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Molecular Identification of a Role for Tyrosine 167 in the Function of the Human Intestinal Proton-Coupled Dipeptide Transporter (hPepT1)

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hPepT1 is a proton-coupled peptide transporter that mediates the absorption of di- and tripeptides. Here we show that tyrosine 167 (Y167) in transmembrane domain 5 (TMD5) of this 12-transmembrane spanning protein contributes to its transport function. We identified this particular amino acid by a computer model of the arrangement of the TMDs of hPepT1 and investigated its role by site-directed mutagenesis and dipeptide uptake studies. [^{3}H]Gly-sar uptake in cells transiently transfected with Y167A-hPepT1 was abolished completely, even though the level of Y167A-hPepT1 expression by Western blot analysis and cell surface expression by immunofluorescence microscopy was similar to those of the wild type. Therefore, mutation affected transport function, but apparently not the steady-state protein level or trafficking of the transporter to the plasma membrane. Moreover, mutation of Y167 into phenylalanine, serine, or histidine all abolished gly-sar uptake in transfected HEK 293 cells. Taken together, these findings suggest that Y167 plays an essential role in hPepT1 function, perhaps due to the unique chemistry of its phenolic side chain.

The intestinal H^+-coupled dipeptide transporter plays an important role in the absorption of di- and tripeptides as well as peptidomimetics such as β-lactam antibiotics and angiotensin converting enzyme inhibitors [for a review, see Ref. (1)]. The cloning of the rabbit intestinal peptide transporter (PepT1) in 1994 (2) represents a major milestone in dipeptide transporter research, but progress in elucidating the structure-function of this 12-transmembrane spanning protein (2, 3) has been slow. Recent findings by Basu *et al.* (4) and Covitz *et al.* (5) support the notion of an intracellular location for the C-terminal end of the transporter (5) and of an extracellular location for the loops between TMD3 and TMD4 (5) and TMD9 and TMD10 (4, 5). Little information is available, however, about constituent amino acids and the functional moieties that govern transporter function (6). Chemical modification of histidine (7,8) and tyrosine residues (8) suggested a possible role for them in dipeptide transporter function. This early finding on the role of histidine was subsequently confirmed by site-directed mutagenesis (9, 10).

The precise tyrosine(s) that are important for dipeptide uptake mediated *via* hPepT1 have not been uncovered. Given the experimental difficulty in purifying and crystallizing membrane proteins for structure-function studies (11), we have developed a sequential approach of identifying key residues by computer simulation of hPepT1, site-directed mutagenesis of one of the identified key residues, tyrosine 167 (Y167), and have tested the prediction by assaying dipeptide uptake in human embryonic kidney (HEK 293) cells transfected with the mutated transporter. Interestingly, this amino acid residue has been shown by multiple sequence alignment analysis (12) to be conserved in the di- and tripeptide transporters from bacteria, fungi, yeast, plant, rabbit and human.

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Abbreviations used: gly-sar, glycyl sarcosine, HEK, human embryonic kidney; MES, 2-[*N*-morpholino]ethanesulfonic acid; TMD, transmembrane domain.

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HEK 293 cells were used for the expression of the wild type and the mutated hPepT1 transporters because of their low endogenous dipeptide transport activity. We discovered that mutation of hPepT1 at tyrosine 167 into alanine abolished the uptake of [^{3}H]gly-sar in transfected HEK 293 cells. Even substitution of this tyrosine to phenylalanine (Y167F an aromatic amino acid lacking only the phenolic hydroxyl group,) or to serine (Y167S an amino acid with aliphatic hydroxyl group,) resulted in a completely inactive transporter. The degree of inactivation by the Y167 mutation is probably due to its effect on transport function rather than folding, since this mutation did not inhibit the synthesis or the surface expression of the transporter protein. The stringent conservation of this tyrosine residue in mammalian H^+/dipeptide cotransporters across species and isoforms (12), and no tolerance to mutation at this position suggests an essential role of this residue in hPepT1-mediated dipeptide uptake.

EXPERIMENTAL PROCEDURES

Computer modeling of hPepT1. A two-step approach was taken to generate a model of hPepT1. First, the most favorable orientation of each of the 12 α-helical TMDs was determined based on pairwise interaction with the adjacent α-helix. Because there are seven possible orientations of α helix along a single face of another helix, there will be 49 possible pairwise alignments for two α helices, excluding translation along the helical axis. By computing the energy of all possible orientations, some simple pairwise relationships were established. Amphipathicity of the helices was determined from the hydropathies of the constituent amino acids using the program HELIXGEN. Based on the pairwise calculations and the amphipathicity, a model was constructed with the TMDs which exhibit highest amphipathicity facing a central channel. This model was visualized using MidasPlus[2] (13), and has allowed us to identify amino acids that might form a putative channel of the dipeptide transporter.

Site-directed mutagenesis and DNA sequencing. The hPepT1 cDNA (nt 719 to nt 2,982) (kindly provided by Dr. Matthias A. Hediger) was subcloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA) by ligating the 2,306 *Kpn*I/*Bam*HI fragment into the multiple cloning sites of pcDNA3. The hPepT1 cDNA in this resultant expression plasmid, pcDNA3-hPepT1, is under the control of the cytomegalovirus (CMV) promotor. The plasmid pcDNA3-hPepT1 was used as a template, whereas a primer that eliminated a unique *Xba*I site in the pcDNA3-hPepT1 plasmid was used as a selection primer in all mutagenesis reactions. A point mutation was introduced using a mutagenic primer bearing the altered amino acid sequence. Mutagenesis procedures were carried out on the wild type hPepT1 using Transformer Site-Directed Mutagenesis Kit from Clontech (Palo Alto, CA) following the manufacturer's protocol. Plasmid DNA from individual clones of mismatch repair-deficient host cell *E. coli* BMH71-18 mut. S. was analyzed by determining the nucleotide sequence at the mutation site according to the dideoxy chain termination method (14) using a Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

Transient transfection and functional characterization of hPepT1 in HEK 293 cells. HEK 293 cells were transiently transfected with the wild type hPepT1, Y167A-hPepT1 or vector pcDNA3 alone following the DEAE-dextran method (15) and studied 48 hr after transfection. For uptake studies, the growth medium was removed from the transfected cells and 0.3 ml of MES-Tris (pH 6) buffer (3), containing 20 μM [^{3}H]gly-sar (specific activity, 30 Ci/mmol; American Radiolabelled Chemicals Inc., St. Louis, MO) was added to each well and incubated at 37°C for varying time periods. After washing three times in ice-cold MES-Tris (pH 6.0) buffer, the cells were lysed in 1.0 ml of lysis buffer (1% SDS in 0.2N NaOH). The protein content of each well was determined using BCA protein assay reagents (Pierce, Rockford, IL) and the cell-associated radioactivity measured in a Beckman LS1801 liquid scintillation counter (Fullerton, CA).

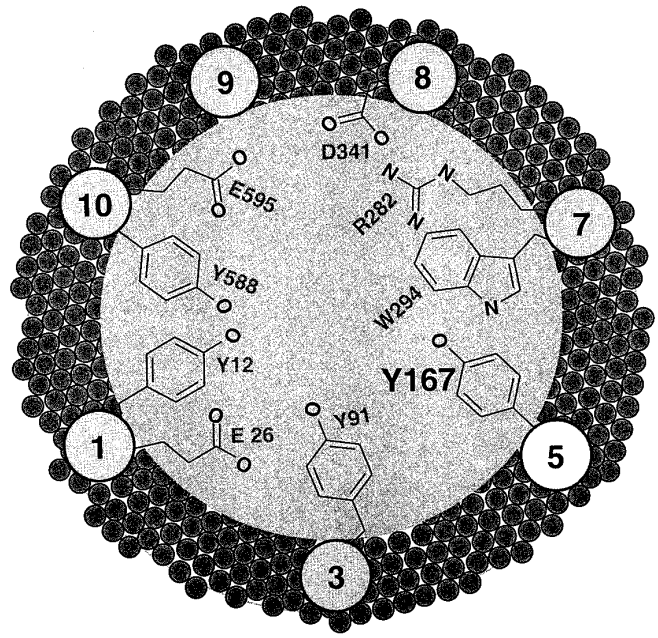

FIG. 1. A schematic view of the computer model of the transmembrane domains (TMDs) of hPepT1. TMDs 7, 8, 9 and 10 form a half-channel and TMDs 1, 3 and 5 constitute the other half. Important residues which project their side chains into the putative channel are Y12 and E26 from TMD1, Y91 from TMD3, Y167 from TMD5, W294 and R282 from TMD7, D341 from TMD8 and Y588 and E595 from TMD10. The polar heads of the lipid bilayer, in which the TMDs are buried, are represented by (●).

Western Blot of hPepT1. Anti-peptide affinity-purified polyclonal antibodies against the C-terminal end (p694-708) of hPepT1 (a generous gift from Drs. Kuang-Ming Covitz and Wolfgang Sadée) were utilized for Western blot analysis. Wild type or Y167A-hPepT1 transfected HEK cells, or nontransfected Caco-2 cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were then incubated in hPepT1-specific rabbit primary antibody at a dilution of 1:1000 overnight at 4°C, and subsequently with HRP-linked goat anti-rabbit secondary antibody at a dilution of 1:20,000 for 1 hr at room temperature. Detection of the transporter protein was performed using enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Arlington Heights, IL). The resulting signal was recorded on a X-ray film (Kodak XAR-2).

Immunofluorescence microscopy staining. Wild type or Y167A-hPepT1 transfected HEK cells were plated on glass coverslips and cultured for 24 hr. After fixation in 3.7% formaldehyde in PBS at room temperature for 20 min, cells were washed 3 times with PBS, and stored in 0.02% NaN_3 in PBS at 4°C. Before staining, cells were

[2] The MidasPlus program is from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH Grant RR-01081).

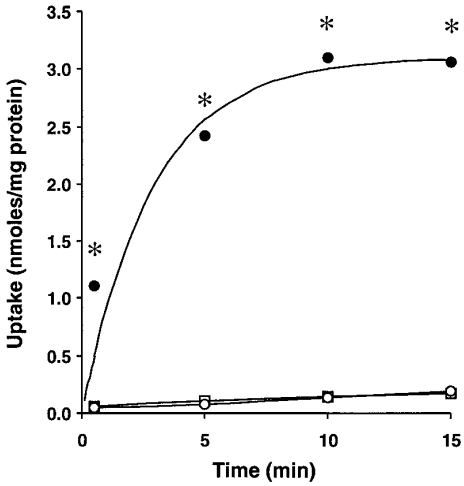

FIG. 2. Uptake of glycyl sarcosine in the wild type or the Y167A-hPepT1 transfected HEK 293 cells. HEK 293 cells were transfected with wild type hPepT1 (●), Y167A-hPepT1 (○), or plasmid vector pcDNA3 alone (□). Uptake of [^{3}H]glycyl sarcosine (20 μM) was measured for different time periods at pH 6.0. Values represent mean ± SEM for three determinations. Asterisks indicate statistically significant difference at $p < 0.05$ compared with uptake via Y167A-hPepT1 at the respective time points. Where no error bar is shown it falls within the symbol.

permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature, blocked with 1% bovine serum albumin and 0.66% gelatin in PBS at room temperature for 30 min, and washed once with 0.05% Tween 20 in PBS (PBST). Cells were then incubated with affinity-purified anti-hPepT1 primary antibodies at a dilution of 1:500 for 2 hr and washed with PBST 3 times. Cells were then incubated with FITC-conjugated secondary antibodies at a dilution of 1:500 for 1 hour. After final washes, coverslips were mounted onto slides with Vectashield (Vector Laboratories, Burlingame, CA), and examined using a Zeiss Axioskop.

RESULTS AND DISCUSSION

Computer model of hPepT1. Based on pairwise interactions of TMDs 7 and 8, 8 and 9, and 9 and 10, we were able to create a model for half of a putative channel formed by hPepT1, comprising these TMDs. Of the remaining TMDs, 1, 3, and 5 have the greatest amphipathicity. We propose that these will complete the channel, with the remaining, largely hydrophobic, TMDs located distal to the channel, and playing a structural role through interactions with the lipid membrane. The proposed channel-forming TMD arrangement is shown schematically in Fig. 1 to illustrate a number of key charged and aromatic amino acids that could be involved in peptide transport, and that are oriented into the channel. The model provided us with the basis for selecting these amino acids for mutagenesis, but not necessarily to discriminate which of the selected amino acids that are most significant in transport. The focus on Y167 in this paper is due to the particular sensitivity of peptide uptake to mutation of this amino acid that is shown in our subsequent experiments. However, most of the other key amino acids identified in Fig. 1 have also been found to be important for peptide transport (manuscript in preparation), which provides us with confidence in the correctness of general features of the model.

Site-directed mutagenesis and uptake characteristics of mutated hPepT1 in HEK 293 cells. To establish a system with which hPepT1 transport function could be measured, pcDNA3-hPepT1 was transiently transfected into HEK 293 cells for measurement of [^{3}H]glysar uptake. [^{3}H]Gly-sar uptake was markedly higher in hPepT1-transfected HEK 293 cells than in the mock-transfected (transfected with the vector pcDNA3 alone) cells, indicating functional expression of hPepT1 (Fig. 2) (3). To investigate the role of Y167, site-directed mutagenesis was used to generate the Y167A-hPepT1. As shown in Fig. 2, uptake mediated *via* Y167A-hPepT1 was similar to the vector-transfected control, and was drastically lower than the wild type hPepT1. To further investigate the obligatory role of Y167, site-directed mutagenesis was used to generate the Y167F-, Y167H- and Y167S-hPepT1 mutations. All three mutations also completely abolished [^{3}H]gly-sar uptake in the transfected HEK 293 cells (Fig. 3).

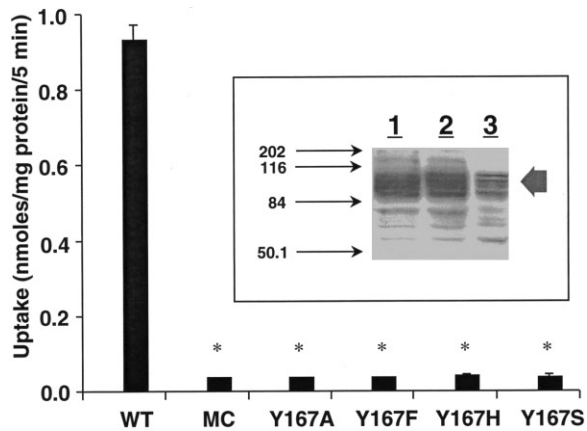

FIG. 3. Uptake of glycyl sarcosine via the wild type or in the various Y167 mutated hPepT1. HEK 293 cells were transfected with wild type hPepT1, pcDNA3 vector alone or the specific Y167 mutated hPepT1. Uptake of [^{3}H]glycyl sarcosine (20 μM) was measured at pH 6.0. Values represent mean ± s.e.m. for three determinations. Key: WT, wild type hPepT1; MC, mock control, i.e., pcDNA3 vector alone. Asterisks indicate statistically significant difference at $p < 0.05$ compared with uptake via wild type hPepT1. Where no error bar is shown it falls within the symbol. (Inset) Western blot analysis of the wild type and Y167A-hPepT1 demonstrating that the reduced uptake in Y167A-hPepT1 was not due to reduced synthesis of the transporter protein. Lane 1, HEK cells transfected with wild type hPepT1; lane 2, HEK cells transfected with Y167A-hPepT1; and lane 3, nontransfected Caco-2 cell lysates (15 μg protein/lane). The predominant species migrated at approximately 110 kDa (arrow). The numbers to the left of the autoradiograph indicate the positions of molecular mass markers in kilodaltons (kD) electrophoresed in parallel.

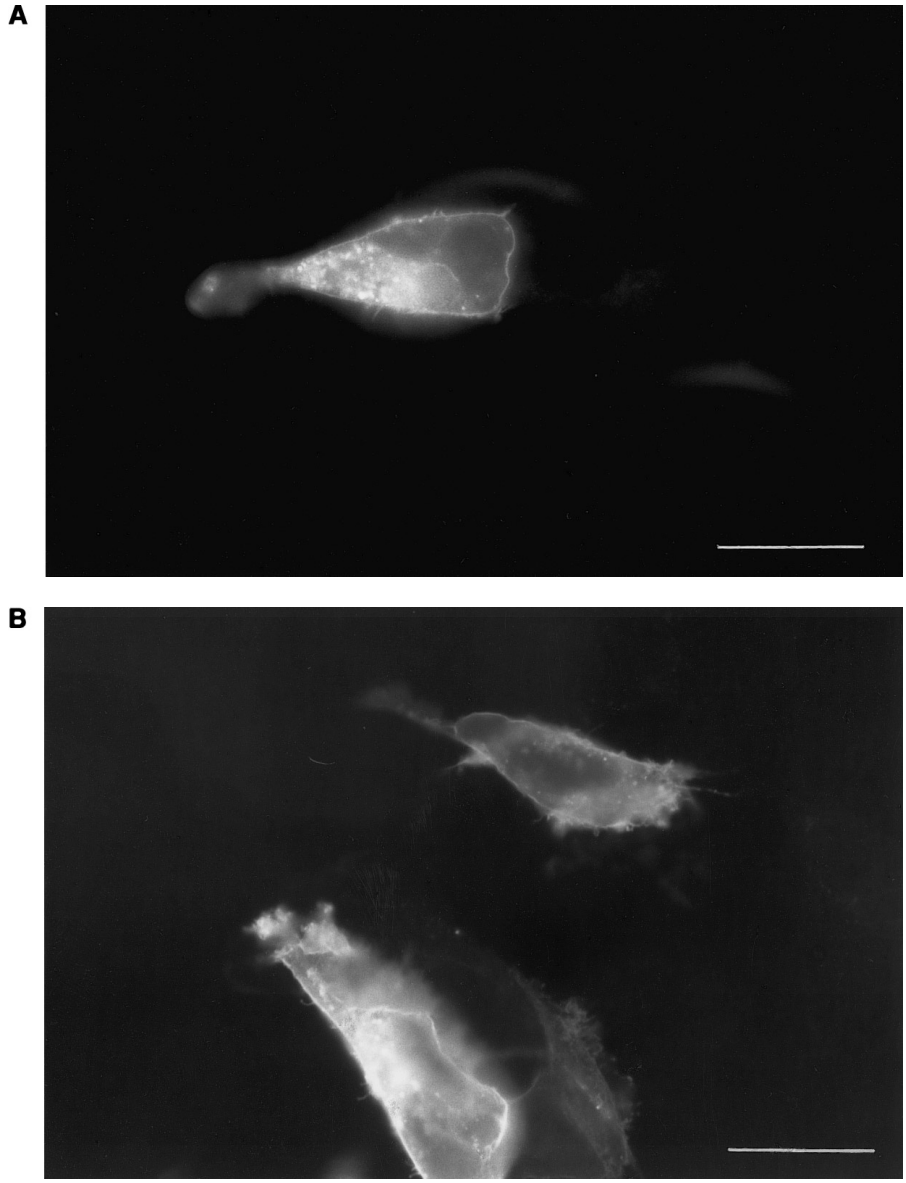

FIG. 4. Immunolocalization of wild type and Y167A-hPepT1 on HEK 293 cell surface. HEK cells were transiently transfected with either wild type hPepT1 (A) or Y167A-hPepT1 (B) cDNAs, grown on glass coverslips, and processed for immunofluorescence microscopy as detailed in the text. Scale bar, 10 μm.

Western blot of the wild type and Y167A-hPepT1. The diminished uptake of gly-sar by Y167A-hPepT1 relative to wild type hPepT1 might result from lower expression of Y167A-hPepT1. To determine the relative levels of Y167A- and wild type hPepT1 in transfected HEK293 cells, Western blot analysis of transfected hPepT1 was performed. Caco-2 cell lysates were used as a positive control (lane 3). As can be seen in the inset of Fig. 3, both wild type (lane 1) and mutated hPepT1 (lane 2) were expressed to comparable levels. Moreover, the relative molecular masses of Y167A-, transfected wild-type, and endogenous hPepT1 are very similar, suggesting that the biosynthesis of Y167A-hPepT1 was not grossly altered.

Immunolocalization of the wild type and Y167A-hPepT1. Although the biosynthesis of Y167A-hPepT1 appears to be normal, this mutation could also result in the misfolding of the transporter. This misfolding might not be detected by Western blots, but could result in the inability of the mutated transporter to be delivered to the plasma membrane. It is known that a misfolded protein would be retained in the endoplasmic reticulum and eventually degraded (16). Thus, the surface expression of Y167A-hPepT1 and wild type

hPepT1 was assayed by immunofluorescence microscopy. As shown in Fig. 4, wild type hPepT1 (Fig. 4A) and Y167A-hPepT1 (Fig. 4B) were expressed at comparable levels at the plasma membrane, suggesting that the diminished uptake of gly-sar by Y167A-hPepT1 is a result of an effect of the mutation on the transport function rather than an effect on protein folding and delivery to the plasma membrane.

Thus, results from the present study indicated a strategic role of Tyr-167 in hPepT1 function which appears to reside in both the chemical and spatial property of its phenolic group. Tyrosine has also been shown to be critical in the uptake of γ-aminobutyric acid (GABA) by the GAT-1 (17) and in the uptake of serotonin (5-HT) by the SERT (18). The role of the phenolic side chain of tyrosine was hypothesized to be liganding the amino group common to all biogenic amines (17).

In summary, an important role for tyrosine 167 in TMD5 for the modulation of dipeptide transport by hPepT1 is predicted by computer modeling and subsequently confirmed by site-directed mutagenesis. Work is in progress to elucidate other amino acid residues that together with tyrosine 167 may constitute the substrate-translocation domain of hPepT1.

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