPT1 examined to date [16,17,21]. Replacement of each histidine residue by glutamine resulted in elimination of the PEPT1 transport activity when expressed in oocytes. This defective activity was not caused by the insufficient targeting of the transporter into the plasma membranes, because these mutants still produced levels of the transporter similar to those of wild-type PEPT1. Therefore, both histidine 57 and histidine 121 were defined to be localized in substrate binding site and/or to be involved in intrinsic transport activity of PEPT1. The protection of DEPC-induced inactivation of ceftibuten transport by glycylsarcosine or cephradine (Fig. 3) suggests that these conserved histidine residues are involved in the interaction of substrates with recognition sites of the transporter. Previously, we reported that histidine residues of the peptide transporter play an important role in transport of β-lactam antibiotics by using DEPC-inactivated intestinal brush-border membrane vesicles [27]. In the present study, it is noteworthy that cephradine but not ceftibuten prevented the DEPC-induced inactivation of glycylsarcosine transport, suggesting differences in the ability of differently charged β-lactams to protect the PEPT1 from modification by DEPC.

Surprisingly, the corresponding histidine residues are also conserved in the putative membrane-spanning α-helices of PEPT2 from the human [19], rabbit [28] and rat [22] kidney. Miyamoto et al. [30] also reported that essential histidine residues might be present at the dipeptide-binding site of the rabbit renal dipeptide transporter. Further studies using site-directed mutagenesis of PEPT2 will provide additional information regarding the roles of these highly conserved histidine residues in substrate recognition by the peptide transporter

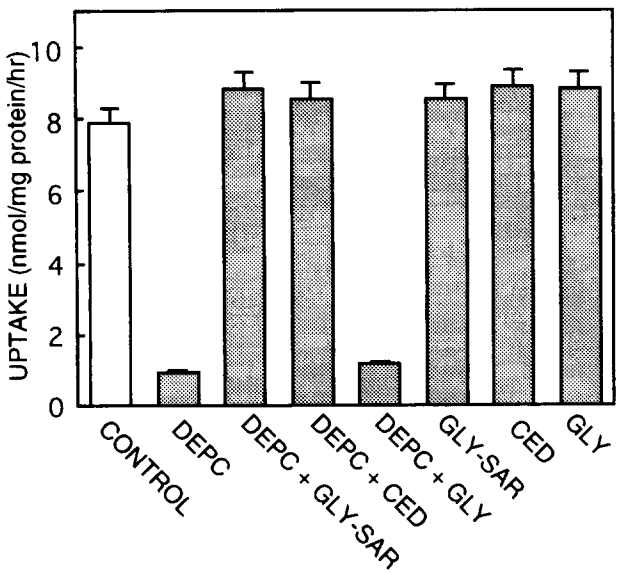

Fig. 3. Effects of DEPC pretreatment on ceftibuten uptake by LLC-rPEPT1 cells. LLC-rPEPT1 cells were preincubated at 25°C for 10 min with 1 mM DEPC (pH 6.0) in the absence or presence of glycylsarcosine, cephradine or glycine at 10 mM. After preincubation, LLC-rPEPT1 cells were rinsed once with incubation medium without drug and then incubated with ceftibuten (1 mM, pH 6.0) for 60 min at 37°C. After incubation, cellular uptake of ceftibuten was measured. Each column represents the mean ± S.E. of three independent monolayers. GLY-SAR, glycylsarcosine; CED, cephradine; GLY, glycine.

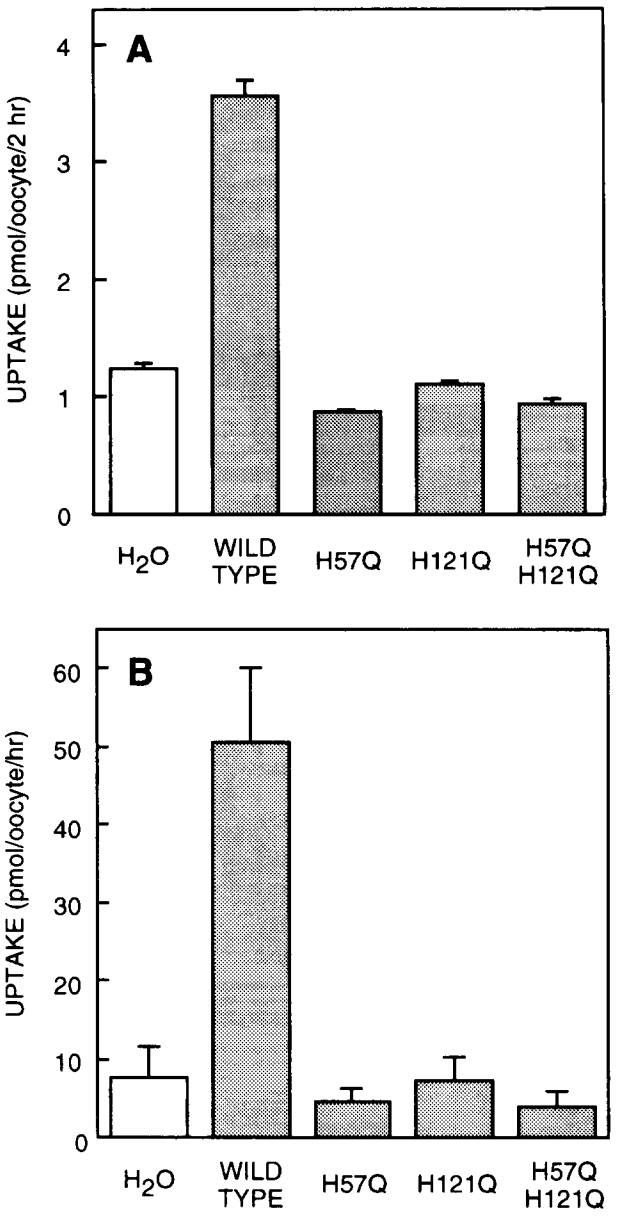

Fig. 4. [^{14}C]Glycylsarcosine (A) and ceftibuten (B) uptake by oocytes expressing wild-type and histidine mutant rat PEPT1. Uptake by oocytes was assayed for 1 or 2 h at 25°C in the presence of either [^{14}C]glycylsarcosine (20 μM, 37 kBq/ml) or ceftibuten (1 mM) 24–48 h after injection of 50 nl of water or cRNA (20 ng). [^{14}C]Glycylsarcosine and ceftibuten taken up by oocytes were measured as described previously [21,22]. Each column represents the mean ± S.E. of 3–5 experiments. Three ([^{14}C]glycylsarcosine uptake) or four (ceftibuten uptake) oocytes were used for each uptake experiment. H57Q, histidine 57 → glutamine; H121Q, histidine 121 → glutamine; H57Q/H121Q, histidines 57 and 121 → glutamine (double mutant).

family. The possibility that these conserved histidines play an important role in the translocation of H^+ cannot be excluded because PEPT1 transporters are coupled with H^+. Recently, Mackenzie et al. [20] reported that glycylsarcosine evoked voltage- and H^+-dependent currents in oocytes expressing human PEPT1, and proposed that H^+ behaves as an essential activator of oligopeptide transport, suggesting an ordered transport model in which H^+ binds first. Histidine

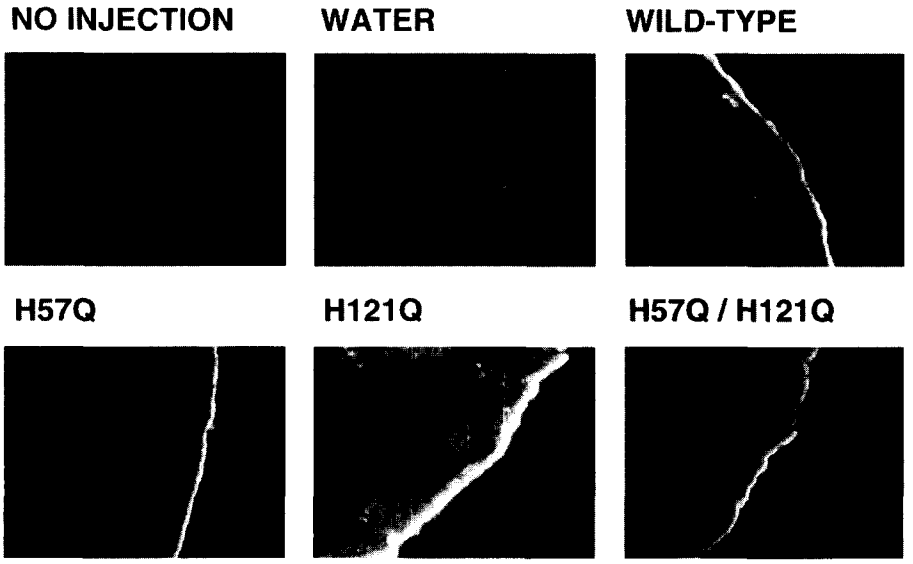

Fig. 5. Expression of wild-type and histidine mutant PEPT1 transporter proteins in oocytes. Oocytes were injected with 50 nl of water or cRNA (20 ng) of wild-type or mutant rat PEPT1. Two days after injection, oocytes were fixed, frozen, sectioned, and stained as described in Section 2. The antiserum (1:100 dilution) for rat PEPT1 was used as the primary antibody. FITC-labeled goat anti-rabbit IgG (5 μg/ml) was used for detection of bound antibodies. Abbreviation for each mutant is the same as described in Fig. 4.

57 and histidine 121 in the PEPT1 protein might serve as proton acceptors. It is very likely that other residues located in transmembrane segments 2 and 4 participate in the transport mechanisms.

In summary, we stably transfected the rat PEPT1 transporter cDNA into LLC-PK_1 kidney epithelial cells, and showed that histidine residues were essential for cephalosporin transport. Site-directed mutagenesis suggested that histidine 57 and histidine 121 in the predicted transmembrane domains 2 and 4, respectively, are involved in substrate binding and/or responsible for intrinsic activity of the transporter. These present findings provide valuable insight into transport mechanisms and structure-function relationships of this transporter family, as well as into drug design to improve intestinal absorption.

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References

[1] Ganapathy, V. and Leibach, F.H. (1985) Am. J. Physiol. 249, G153–G160.
[2] Hoshi, T. (1985) Jpn. J. Physiol. 35, 179–191.
[3] Ganapathy, V. and Leibach, F.H. (1983) J. Biol. Chem. 258, 14189–14192.
[4] Takuwa, N., Shimada, T., Matsumoto, H., Himukai, M. and Hoshi, T. (1985) Jpn. J. Physiol. 35, 629–642.
[5] Okano, T., Inui, K., Maegawa, H., Takano, M. and Hori, R. (1986) J. Biol. Chem. 261, 14130–14134.
[6] Okano, T., Inui, K., Takano, M. and Hori, R. (1986) Biochem. Pharmacol. 35, 1781–1786.
[7] Tsuji, A., Terasaki, T., Tamai, I. and Hirooka, H. (1987) J. Pharmacol. Exp. Ther. 241, 594–601.
[8] Inui, K., Okano, T., Maegawa, H., Kato, M., Takano, M. and Hori, R. (1988) J. Pharmacol. Exp. Ther. 247, 235–241.
[9] Muranushi, N., Yoshikawa, T., Yoshida, M., Oguma, T., Hirano, K. and Yamada, H. (1989) Pharm. Res. 6, 308–312.
[10] Inui, K., Yamamoto, M. and Saito, H. (1992) J. Pharmacol. Exp. Ther. 261, 195–201.
[11] Matsumoto, S., Saito, H. and Inui, K. (1994) J. Pharmacol. Exp. Ther. 270, 498–504.
[12] Matsumoto, S., Saito, H. and Inui, K. (1995) Pharm. Res. 12, 1483–1487.
[13] Saito, H. and Inui, K. (1993) Am. J. Physiol. 265, G289–G294.
[14] Hu, M. and Amidon, G.L. (1988) J. Pharm. Sci. 77, 1007–1011.
[15] Swaan, P.W., Stehouwer, M.C. and Tukker, J.J. (1995) Biochim. Biophys. Acta 1236, 31–38.
[16] Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S.K., Boron, W.F. and Hediger. M.A. (1994) Nature 368, 563–566.
[17] Liang, R., Fei, Y.-J., Prasad, P.D., Ramamoorthy, S., Han, H., Yang-Feng, T.L., Hediger, M.A., Ganapathy, V. and Leibach, F.H. (1995) J. Biol. Chem. 270, 6456–6463.
[18] Ganapathy, M.E., Brandsch, M., Prasad, P.D., Ganapathy, V. and Leibach, F.H. (1995) J. Biol. Chem. 270, 25672–25677.
[19] Liu, W., Liang, R., Ramamoorthy, S., Fei, Y.-J., Ganapathy, M.E., Hediger, M.A., Ganapathy, V. and Leibach, F.H. (1995) Biochim. Biophys. Acta 1235, 461–466.
[20] Mackenzie, B., Loo, D.D.F., Fei, Y.-J., Liu, W., Ganapathy, V., Leibach, F.H. and Wright, E.M. (1996) J. Biol. Chem. 271, 5430–5437.
[21] Saito, H., Okuda, M., Terada, T., Sasaki, S. and Inui, K. (1995) J. Pharmacol. Exp. Ther. 275, 1631–1637.
[22] Saito, H., Terada, T., Okuda, M., Sasaki, S. and Inui, K. (1996) Biochim. Biophys. Acta 1280, 173–177.
[23] Ogihara, H., Saito, H., Shin, B.-C., Terada, T., Takenoshita, S., Nagamachi, Y., Inui, K. and Takata, K. (1996) Biochem. Biophys. Res. Commun. 220, 848–852.
[24] Saito, H., Yamamoto, M., Inui, K. and Hori, R. (1992) Am. J. Physiol. 262, C59–C66.
[25] Brewer, C.B. (1994) Meth. Cell Biol. 43, 233–245.
[26] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[27] Kato, M., Maegawa, H., Okano, T., Inui, K. and Hori, R. (1989) J. Pharmacol. Exp. Ther. 251, 745–749.
[28] Boll, M., Herget, M., Wagener, M., Weber, W.M., Markovich, D., Biber, J., Clauss, W., Murer, H. and Daniel, H. (1996) Proc. Natl. Acad. Sci. USA 93, 284–289.
[29] Yoshikawa, T., Muranushi, N., Yoshida, M., Oguma, T., Hirano, K. and Yamada, H. (1989) Pharm. Res. 6, 302–307.
[30] Miyamoto, Y., Ganapathy, V. and Leibach, F.H. (1986) J. Biol. Chem. 261, 16133–16140.