Identification of the Histidyl Residue Obligatory for the Catalytic Activity of the Human H^+/Peptide Cotransporters PEPT1 and PEPT2[†]

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Received August 15, 1996; Revised Manuscript Received October 24, 1996[⊗]

ABSTRACT: Histidyl residues are known to be essential for the catalytic function of the H^+-coupled peptide transporters expressed in the intestine and the kidney, most likely participating in the binding and translocation of H^+. Three histidyl residues are conserved among the intestinal and renal peptide transporters (PEPT1 and PEPT2, respectively) from different animal species. In hPEPT1, these residues are His-57, His-121, and His-260. The corresponding residues in hPEPT2 are His-87, His-142, and His-278. We have individually mutated each of these histidyl residues in hPEPT1 and in hPEPT2 and compared the catalytic function of the mutants with that of their respective wild type transporters by expressing the transporters in *Xenopus laevis* oocytes and also in HeLa cells. His-57 in hPEPT1 and His-87 in hPEPT2 were found to be absolutely essential for catalytic activity because the corresponding mutants had no detectable peptide transport activity. His-121 in hPEPT1 is not essential since mutation of this residue did not impair transport function. His-142 in hPEPT2 was found to play a significant role in the maintenance of transport function but was not found to be obligatory because the mutant had appreciable transport activity. The obligatory histidyl residue (His-57 in hPEPT1 and His-87 in hPEPT2) is located in an almost identical topological position in both transporters, near the extracellular surface of the second putative transmembrane domain. The second conserved histidyl residue is located in the fourth putative transmembrane domain in hPEPT1 as well as in hPEPT2. The third conserved histidyl residue is present in the cytoplasmic loop between the transmembrane domains 6 and 7 and is unlikely to play any significant role in the binding and translocation of H^+ and this was supported by the findings that mutation of this histidyl residue in hPEPT1 did not interfere with transport function. The loss of transport function of hPEPT1 and hPEPT2, when His-57 in hPEPT1 and His-87 in hPEPT2 were mutated, was not due to alterations in protein expression because the expression levels of these mutants were similar to those of the respective wild type transporters in HeLa cells as assessed by immunoblot analysis. Confocal analysis of immunofluorescence in *X. laevis* oocytes expressing the wild type and the three histidine mutants of hPEPT1 showed that the transporter protein is expressed exclusively in the plasma membrane and that the level of expression is comparable among the wild type and the three mutants. These site-directed mutagenesis studies clearly show that His-57 in hPEPT1 and His-87 in hPEPT2 are the most critical histidyl residues necessary for the catalytic function of these transporters.

Recent molecular cloning studies have established that the H^+-coupled peptide transporters expressed in the mammalian small intestine and kidney are members of a distinct family of transport proteins [for reviews, see Ganapathy and Leibach (1996) and Leibach and Ganapathy (1996)]. The role of a transmembrane H^+ gradient as the driving force for the intestinal and renal peptide transporters was originally delineated in studies with isolated brush border membrane vesicles from these two tissues (Ganapathy & Leibach, 1983; Ganapathy *et al*., 1984; Miyamoto *et al*., 1985; Takuwa *et al*., 1985). The mechanisms responsible for the generation of the H^+ gradient across the intestinal and renal brush border membranes have been recently reviewed (Ganapathy & Leibach, 1991; Ganapathy *et al*., 1994). Several transport systems for which H^+ is a cotransported ion are known to contain specific histidyl residues that are essential for the catalytic activity of these transport systems. Examples of these transport systems include the Na^+/H^+ exchanger (Grillo & Aronson, 1986; Ganapathy *et al*., 1987), the organic cation/H^+ antiporter (Hori *et al*., 1989), and the folate transporter (Said & Mohammadkhani, 1993) in mammals. The function of specific histidyl residues in H^+ binding and translocation has been investigated in great detail in the case of the *Escherichia coli* Lac permease, a prototypical H^+-coupled solute transport system (Kaback, 1987). Work from our laboratory, described several years ago (Miyamoto *et al*., 1986), has led to the identification of a similar essential function for specific histidyl residues in the catalytic activity of the renal peptide transport system. Subsequently, the peptide transport system expressed in the small intestine has also been shown to possess specific histidyl residues that are critical for the catalytic activity (Kramer *et al*., 1988; Kato *et al*., 1989).

[†] This work was supported by National Institutes of Health Grant DK 28389.

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[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1997.

S0006-2960(96)02058-2 CCC: $14.00

There is evidence at the molecular level that the intestinal and renal peptide transporters are distinct proteins (Fei *et al*., 1994; Boll *et al*., 1994, 1996; Liang *et al*., 1995; Liu *et al*., 1995; Saito *et al*., 1995, 1996; Miyamoto *et al*., 1996), even though both transport systems mediate active transport of dipeptides and tripeptides energized by a transmembrane H^+ gradient. PEPT1 is a protein consisting of 707−710 amino acids, depending on the animal species, and is primarily expressed in the small intestine and, to a lesser extent, in the kidney. PEPT2 consists of 729 amino acids and is prominently expressed in the kidney but is absent in the small intestine. Even though PEPT1 and PEPT2 are similar in several aspects of their transport function, significant differences do exist between these transporters in terms of substrate affinity (Ramamoorthy *et al*., 1995) and substrate specificity (Ganapathy *et al*., 1995).

We have recently reported cloning and characterization of the human PEPT1 and PEPT2 (Liang *et al*., 1995; Liu *et al*., 1995). The present investigation was undertaken to identify the histidyl residues that are critical for the catalytic activity of these two transporters using site-directed mutagenesis. Results of this investigation demonstrate that His-57 in human PEPT1 and His-87 in human PEPT2 are obligatory for transport function. Each of these histidyl residues is present in an almost identical location in the second putative transmembrane domain of the respective transport protein.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The Transformer Site-Directed Mutagenesis Kit (Clontech) was used to mutate the histidine codon in hPEPT1 and hPEPT2 (CAT or CAC) to an asparagine codon (AAT or AAC) or a glutamine codon (CAA) according to the manufacturer's protocol. There are three highly conserved histidyl residues in hPEPT1 and hPEPT2 (see Results and Discussion). The mutagenic primers used to mutate these histidyl residues in the present study are as follows: 5′-CACCGCCATCTACAATACGTTTGTGGC-3′ (hPEPT1, H57N), 5′-CGCCATCTACCAAACGTTTGTGGCTCT-3′ (hPEPT1, H57Q), 5′-GCCTTCCTGTGCAAGTGGTGCTGTCCT-3′ (hPEPT1, H121Q), 5′-ACAGCCTTCCTGTGAACGTGGTGCTGT-3′ (hPEPT1, H121N), 5′-CCCAAGAGGGAGCAATGGCTGGACTGG-3′ (hPEPT1, H260Q), 5′-CCCAAGAGGGAGAACTGGCTGGACTGG-3′ (hPEPT1, H260N), 5′-CCACATCTATATACAATGCCTTCAGCAGCC-3′ (hPEPT2, H87N), and 5′-GGAGGACAAGTGGTAAACACAGTCCTATCA-3′ (hPEPT2, H142N). The selection primers used in this study are 5′-GCTCATCATTGGATATCGTTCTTCGGG-3′ for hPEPT1 mutagenesis and 5′-GTGACTGGTGAGATCTCAAACAAGTC-3′ for hPEPT2 mutagenesis. A unique *Xmn*I restriction site was chosen as the target site for selection for site-directed mutagenesis with hPEPT1. There is a single site for this enzyme in the pBluescript vector carrying the hPEPT1 cDNA insert. The cDNA insert lacks this site. In the selection primer used for mutagenesis with hPEPT1, the *Xmn*I site was changed to an *Eco*RV site. For mutagenesis with hPEPT2, a unique *Sca*I site was chosen as the target site for selection. There is a single site for this restriction enzyme in the pBluescript vector carrying the hPEPT2 cDNA insert. The cDNA insert lacks this site. In the selection primer used for mutagenesis with hPEPT2, the *Sca*I site was changed to an *Bgl*II site. The selection primer chosen for mutagenesis with hPEPT1 could not be used for mutagenesis with hPEPT2 because of the presence of sites for *Xmn*I in the hPEPT2 cDNA. The presence of the specific mutation introduced into the hPEPT1 cDNA or into the hPEPT2 cDNA by the specific mutagenic primers was confirmed by sequencing. DNA sequencing was done by the dideoxy chain termination method (Sanger *et al*., 1977).

cRNA Synthesis. Wild type and mutant plasmids were linearized with *Bam*HI, and the cDNA inserts were transcribed *in vitro* using T7 RNA polymerase in the presence of RNase inhibitor and RNA cap analog. The Ambion MEGAscript kit was employed for this purpose. The resultant cRNAs were each phenol−chloroform extracted and ethanol precipitated. The RNA concentration was determined by UV spectrophotometry, and the integrity of the RNA was verified by denaturing 1% formaldehyde-agarose gel electrophoresis and visualization using ethidium bromide fluorescence.

Oocyte Expression and Electrophysiological Studies. Oocytes, isolated from *Xenopus laevis* (Nasco, Fort Atkinson, WI), were subjected to digestion with collagenase A (Boehringer-Mannheim) (1.6 mg/mL) in a Ca^{2+}-free buffer for 30 min at room temperature, and manually defolliculated. Mature (stage V−VI), defolliculated oocytes were selected and maintained at 18 °C in modified Barth's medium (Parent *et al*., 1992) with 10 mg/L gentamycin sulfate. Oocytes were injected 1 day after isolation with approximately 50 ng of wild type or mutant hPEPT1 cRNA and incubated at 18 °C. The oocytes were used for electrophysiological studies 5−7 days after cRNA injection.

A two-microelectrode voltage-clamp system (Loo *et al*., 1993) was used to measure steady-state currents associated with wild type or mutant hPEPT1 expressed in oocytes. Oocytes were superfused at room temperature first with a pH 7.5 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/Tris), followed by a pH 5.5 buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 3 mM Hepes, 3 mM Mes, and 3 mM Tris) and then by Gly-Sar solutions of varied concentrations made in the same pH 5.5 buffer. Test solutions were always washed out by superfusing the oocyte with Gly-Sar-free, choline chloride-containing medium, pH 7.5. The holding membrane potential was −50 mV. For determination of the *I*/*V* (current/membrane potential) relationship, step changes in membrane potential (V_{test}) were applied, each for a duration of 100 ms (+50 to −150 mV in 20-mV increments) using a voltage clamp amplifier (GeneClamp 500) controlled by the computer software program pCLAMP 6.0 (Axon Instruments, Foster City, CA). The currents were averaged over three sweeps and low-pass filtered at 500 Hz by an eight-pole Bassel filter. Steady-state data were fitted to the following equation:

$$I = \frac{I_{max} S^n}{K_{0.5}^n + S^n}$$

where I is the Gly-Sar-evoked current (i.e., the difference between the steady-state currents measured in the presence and absence of Gly-Sar), I_{max} is the derived current maximum, S is the concentration of Gly-Sar or H^+, n is the Hill coefficient, and $K_{0.5}$ is the concentration of Gly-Sar or H^+ at which the current is half-maximal. When S is H^+, the Hill coefficient n represents the H^+/Gly-Sar coupling ratio.

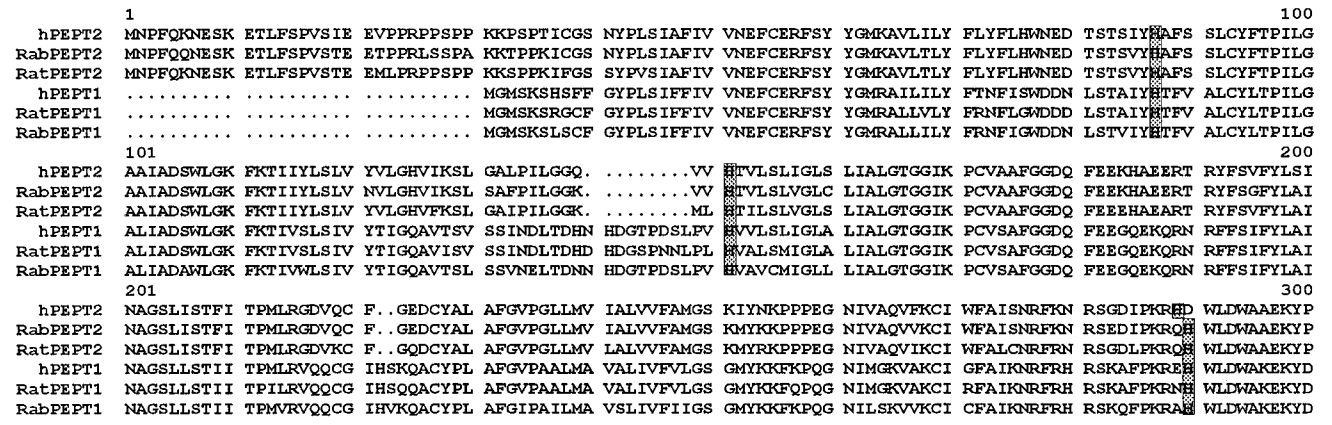

FIGURE 1: Comparison of partial amino acid sequences among the cloned human, rabbit, and rat PEPT1 and PEPT2 (hPEPT1, rabPEPT1, ratPEPT1, hPEPT2, rabPEPT2, and ratPEPT2, respectively).

Expression of hPEPT1 and hPEPT2 in HeLa Cells. This was done using the vaccinia virus expression system as described previously (Liang *et al*., 1995; Liu *et al*., 1995; Ramamoorthy *et al*., 1995). The wild type and mutant cDNAs are located downstream of the T7 promotor present in the pBluescript vector. Thus, the transcription of the cDNA inserts is under the control of T7 promotor. The vaccinia virus expression system utilizes a recombinant (VTF_{7-3}) vaccinia virus encoding T7 RNA polymerase (Blakely *et al*., 1991). When HeLa cells are infected first with the recombinant virus and then transfected with the plasmids containing either the hPEPT1 cDNA insert (phPEPT1) or the hPEPT2 cDNA insert (phPEPT2), the cells acquire the ability to functionally express the cDNA inserts using the virus-encoded T7 RNA polymerase. Using this technique, PEPT1- or PEPT2-mediated transport activity was measured in the cells after 10−12 h post-transfection by assaying the uptake of [2-^{14}C]glycyl[1-^{14}C]sarcosine (specific radioactivity, 109 mCi/mmol; Cambridge Research Chemicals, U.K.). The uptake medium was 25 mM Mes/Tris (pH 6.0), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. The time of incubation was 3 min, and the concentration of Gly-Sar was 20 μM for PEPT1. For PEPT2, the time of incubation was 3 min, and the concentration of Gly-Sar was 60 μM. Nonspecific transport was determined in parallel experiments with the pBluescript vector. HeLa cells do not possess detectable levels of endogenous carrier-mediated Gly-Sar transport activity. Therefore, the Gly-Sar uptake measured in cells transfected with the empty vector represents diffusion of Gly-Sar. This component was subtracted from the uptake measured in cells transfected with phPEPT1 or phPEPT2 to determine uptake that was specific to the cDNA inserts.

Immunodetection. Antipeptide antibodies were raised against an 18-amino acid peptide sequence in hPEPT1. The peptide, RFRHRSKAFPKREHWLDW, corresponds to position 247−264 in the hPEPT1 amino acid sequence and is located in the large putative intracellular loop between transmembrane domains 6 and 7. A cysteine residue was added to the C-terminus of this peptide for coupling to keyhole lympet hemocyanin. Polyclonal antibodies against this peptide−keyhole lympet hemocyanin conjugate were raised in rabbits. The antibodies were affinity-purified by passing through a column of Sepharose 4B to which the 18-amino acid peptide had been coupled by the cyanogen bromide activation method.

Plasma membranes isolated from human intestinal cell lines HT-29 and Caco-2 and crude membranes prepared from transfected HeLa cells were probed with the antibody by immunoblotting. The proteins separated by SDS−PAGE were transferred onto an activated nylon membrane (Hybond N^+, Amersham). The membrane was blocked with 1% nonfat dry milk, 3% fetal bovine serum, and 0.5% Tween-20 and then probed with the antibody (1 μg/mL). Immunoreactive protein bands were identified by the Enhanced ChemiLuminescence Western blotting detection system (Amersham). In the case of *X. laevis* oocytes expressing the wild type or the mutant hPEPT1s, confocal analysis of immunofluorescence was used to detect the hPEPT1 protein. Oocytes injected with water (control) or hPEPT1 cRNAs were fixed in ethanol/acetic acid (95:5, v/v) at 4 °C for 1 h and then washed three times in phosphate-buffered saline at room temperature. Washed oocytes were placed in 10% goat serum for 10 min and then incubated with antipeptide antibody (1:100 dilution) at 4 °C overnight. This was followed by washing the oocytes three times in phosphate-buffered saline. Oocytes were then incubated with fluorescein isothiocyanate-labeled AffiniPure goat antirabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 h in dark. After washing the oocytes again in phosphate-buffered saline, immunofluorescence was detected by confocal microscopy (Bio-Rad, model MRC-600).

RESULTS AND DISCUSSION

Conserved Histidyl Residues in PEPT1 and PEPT2. PEPT1 and PEPT2 have been cloned from three different animal species, namely rat, rabbit, and human (Fei *et al*., 1994; Boll *et al*., 1994, 1996; Liang *et al*., 1995; Saito *et al*., 1995, 1996; Liu *et al*., 1995; Miyamoto *et al*., 1996). A partial alignment of amino acid sequences of all these six peptide transporters is given in Figure 1, identifying the conserved histidyl residues. There are three of them, His-57, His-121, and His-260 in the human PEPT1 and His-87, His-142, and His-278 in the human PEPT2. His-57 and His-87 are located in the second putative transmembrane domain near the extracellular surface in the respective proteins. His-121 and His-142 are located in the fourth putative transmembrane domain in the respective proteins. However, the position of His-121 in hPEPT1 is much closer to the extracellular surface than that of His-142 in hPEPT2. The third histidyl residue (His-260 in hPEPT1 and His-278 in

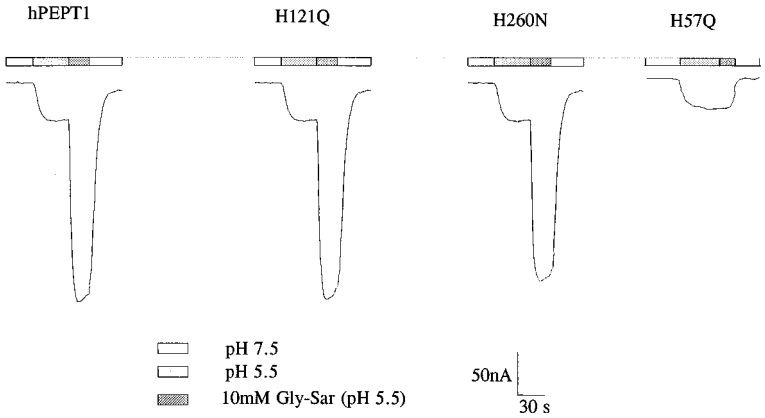

FIGURE 2: Representative recording of Gly-Sar-evoked inward currents in oocytes expressing the wild type (hPEPT1) and mutant (H121Q, H260N, and H57Q) PEPT1s. The oocytes were superfused first with a pH 7.5 buffer, followed by a pH 5.5 buffer and then by a 10 mM Gly-Sar solution prepared in the same pH 5.5 buffer. Recordings were done on the fifth day following injection of the respective cRNA.

hPEPT2) is present in the large intracellular loop between the putative transmembrane domains 6 and 7 in the respective proteins.

Generation of hPEPT1 and hPEPT2 Mutants. Six different hPEPT1 mutants were generated by converting each of the three conserved histidyl residues to asparagine or glutamine. These mutants are designated as H57N, H57Q, H121N, H121Q, H260N, and H260Q. Two different hPEPT2 mutants were generated by converting the first two of the three conserved histidyl residues to asparagine. These mutants are designated as H87N and H142N. Asparagine and glutamine were selected for substitution because replacement of histidine with either of these amino acids would have minimal steric effect and charge effect on the resultant mutant protein.

Functional Analysis of Wild Type hPEPT1 and Mutant hPEPT1s. The function of the wild type and six mutant hPEPT1s was tested in oocytes expressing these proteins by analyzing Gly-Sar-evoked currents at a holding potential of −50 mV. When the pH of the superfusing buffer was changed from 7.5 to 5.5, there was a significant inward current associated with the change. Addition of 10 mM Gly-Sar to the superfusing medium at pH 5.5 further evoked a marked inward current in oocytes expressing the wild type hPEPT1 (Figure 2). Comparable inward currents were observed in the case of four hPEPT1 mutants in which His-121 or His-260 was changed to asparagine or glutamine. Representative traces for the mutants H121Q and H260N are given in Figure 2. In contrast, mutants in which His-57 was changed to asparagine or glutamine failed to evoke detectable inward currents upon addition of Gly-Sar. A representative trace for the H57N mutant is given in Figure 2. These results were reproducible in a number of different oocytes. These data suggest that His-57 is absolutely essential for the transport activity of hPEPT1. The other two conserved histidyl residues, His-121 and His-260, do not appear to be critical for the activity.

Since the four hPEPT1 mutants (H121N, H121Q, H260N, and H260Q) showed comparable Gly-Sar-evoked currents as the wild type hPEPT1, we investigated the characteristics of these currents in detail to see whether there are differences in various kinetic parameters of the evoked currents as a consequence of substitution of His-121 and His-260. The characteristics of Gly-Sar-evoked currents in oocytes expressing the wild type hPEPT1 have been recently reported (Mackenzie *et al*., 1996). The following are the salient features: (i) the currents evoked by Gly-Sar at saturating concentrations are dependent upon membrane potential between −150 and +50 mV, but with no reversal of the current within this range of membrane potential; (ii) the currents increase hyperbolically with increasing extracellular H^+ concentration, with a H^+/Gly-Sar coupling ratio of approximately 1 and an apparent affinity constant ($K_{0.5}$) for H^+ in the range of 0.05−1 μM; and (iii) the $K_{0.5}$ for Gly-Sar is markedly dependent upon membrane potential at pH 5.0, the $K_{0.5}$ being minimal (0.3−0.7 mM) at the membrane potential range of −50 to +50 mV but increasing substantially with hyperpolarizing membrane potential. Similar studies done with the four hPEPT1 mutants showed that the characteristics of Gly-Sar-evoked currents in oocytes expressing these hPEPT1 mutants were comparable to the characteristics described previously for the wild type hPEPT1. The data given in Figures 3 and 4 were obtained with the H260N mutant of hPEPT1. Similar data were obtained with each of the other three mutants (data not shown). Figure 3 describes the current/membrane potential relationship for the H260N mutant at increasing concentrations of Gly-Sar (0.25−20 mM) and at pH 5.5. At lower, subsaturating concentrations of Gly-Sar, the evoked currents were not very much dependent upon membrane potential. However, when the concentrations of Gly-Sar were increased, the dependence of the currents upon membrane potential became clearly evident. There was no reversal of the currents between membrane potential −150 and +50 mV at all concentrations of Gly-Sar tested.

The dependence of Gly-Sar (10 mM)-evoked currents on extracellular H^+ concentration is described in Figure 4A. The currents were hyperbolically related to H^+ concentration. The H^+/Gly-Sar coupling ratio was 0.8 ± 0.1 and the $K_{0.5}$ for H^+ was 0.08 μM. Figure 4B shows the dependence of $K_{0.5}$ for Gly-Sar at pH 5.5 on membrane potential. This was done by determining the evoked currents over a range of Gly-Sar concentrations (0.25−20 mM) at different membrane potential and using the data to determine $K_{0.5}$ at each membrane potential. The $K_{0.5}$ for Gly-Sar was markedly dependent upon membrane potential. The $K_{0.5}$ was minimal at −10 mV but increased several-fold as membrane potential was hyperpolarized.

Taken collectively, these results show that mutation of His-121 and His-260 in hPEPT1 to asparagine or glutamine does

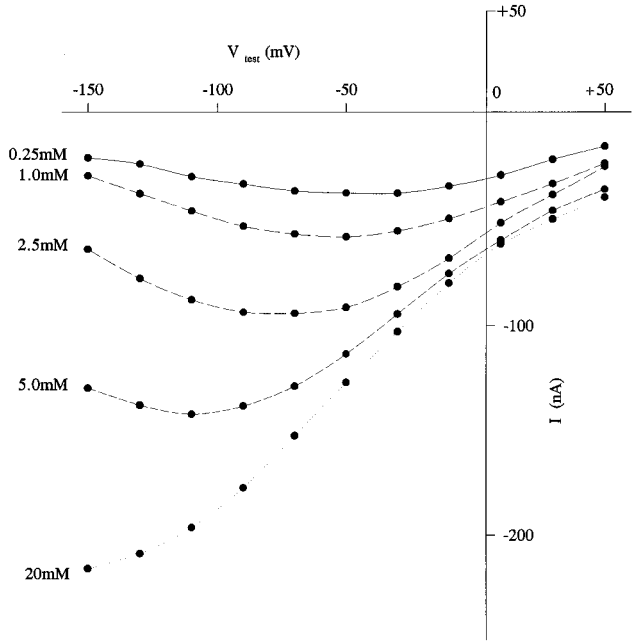

FIGURE 3: Voltage-dependence of Gly-Sar-evoked currents for the hPEPT1 mutant H260N. Recordings were done on the 5th day following injection of cRNA. The oocyte was superfused first with a pH 5.5 buffer and then with increasing concentrations (0.25−20 mM) of Gly-Sar prepared in the same pH 5.5 buffer. The pulse protocol was applied when the Gly-Sar-evoked current reached the maximum at each testing concentration of Gly-Sar. The Gly-Sar-evoked current (I) is plotted against membrane potential (V_{test}) for each concentration of Gly-Sar.

not have any significant influence on the catalytic activity of hPEPT1. With respect to important parameters of the catalytic activity such as current/ membrane potential relationship, H^+/Gly-Sar coupling ratio, apparent affinity ($K_{0.5}$) for H^+ and Gly-Sar and dependence of $K_{0.5}$ for Gly-Sar on membrane potential, all four mutants (H121N, H121Q, H260N, and H260Q) functioned exactly as the wild type hPEPT1. The findings that His-260 is not essential for the catalytic activity of hPEPT1 are not surprising because this residue is located in one of the putative cytoplasmic loops of the transport protein and thus is the least likely residue among the three conserved histidyl residues to play a role in the binding and translocation of H^+. His-57 and His-121 are, on the other hand, located in putative transmembrane domains and thus either one or both of the residues may potentially be involved in H^+ binding and translocation. However, the data described above clearly demonstrate that His-57 which is located in the second transmembrane domain is the only histidyl residue that is absolutely essential for the catalytic activity of hPEPT1. His-121 which is located in the fourth putative transmembrane domain does not appear to play an essential role in the catalytic activity.

We have also employed a different functional expression technique, namely the vaccinia virus expression system, to corroborate the findings from the oocyte expression system with respect to the obligatory role of His-57 in hPEPT1 function. HeLa cells were transfected with either the wild type hPEPT1 cDNA or the mutant hPEPT1 cDNA (H57N or H121N) using this transient expression technique and the cDNA-induced transport activity was measured in the cells by assaying Gly-Sar transport at pH 6.0. Cells transfected with empty pBluescript vector served as control. The results given in Figure 5 show that Gly-Sar transport in cells transfected with wild type hPEPT1 cDNA was about 20-fold higher than in cells transfected with the empty vector. In contrast, Gly-Sar transport in cells transfected with the H57N mutant hPEPT1 cDNA was not different from that in cells transfected with the empty vector. These data show that the H57N mutant has no detectable transport activity, thus confirming the observations from oocyte expression. We have also expressed the H121N mutant in HeLa cells. The transport activity of this mutant was almost comparable (80%) to that of the wild type hPEPT1.

Functional Analysis of Wild Type hPEPT2 and Mutant hPEPT2s. Even though the recently cloned rabbit PEPT2 induces considerable substrate-evoked currents in oocytes expressing this transporter (Boll *et al.*, 1996), hPEPT2 evokes negligible Gly-Sar-evoked currents (∼5 nA) in oocytes. This precluded the use of the oocyte expression system to analyze the catalytic activity of hPEPT2 and its mutants. However, hPEPT2 could be studied with the vaccinia virus expression

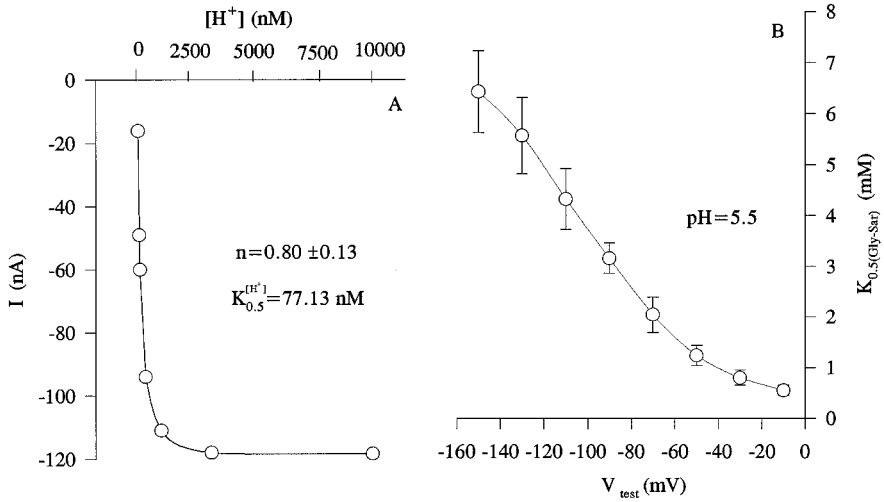

FIGURE 4: Dependence of Gly-Sar-evoked currents on H^+ concentration (A) and dependence of the apparent affinity for Gly-Sar ($K_{0.5}$) on membrane potential (B) for the hPEPT1 mutant H260N. (A) At a Gly-Sar concentration of 10 mM, Gly-Sar-evoked currents (I) were measured at a fixed membrane potential (−50 mV) but at varying concentrations of H^+ (pH range, 5.0−8.0). (B) $K_{0.5}$ was determined by measuring Gly-Sar-evoked currents over a Gly-Sar concentration range of 0.25−20 mM at each indicated membrane potential (V_{test}) and by fitting the data to the Michaelis−Menten equation. The pH of the superfusion medium was 5.5.

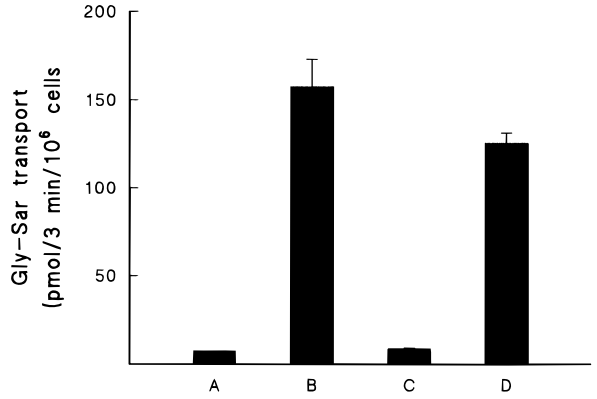

FIGURE 5: Transport function of the wild type hPEPT1 and the H57N and H121N mutants of hPEPT1 in HeLa Cells. The cDNAs were expressed in HeLa cells by using the vaccinia virus expression technique. Cells were transfected with one of the following: pBluescript (pBS) without cDNA insert (A), pBS-hPEPT1 cDNA (B), pBS-H57N hPEPT1 cDNA (C), or pBS-H121N hPEPT1 cDNA (D). Transport of 20 μM Gly-Sar was measured in these cells at pH 6.0 with an incubation period of 3 min.

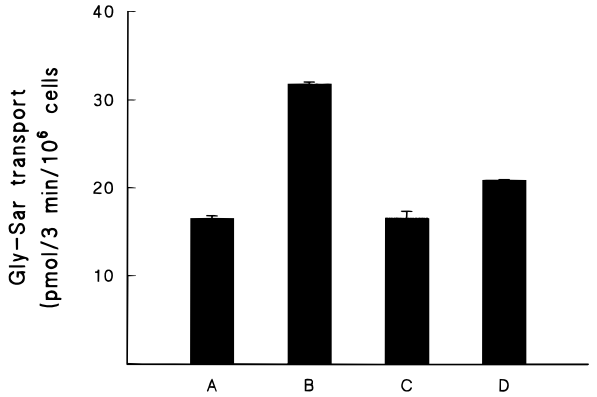

FIGURE 6: Transport function of the wild type hPEPT2 and the H87N and H142N mutants of hPEPT2 in HeLa cells. The cDNAs were expressed in HeLa cells by using the vaccinia virus expression technique. Cells were transfected with one of the following: pBluescript (pBS) without cDNA insert (A), pBS-hPEPT2 cDNA (B), pBS-H87N hPEPT2 cDNA (C), or pBS-H142N hPEPT2 cDNA (D). Transport of 60 μM Gly-Sar was measured in these cells at pH 6.0 with an incubation period of 3 min.

technique (Liu *et al*., 1995; Ramamoorthy *et al*., 1995). We generated only two mutants of hPEPT2 (H87N and H142N) for functional analysis. Because of the cytoplasmic location of His-278 in hPEPT2, which makes it the least likely candidate for the essential histidyl residue, mutants involving this particular residue were not generated in the present study. The results given in Figure 6 show that Gly-Sar transport in HeLa cells transfected with wild type hPEPT2 cDNA was about 2.5-fold greater than transport in cells transfected with empty pBluescript vector. In contrast, Gly-Sar transport in cells transfected with the H87N mutant was the same as that in cells transfected with the empty vector, clearly demonstrating that the H87N mutant has no detectable catalytic activity. The H142N mutant was found to induce Gly-Sar transport activity in HeLa cells to a significant extent, indicating that this particular mutant does have measurable catalytic activity. However, the activity was found to be only about 30% of the activity induced by the wild type hPEPT2. It is concluded from these results that His-87, which is located in the second putative transmembrane domain of hPEPT2, is absolutely essential for catalytic activity. Although His-147 which is located in the fourth

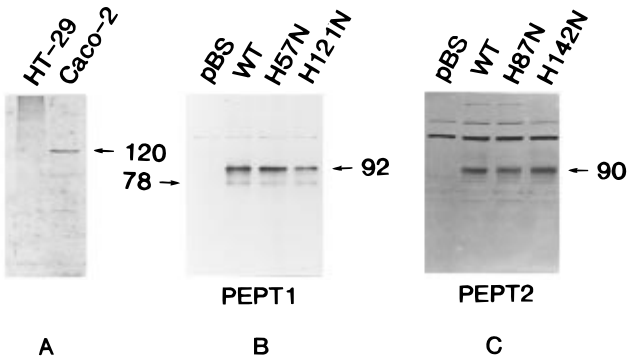

FIGURE 7: Immunoblot analysis of protein expression with wild type and mutant hPEPT1s and hPEPT2s. The polyclonal antibodies used here were raised against an 18-amino acid peptide sequence in hPEPT1. (A) Plasma membranes prepared from HT-29 cells (peptide transport-negative) and Caco-2 cells (peptide transport-positive) were probed with the antibodies. (B) HeLa cells were transfected with one of the following using the vaccinia virus expression technique: pBluescript (pBS) without cDNA insert, pBS-hPEPT1 cDNA (wild type, WT), pBS-H57N hPEPT1 cDNA, or pBS-H121N hPEPT1 cDNA. Crude membranes were prepared from cell lysates and probed with the antibodies. (C) HeLa cells were transfected with one of the following using the vaccinia virus expression technique: pBluescript (pBS) without cDNA insert, pBS-hPEPT2 cDNA (wild type, WT), pBS-H87N hPEPT2 cDNA, or pBS-H142N hPEPT2 cDNA. Crude membranes were prepared from cell lysates and probed with the antibodies. Arrows indicate the molecular mass of the proteins in kDa.

putative transmembrane domain is not absolutely essential, this residue does appear to play a significant role in the maintenance of the catalytic activity of hPEPT2.

The results described thus far demonstrate that the histidyl residue which is obligatory for catalytic function of hPEPT1 and hPEPT2 is the residue that is present in the second putative transmembrane domain of these proteins, namely His-57 in hPEPT1 and His-87 in hPEPT2. The relative location of these hisitidyl residues in the membrane topology of these proteins predicted from their amino acid sequence is identical. In both of these proteins, this particular histidyl residue is located near the extracellular surface of the second transmembrane domain. This residue is most likely involved in the binding and translocation of H^+. With respect to the role of the second conserved histidyl residue that is located in the fourth putative transmembrane domain, there is a significant difference between hPEPT1 and hPEPT2. In hPEPT1, there is no indication that this residue (His-121) is essential for catalytic function to any significant extent. On the other hand, mutation of the corresponding histidyl residue in hPEPT2 (His-142) significantly reduces the catalytic activity, suggesting a potential functional role for this residue.

Immunodetection. The results of the present study strongly suggest, but do not prove, that His-57 in hPEPT1 and His-87 in hPEPT2 are obligatory for the transport function of these proteins. It could be argued that the lack of detectable transport activity with the hPEPT1 and hPEPT2 mutants in which these histidyl residues are mutated may be due to interference of these mutations with protein stability rather than due to the obligatory nature of the histidyl residues. Therefore, we investigated the expression of these proteins in HeLa cells transfected with wild type and mutant cDNAs by immunoblot analysis. The antibodies used for this purpose were raised against an 18-amino acid peptide sequence in hPEPT1. The specificity of affinity-purified antibodies toward the peptide transporter was first probed

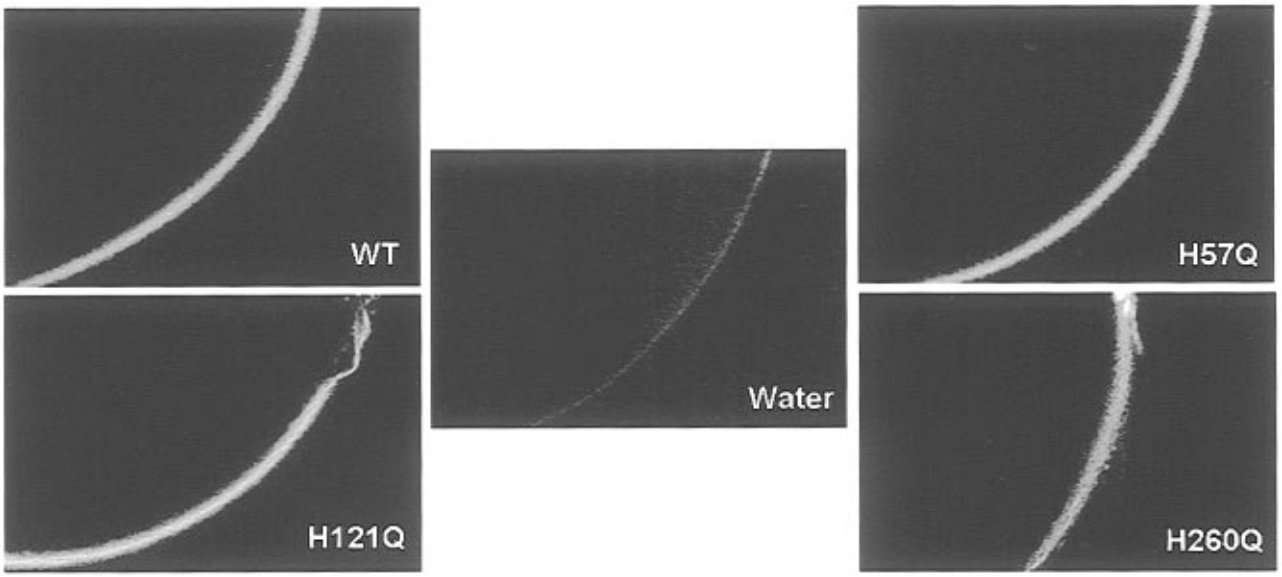

FIGURE 8: Confocal analysis of hPEPT1-specific immunofluorescence in *X. laevis* oocytes. Oocytes were injected with water (control) or with cRNAs of the wild type and mutant hPEPT1s. A polyclonal antipeptide antibody raised in rabbits against an 18-amino acid peptide sequence of hPEPT1 was used as the primary antibody, and fluorescein isothiocyanate-labeled goat antirabbit IgG was used as the secondary antibody. Immunofluorescence was detected by confocal microscopy.

by immunoblot analysis with plasma membranes isolated from two human colon carcinoma cell lines, HT-29 and Caco-2. The HT-29 clone used in our laboratory does not possess detectable H^+-coupled peptide transport activity, whereas the Caco-2 cell clone expresses a H^+/peptide cotransporter which is structurally and functionally identical to hPEPT1 (Brandsch *et al*., 1994; Ganapathy *et al*., 1995; Muller *et al*., 1996). Plasma membranes were isolated from these two cell lines by Mg^{2+}-aggregation technique (Jayanthi *et al*., 1994) and used for analysis with the antibodies. A major protein corresponding to a molecular mass of 120 kDa was detectable with these antibodies in plasma membranes from Caco-2 cells (Figure 7A). This protein was absent in plasma membranes which were obtained from HT-29 cells. These data show that the antibodies used in this study are specific for the peptide transporter. These antibodies were subsequently used for immunoblot analysis of membrane fractions obtained from HeLa cells transfected with empty pBluescript vector, wild type hPEPT1 cDNA or mutant hPEPT1 cDNAs (Figure 7B). A prominent protein band of 92 kDa and a much less prominent protein band of 78 kDa were detected with the antibodies in HeLa cells transfected with wild type hPEPT1. These two proteins were not present in membrane fractions obtained from HeLa cells transfected with the empty vector, suggesting that the proteins are related to hPEPT1. A more relevant finding was that these two proteins were expressed in HeLa cells transfected with the H57N hPEPT1 and H121N hPEPT1 mutant cDNAs to levels comparable to those in cells expressing the wild type hPEPT1. There was a minor protein band with a molecular mass of 140 kDa detectable by the antibodies, but this protein is unrelated to hPEPT1 because it was also present in HeLa cells transfected with the empty vector. These data show that mutation of His-57 or His-121 in hPEPT1 does not interfere with protein stability. Therefore, lack of detectable transport activity with the H57N hPEPT1 mutant indicates the obligatory role of His-57 in the catalytic function of hPEPT1. The difference in the molecular mass of the proteins detected by the antibodies between Caco-2 cells and HeLa cells expressing hPEPT1 is probably due to difference in glycosylation. hPEPT1 has seven putative N-glycosylation sites. Caco-2 cells express hPEPT1 endogenously, and therefore hPEPT1 in these cells is expected to be optimally glycosylated. In contrast, hPEPT1 is only transiently expressed in HeLa cells, and the expressed proteins are most probably underglycosylated contributing to the relatively smaller molecular mass.

We do not have antipeptide antibodies against hPEPT2. However, we found that the antibodies raised against the peptide sequence in hPEPT1 do crossreact with hPEPT2. Comparison of amino acid sequences between hPEPT1 and hPEPT2 reveals that hPEPT2 possesses a region highly homologous to the 18-amino acid peptide sequence of hPEPT1 against which the antibodies were raised. The amino acid identity in this region of hPEPT2 and the 18-amino acid peptide of hPEPT1 is 61%. This homologous region in hPEPT2 is located in the large cytoplasmic loop between transmembrane domains 6 and 7 which is the same as the location of the 18-amino acid peptide sequence in hPEPT1. Therefore, it is not surprising that the polyclonal antibodies raised against the 18-amino acid peptide crossreact with hPEPT2. This enabled us to perform immunoblot analysis of the expression of hPEPT2 in HeLa cells transfected with wild type and mutant hPEPT2 cDNAs (Figure 7C). A major protein with a molecular mass of 90 kDa was detected in HeLa cells expressing the wild type hPEPT2. This protein was absent in HeLa cells which were transfected with the empty vector. The level of expression of this 90 kDa protein was similar in cells transfected with the wild type hPEPT2 cDNA or with the mutant (H87N and H142N) hPEPT2 cDNAs. There were a number of other proteins in HeLa cells which were detected by the antibodies, but these proteins were unrelated to hPEPT2 as they were also present in cells transfected with the empty vector. The unrelated proteins are more numerous in Figure 7C than in Figure 7B because the time of exposure of the autoradiographic film to detect hPEPT2 was much longer than to detect hPEPT1 with these antibodies. These results show that mutation of

His-87 or His-142 in hPEPT2 does not interfere with protein stability. Therefore, lack of detectable transport activity with the H87N hPEPT2 mutant indicates the obligatory role of His-87 in the catalytic function of hPEPT2. Even though the transport activity of the H142N hPEPT2 mutant was only 30% of the transport activity of the wild type hPEPT2, the level of expression of the hPEPT2 protein was comparable in both cases. This shows that His-142 does indeed play a significant function in the maintenance of the transport activity. It has to be pointed out here that the molecular size of the hPEPT2 protein detected in HeLa cells is much smaller than the molecular size of the rabbit homolog (glycosylated form) (Boll *et al*., 1996). The rabbit PEPT2 consists of 729 amino acids just as hPEPT2 does and the molecular size of the protein core is ~82 kDa in both cases. The molecular size of the glycosylated form of rabbit PEPT2 is 107 kDa (Boll *et al*., 1996) which is higher than that of hPEPT2 found in HeLa cells. It is not known whether this difference is due to species-specific variations in the extent of glycosylation between the two PEPT2s or due to incomplete glycosylation of hPEPT2 when transiently expressed in HeLa cells.

The experiments described above were done with crude membrane preparations obtained from transfected HeLa cells. Even though the results clearly show that the total cellular expression of the transporter protein is comparable among the wild type and mutant PEPT1s and PEPT2s, it can be argued that trafficking of the transporter protein to the plasma membrane may not be the same. We addressed this issue by confocal analysis of immunofluorescence in *X. laevis* oocytes expressing the wild type and mutant hPEPT1s (Figure 8). The hPEPT1 protein was detected using the antipeptide antibody and fluorescein isothiocyanate-labeled goat antirabbit IgG. Immunofluorescence was analyzed by confocal microscopy. The hPEPT1 cRNA-induced protein was found only in the plasma membrane of the oocyte. Moreover, the level of expression was comparable among the wild type and mutant hPEPT1s. There was very little immunofluorescence associated with the plasma membrane of water-injected oocytes. These data show that mutation of the three histidyl residues does not interfere with protein trafficking to the plasma membrane.

In the case of hPEPT2, functional studies in cDNA-transfected HeLa cells have clearly shown that His-87 is absolutely essential for the transport activity. Total expression of the mutant hPEPT2 protein was found to be comparable to that of the wild type hPEPT2 in these cells, making it very unlikely that defects in protein trafficking to the plasma membrane are responsible for the observed total loss of transport function. The H142N mutant of hPEPT2, on the other hand, possesses 30% of transport activity compared to the wild type hPEPT2. It is possible, but unlikely, that the decrease in transport function resulting from the mutation of His-142 is due to a defect in protein trafficking to the plasma membrane. However, quantification of the plasma membrane expression of the wild type and mutant hPEPT2 using confocal analysis is necessary to address this issue unequivocally. This approach would require availability of antibodies which are much more specific towards hPEPT2 than the hPEPT1 antibodies used in the current study.

In summary, we were able to identify the histidyl residue that is obligatory for catalytic function of the human H^+/peptide cotransporters PEPT1 and PEPT2. This was accomplished by site-directed mutagenesis of specific histidyl residues and by comparative analysis of transport function and protein expression of the wild type and mutant cDNAs. The results of the investigation show that His-57 in hPEPT1 and His-87 in hPEPT2 are absolutely essential for the transport function of these proteins. These two critical histidyl residues are located in similar topological positions in these proteins, i.e., near the extracellular surface of the second putative transmembrane domain. These histidyl residues are most likely involved in the binding and translocation of H^+ associated with the peptide transport activity of hPEPT1 and hPEPT2.

ACKNOWLEDGMENT

We thank Ida O. Walker and Lisa Young for excellent secretarial assistance.

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BI962058P