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The functional evaluation of human peptide/histidine transporter 1 (hPHT1) in transiently transfected COS-7 cells

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ABSTRACT

Recently, the expression of the human peptide/histidine transporter (hPHT1, SLC15A4) mRNA was observed in the GI tract and in Caco-2 cells, suggesting that it may participate in the intestinal absorption of peptide-based agents. This study aims to elucidate the: (i) protein expression pattern of hPHT1 (SLC15A4) in human small intestine; (ii) cloning of the hPHT1 full-length sequence; (iii) functional characterization of hPHT1 in transiently transfected COS-7 cells. The expression of hPHT1 was measured using Western blot and immunohistochemical analysis. The hPHT1 full-sequence was amplified from BeWo cells, inserted into the pcDNA3.1–V5/His TOPO® plasmid and transiently transfected into COS-7 cells to investigate the uptake kinetics of [^{3}H]histidine and [^{3}H]carnosine. Time, pH and sodium-dependent uptake studies were performed in mock (empty vector) and hPHT1–COS-7 cells. Results demonstrated hPHT1 protein expression in different intestinal regions. Histidine and carnosine uptake was linear in hPHT1–COS-7 cells over 15 min and was found to be pH-dependent. These substrates and valacyclovir showed significantly higher uptake at pH 5.0 in the hPHT1 transients when contrasted to the mock COS-7 cells, whereas glycylsarcosine uptake was significantly lower and unaffected by pH. Other di- and tripeptides also showed affinity for hPHT1. This study presents the initial functional characterization, the protein expression of the hPHT1 transporter and provides insight into a potentially different route for increasing peptide and peptide-based drug transport.

1. Introduction

The Proton-coupled Oligopeptide Transporter (POT) superfamily comprises peptide transporters that utilize a proton-dependent mechanism for the transmembrane transport of primarily di- and tripeptide-based substrates (Herrera-Ruiz and Knipp, 2003; Daniel and Kottra, 2004). POT family members have been reported in mammals, bacteria, fungi, yeast and plants (Meredith and Boyd, 2000). A large number of genes that encode components of oligopeptide transport systems in bacteria have been cloned and sequenced. In contrast, a minimal number of eukaryotic and mammalian peptide transporters have been reported (Meredith and Boyd, 2000). In humans, the primary POT isoforms studied have been PepT1 (SLC15A1) and PepT2 (SLC15A2), both of which have been widely demonstrated to actively transport numerous

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peptide-based substrates including di- and tripeptides derived from the 20 proteinogenic L-amino acids (Herrera-Ruiz and Knipp, 2003; Daniel and Kottra, 2004). Comparatively, PepT1 is mainly expressed in epithelial cells in the intestine, S1 segment of renal proximal tubular cells, the liver and in bile duct epithelial cells (Shen et al., 1999; Herrera-Ruiz et al., 2001; Knutter et al., 2002; Herrera-Ruiz and Knipp, 2003), while PepT2 is primarily expressed in the S2 and S3 segments of the apical membranes in kidney tubular cells, brain astrocytes, epithelial cells of choroids plexus, mammary gland and bronchial epithelial cells (Berger and Hediger, 1999; Groneberg et al., 2001, 2002; Izzedine et al., 2005).

Recently, two additional putative peptide/histidine transporters (PHT1 [SLC15A4] and PHT2 [SLC15A3]) and their different splice variants were identified in the human genome and databases (Botka et al., 2000; Herrera-Ruiz and Knipp, 2003; Knipp and Herrera-Ruiz, 2004). Mammalian PHT1 was originally cloned from a rat brain cDNA library and characterized in rat tissues, demonstrating a high affinity for histidine and the histidine-containing dipeptide, carnosine (Yamashita et al., 1997). The rPHT1 cDNA had an open reading frame of 1719 bp, encoding a 572 amino acid protein with a predicted molecular mass of 64.9 kDa, 12 predicted transmembrane domains (TMD) and the POT family member signatures. Yamashita et al. (1997) also analyzed the rPHT1 mRNA expression in several tissues by Northern blot analysis, with expression shown primarily in the brain, eye, lung and spleen. Additional in situ hybridization studies using a ^{35}S-labeled cRNA probe revealed rPHT1 localization in several regions of the rat brain, such as the hippocampus, cerebellum and pontine nucleus, and in lower levels of the cerebral cortex, brain stem, thalamus and hypothalamus. Moreover, RT-PCR and Southern blot analyses have also demonstrated rPHT1 mRNA expression throughout the rat GI-tract and placenta (Herrera-Ruiz et al., 2001). Overall, the reported putative hPHT1 sequence (GI:21717816) is 86.5% identical to the rPHT1 isoform and 48.4% identical to rPHT2. Further comparison of the hPHT1 protein sequence with other proteins revealed that hPHT1 contains conservative domains found in several protein sequences relevant to the POT family (Botka et al., 2000; Herrera-Ruiz and Knipp, 2003; Daniel and Kottra, 2004).

While the functional analysis of rPHT1 has been characterized, the identity and functional characterization of the human orthologue have not yet been widely studied. Our objective was to clone and characterize the human orthologue of the peptide/histidine transporter 1 (hPHT1) as a potential intestinal transporter of peptide-based nutrients and/or drugs. To accomplish this, the full length human PHT1 sequence was amplified from the human placental trophoblast BeWo cell line, inserted into a pcDNA3.1–V5/His TOPO® plasmid and transiently transfected into COS-7 cells, which were subsequently used to characterize the uptake kinetics of [^{3}H]histidine and [^{3}H]carnosine, as well as study the uptake of [^{3}H]valacyclovir and [^{3}H]glycylsarcosine (GlySar), in general. Several experiments have shown that COS-7 cells can be used to express biologically active cell-surface and secreted proteins (Rose and Bergmann, 1982; Hosokawa et al., 1997; Sakai et al., 2000). We also generated a rabbit polyclonal antibody to hPHT1 and investigated its expression in the stomach, colon and small intestinal segments of three Asian male intestinal tissue protein lysates as well as its cellular localization in the small intestine. Thus, the complete cDNA cloning of human PHT1, its expression in the gastrointestinal tract and the determination of its functional activity are reported below. These results may serve as a template for further assessing the relative physiological significance of this transporter.

2. Materials and methods

2.1. Materials

TRIzol® Reagent was obtained from Sigma Chemical Company (St. Louis, MO). Reverse transcriptase-polymerase chain reaction kits, LipofectAMINE® Plus and OPTIMEM were obtained from Invitrogen, Inc. (Carlsbad, CA). The long and accurate polymerase enzyme was obtained from Panvera (Madison, WI). The North2South Biotin Random Prime Kit used in the preparation of DNA probes and the North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit used for Southern analysis were purchased from Pierce Chemical Company (Rockford, IL). Dulbecco's Modified Eagle's Medium (D-MEM, 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate) was obtained from the American Type Culture Collection (Manassas, VA) and fetal bovine serum (FBS) from Mediatech Inc. (Herndon, VA). [^{3}H]histidine, [^{3}H]carnosine, [^{3}H]glycylsarcosine and [^{3}H]valacyclovir were purchased from Moravek Biochemicals (Brea, CA). All other chemicals were obtained from Sigma Chemical Company.

2.2. Analysis of protein expression

2.2.1. Western blotting

Protein lysate samples of the stomach, small intestinal segments and the colon of three Asian male donors were commercially obtained (Biochain Institute Inc., Hayward, CA). Sixty micrograms of protein was loaded for electrophoresis and the fractions were separated on 10% SDS polyacrylamide gels, and electrophoretically blotted onto PVDF membranes. The membranes were blocked with 5% powdered non-fat milk in PBST (1× PBS:0.5% Tween 20) and subsequently probed with custom designed rabbit anti-PHT1 polyclonal antibodies (1:1000) generated by Invitrogen, Inc. Detection of the immunoreactions was accomplished using a 1:2000 dilution of a goat HRP–anti-rabbit monoclonal antibody (Sigma Chemical Company) and the Femto Super Signal Western detection kit (Pierce Chemical Company), recorded by the NucleoTech 920 Chemiluminescence image detection system (NucleoTech Corporation, San Mateo, CA), equipped with an 8-bit, Peltier-cooled digital camera. Band signal intensities were quantified using the GelExpert 4.0 program (Nucleotech). The Western blots were subsequently stripped and reprobed with a human β-actin antibody (Santa Cruz Antibody Company, Santa Cruz, CA) to serve as a positive control and to confirm protein integrity.

2.2.2. Immunohistochemical staining of human small intestine

The immunohistochemical staining of human intestinal tissue sections was performed as reported previously (Wang et al., 2005). Briefly, the slides were deparaffinized in xylene,