Characterization of the human peptide transporter PEPT1 promoter: Sp1 functions as a basal transcriptional regulator of human PEPT1

Jin Shimakura, Tomohiro Terada, Toshiya Katsura, andKen-Ichi Inui

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyoku, Japan

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Shimakura, Jin, Tomohiro Terada, Toshiya Katsura, and Ken-Ichi Inui. Characterization of the human peptide transporter PEPT1 promoter: Sp1 functions as a basal transcriptional regulator of human PEPT1. Am J Physiol Gastrointest Liver Physiol 289: G471–G477, 2005. First published May 19, 2005; doi:10.1152/ajpgi.00025.2005.—H+-coupled peptide transporter 1 (PEPT1, SLC15A1) localized at the brush-border membranes of intestinal epithelial cells plays an important role in the intestinal absorption of small peptides and a variety of peptidemimetic drugs. PEPT1 is regulated by various factors, including hormones, dietary conditions, some pharmaceutics, and diurnal rhythm. But there is little information about the transcriptional regulation of PEPT1. In the present study, therefore, we cloned the human (h)PEPT1 promoter region and examined its promoter activity using a human intestinal cell line, Caco-2. Deletion analysis of the hPEPT1 promoter suggested that the region spanning −172 to −35 bp was essential for basal transcriptional activity. This region lacked a TATA-box but contained some GC-rich sites that supposedly bind with the transcription factor Sp1. Mutational analysis revealed that three of these putative Sp1 sites contributed to the transcriptional activity. EMSA showed that Sp1 bound to two GC-rich sites. Furthermore, inhibition of Sp1 binding by mithramycin A treatment significantly reduced the transcriptional activity. Finally, overexpression of Sp1 increased the transcriptional activity in a dose-dependent manner. This study reports the first characterization of the hPEPT1 promoter and shows the significant role of Sp1 in the basal transcriptional regulation of hPEPT1.

Caco-2; SLC15A1; small intestine

DIETARY PROTEINS ARE DEGRADATED into a mixture of free amino acids and small peptides. Cellular uptake of di- and tripeptides is mediated by H+-coupled peptide transporter 1 (PEPT1, SLC15A1) located at the brush-border membranes of intestinal epithelial cells (8). Because of its broad substrate specificity, PEPT1 can accept several peptidemimetic drugs such as oral β-lactam antibiotics, the anticancer agent bestatin, and angiotensin-converting enzyme inhibitors (28). Thus PEPT1 plays important roles not only as a nutrient transporter but also as a drug transporter. It has been reported that intestinal PEPT1 is regulated by various factors (1), including hormones [insulin (11), thyroid hormone (2)], dietary conditions (17, 24), some pharmacological agents (3, 10), and diurnal rhythm (19). Although the elucidation of these regulatory mechanisms is quite important for nutritional therapy for absorptive disorders and for the efficient oral delivery of peptidemimetic drugs in a clinical situation, studies that address this point are limited. Shiraga et al. (24) has cloned the 5′-flanking region of rat PEPT1 and revealed that the rat PEPT1 promoter was transcriptionally regulated by some amino acids via the amino acid responsible element. In the mouse PEPT1 promoter, a functional promoter analysis demonstrated that essential promoter/enhancer sites were present within 1140 bp upstream of the transcription start site (9). Nevertheless, cis elements and/or trans factors, which are critical for basal transcriptional regulation, have not been identified in these studies. As for human (h)PEPT1, a computational sequence analysis but not a functional analysis has been conducted (29).

In the present study, to fully understand the transcriptional regulation of PEPT1, we cloned the 5′-flanking region of the hPEPT1 gene and identified the minimal region and cis-regulatory elements required for the basal hPEPT1 promoter activity. In addition, the results provide evidence for the involvement of Sp1 in the regulation of basal promoter activity.

MATERIALS AND METHODS

Materials. γ-32P]ATP was obtained from Amersham Biosciences (Buckinghamshire, UK). Anti-human Sp1 was purchased from Upstate (Charlottesville, VA). Restriction enzymes were from New England BioLabs (Beverly, MA). Mithramycin A was purchased from Sigma-Aldrich (St. Louis, MO). CMV-Sp1 plasmid was kindly provided by Dr. Robert Tjian (University of California, Berkeley, CA). All other chemicals used were of the highest purity available.

Cloning of the 5′-regulatory region of hPEPT1 gene. The 2940-bp flanking region upstream of the transcription start site, which was indicated in the literature (29), was cloned using primers (hPT1proSacI-F, hPT1proXhoI-R) shown in Table 1 and human genomic DNA (Promega, Madison, WI). The primers were designed based on the genomic sequence deposited in the literature (29). The PCR conditions were denaturing at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 4 min, before a final extension at 72°C for 10 min. The PCR product was isolated by electrophoresis and subcloned into the firefly luciferase reporter vector, pGL3-Basic (Promega), at SacI and XhoI sites. This full-length reporter plasmid is hereafter referred to as −2940/+60.

Preparation of deletion reporter constructs. The 5′-deleted constructs (−1111/+60, −960/+60, −401/+60, −247/+60, −172/+60, −89/+60, −21/+60 constructs) were generated by digestion of the −2940/+60 construct with HindIII and each of the following enzymes: NheI, KpnI, PshIAl, PvuII, AapI, XmnI, and AatII, respectively. The ends were blunt with T4 DNA polymerase and then self-ligated. The −35/+60 construct was generated by PCR with primers containing a SacI site and XhoI site (Table 1). The site-directed mutations in putative Sp1-binding sites were introduced into the −172/+60 construct with a Quik Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in Table 1. The nucleotide sequences of these deleted or mutated constructs were

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confirmed using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Cell culture, transfection, and reporter gene assay. Caco-2 cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% nonessential amino acid mixture and 2.5 ng of the plasmid DNA per well. Cells were treated with 50, 100, and 250 nM of mithramycin A at the time of transfection of the reporter constructs. The results were expressed relative to the pGL3-basic vector (Promega). The binding mixture consisted of 1 μg Caco-2 nuclear extract, 0.5 μg poly(dI-dC), and unlabeled competitor probes in buffer solution containing (in mM) 10 Tris-HCl (pH 7.5), 50 NaCl, 1 MgCl₂, 0.5 EDTA, and 0.5 DTT, with 4% glycerol. After preincubation at room temperature for 10 min, labeled probes (~0.4 ng) were added and the binding mixture was incubated for a further 20 min. For supershift assays, 1 μg Sp1 antibody was added 10 min before the addition of the labeled probes. The volume of the binding mixture was 10 μl throughout the experiments. The DNA-protein complex was then separated by 4% polyacrylamide gel at room temperature in 0.5 Tris-borate-EDTA buffer.

Determination of minimal hPEPT1 promoter. To determine the minimal region required for basal activity of the promoter, a series of deletion constructs was transfected into Caco-2 cells and luciferase activity was measured (Fig. 1). The transfection efficiency was determined by the green fluorescence of Renilla reniformis luciferase activity, and the results were expressed as a fold change compared with the empty pGL3 vector. The transfection efficiency was confirmed to be over 90% in each experiment.

### Table 1. Oligonucleotide sequences of primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
</tr>
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<tr>
<td>Primers for cloning of the hPEPT1 promoter</td>
<td>AGGAACCTTTTCTCCTAAGGACAGCAG</td>
<td>−2940 to −2920</td>
</tr>
<tr>
<td>hPEPT1proSacI-F</td>
<td>ACGCTAGGGCGATGGGCTCTCGCAG</td>
<td>+60 to +60</td>
</tr>
<tr>
<td>hPEPT1proXhoI-R</td>
<td>ACGCTAGGGCAGATGGGCTCTCGCAG</td>
<td>+60 to +60</td>
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<tr>
<td>Primers for the −35/+60 deletion construct</td>
<td>AGGAACCTTTTCTCCTAAGGACAGCAG</td>
<td>−35 to −20</td>
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<tr>
<td>hPEPT1pro-35SacI-F</td>
<td>ACGCTAGGGCAGATGGGCTCTCGCAG</td>
<td>+60 to +60</td>
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<tr>
<td>Primers for the site-directed mutagenesis</td>
<td>AGGAACCTTTTCTCCTAAGGACAGCAG</td>
<td>+60 to +60</td>
</tr>
<tr>
<td>Mut A-F</td>
<td>GGTGAGGCGGCGGCAAGCAGGACAGCTGG</td>
<td>−85 to −52</td>
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<tr>
<td>Mut A-R</td>
<td>CGGAGCTCTGGAGTGGTGGGCTCGCAG</td>
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<tr>
<td>Mut B-F</td>
<td>CGGAGCTCTGGAGTGGGCTCTCGCAG</td>
<td>−97 to −67</td>
</tr>
<tr>
<td>Mut B-R</td>
<td>CGGAGCTCTGGAGTGGGCTCTCGCAG</td>
<td>−97 to −67</td>
</tr>
<tr>
<td>Mut C-F</td>
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<td>−107 to −79</td>
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<tr>
<td>Mut C-R</td>
<td>CTCCGCCAGACCGGAATCCGCTGGGAG</td>
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<tr>
<td>Mut D-F</td>
<td>CACGCAGCTTGAACAGGGCTGGTTCCGGGCC</td>
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<tr>
<td>Mut D-R</td>
<td>GCGGCTGGACAGCAGGGCTTGGGAGGAGG</td>
<td>−167 to −133</td>
</tr>
<tr>
<td>Oligonucleotide for EMSA</td>
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<tr>
<td>Probes +77/+54-F1</td>
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<td>−77 to −54</td>
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<tr>
<td>Probes +102/+75-F1</td>
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<tr>
<td>Sp1-consensus-F1</td>
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<td>−102 to −75</td>
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<tr>
<td>Sp1-consensus-R1</td>
<td>GGTGAGGCGGCGGCAAGCAGGACAGCTGG</td>
<td>−102 to −75</td>
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SacI and XhoI sites are underlined. Mutations introduced into the oligonucleotides are shown in bold. hPEPT1, human peptide transporter; Mut, mutation.
pGL3-Basic. The -1111/+60 construct reduced luciferase activity, suggesting the existence of some positive regulatory sites between -2940 and -1111. Deletion of the sequence from nucleotides -1111 to -960 and further deletion from -960 to -401 resulted in an increase in promoter activity, suggesting the presence of repressive elements in these regions. The -401/+60 construct had the strongest promoter activity, i.e., a sixfold increase in luciferase activity compared with pGL3-Basic. Serial 5′-deletions of the construct from -401 to -247, -172, -89, and -35 gradually reduced the activity, which was completely abolished with the -35/+60 construct. Thus the elements important for the basal promoter activity were shown to be located between -401 and -35. We considered the region between -172 and -35 to be the minimal promoter, because the -172/+60 construct retained fourfold greater activity than pGL3-Basic, which is almost the same level of activity as the longest construct, -2940/+60.

We performed a computational sequence analysis on the -401/-1 region of the hPEPT1 promoter, which had the strongest activity, using TFSEARCH at www.cbrc.jp/research/db/TFSEARCH.html (Fig. 2). This analysis revealed that the region proximal to the transcription start site lacks canonical TATA or CAAT boxes. Instead, several GC-rich sites were observed, suggesting a possible contribution of Sp1 to the transcriptional regulation of hPEPT1. This promoter region also had putative binding sites for AP-1, CREB, myeloid zinc finger 1, and caudal-related homeobox transcription factor Cdx A. Considering that the minimal regulatory elements are located downstream of -172, we subsequently focused on these putative Sp1-binding sites between -172 and -35.

**Mutagenesis of the Sp1 sites.** The putative Sp1-binding sites located between -172 and -35 were designated Sp-A, Sp-B, Sp-C, and Sp-D, as shown in Fig. 3A. To determine whether these sites were important for hPEPT1 promoter activity, mutations in these sites (mut A, mut B, mut C, and mut D, respectively) were introduced in the -172/+60 construct (Fig. 3A) and transfected into Caco-2 cells. As shown in Fig. 3B, mut A reduced the luciferase activity to one-third of the wild-type (WT) level. The luciferase activity was also markedly reduced by introducing mut B or C, whereas little effect was observed with mut D. These results suggest that the Sp-A, -B, and -C sites play an important role in regulating the hPEPT1 promoter activity.

**EMSA.** Next, we performed an EMSA using two oligonucleotide probes, probe -77/-54 and probe -102/-75, and nuclear extract from Caco-2 cells to determine directly whether Sp1 binds to the promoter. Probe -77/-54 formed three DNA-protein complexes, that is, complex I, II, and III, with the

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**Fig. 1.** Identification of transcriptional activity and deletion analysis of the human peptide transporter (hPEPT1) promoter in Caco-2 cells. A series of deleted promoter constructs (equimolar amounts of the -2940/+60 construct (500 ng)) were transfected into Caco-2 cells for luciferase assays. Firefly luciferase activity was normalized to Renilla luciferase activity. Data are reported as the relative fold increase compared with pGL3-Basic vector and represent the means ± SE of 3 replicates.

**Fig. 2.** Nucleotides sequence of the promoter region (-401 to -1) that had the highest basal activity. Numbering is relative to the transcription start site. The putative binding sites for the transcription factors are indicated on the sequence (the arrows indicate the direction). MZF-1, myeloid zinc finger 1.
nuclear extract, whereas no complex was formed in the absence of nuclear extract (Fig. 4, lanes 1 and 2). The formation of all three complexes was completely competed away by the addition of an excess amount of unlabeled WT oligonucleotide but not by the mut A oligonucleotide (Fig. 4, lanes 3 and 4), suggesting that all these complexes bind to the Sp-A site. Moreover, Sp1 oligonucleotide competed the formation of complex I and II (Fig. 4, lane 5), further suggesting the identity of these factors as Sp1-like proteins. Sp1 appeared to exist in complex I because of the reduction of corresponding band and formation of supershifted band on incubation with anti-Sp1 antibody (Fig. 4, lane 6).

Probe −102/−75 formed three DNA-protein complexes (complexes IV, V, and VI) with the nuclear extract, whereas no complex was formed in the absence of nuclear extract (Fig. 4, lanes 7 and 8). The formation of the complex IV was completely diminished by the addition of an excess amount of unlabeled WT or mut B oligonucleotide but not by mut C oligonucleotide (Fig. 4, lanes 9–11). These results suggest that Sp-C is a more important region because the mut B oligonucleotide retains an intact Sp-C site, whereas the mut C oligonucleotide retains an intact Sp-B site. Complex IV was also competed by the addition of Sp1 consensus oligonucleotide and, most importantly, formed a supershifted band on incubation with anti-Sp1 antibody (Fig. 4, lanes 12 and 13). Complex V was so faint that its nature was not clear. Although complex VI was clearly detectable when the probe was incubated with nuclear extract only, moderate bands were also observed in every competitor used. This complex VI might be nonspecific, because there was no difference in the intensity of the bands between each competitor.

Inhibition of Sp1-binding by Sp1-specific chemical inhibitor mithramycin A. Mithramycin A is known to bind to the GC box and inhibit Sp1-binding (6, 21). The effect of mithramycin A on the hPEPT1 promoter activity was investigated with the −401/+60 construct in Caco-2 cells (Fig. 5). Treatment with mithramycin A led to a significant decrease in the promoter activity in a dose-dependent manner.

Transactivation of promoter activity by Sp1 overexpression. Finally, we investigated the effect of Sp1 overexpression on the hPEPT1 promoter activity (Fig. 6). The −401/+60 construct was cotransfected into Caco-2 cells with the CMV-Sp1 expression vector. The luciferase activity showed a dose-dependent increase on cotransfection of CMV-Sp1, providing direct evidence that Sp1 enhanced the promoter activity.
DISCUSSION

In the present study, we cloned the 5’-flanking region of the hPEPT1 gene and investigated its transcriptional regulation. When subcloned into a luciferase vector and transfected into Caco-2 cells, the 5’-flanking region showed considerable promoter activity. We used Caco-2 cells because a significant amount of hPEPT1 is expressed constitutively in these cells (2), and the transcription factors and/or cofactors required for the expression exist intrinsically in these cells. In the deletion analysis, the promoter activity was highest with the −401/+60 region, and the minimal promoter was considered to be located in the −172/+35 region. Computational analysis showed the lack of a TATA box and a CAAT box near the transcription start site but the presence of several GC-rich regions. This feature was similar to the mouse PEPT1 promoter, in which a TATA box was not located near the transcription start site, whereas some GC-rich elements were located in the proximal region (9). In such a TATA-less promoter, Sp1 binds to the GC-rich region and this Sp1 site has been shown to be responsible for recruiting TATA-binding protein (20) and fixing the transcription start site (5). Furthermore, the promoter activity is enhanced if multiple Sp1-binding sites exist (14). Thus Sp1 is speculated to play a significant role as a basal regulator of hPEPT1 expression.

Fig. 4. EMSA of Caco-2 nuclear proteins binding to the probes containing putative Sp1-binding elements. Nuclear extract from Caco-2 cells was incubated with the 32P-labeled oligonucleotide probes (probe −77/−54 and probe −102/−75) alone (lanes 2 and 8) or in the presence of excess unlabeled WT oligonucleotide (lanes 3 and 9), mutated oligonucleotide (lanes 4, 10, and 11), Sp1 consensus oligonucleotide (lanes 5 and 12), and anti-Sp1 antibody (lanes 6 and 13). In lanes 1 and 7, nuclear extract was not added. Arrows indicate the supershifted complexes.

Fig. 5. Inhibition of the hPEPT1 transcriptional activity by mithramycin A. Caco-2 cells were transiently transfected with the −401/+60 construct. Mithramycin A was added to the cells 2 times, just after transfection and after the medium change at 24 h. Firefly luciferase activity was normalized to Renilla luciferase activity. Data are reported as the relative fold increase compared with pGL3-Basic vector and represent the means ± SE of 3 replicates. Symbols show significant difference from control (without mithramycin A; *P < 0.05 and **P < 0.01).

Fig. 6. Effect of Sp1 overexpression on hPEPT1 transcriptional activity. Caco-2 cells were transiently transfected with 250 ng of the −401/+60 construct and 500, 750, and 1,000 ng of the CMV-Sp1 expression vector. The total amount of transfected DNA was kept constant by adding empty vector. Data are reported as the relative fold increase compared with no CMV-Sp1 and represent the means ± SE of 3 replicates. *Significantly different from control (no CMV-Sp1; P < 0.05).
transcription factor through these GC-rich sites in the case of the hPEPT1 promoter.

Mutational analysis of these putative Sp1 sites revealed that mutation of Sp-A, Sp-B, or Sp-C site reduced the promoter activity. The EMSA experiment demonstrates that Sp1 binds to Sp-A and Sp-C but not to Sp-B. Collectively, these results suggest that Sp1 binds to both Sp-A and Sp-C sites and significantly contributes to the promoter activity. Although we failed to obtain evidence of the formation of a complex at the Sp-B site in EMSA, some transcription factors may interact with Sp-B and play a role in the basal promoter activity of hPEPT1. The contribution of Sp1 to the promoter activity was confirmed by a different approach, inhibition of Sp1-binding by mithramycin A and the transactivation of the promoter by overexpressed Sp1. Mithramycin A clearly reduced the promoter activity in a dose-dependent manner. Overexpression of Sp1 increased the promoter activity 2.5-fold. These results strongly indicate that Sp1 plays an essential role in the basal transcriptional regulation of hPEPT1. This regulatory mechanism for hPEPT1 was found to be similar to that of other intestinal nutrient transporters, such as Na\(^{+}\)-glucose transporter (SGLT1) (13) and thiamin transporter (16). In both these studies, Sp1 was shown to play a critical role through the GC-box using Caco-2 cells.

Although the present results implicated Sp1 in the basal transcriptional activity of the hPEPT1 promoter, Sp1 is not the only protein acting through GC-rich sites. Other Sp family transcription factors, such as Sp2, Sp3 and Sp4, also interact with GC-rich sites. Among them, Sp3 is ubiquitously expressed in mammalian cells (12) and has a similar affinity for the Sp1-binding site. The present results do not exclude the possibility that Sp3 might also be responsible for the transcriptional regulation of hPEPT1. In addition to Sp family proteins, Krüppel-like factor family proteins (KLFs) also bind with different affinities to GC or GT box (4). Among the Sp family, Sp1, Sp3, and Sp4 have a higher affinity for the GC box than GT box, whereas many of the KLFs bind preferentially to the GT box (4). The Sp-B site has a GT box; thus some KLFs such as GKLFLF, which is highly expressed in terminally differentiated epithelial cells of the intestine (23), might interact with Sp-B.

hPEPT1 protein is expressed mainly in the small intestine and, to a lesser extent, in the kidney. Although the present study revealed the contribution of Sp1 to the transcriptional regulation of hPEPT1, the mechanism of this tissue-specific expression has not been clarified yet. Computational analysis showed the presence of a binding site for a caudal related homeobox factor, Cdx, within 500 bases upstream of the transcription start site. Cdx-2 is involved in the early differentiation, proliferation, and maintenance of intestinal epithelial cells (25, 27) and in the transcription of intestinal genes, such as the sucrase-isomaltase (26), lactase-phlorizin hydrolase (15), and claudin-2 (22) genes. Although more studies are needed, Cdx-2 may be responsible for the tissue specificity of hPEPT1 expression.

In conclusion, the present results indicate that Sp1 functions as a basal transcriptional regulator of the hPEPT1 gene, and this is the first demonstration to identify the cis elements and trans factors for the regulation of a human peptide transporter. These findings should serve as a basis for future investigation into the molecular regulation of the transport of nutrient peptides and some pharmaceuticals in the human intestine and other tissues.

ACKNOWLEDGMENTS

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GRANTS

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