Immunolocalization and pharmacological relevance of oligopeptide transporter PepT1 in intestinal absorption of β-lactam antibiotics

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Abstract A polyclonal antibody (anti-PepT1/C) was raised against the rabbit intestinal H^+-coupled oligopeptide transporter, PepT1. Anti-PepT1/C detected 70–80-kDa protein in crude membranes obtained from rabbit duodenum, jejunum and ileum. PepT1 was localized in the brush-border of the absorptive epithelial cells by subcellular fractionation of membranes on a sucrose density gradient and by immunohistochemistry using light and electron microscopy. Transport activity for cephalosporins and dipeptide expressed in *Xenopus laevis* oocytes injected with total mRNA obtained from rabbit small intestine was eliminated completely by prehybridization of the mRNA with antisense oligonucleotide against the 5′-coding region of rabbit PepT1 cDNA.

Key words: Peptide transport; β-Lactam antibiotic; Intracellular localization; Basolateral membrane; *Xenopus* oocyte; Hybrid depletion

1. Introduction

Intestinal transport of oligopeptides generated by digestion of dietary protein involves two distinctive transcellular membrane transport processes: entry into the enterocyte across the brush-border membrane (BBM) and discharge to the bloodstream across the basolateral membrane (BLM). A comprehensive description of intestinal absorption requires characterization of the transport systems in both the brush-border and the basolateral membranes of epithelial cells. Previous studies of oligopeptide transporter(s) in the small intestine have been restricted exclusively to functional characterization of transport activities of BBM vesicles [1–8]. However, the presence of a basolateral peptide transport activity has also recently been demonstrated by using a BLM vesicle preparation [9]. Saito et al. also examined the basolateral-to-apical transcellular transport in an intestinal epithelial cell line, Caco-2, and suggested the existence of a basolateral dipeptide transporter [10]. However, no evidence for a possible identity of BBM and BLM peptide transporters at the molecular level is available, so it remains to be seen whether the transport activities in the two membranes are expressed by the same protein.

Recently, cDNA clones encoding an H^+-coupled peptide transporter have been isolated from intestine or kidney of rabbits, humans and rats [11–16], including a report from our laboratory [17]. The first report concerned rabbit intestine, and the predicted protein, termed PepT1 [11], consists of 707 amino acids and has 12 membrane-spanning domains. On the basis of in vitro translation, the apparent molecular mass of the product is 71 kDa.

In the present study, we raised a polyclonal antibody against a synthetic peptide corresponding to a fragment of the deduced amino acid sequence of rabbit PepT1. Using this antibody, we determined the distribution of PepT1 protein between BBM and BLM in rabbit intestinal epithelial cells as an approach to clarify whether or not the peptide transporters in the two membranes are identical. Oligopeptide transporter is considered to have enormous potential as an oral drug delivery system, because this transporter mediates the intestinal absorption of not only oligopeptides, but also peptidomimetic drugs including orally active β-lactam antibiotics [8,25,26]. So, the ability of PepT1 to transport orally active β-lactam antibiotics was also examined by expression of the mRNA in *Xenopus* oocytes.

2. Materials and methods

2.1. Preparation of anti-PepT1 polyclonal antibody

Rabbit anti-PepT1 polyclonal antibody (anti-PepT1/C) was raised against a synthetic peptide (CYPSLAPVSQTQM) corresponding to the deduced C-terminal amino acid sequence of PepT1 [11]. The peptide was conjugated to keyhole limpet hemocyanin (Sigma, St. Louis, MO, USA), emulsified in an equal volume of Freund's adjuvant (Difco, Detroit, MI, USA), and injected subcutaneously into New Zealand White rabbits.

2.2. Subcellular fractionation

Separation of BBM and BLM was achieved by a modification of the method of Knickelbein [18]. The mucosal scrapings from the proximal 1.2 m of small intestine of a fasted rabbit (Japanese White, male, 2.5 kg body weight) were homogenized in sucrose buffer (250 mM sucrose, 12 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), 16 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 1 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)), and crude membranes were prepared by successive centrifugation at $3000\times g$ for 10 min and $41\,000\times g$ for 30 min. The resulting pellet was suspended in 60% sucrose, and was layered at the bottom of a 28–48% continuous sucrose gradient, then centrifuged at $111\,700\times g$ for 3.5 h. Ten fractions of similar volume were collected and used for enzyme assays and immunoblotting.

2.3. Enzyme assays

Alkaline phosphatase and Na^+/K^+-ATPase were assayed as described by Pekarthy et al. [19] and Scharschmidt et al. [20], respectively.

2.4. Immunoblotting

Membrane proteins were separated by 10% SDS-polyacrylamide-gel electrophoresis, followed by transfer to PVDF membrane (Millipore, Bedford, MA, USA). The blot was immunostained with anti-PepT1/C

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Abbreviations: BBM, brush-border membrane; BLM, basolateral membrane

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Table 1
Hybrid depletion of rabbit intestinal mRNA-induced uptake of substrates by *Xenopus laevis* oocytes with PepT1 antisense oligonucleotide

Substrate	Uptake (pmol/h/oocyte)			
	Water-injected	mRNA-injected		
		Control	+Sense	+Antisense
[^{14}C]GlySar	0.63 ± 0.07*	4.53 ± 0.18	5.79 ± 0.63	1.25 ± 0.13*
Ceftibuten	0.89 ± 0.25*	54.16 ± 11.86	56.40 ± 5.33	0.99 ± 0.47*
Cefadroxil	0.07 ± 0.04*	107.55 ± 16.48	100.36 ± 16.88	4.99 ± 3.12*
[^{3}H]Leucine	3.29 ± 0.18*	9.10 ± 0.72	7.54 ± 1.13	9.10 ± 1.22

Oocytes were injected with 50-nl aliquots of rabbit intestinal total mRNA (25 ng) or water. Prior to injection, total mRNA was incubated with PepT1 sense or antisense oligonucleotide (0.25 ng) as described in Section 2. Uptake of [^{14}C]glycylsarcosine (50 μM), ceftibuten (2 mM), cefadroxil (2 mM) and [^{3}H]L-leucine (50 nM) was measured at 25°C at pH 5.5. Each datum represents the mean ± S.E.M. of 5–9 determinations.
*Significantly different ($P < 0.05$) from the mRNA-injected control value by Student's *t*-test.

and developed with the BICP/NBT phosphatase substrate system (Kirkegaard and Perry Lab., Gaithersburg, MD, USA).

2.5. Immunohistochemistry

Rabbits were fixed by transcardial perfusion with 4% paraformaldehyde and portions of the intestine were dissected out as described by Amano et al. [21]. For light microscopic immunohistochemistry, the cryoprotected tissue blocks were frozen and sectioned on a cryostat. Following successive pretreatments with 0.3% Tween 20 in phosphate-buffered saline (PBS), 0.3% H_2O_2 in methanol and 5% normal porcine serum, the sections were incubated with anti-PepT1/C serum. They were washed with PBS, and the immunoreaction was visualized by incubating the sections successively with biotinylated anti-rabbit IgG (Dakopatts, Glostrup, Denmark), streptavidin-conjugated horseradish peroxidase (HRP) (Dakopatts), and 3′,3′-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.01% hydrogen peroxide. Nuclei were counter-stained with methyl green.

For immuno-electron microscopic analysis, the sections prepared for light-microscopic immunohistochemistry were postfixed with 0.5% OsO_4. After block-staining with uranyl acetate, the sections were dehydrated in graded ethanols, embedded in Epon 812, and cut into ultrathin sections.

2.6. Transport studies

Xenopus laevis oocytes were injected with total mRNAs extracted from rabbit small intestine as described previously [22,23]. Transport activity was measured in buffer containing 2 mM β-lactam antibiotics (ceftibuten or cefadroxil), 50 μM [^{14}C]glycylsarcosine or 50 nM [^{3}H]leucine at 25°C. β-Lactam antibiotics and radioactive compounds taken up by oocytes were analyzed by HPLC and with a liquid scintillation counter, respectively. For the hybrid depletion study, total mRNAs were prehybridized with sense or antisense oligonucleotide corresponding to the 5′-coding region of rabbit PepT1 cDNA, and then injected into oocytes.

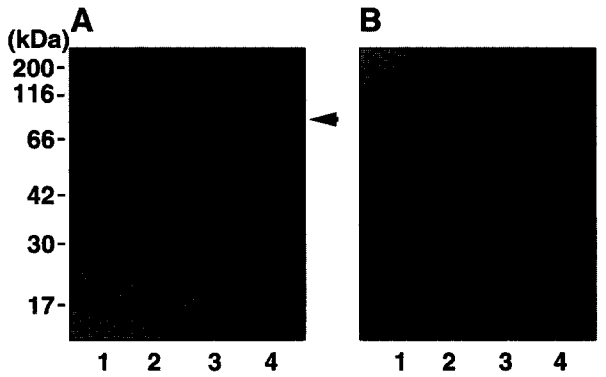

Fig. 1. Western blot analysis of total membranes from rabbit large and small intestine. Total membrane proteins (25 μg/lane) from the duodenum (lane 1), jejunum (lane 2), ileum (lane 3) and colon (lane 4) were subjected to 10% SDS-polyacrylamide-gel electrophoresis followed by transfer to PVDF membrane. The blot was immunostained with anti-PepT1/C antiserum (A) or preimmune serum (B).

3. Results and discussion

3.1. Hybrid depletion of intestinal oligopeptide transporter using antisense oligonucleotide

The pharmacological relevance of PepT1 in the intestinal absorption of peptidomimetic β-lactam antibiotics was evaluated by measuring the transport activity of *Xenopus laevis* oocytes injected with total mRNAs obtained from rabbit intestinal epithelial cells in the presence of an inwardly directed H^+-gradient (Table 1). An antisense oligonucleotide against the 5′-coding region of the PepT1 cDNA clone effectively eliminated uptake of the dipeptide [^{14}C]glycylsarcosine, while a sense oligonucleotide was without effect. These oligonucleotides hardly affected the uptake of leucine, proving the specificity of hybrid depletion of the oligopeptide transporter. This antisense oligonucleotide virtually eliminated uptake of the two β-lactam antibiotics, ceftibuten and cefadroxil, suggesting that these β-lactam antibiotics are almost exclusively transported via PepT1 in intestinal epithelium. Therefore, PepT1 is suggested to be the major participant in intestinal absorption of many peptidomimetic drugs.

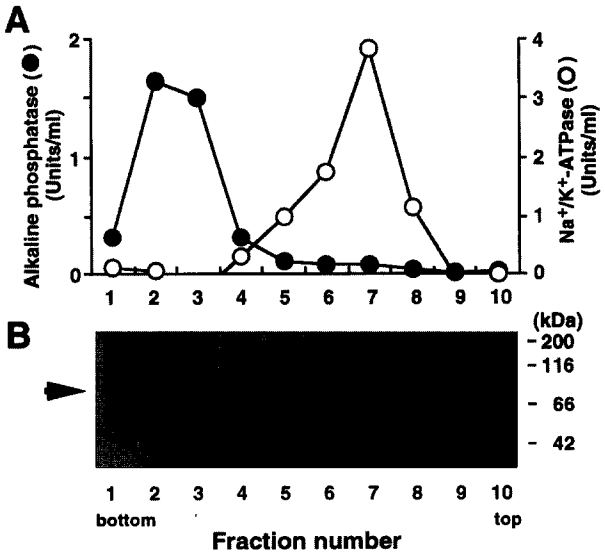

Fig. 2. Distribution of PepT1 protein between brush-border and basolateral membranes. Crude membrane fraction obtained from rabbit jejunum was separated on a 28–48% continuous sucrose density gradient. Ten fractions of similar volume were collected and analyzed for the indicated marker enzymes (A) and subjected to Western blotting with anti-PepT1/C antiserum (B) as described in Section 2.

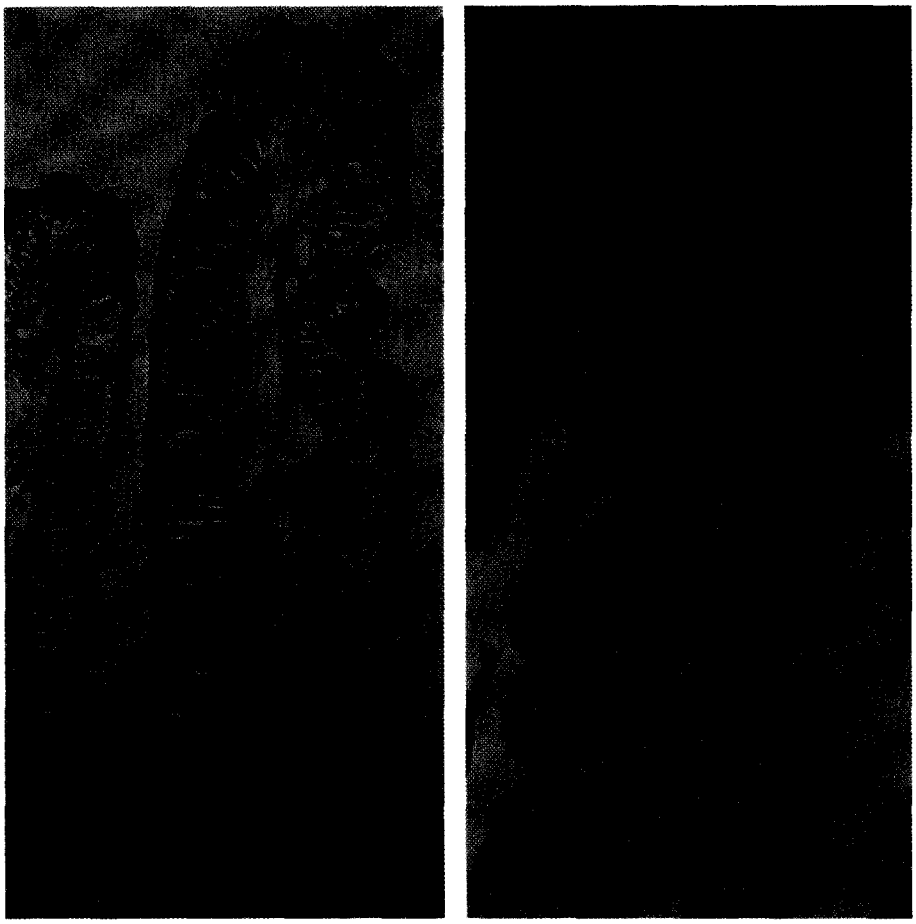

Fig. 3. Immunohistochemical localization of PepT1 in rabbit jejunum. Frozen sections were stained with anti-PepT1/C antiserum in the absence (A) or presence of 4 μM peptide antigen (B), and detected with horseradish peroxidase and DAB (brown). Both sections were counter-stained for nuclei with methyl green (green).

3.2. Intestinal distribution of PepT1 in rabbit

A polyclonal antibody (anti-PepT1/C) was raised against a synthetic peptide corresponding to the carboxy-terminal residues 696–707 of the predicted PepT1 protein. Immunoblotting using anti-PepT1/C was carried out with total membrane fractions obtained from mucosa of large and small intestine. Fig. 1 shows that anti-PepT1/C, but not preimmune serum, recognized a 70–80-kDa protein in the total membrane fraction obtained from duodenum, jejunum and ileum. To confirm the specificity of this immunostaining, the blots were incubated with anti-PepT1/C in the presence of the peptide antigen that had been used to generate the anti-PepT1/C or in the presence of irrelevant mouse anion-exchanger (band III) peptide (CTFSKLIKIFQDY) [24]. The antigen peptide eliminated the staining of 70–80-kDa protein, while the irrelevant peptide was without effect (data not shown).

The apparent molecular mass of PepT1 protein detected in total membranes obtained from duodenum, jejunum and ileum was in the range of 70–80 kDa. This value is consistent with that of an in vitro translation product (ca. 71 kDa) and also with that of the deduced amino acid sequence of 707 amino acids (~79 kDa) with *N*-glycosylation (~11 kDa) [11]. The small difference in size presumably represents the post-translational modification or degradation of PepT1 protein in vivo. Fei et al. [11] studied the tissue distribution of PepT1 by Northern hybridization using full-length PepT1 cDNA as a probe. They found PepT1 mRNA in intestine, kidney and liver. The abundance of PepT1 protein in duodenum, jejunum and ileum (Fig. 1) is consistent with these segments being the principal sites of intestinal absorption of digestion products, but we failed to detect PepT1 protein in liver and kidney (data not shown). It is possible that multiple peptide transporters modified in the carboxy-terminus exist in such tissues. To solve this problem, a hybrid depletion study using antisense oligonucleotide against the C-terminal region should be effective.

3.3. Distribution of PepT1 between brush-border and basolateral membranes

To examine the distribution of PepT1 between BBM and BLM, a crude membrane preparation was fractionated on a 28–48% continuous sucrose density gradient. Alkaline phosphatase and Na^+/K^+-ATPase were used as brush-border and basolateral enzyme markers, respectively, and good separation of the membranes was confirmed (Fig. 2A). In this preparation, PepT1 protein was distributed to the bottom fraction, and its distribution profile showed good correspondence to that of alkaline phosphatase activity, the BBM marker (Fig. 2B).

3.4. Light microscopical localization of PepT1

In the small intestine, immunoreactivity for PepT1 was lo-

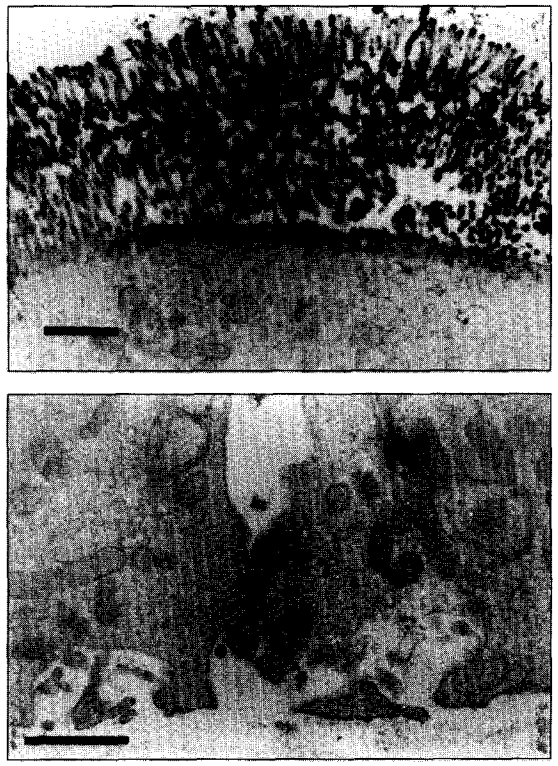

Fig. 4. Ultrastructural localization of PepT1 in absorptive epithelial cells of villi in rabbit jejunum. Frozen sections were stained for PepT1 by an immuno-enzyme method and processed for electron microscopy. A: The apical brush border of absorptive epithelial cell. PepT1 immunoreactivity (dark black stain) is seen along the microvillous plasma membrane. B: The basal and lateral portions of absorptive epithelial cells. No label is seen in the plasma membrane or the membranes of cytoplasmic vesicles. Scale bar, 1 μm.

calized at the brush-border of the absorptive epithelial cells in the villi (Fig. 3A). Specificity of this staining was evident from immunoabsorption studies, in which the antigen peptide and an irrelevant peptide completely blocked (Fig. 3B) and hardly affected (not shown) the staining, respectively. In the control experiments, weak staining was found in other portions of the small intestine, e.g. the lamina propria, the submucosa, and the smooth muscle coat. This occurred, however, even in the presence of pre-immune serum instead of the anti-PepT1/C (data not shown).

3.5. Electron microscopical localization of PepT1

The ultrastructural localization of PepT1 in the jejunum was examined by immuno-electron microscopy. In the absorptive epithelial cells in the jejunum, numerous microvilli were found at the apical portion of the cells (Fig. 4A). Dark black deposits representing the PepT1 immunoreactivity were localized exclusively at such apical plasma membranes. In contrast, immunoreaction was hardly detected on the lateral or basal plasma membrane of the absorptive epithelial cells (Fig. 4B), or on other intracellular organelles including the endoplasmic reticulum, mitochondria and nuclei.

All of these data from immunoblotting and immunohistochemistry indicate that the cellular distribution of PepT1 protein is restricted to the brush-border membrane of absorptive epithelial cells. This is the first demonstration of the cellular localization of an H^+-coupled oligopeptide transporter in intestinal epithelial cells at the molecular level. Further, it is established that the basolateral oligopeptide transporter is distinct from PepT1, at least in its carboxy-terminal immunoreactivity.

In conclusion, PepT1 is considered to play a key role in intestinal absorption of orally active β-lactam antibiotics and other peptidomimetic drugs, especially in their passage across the brush-border membrane into the epithelial cells. Although PepT1 was localized in the brush-border membranes of rabbit intestine, Dyer et al. demonstrated the presence of a basolateral peptide transport activity [9]. This implies the existence of another peptide transporter on the basolateral membrane, although the possibility is not completely excluded that their results were due to brush-border membrane contamination. To clarify this problem, further studies including molecular identification of the basolateral transporter molecule are required.

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