Development and Utility of Anti-PepT1 Anti-Peptide Polyclonal Antibodies

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INTRODUCTION

Dipeptide transporters may play an important role in the intestinal absorption of penicillins, cephalosporins, angiotensin-converting enzyme (ACE) inhibitors, renin inhibitors, and human immune deficiency virus (HIV) protease inhibitors. Expression cloning has now revealed the primary structure of the rabbit intestinal dipeptide transporter PepT1 (1,2), and a topological model has been proposed on the basis of hydropathy analysis of the cDNA-deduced primary structure of PepT1 (1) and its human (3) and rat (4,5) homologs. All members of this family share three common structural features suggested by their primary sequence (Fig. 1): (a) Twelve α-helical transmembrane domains that are interrupted by alternating intra- and extracellular loops, (b) One large extracellular loop that is positioned between transmembrane domain 9 (TM9) and TM10, which possesses multiple *N*-glycosylation sites, and (c) Intracellular location of both N- and C-terminus amino acids.

Anti-peptide antibodies to transporters for demonstrating their presence (4), topology (6,7) as well as cellular localization and surface expression (8,9) have been reported previously. In particular, anti-PepT1 polyclonal antibodies against synthetic peptides corresponding to the C-terminal 13–15 amino acids of the transporter have been used in immunoblotting and immunohistochemistry studies (4,8,9). However, no attempt has been made to verify the hydropathy-generated model of PepT1. While monoclonal antibodies are exquisitely specific, generally they are much more difficult to develop. Moreover, monoclonal antibodies recognize peptides in a specific conformation and therefore, may not necessarily recognize the transporter protein in its native conformation (6). Davies *et al.* (6) have reported on the utility of monoclonal anti-peptide antibodies against cytoplasmic peptide segments of the human erythrocyte glucose transporter for determining its topology and structure-function relationship. There is no report on the development of monoclonal antibodies against the dipeptide transporter.

Here we have chosen to use polyclonal antibodies as biochemical and structural probes of the PepT1 transporter protein. We have raised antibodies against two peptide segments of the large, presumably extracellular, loop as shown in Fig. 1, and characterized both antibodies using indirect ELISA, and one of them also using membrane ELISA, cell ELISA and Western blot. The tools developed in this research may be used to generate critical information about the precise isoform of the transporter protein and folding of the dipeptide transporter. Detection of cell surface exposure of putative extracellular loops would be of considerable interest in examining the predicted structure and the surface expression of PepT1.

MATERIALS AND METHODS

Materials

The R[a]MPS multiple peptide synthesis system (the solid phase peptide synthesis apparatus) and Wang resin cartridges were purchased from E. I. Du Pont (Boston, MA). Amino acids were obtained either from Du Pont (Boston, MA) or Advanced ChemTech (Louisville, KY). Sephadex PD-10 columns were purchased from Pharmacia Biotech (Piscataway, NJ). BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used for immunization. The β-galactosidase (β-GAL) conjugated goat anti-mouse secondary antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). Chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). High protein binding 96-well flat-bottom cluster plates (Immulon 4) were purchased from Dynatech Laboratories (Chantilly, VI). Tissue culture treated flat-bottom and untreated round-bottom 96-well cluster plates were obtained from Costar (Cambridge, MA). All other chemicals were of the highest purity available commercially.

Methods

Selection and Synthesis of Antigenic Peptide

Two peptide segments were selected from the primary sequence of PepT1 based on hydrophilicity, flexibility, and accessibility considerations (Fig. 1). The uniqueness of each peptide segment was investigated using the sequence similarity program BLAST (10) to search the National Center for Biotechnology Information (NCBI) combined molecular biology database. Peptides were synthesized using the solid-phase FMOC technique (11). Each resulting peptide was subjected to amino acid analysis at the Norris Microchemical Core Facility, University of Southern California Comprehensive Cancer Center (Los Angeles, CA) prior to the coupling with carrier protein.

Immunization and Preparation of Antisera

Peptides were coupled to the carrier protein Keyhole Limpet Hemocyanin (KLH) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) as the linking agent. BALB/c mice

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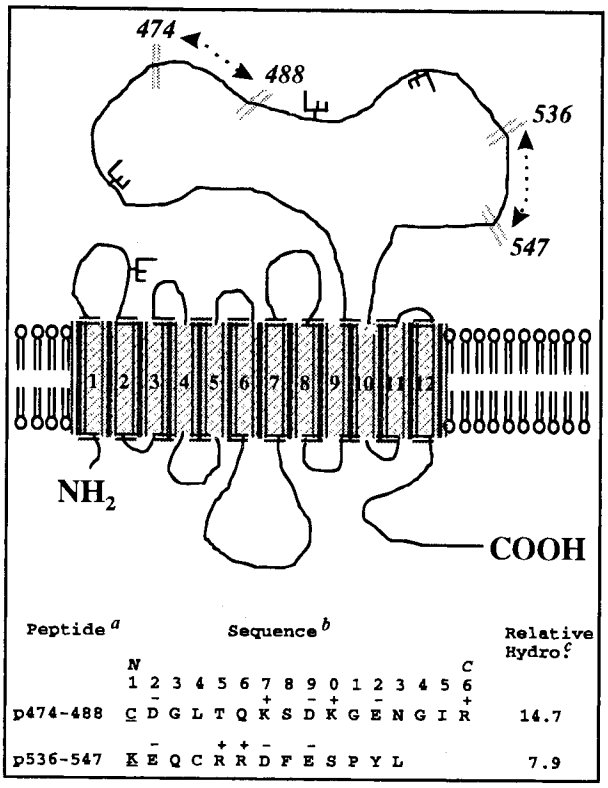

Fig. 1. Schematic model for PepT1. The model shows the topology as predicted by Fei *et al.* (1). Locations of the two chosen peptide segments for anti-peptide antibody development are shown with arrows. Locations of the putative glycosylation sites are shown as Ψ. [a]Peptides are identified by the number of their N- and C-terminal amino acid residues in the rabbit PepT1 sequence. The same numbering system is used to identify the antipeptide antibodies. [b]The starting amino acid when underlined indicates that it is not a part of PepT1, but was added to conjugate the peptide with the carrier protein. Positively and negatively charged amino acids are indicated with + or – signs, respectively. [c]Relative hydrophilicity of the peptide segments was calculated using the hydrophilicity values of the constituent amino acids reported by Hopp and Woods (16). Higher values mean higher hydrophilicity.

were immunized once with peptide-KLH conjugate in complete Freund's Adjuvant and boosted twice with the same antigen in incomplete Freund's Adjuvant at biweekly time intervals. Mice were bled and the antisera were prepared. Prior to the immunization procedure, mice were bled to obtain preimmune sera. Aliquots of the preimmune sera and anti-peptide antisera were stored at –70°C.

Enzyme-linked Immunosorbent Assays (ELISA)

Indirect ELISA (Peptide ELISA and Membrane ELISA). Anti-peptide antisera were characterized by indirect ELISA using sera from preimmune mice as the controls. Peptide or peptide coupled with ovalbumin (Pep-Ova) was used to coat 96-well high protein binding flat-bottom cluster plates (Immulon 4) for performing ELISA. For Membrane ELISA membrane fractions (see below for preparation of membrane fractions) were used for coating the assay plates. Antigen solutions were prepared by dissolving antigens (usually 0.2 to 10.0 μg/ml) in phosphate buffered saline containing 0.05% sodium azide (PBSN). Fifty microliter of antigen solution was dispensed into each well of the 96-well flat-bottom plate, and was evenly distributed over the bottom of each well by gentle tapping. The cluster plates were wrapped with polyvinyl chloride wrapping film and incubated overnight at 4°C. The coated wells were washed three times with deionized water. For blocking of excess antigen binding sites, each well was filled with blocking buffer (borate-buffered saline at pH 8.5 containing 0.05% Tween 20, 1 mM EDTA, 0.25% bovine serum albumin (BSA), and 0.05% NaN_3), incubated at room temperature for 30 min, and washed again three times with deionized water. For primary antibody incubation, 50 μl of either preimmune serum, sera from mock immunized animals (using the carrier protein KLH alone as the antigen), or immune sera were added at serial dilutions to each well, followed by incubating for 4 hr at 4°C. The wells were then washed three times with deionized water. Captured antibody was detected using 50 μl of β-GAL-conjugated goat anti-mouse antibody (1:200 dilution) as the secondary antibody. β-galactosidase activity was quantitated by monitoring the kinetics of color development of the chromogenic substrate CPRG (2 mg/ml, 100 μl) at 570 nm using a Cambridge 7520 microplate reader (Cambridge, MA).

Cell ELISA. The polyclonal antibodies raised were also tested using the cell ELISA method developed by Gurlo and von Grafenstein (details of this method will be published elsewhere). Briefly, Caco-2 cells grown on tissue culture-treated 96-well plates were washed with appropriate washing buffer, and incubated with primary antibodies (preimmune or immune serum) for 30 min or 60 min on ice. After gentle washing the cells were incubated with β-GAL-labeled goat anti-mouse human-absorbed secondary antibody for 30 min on ice, and washed again. Following the addition of CPRG, β-galactosidase activity was quantitated as described above for the indirect ELISA.

Western Blot of PepT1 from Caco-2 Membrane Fraction

The dipeptide transporter PepT1 is a transmembrane protein (1,3). Therefore, a membrane fraction was prepared and used as a source of PepT1 protein rather than whole cell lysate. Crude Caco-2 cell membranes were prepared from Caco-2 cells grown on Transwells for 21 days. The cells were detached from the filters by trypsinization. Cells were homogenized in a Downce homogenizer using ice-cold MSEP buffer at pH 6.7 (125 mM mannitol, 40 mM sucrose, 1 mM EDTA-Tris, and 5 mM PIPES-Tris) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitory cocktail (PIC) comprising 75 μg/ml benzamidine, 10 μg/ml chymotrypsin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. Following centrifugation at 5,000× g for 10 min, the pellet (P_1, the nuclear fraction) was discarded. The post-nuclear supernatant (S_1) was centrifuged at 50,000× g for 2 hr and the resulting pellet (P_2) containing crude cell membranes was resuspended in 0.5 mL MSEP buffer. Fifty microliter aliquots of membrane preparation were stored at –70°C.

For immunoblotting, Caco-2 membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were then incubated either in dipeptide transporter-specific mouse primary antibody (immune serum p474–488) or control antibody

(preimmune serum) at a dilution of 1:500 overnight at 4°C. Specific protein bands were detected by immunoblotting with HRP-linked goat anti-mouse secondary antibody at a dilution of 1:20,000 for 1 hr at room temperature. The 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).

RESULTS AND DISCUSSION

We have used several methods to assess the presence, membrane localization, surface expression and folding topology of PepT1. An overview of the various techniques employed and their utility is shown in Table I.

ELISA

Two polyclonal antibodies were developed against peptide segments of the large extracellular loop of PepT1 (p474–488 and p536–547). These antibodies showed a 3.3–11.3-fold stronger signal compared to preimmune serum in indirect ELISA performed in plates coated with the peptide conjugated with ovalbumin (p474-488-Ova) (Fig. 2a) and (p536-547-Ova) (Fig. 2b), respectively ("Peptide ELISA"). Color development in the peptide-coated plates was ~50% less when compared to that of p474–488-Ova coated plates, possibly indicating lesser binding of the nonconjugated peptide to the plate or lesser binding of antibody to peptide-coated plates due to steric hindrance (Fig. 2a). The assay was sensitive to nanogram quantities of immunogenic peptide and was reproducible (standard error being <10%). The signal was specific since color development was inhibited by 70% by excess p536-547-Ova (Fig. 2b). However, the signal was not abolished probably due to the monovalent nature of the soluble peptide used for competitive inhibition compared to the multivalent nature of the peptide coated on the cluster plate. The latter is able to engage both binding sites of the antibody in a cooperative manner.

It should be noted that p474-488 shares an overall 87% sequence similarity with the homologous segment in human PepT1 (hPepT1) (NH_2-DGLNQKP*E*KGENGIR-COOH; 12 amino acids identical, 1 amino acid similar) (*underlined letters indicate identical and letters in italics indicate similar amino acids within the homologous segment of rabbit PepT1*). By contrast, p536–547 shares only an overall 50% sequence similarity (NH_2-POCQPNFN*T*FYL-COOH; 5 amino acids identical, 1 amino acid similar) (Fig. 1) (1,3). Therefore, we used polyclonal antibodies against p474-488 for further investigation.

Table I. An Overview of the Various Techniques Employed and Their Utility

Methods	Utility
ELISA	Detects immunizing antigens (peptide).
Western blot (denaturing conditions, e.g., SDS PAGE)	Detects peptide within unfolded protein (linearized).
Membrane ELISA	1. Suggests localization in membrane, 2. Detects peptide epitope in folded protein.
Cell ELISA	Detects: 1. peptide epitope in folded protein, 2. surface expression on plasma membrane, 3. folding topology, i.e., extracellular localization of peptide epitope containing loop.

Fig. 2a shows that the polyclonal antibodies developed against p474-488 were able to recognize the synthetic peptide. This prompted us to use ELISA with membrane proteins derived from Caco-2 and rabbit jejunal cells to investigate whether these antibodies can also recognize PepT1 in its native conformation ("Membrane ELISA"). Significant signals compared to preimmune serum for both Caco-2 and jejunal membrane proteins were seen (Fig. 2c), indicating that the antibodies can indeed recognize the dipeptide transporter in its native conformation.

To confirm the results of ELISA using membrane proteins, we performed cell ELISA. The preliminary results of cell ELISA following 30 and 60 min of primary antibody incubation both yielded strong signals, and color development occurred within minutes. A problem associated with 60 min incubation was substantial detachment of cells. Unfortunately, treatment of cells with 1% glutaraldehyde for 10 min as a possible means to fix the cells yielded no signal in the ELISA (data not shown). A possible reason may be that glutaraldehyde, at the concentration used, had modified PepT1 epitopes, thereby interfering with their recognition by the antibody. As a compromise, 30 min incubation time was chosen and gluteraldehyde fixation was omitted. The cell ELISA performed with 30 min primary antibody (antiserum p474-488) incubation showed a 2–3-fold stronger signal compared to control at 1:10 or 1:30 dilutions (Fig. 2d), further corroborating the recognition of PepT1 in its native conformation by the anti-peptide antibodies.

Western Blot

Immunoblot of Caco-2 membrane protein preparation using the anti-peptide antibody (anti-p474-488 antiserum) revealed a positive band at an apparent molecular weight of ~110 kDa (Fig. 3). This major band was absent when the Western blot was performed using preimmune serum. Caco-2 cells are known to express H^+/dipeptide cotransporter activity (12–14). Furthermore, Ganapathy *et al.* (14) have also shown at the mRNA level that the dipeptide transporter expressed in Caco-2 cells is PepT1. Therefore, it is most likely that the ~110 kDa protein represents PepT1. The cloned PepT1 predicts a molecular size of ~79 kDa based upon the primary amino acid sequence. However, the hydrophilic segment between the TM9 and TM10 contains several putative *N*-glycosylation sites. The possible glycosylation of PepT1 most likely explains the larger molecular size of the protein detected in Western blot. Moreover, Fei et al. (15) has recently reported a 120 kDa protein band for PepT1 from Caco-2 cells using antipeptide (corresponding to amino acids 247–264 of PepT1, part of the intracellular loop between the TM6 and TM7) polyclonal antibodies. Taken together, these data suggest that the antibodies used in this study are specific for the dipeptide transporter PepT1.

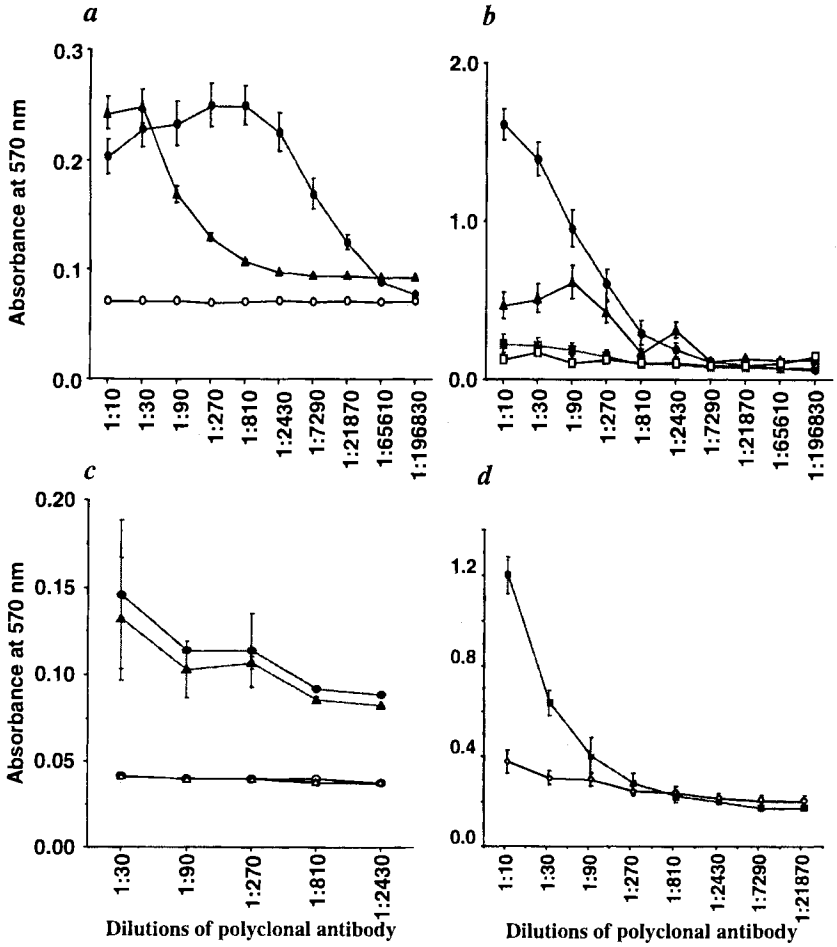

Fig. 2. ELISA protocols designed for assay of peptide antigens or peptide epitope within membrane fractions and intact cells. *Panel a*, ELISA plate coated with p474-488-Ova (●), or p474-488 alone (▲) incubated with immune serum anti-p474-488. Incubation with preimmune serum (○) was used as the control. *Panel b*, ELISA plate coated with p536-547-Ova, incubated with either preimmune serum (□), serum from mock immunized animals (■), anti-p536-547 antiserum (●) or anti-p536-547 antiserum with excess p536-547-Ova (▲). *Panel c*, ELISA plate coated with membrane protein at a particular concentration, incubated with either preimmune (○, rabbit jejunal membrane protein; Δ, Caco-2 membrane protein) or immune (p474-488) serum (●, rabbit jejunal membrane protein; and ▲, Caco-2 membrane protein). Rabbit jejunal membrane protein was prepared from freshly isolated intestinal tissue using similar procedure described for Caco-2 cells. *Panel d*, Caco-2 cells grown on tissue culture treated 96-well plates, incubated with either preimmune serum (○) or immune serum (anti-p474-488) (■). Each point with bar represents mean ± s.e.m. of 4–6 independent observations. Where no error is shown it falls within the symbol.

CONCLUSIONS

The purpose of this study was to develop anti-peptide polyclonal antibodies against the rabbit intestinal dipeptide transporter PepT1 and to evaluate their utility using a variety of assay techniques. Peptide segments were selected from the primary sequence of PepT1 based on hydrophilicity and accessibility considerations. Anti-peptide antisera were characterized using Western blotting and various ELISA protocols designed for assay of peptide antigen, membrane fractions and intact cells ("Peptide ELISA", "Membrane ELISA" and "Cell ELISA"). Two polyclonal antibodies (anti-p474-488 and anti-p536-547 antisera) developed against the extracellular domain of PepT1 showed significant signals compared to preimmune serum in Peptide ELISA (using peptide-coated or Pep-Ova coated ELISA plates). Color development was markedly inhibited by excess Pep-Ova. Antibody reactivity was positive when tested by Membrane ELISA i.e., indirect ELISA using total membrane protein as antigen and by cell ELISA with Caco-2 cells. Western blotting of a Caco-2 membrane protein preparation using the anti-peptide antibody revealed a positive band at an apparent molecular weight of ~110 kDa. These data suggest that the anti-PepT1 antibodies developed against the extracellular domain of PepT1 recognize the transporter molecule in both the linear form and its native conformation. In conclusion, these data confirm the surface expression of the transporter protein. Moreover, these data also validate the extracellular assign-

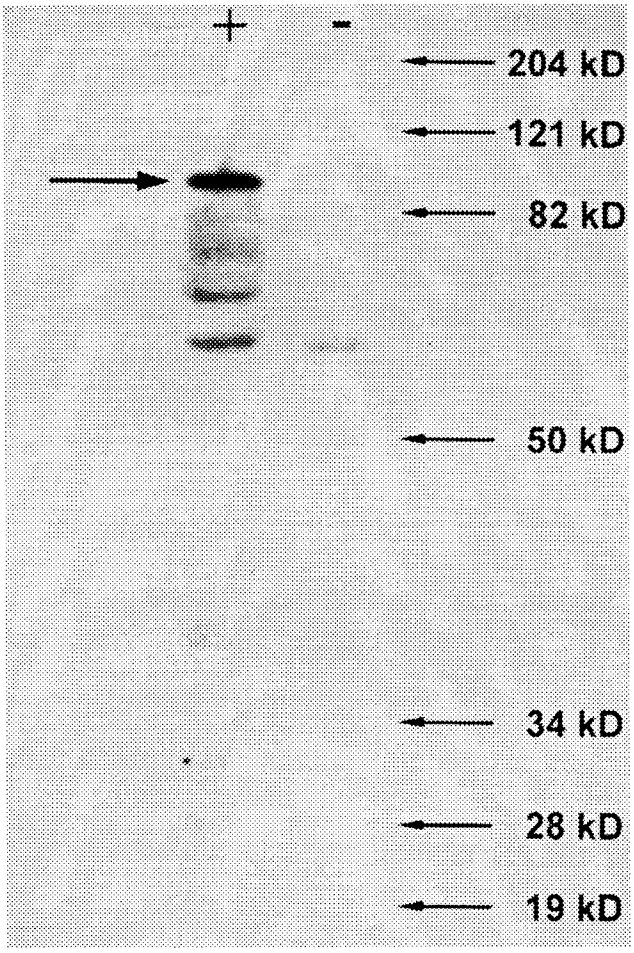

Fig. 3. Western Blot of PepT1 from Caco-2 membrane proteins using either anti-p474-488 antiserum (+) or preimmune serum (–) as the primary antibody. The predominant species migrated at approximately 110 kDa (*arrow*). A consistent background band (e.g., 60 kDa) confirmed that roughly equal amounts of protein were loaded in both lanes (25 μg). The numbers to the right of the blot indicate the positions of molecular mass markers in kilodaltons (kD) electrophoresed in parallel. The proteins used as molecular mass standards were myosin (204 kD), β-galactosidase (121 kD), bovine serum albumin (82 kD), ovalbumin (50.2 kD), carbonic anhydrase (34.2 kD), soybean trypsin inhibitor (28.1 kD) and lysozyme (19.4 kD) prestained SDS-PAGE standards, broad range (Bio-Rad, Hercules, CA).

ment by Fei *et al.* (1) of the domain between TM9 and TM10 in their proposed PepT1 model. This model can be further confirmed and/or refined using antibodies specific to other intra- and extracellular peptide segments of the transporter.

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