Stable Transfection of MDCK Cells with Epitope-Tagged Human PepT1

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Purpose. Establish and characterize a MDCK cell line that stably expressed PepT1.

Methods. MDCK cells stably transfected with N-terminal HA-tagged hPepT1 were examined by immunostaining using antibodies to HA and hPepT1. HA-hPepT1 expression was verified by immunoprecipitation and Western blotting from cell lysates. The function of the expressed HA-hPepT1 was evaluated by glycyl-sarcosine uptake. The half-life of PepT1 on the cell surface was measured by biotinylation and chase-labeling of the cells, followed by immunoprecipitation with the anti-PepT1 antibody and blotting with HRP-streptavidin.

Results. Immunolabeling with antibodies to HA and PepT1 showed fluorescence colocalization. Immunoprecipitation with the anti-HA antibody and Western blotting with the anti-PepT1 antibody showed a broad 90–105 kDa band, and vice versa when the antibodies were utilized in the reverse order. Glycyl-sarcosine uptake was increased in the stably transfected cells by 2–5 fold over untransfected control cells. The Km value of 375 μM accurately reflected the characteristic low affinity of PepT1. Finally, the half-life of the surface biotinylated HA-hPepT1 was measured to be 22 hrs.

Conclusions: A MDCK cell line stably expressed intact and functional HA-tagged hPepT1 on its cell surface. This cell line represents an alternative to the use of Caco-2 cells to evaluate PepT1 function.

KEY WORDS: epitope tagging; MDCK; PepT1; stable transfection; transporter.

INTRODUCTION

PepT1 (SLC15A1) is a mammalian member of the proton-coupled oligopeptide transporter family SLC15, which transports short-chain peptides and peptidomimetics across the cell membrane (1). PepT1 was identified in the intestinal epithelium as playing a role in the intestinal absorption of peptide nutrients and peptidomimetic compounds (2). Compared to the renal isoform PepT2 (SLC15A2), PepT1 is a lower affinity, higher capacity isoform (2). Besides all natural di-/tri-peptides, PepT1 substrates also include β-lactam antibiotics, angiotensin-converting enzyme inhibitors, 4-aminophenylacetic acid, L-valine esters, and ω-amino fatty acids (1,2). Recently, amino acid groups were conjugated to hydrophilic drugs, resulting in the increase of intestinal permeability of their parent drugs several-fold (3).

Because of its unusually broad substrate selectivity, large transport capacity, and significance in prodrug design, intensive investigation has been focused on the substrate requirements, tissue distribution, protein structure, and mechanisms of regulation of PepT1. However, these studies are often hindered by the absence of a highly specific PepT1 antibody, as well as a good cell culture model to test PepT1-dependent transport.

We found that PepT1 retained its dipeptide transport function after fusing the influenza hemagglutinin (HA) epitope, a nine-amino-acid sequence, at the N-terminus. Therefore, specific high-affinity monoclonal antibodies against HA could be used for the detection of HA-tagged PepT1. Experimentally, we constructed and characterized a Madin-Darby canine kidney (MDCK) cell line that stably expressed HA-tagged hPepT1 on the cell surface. MDCK cells were chosen because they are one of the best-characterized epithelial cell lines (4) and form a cell monolayer with a resistance comparable to the intestinal epithelial Caco-2 cell line (5). Availability of an MDCK clone that stably expresses hPepT1 would represent a valuable model system to test PepT1 substrates.

MATERIALS AND METHODS

Materials

MDCK and HEK293 (human embryonic kidney) cells were purchased from American Type Culture Collection. Polymerase chain reaction (PCR) primers, Taq polymerase, T4 ligase, endonucleases Kpn I and Sal I, Dulbecco's Modified Eagle's Medium (DMEM), LipofectAMINE 2000 reagent, protein ladders, Protein A Sepharose CL-4B, and geneticin were obtained from Invitrogen (Carlsbad, CA, USA). Endonucleases Pml I was purchased from New England Biolabs (Beverly, MA, USA). Mouse monoclonal anti-HA antibody, glycyl-sarcosine (gly-sar), Sepharose CL-2B, horseradish peroxidase (HRP)-conjugated streptavidin, and PMA (phorbol 12-myristate 13 acetate) were purchased from Sigma (St. Louis, MO, USA). The rabbit anti-hPepT1 antibody was developed by Sigma Genosys (Houston, TX, USA) using the hPepT1 C-terminal peptide LEKSNPYFMSGANSQKQM (6). Donkey or goat anti-mouse or anti-rabbit IgG conjugated to HRP, fluorescein (FITC), or rhodamine red-X (RRX) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Sulfo-NHS-biotin, Western blot stripping buffer, and chemiluminescent substrates were obtained from Pierce (Rockford, IL, USA). DC protein Assay Kit was obtained from BioRad (Hercules, CA, USA). [^{3}H]gly-sar was purchased from Moravek (Brea, CA, USA). Antifade mounting media was obtained from Molecular Probes (Eugene, OR, USA).

Construction of HA-Tagged hPepT1

The DNA sequence encoding the HA epitope was fused to the N-terminus of the hPepT1 cDNA by PCR method. Oligo CAGGTACCATGTACCCATACGATGTTCCTGACTATGCGGGTATGGGAATGTCCAAATCACACAGT was the 5′-primer, oligo GCGAATTCCAAACTTCCAGCATTAATAGC the 3′-primer, and hPepT1-pcDNA3 (7) the template. The PCR product was cut with restriction endonucleases Kpn I and Pml I, and inserted

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ABBREVIATIONS: HEK, human embryonic kidney; HA, influenza hemagglutinin; gly-sar, glycyl-sarcosine; MDCK, Madin-Darby canine kidney; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; PKC, protein kinase C.

into the Kpn I/Pml I digested hPepT1-pcDNA3. The above insertion was verified by DNA sequencing.

Transfection

Transient transfection of HEK cells was performed as described (7). For generation of stable transfectants, MDCK cells were transfected with the HA-hPepT1-pcDNA3 plasmid using LipofectAMINE 2000 reagent, and were grown in 0.5 mg/ml geneticin in DMEM (high glucose) for 4 weeks. Ten surviving cell colonies were randomly selected and examined by immunostaining using the anti-HA antibody as described in Ref. 7. One cell colony that exhibited the highest surface HA staining was chosen for the following experiments. The expression of HA-hPepT1 in this cell line persisted after 3 months of continuous culture.

Uptake

Cells grown on 24-well cluster plates were incubated with gly-sar solution (10 μM, pH 6.0) spiked with 1.2 μCi/ml [^{3}H]gly-sar at 37°C for 10 min. After washing three times with ice-cold PBS, the cells were lysed with 0.5% Triton X-100. Protein concentration of the cell lysates was analyzed with the DC protein Assay Kit, and the radioactivity was measured by scintillation counting.

Immunoprecipitation

Immunoprecipitation was performed as described (8). Briefly, cell lysates in Triton Dilution Buffer (2.5% Triton X-100, 100 mM triethanolamine, and 100 mM NaCl) were pre-cleared twice with Sepharose CL-2B, and incubated with the antibody and Protein-A Sepharose CL-4B at 4°C overnight. After washing, the beads were boiled in 10% SDS buffer for 5 min and centrifuged for 30 s. The supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filter, and then subjected to Western blot analysis using the appropriate primary and secondary antibodies as indicated in the text.

Detection of Cell Surface PepT1

Three days after seeding on 60-mm dishes, both transfected and untransfected MDCK cells reached confluence. The cells were pre-chilled and incubated with 0.5 mg/ml sulfo-NHS-biotin in HBSS/20 mM Hepes (pH 7.4) on ice for 2 × 15 min. After washing, residual biotin was quenched by 30 min incubation with 50 mM glycine in Hanks-buffered MEM containing 0.6% BSA and 20 mM Hepes on ice. The cells were then cultured at 37°C for 0, 1, 5, 22, 30, and 48 h. Cell lysates were immunoprecipitated with 2 μl of the anti-PepT1 C-terminus antibody, and subjected to SDS-PAGE and electrotransfer similar to the Western blot procedure, except that HRP-conjugated streptavidin instead of a secondary antibody was used to detect the biotinylated proteins by enzyme-linked chemiluminescent assay. The relative abundance of PepT1 protein on blots was quantified using NIH Image.

RESULTS

Visualization of the HA-Tagged hPepT1 Transiently Expressed in HEK293 Cells

HEK293 cells were transiently transfected with the pcDNA3 plasmid containing the N-terminal HA-tagged hPepT1. Immunostaining showed HA staining on the cell border and within punctuate intracellular structures (Fig. 1A), suggesting expression of HA epitope on the cell surface and within intracellular membrane compartments. Immunostaining with the C-terminus PepT1 antibody exhibited the same staining pattern (Fig. 1B). The anti-PepT1 staining completely overlapped with the anti-HA staining (Fig. 1C), suggesting that the HA-tagged protein was in fact hPepT1 and further that intact HA-tagged hPepT1 was expressed on the cell surface.

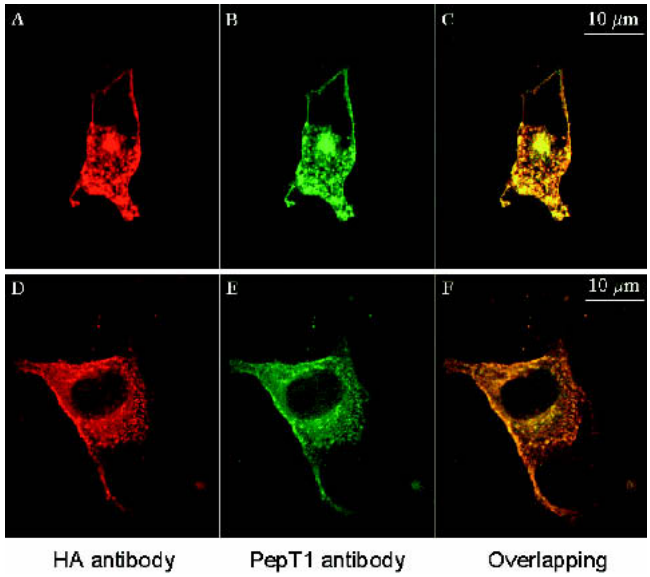

Fig. 1. Visualization of the HA-tagged hPepT1. Cells were double-labeled with the C-terminus PepT1 antibody and a monoclonal antibody against HA epitope, as well as appropriate secondary fluorescent antibodies. (A–C) HEK293 cells transiently transfected with HA-hPepT1. (D–F) MDCK cells stably expressing HA-PepT1. (A and D) Anti-HA staining; (B and E) anti-PepT1 staining; (C and F) overlapping of the anti-HA staining and the anti-PepT1 staining.

Expression of Intact HA-Tagged hPepT1 in Stably Transfected MDCK Cells

After screening of the stably transfected HA-hPepT1-MDCK cells, cells of the selected clone that exhibited the highest HA staining (see Materials and Methods) were subjected to the double immunolabeling procedure conducted with the transfected HEK293 cells in Figs. 1A–1C. Surface labeling and some intracellular punctuate labeling were observed (Figs. 1D and 1E), and the anti-HA staining overlapped with the anti-PepT1 staining (Fig. 1F).

To verify whether the hPepT1 expressed in the stably transfected MDCK cells contained the HA epitope tag, cell lysates were immunoprecipitated with the anti-PepT1 antibody. A broad 90–105 kDa band was recognized on Western blots by the HA antibody in the immunoprecipitates of the transfected cells, but not in that of the untransfected cells (Fig. 2A). In a parallel experiment, the same cell lysates were immunoprecipitated with the HA antibody, and the same protein band was recognized by the anti-PepT1 antibody (Fig. 2B). The use of two antibodies identifying two different epitopes within the HA-hPepT1 confirmed the expression of HA-tagged hPepT1 as an intact protein.

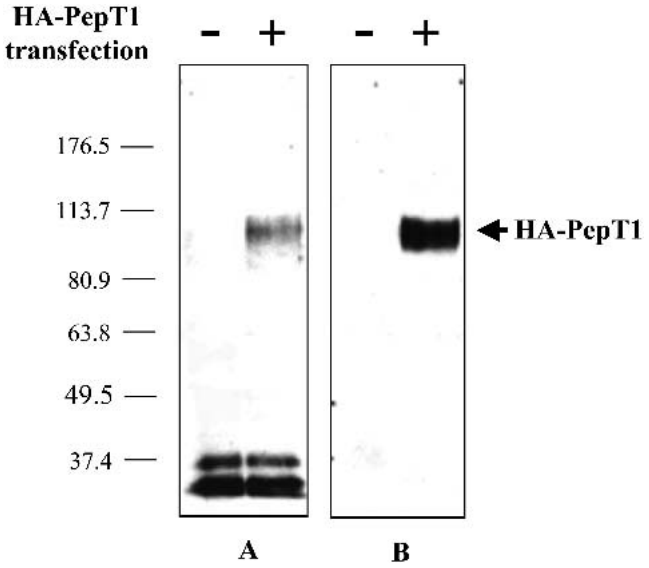

Fig. 2. Intact HA-tagged hPepT1 is expressed in the stably transfected HA-PepT1-MDCK cells. (A) Lysates of transfected and untransfected cells were immunoprecipitated with the anti-PepT1 antibody and examined by Western blot analysis using the anti-HA antibody. (B) The same cell lysates were immunoprecipitated with the anti-HA antibody and examined with the anti-PepT1 antibody by Western blotting. Overlap of the protein bands in (A) and (B) indicated that these bands represented the same protein, HA-hPepT1.

Functional Expression of the HA-hPepT1 in the Stably Transfected MDCK Cells

Transfected- and untransfected-MDCK cells were grown in 24-well plates for 3 days and incubated with a range of concentrations of gly-sar (0, 5, 20, 100, 200, 500, and 1000 μM) spiked with 1.2 μCi/ml [^{3}H]gly-sar for 10 min. As shown in Fig. 3, gly-sar uptake increased 2- to 5-fold after transfection, indicating the functional expression of the HA-hPepT1.

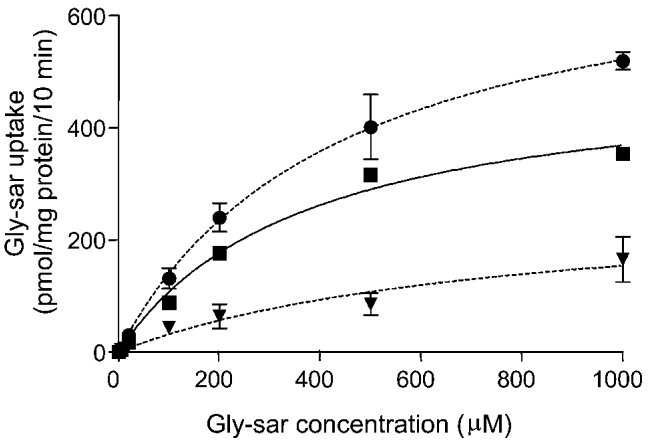

Fig. 3. Kinetics of gly-sar uptake of the HA-tagged hPepT1. Transfected and untransfected MDCK cells were grown in 24-well plates for 3 days and incubated with increasing concentrations of gly-sar (0, 5, 20, 100, 200, 500, and 1000 μM) spiked with 1.2 μCi/ml [^{3}H]gly-sar for 10 min. ● Transfected cells; ▼ untransfected cells; ■ PepT1-dependent gly-sar uptake. V_{max} and K_m were evaluated by Prizm3 (n = 6).

Characteristics of PepT1 in the Stably Transfected MDCK Cells

After subtracting the uptake value of the untransfected cells from the total uptake in transfected cells, V_{max} of the transfected PepT1-dependent gly-sar uptake was calculated to be 507 ± 44 pmol · mg protein^{-1} · 10 min^{-1} by Prizm3 and the K_m 375 ± 78 μM (Fig. 3). The K_m value for gly-sar uptake has been reported at 290 μM for hPepT1 (9) and 74 μM for hPepT2 (10). Apparently, a low-affinity dipeptide transporter, such as PepT1, is the major contributor to gly-sar uptake in the transfected cells.

Brandsch *et al.* have reported that gly-sar uptake in Caco-2 cells, a colonic cell line that endogenously expresses PepT1 (11), decreased by 48% after 2 h-treatment of 1 μM PMA (12) in a process likely dependent on protein kinase C activation. In the MDCK cell line stably expressing HA-tagged hPepT1, we measured the gly-sar uptake (10 μM cold gly-sar spiked with 1.2 μCi/ml [^{3}H]gly-sar) before and after PMA treatment (1 μM, 2 h) as 6.92 ± 0.33 pmol · mg protein^{-1} · 10 min^{-1} and 5.87 ± 0.36 pmol · mg protein^{-1} · 10 min^{-1}, respectively. PMA slightly (15%) but significantly ($n = 6$, $p \leq 0.05$) reduced the gly-sar uptake in the stably transfected HA-hPepT1-MDCK cells, consistent with the inhibitory effect of PMA on PepT1 activity in other two kinds of cells that have been tested.

Half-life of the Surface HA-hPepT1 in the Stably Transfected MDCK Cells

After cell surface biotinylation, the labeled cells were incubated at 37°C for 0–48 h (see Materials and Methods), lysed, and HA-hPepT1 immunoprecipitated with the anti-PepT1 antibody. The immunoprecipitated protein was probed with HRP-streptavidin to quantify biotinylated PepT1. Figure 4A shows that biotinylated PepT1 started to disappear several hours after biotinylation. The half-life of the biotinylated HA-hPepT1 in the stably transfected MDCK cells was calculated to be 22 h. Reprobing of the blot shown in Fig. 4A with the HA antibody enabled quantitation of the total HA-hPepT1 in the immunoprecipitates, whose signal (Fig. 4B) overlapped with the bands in Fig. 4A. Although amount of the biotinylated PepT1 decreased during chase-labeling of the surface PepT1, the amount of total PepT1 in the cells increased, possibly as the result of cell proliferation during the assay. Untransfected MDCK cells were processed

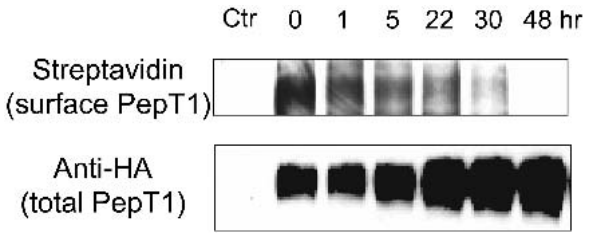

Fig. 4. Half-life of the surface PepT1. The stably transfected HA-PepT1-MDCK cells were grown in 60 mm dishes, incubated with 0.5 mg/ml sulfo-NHS-biotin for 2 × 15 min, and immunoprecipitated with 1.5 μl of the anti-PepT1 antibody. (A) Immunoprecipitates examined using the ECL blotting method with HRP-conjugated streptavidin. (B) The same blot was examined by Western blotting using the HA antibody. Untransfected MDCK cells served as the negative control.

in parallel as another control, in which no PepT1 band was detected.

DISCUSSION

Colonic adenocarcinoma cell line Caco-2 serves as a model to examine paracellular transport across the intestinal epithelium (13), because it exhibits morphologic and biochemical characteristics of the columnar enterocytes of small intestines (5). Another important characteristic in which Caco-2 cells resemble epithelial cells of small intestines is the expression of endogenous PepT1 (11). Caco-2 monolayer has widely been used to study the transepithelial transport activity of drugs that are prospective substrates of PepT1 (2,3), as well as the regulation of the dipeptide transport activity of PepT1 (11,12). Those investigations are often hampered by insufficient specificity and affinity of the currently available PepT1 antibodies and a good cell culture model system.

Epitope tagging technique enables the use of specific high-affinity antibodies to probe proteins such as PepT1 that are lacking specific antibodies. Sun *et al.* reported that PepT1 retains dipeptide transport function after fusing to green fluorescence protein (GFP) at the N-terminus (14). To minimize the interference of the epitope tag to the structure and subcellular sorting of the transporter, we selected the HA sequence, one of the shortest epitope, for tagging. After confirming the expression of intact functional HA-tagged hPepT1 in an unpolarized epithelial cell type HEK293, we stably transfected MDCK, a polarized epithelial cell line that could form a monolayer and develop transepithelial resistance (4), and obtained a cell line that could express HA-tagged hPepT1 on the cell surface.

Using this cell line, we successfully measured the half-life of cell surface PepT1 by labeling cell surface PepT1 with biotin. The number, 22 h, is typical among membrane proteins (15), suggesting the HA tag does not alter the normal turnover of PepT1 on the cell surface. However, the half-life of endogenous PepT1 on the cell surface of Caco-2 or other intestinal epithelial cells has not yet been reported.

Using this cell line, we expect to be able to investigate subsequently the subcellular distribution and intracellular trafficking of hPepT1 in the MDCK monolayer using the HA antibody. Moreover, with untransfected MDCK cells as the negative control, this cell line may serve as a better culture model than do Caco-2 cells for PepT1-mediated transport studies. The contribution of PepT1 in epithelial permeability of a drug can easily be evaluated by comparing two epithelial monolayers with or without the expression of PepT1.

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