Targeted Disruption of the PEPT2 Gene Markedly Reduces Dipeptide Uptake in Choroid Plexus*

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Dipeptide uptake in choroid plexus was essentially ablated (10). These transporters (POT)1 have been cloned from a variety of animal species, and are operationally similar in that they are electrogenic, coupling substrate influx to proton movement down an inwardly directed electrochemical proton gradient. Homozygous PepT2 null mice lacked expression of PEPT2 mRNA and protein in choroid plexus and kidney, tissues in which PepT2 is normally expressed, whereas heterozygous mice displayed PepT2 expression levels that were intermediate between those of wild-type and homozygous null animals. Mutant PepT2 null mice were found to be viable, grew to normal size and weight, and were without obvious kidney or brain abnormalities. Notwithstanding the lack of apparent biological effects, the proton-stimulated uptake of 1.9 μM glycylsarcosine (a model, hydrolysis-resistant dipeptide) in isolated choroid plexus was essentially ablated (i.e. residual activity of 10.9 and 3.9% at 5 and 30 min, respectively). These novel findings provide strong evidence that, under the experimental conditions of this study, PEPT2 is the primary member of the peptide transporter family responsible for dipeptide uptake in choroid plexus tissue.

Four mammalian proton-dependent oligopeptide transporters (POT)1 have been cloned from a variety of animal species, including rabbit (1, 2), rat (3–6), human (7, 8), and mouse (9, 10). These transporters (i.e. PEPT1, PEPT2, PHT1, and PHT2) are operationally similar in that they are electronegic, coupling substrate influx to proton movement down an inwardly directed electrochemical proton gradient. PEPT1 was the first one cloned, from a rabbit intestinal cDNA library (1), and was shown to be of high capacity and low affinity for di- and tripeptides (11, 12). This is the only POT in the intestine and is responsible for the absorption of small peptides arising from digestion of dietary proteins. PEPT2 was subsequently cloned from a human kidney cDNA library (8) and, in contrast to PEPT1, was found to be a low capacity, high affinity transporter (13). Although both PEPT1 and PEPT2 are expressed in kidney (14, 15), PEPT2 is believed to play a more dominant role with respect to conservation of peptide-bound amino acids. PEPT1 and PEPT2 display overlapping, broad substrate selectivity and have pharmacologic significance in their ability to facilitate the movement of peptidomimetic drugs (e.g. β-lactam antibiotics, angiotensin-converting enzyme inhibitors, and antiviral nucleoside prodrugs) across biological membranes (11, 12, 16–18).

Two new members of the POT family were cloned from a rat brain cDNA library, the peptide/histidine transporters PHT1 (5) and PHT2 (6). These transporters are unique in that they transport both small peptides and histidine. PHT1 displayed a high affinity for histidine when expressed in Xenopus oocytes and was expressed strongly in the brain and eye. In contrast, PHT2 was expressed primarily in the lymphatic system and detected faintly in the brain. Their homology to either rat PEPT1 or PEPT2 was weak (less than 25% amino acid identity). Interestingly, a third peptide transporter was cloned from rat brain but found to be identical to that of rat kidney PEPT2 (19). Still, the physiological role of POT members in the brain remains to be determined.

The presence of multiple peptide transporters within the brain has generated substantial interest regarding their precise anatomical location, role in neuropeptide homeostasis, pharmacologic potential, and relative importance. Preliminary studies from our laboratory indicate that PEPT2, but not PEPT1, is present in rat choroid plexus epithelial cells in primary culture and that PEPT2 is functionally active at the apical membrane surface (20). In addition, our studies in isolated rat choroid plexus suggest that PEPT2 has a major role in the uptake of 5-aminolevulinic acid (21), GlySar (22), and carcinosine (23) (i.e. on the order of 30–60%). However, these results are far from conclusive. In this regard, targeted disruption of the PEPT2 gene in mouse would allow one to determine the significance of this POT relative to others present in brain (i.e. PHT1 and PHT2 but not PEPT1). The generation of transgenic PEPT2-deficient mice would also provide a powerful tool to probe the physiological and pharmacological functions of PEPT2 in other cells types or tissues.

In this study, we have developed heterozygous and homozygous PEPT2-deficient mice, and we clearly demonstrated that PEPT2−/− mice have impaired uptake of dipeptide in choroid plexus relative to that observed in wild-type animals. Almost all of the uptake of GlySar was eliminated in null mice, demonstrating for the first time that, under the experimental conditions of this study, PEPT2 is the primary member of the

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‡ The abbreviations used are: POT, proton-dependent oligopeptide transporter; CSF, cerebrospinal fluid; GlySar, glycylsarcosine; MES, 2-(N-morpholino)ethanesulfonic acid; RT, reverse transcription; ES, embryonic stem.
peptide transporter family responsible for trafficking of peptides/mimetics at the blood-cerebrospinal fluid (CSF) barrier.

**MATERIALS AND METHODS**

**Disruption of the PEPT2 Gene in Embryonic Stem (ES) Cells**—To clone the mouse PEPT2 gene, a partial complementary DNA probe was prepared by PCR after reverse transcription of total RNA isolated from mouse kidney. The PCR primer sequences were designed on the basis of the homology between human, rat, and rabbit PEPT2 cDNA sequences (2, 4, 8). 5’ primer (ATTGCCCTATGGTTGGAATGGATCTGGCGA) and 3’ primer (ACCAAGAACATGAAGGTCATGATGGTCGCT). The resulting 2065-bp partial cDNA fragment corresponds to the region of the PEPT2 transporter protein extending from the putative first membrane-spanning domain through the carboxyl terminus.

This probe was used to screen a mouse ES-129/SvJ BAC genomic library (Genome Systems, St. Louis). Hybridizing BAC clones were isolated, restriction-mapped, and partially sequenced. To create a targeted PEPT2 gene with an in-frame lacZ reporter gene under the control of the endogenous PEPT2 gene promoter, a 3.5-kb EcoRI fragment containing exon 1 was subcloned into pBluescript KS (Stratagene Inc., La Jolla, CA). Then a 300-bp PCR fragment (ClaI-KpnI site) was generated to create a novel KpnI site in the translation start codon (ATG) of the PEPT2 gene. DNA sequence of the PCR fragment was identical to the original sequence of PEPT2 gene genomic clone except for the KpnI site. The 300-bp PCR fragment (ClaI-KpnI) was utilized as a linker to the upstream 2.9-kb PEPT2 gene fragment (EcoRI-ClaI). The entire 3.2-kb 5′ homologous arm of PEPT2 gene was then inserted into the KpnI-NheI sites upstream of the lacZ reporter gene in the pNZTK2 vector (gift of Dr. Richard Palmiter). The 4.4-kb 3′ PEPT2 genomic fragment, including exons 4 and 5, was cloned downstream of the Neo cassette in pNZTK2. Thus, genomic sequence from the amino terminus to the second transmembrane domain (5.2-kb fragment) was deleted in the PEPT2 gene targeting vector.

The targeting vector was linearized at a unique Ase1 site and then electroporated into 129/SvJ ES cells (Stem Cell Facility of the University of Cincinnati, OH) according to the manufacturer (Stratagene Inc.). PCR-positive clones were expanded in G418 and ganciclovir. Targeted ES cell clones were identified by the presence of a 3.3-kb PCR product using the PCR primers described previously (24). Briefly, total RNA was isolated from mouse kidney along the apical membrane of epithelia in the proximal tubule, whereas PEPT2 is expressed in kidney brush border vesicle preparations from Pept2−/− animals. Protein levels in renal apical membranes of Pept2−/− mice were ~50% of that found in Pept2+/+ animals (Fig. 2C). The amount of protein obtained from the mouse choroid plexus was too small to evaluate by immunoblotting. The probe contains a 5′ rabbit polyclonal anti-PEPT2 antibody raised against a purified PEPT2 GST fusion protein, as described previously (15).

**Oligopeptide Uptake Assay**—Whole tissue choroid plexus uptake studies were performed in 30–50-day-old-mice, as described previously in rats for 5-aminolevulinic acid (21) and GlySar (22). Lateral ventricle and fourth ventricle choroid plexuses were harvested from anesthetized mice, weighed, and transferred to bicarbonate artificial cerebrospinal fluid buffer (127 mM NaCl, 20 mM NaHCO3, 2.4 mM KCl, 0.5 mM KH2PO4, 1.1 mM CaCl2, 0.85 mM MgCl2, 0.5 mM Na2SO4, and 5 mM glucose (pH 7.4)) which was continuously bubbled with 5% CO2, 95% O2. After 5 min of recovery, the choroid plexuses were transferred to 0.95 ml of Tris-MES buffer (147 mM NaCl, 2.4 mM KCl, 0.5 mM KH2PO4, 1.1 mM CaCl2, 0.85 mM MgCl2, 0.5 mM Na2SO4, and 50 mM Tris, and/or MES (pH 6.5)) and continuously bubbled with 100% O2 for 5 min. Transport was initiated by a 0.05 ml of Tris-MES buffer which contained ~0.2 μCi of [3H]mannitol (an extracellular marker), resulting in a final GlySar concentration of 1.9 μM. Transport was terminated after 5 or 30 min by transferring the plexuses to ice-cold buffer and filtering under reduced pressure. The filters (118-μm mesh, Teto, Kansas City, MO) were washed three times with the same buffer. The filters and choroid plexuses were then soaked in 0.33 M l-hydroxy hydrazide (a tissue solubilizer) for 30 min before the addition of scintillation mixture (Cytoscan) and counting with a dual-channel liquid scintillation counter (Beckman LS 3801; Fullerton, CA).

**Statistical Analysis**—Data are expressed as the mean ± S.E. of four experiments, unless otherwise noted. Each experiment consisted of choroid plexuses (i.e. lateral and fourth) pooled from two animals. Statistical comparisons were made between litter-mate wild-type (Pept2+/+) and homozygous transgenic mice (Pept2−/−) at each time point (i.e. 5 and 30 min) using a two-sample t test. A p value of ≤ 0.05 was considered significant.

**RESULTS**

**Targeted Disruption of the PEPT2 Gene**—The Pept2 gene spanned ~33 kb as reported previously by Rubio-Aliaga et al. (10) and was totally contained within the BAC construct 220211 (Fig. 1A). To inactivate the Pept2 gene, we constructed a targeting vector in which the 3.2 kb of 5′ promoter/enhancer and untranslated sequence and 4.4 kb of sequence encoding part of intron 3, exons 4 and 5, and part of intron 5 of the murine Pept2 gene were cloned into the appropriate sites of pNZTK2 (Fig. 1B). A Lacz construct was inserted immediately 3′ to the Pept2 promoter/enhancer region. The targeted construct was used to disrupt the Pept2 gene in 129/SvJ/R1 ES cells (Fig. 1, C and D). The resulting disrupted gene lacked the sequence that encoded the first 112 amino acids of the PEPT2 polypeptide. Germ line transmission of the targeted gene resulted in mice heterozygous for the disrupted Pept2 gene on a mixed background of C57BL/6 and 129/SvJ/R1. The heterozygote animals were mated to produce Pept2 null animals (Fig. 1E). Of the initial 182 pups born, 44, 100, and 38 animals had Pept2+/−, Pept2+/+, and Pept2−/− genotypes, respectively (i.e. a ratio of 24:55:21%). Average litter size was 10 pups, with normal gender distribution.

**Pept2 Expression Levels**—Northern blot analysis demonstrated that Pept2 mRNA expression was absent in the kidneys from Pept2−/− mice and that expression in the Pept2+/+ mice was ~50% of the levels found in Pept2+/+ mice (Fig. 2A). Similar, RT-PCR analysis showed no detectable Pept2 mRNA in choroid plexus from Pept2−/− mice (Fig. 2B). Immunoblots showed complete absence of immunoreactive Pept2 in kidney brush border vesicle preparations from Pept2−/− animals. Protein levels in renal apical membranes of Pept2−/− mice were ~50% of that found in Pept2+/+ animals (Fig. 2C). The amount of protein obtained from the mouse choroid plexus was too small to evaluate by immunoblotting.

**Pept1 Expression Levels**—Pept1 is an oligopeptide transporter with ~50% homology to Pept2. Pept1 is expressed in kidney along the apical membrane of epithelia in the proximal portion of the proximal tubule, whereas Pept2 is expressed in
**Fig. 1. Targeted disruption of the PEPT2 gene.** A, partial restriction map of the murine Pept2 gene (top), the relation to the cDNA probe used to clone the gene (mid), and PEPT2 protein domains. All HindIII (H) and XbaI (X) restriction sites and the primers used in the RT-PCR analyses are indicated. B, targeting of Pept2 by homologous recombination. The top line represents the normal Pept2 allele, the middle line the
Targeted Disruption of the PEPT2 Gene

4789

FIG. 2. PEPT2 gene expression. A, Northern blot analysis of total RNA from kidney of Pept2+/+, Pept2+/−, and Pept2−/− mice. Total RNA (25 μg) was blotted onto a nylon membrane and hybridized sequentially with 32P-labeled mouse PEPT2 (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lower panel) cDNA probes. B, RT-PCR of total choroid plexus RNA from Pept2+/+, Pept2+/−, and Pept2−/− mice. Total RNA (2 μg) was reverse-transcribed and PCR-amplified. An aliquot of each PCR product was electrophoresed on 1% agarose gels and visualized with ethidium bromide. C, immunoblot analysis of kidney apical membrane vesicles from Pept2+/+, Pept2+/−, and Pept2−/− mice and a Sprague-Dawley rat. Apical membrane vesicles were pooled from five 8-week-old mice kidneys (100 μg) or made from a single rat kidney (10 μg), subjected to SDS-PAGE, transferred to nitrocellulose, and the blots incubated with a rabbit polyclonal anti-rat PEPT2 antibody. Molecular mass standards are indicated.

the more distal portion of the proximal tubule (15). We investigated whether there was any compensatory up-regulation of the Pept1 gene in Pept2−/− animals. Immunoblotting showed only a minor increase in PEPT1 expression between Pept2−/− and Pept+/+ animals (Fig. 3).

Initial Phenotypic Analysis—Pept2−/− mice appeared normal and grew to normal adult weight (Table I). Base-line blood and urine chemistries were comparable with those from Pept2+/+ animals (Table I). There was no obvious behavioral or neurological phenotype. Gross and light microscopic morphology of the kidneys and choroid plexus appeared normal. Choroidplexus weights did not vary between Pept2−/− and Pept+/+ mice.

Diipeptide Uptake in Choroid Plexus from PEPT2−/− and PEPT2+/+ Animals—Based on functional studies of GlySar uptake in rat choroid plexus whole tissue (22), phenotypic analyses were performed at either 5 or 30 min to approximate linear and plateau conditions, respectively. As also shown in the previous study, a Tris-MES buffer (pH 6.5) is optimal to probe the proton-dependent uptake mechanisms of relevant oligopeptide transporters. As shown in Fig. 4, virtually all of the uptake of GlySar in choroid plexus tissue was eliminated in Pept2−/− mice. In this regard, only 10.9% and 3.9% of the residual activity was still present at 5 and 30 min, respectively (p < 0.001 for both analyses).

FIG. 3. PEPT1 expression in kidney. PEPT1 immunobLOTS of kidney apical membrane vesicles were performed as in Fig. 2C except that a rabbit polyclonal anti-rat PEPT1 antibody was utilized.

DISCUSSION

Cellular, molecular, and physiological studies have made major contributions toward our understanding of transport phenomena and the role of membrane transporters. However, these experimental approaches are often limited by their in vitro design and lack of blood supply, overlapping substrate specificities, and contribution of multiple transport systems, some of which are unknown at the time of study. As a result, it is difficult, if not impossible, to define the function of a single specific protein and its significance in relation to other possible proteins that are present in the tissue or organ of interest. Such is the situation in brain in which multiple oligopeptide transporters are present (e.g. PEPT2, PHT1, and PHT2).

A detailed description of PEPT2 mRNA distribution in the rat nervous system was first reported by Berger and Hediger (27). By using nonspecific in situ hybridization techniques,
Targeted Disruption of the PEPT2 Gene

Table I

|                      | Pept2+/+ (n) | Pept2+/- (n) | Pept2-/- (n) |
|----------------------|-------------|-------------|-------------|
| Body Weight          |             |             |             |
| Male, 7–8 weeks (g)  | 25.6 ± 0.7  | 24.9 ± 0.8  | 25.3 ± 0.5  |
| Female, 7–8 weeks (g)| 19.9 ± 0.4  | 19.5 ± 0.4  | 19.9 ± 0.6  |
| Serum                |             |             |             |
| Glucose (mg/dl)      | 146.7 ± 8.0 | 191.4 ± 11.3| 168.1 ± 6.4 |
| Sodium (mmol/liter)  | 152.1 ± 0.6 | 149.8 ± 1.1 | 153.4 ± 0.8 |
| Potassium (mmol/liter)| 5.2 ± 0.9   | 6.2 ± 0.5   | 5.8 ± 0.3   |
| Chloride (mmol/liter)| 116.9 ± 0.9 | 116.5 ± 0.8 | 119.0 ± 0.5 |
| CO₂ (mmol/liter)     | 14.5 ± 0.8  | 15.6 ± 1.2  | 15.7 ± 0.6  |
| Alkaline Phosphatase | 100.2 ± 8.8 | 88.0 ± 7.8  | 96.3 ± 6.9  |
| ALT (units/liter)    | 79.9 ± 27.6 | 79.0 ± 22.4 | 74.1 ± 11.0 |
| Calcium (mg/dl)      | 8.5 ± 0.1   | 8.8 ± 0.1   | 8.9 ± 0.1   |
| Protein (g/dl)       | 5.0 ± 0.1   | 4.8 ± 0.1   | 4.9 ± 0.1   |
| Bilirubin (mg/dl)    | 0.5 ± 0.1   | 0.5 ± 0.1   | 0.6 ± 0.1   |
| AST (units/liter)    | 215 ± 24    | 217 ± 25    | 225 ± 29    |

Fig. 4. Oligopeptide uptake. Uptake of 1.9 μM [14C]GlySar was performed for 5 and 30 min in choroid plexuses pooled from Pept2+/+ and Pept2-/- mice. Uptakes were performed on four different pools (n = 4). * p < 0.001 versus Pept2 +/+ uptake.

They found that PEPT2 was expressed in brain by astrocytes, subependymal cells, ependymal cells, and epithelial cells of choroid plexus. In a subsequent study using immunoblot analyses and immunofluorescent confocal microscopy, Shu et al. (20) demonstrated that PEPT2, but not PEPT1, protein was present in the apical membrane of rat choroid plexus epithelial cells. They also observed that GlySar accumulation and transepithelial transport were 3 to 4 times higher when introduced from the apical as opposed to the basal side of the cell monolayers. This study and other studies from our laboratory (21–23) suggest that PEPT2 may have a role in the efflux of neu-ropetides, peptide fragments, and peptidomimetics from cerebrospinal fluid to the blood. Based on whole tissue studies, in the presence and absence of sodium, it has been estimated that PEPT2-mediated transport may account for about 30–60% of the total transport processes in rat choroid plexus. However, this estimate is far from certain because PHT1 (5) and PHT2 (6) transcripts have been reported in the brain, with PHT1 being especially abundant in the hippocampus, choroid plexus, cerebellum, and pontine nucleus. Moreover, it is unknown if other as yet unidentified peptide transporters are expressed and functionally active in brain. Thus, a transgenic PEPT2-deficient model would be a valuable tool in delineating the role and relative importance of this specific transporter at the blood-CSF interface.

In the present study, a PEPT2-deficient mouse model was successfully generated by targeted gene disruption in embryonic stem cells. The Southern analyses confirm that this novel mutant mouse strain does not produce the gene for PEPT2. Moreover, RT-PCR analyses confirm that PEPT2 transcripts are absent from the choroid plexus, kidney, and brain of null mice, while being retained in wild-type and to a lesser extent in PEPT2+/− mice. As expected, the low affinity peptide transporter, PEPT1, was retained in kidney. Surprisingly, PEPT2 null mice were viable and grew at a normal rate. There were no obvious biochemical changes nor morphologic abnormalities in the kidneys and choroid plexus of PEPT2−/− animals. Despite the large number of possible di- and tripeptide substrates in brain, and the putative physiological role of PEPT2 as an efflux pump for neuropeptides (and/or fragments), no apparent pathologies were observed. Nevertheless, dipeptide uptake studies in isolated rat choroid plexus revealed a markedly dysfunctional process. As clearly demonstrated, the transport activity of GlySar in PEPT2−/− mice was essentially ablated, with less than 10% residual function on average.

Several factors may explain the 10% residual activity for dipeptide uptake in PEPT2 null mice. Most notably, the activity may reflect the presence of a low affinity carrier. This possibility is consistent with the study of Shu et al. (20), in which a pH-independent, low affinity transporter (i.e. Km of 1.4 mM for GlySar) was observed at the basolateral membrane of rat choroid plexus epithelial cells in primary culture. Although the molecular properties of this protein were not delineated, the basolateral carrier was distinct from that of PEPT1 and PEPT2 and was involved in the coordinated efflux of GlySar across the cell monolayers. We subsequently performed a temperature-dependent study in null mice and found that 1.9 μM GlySar uptake was reduced at 5 min (0.111 ± 0.013 μM/g at 37 °C versus 0.069 ± 0.007 μM/g at 4 °C; p < 0.05). Thus, there is some temperature-dependent uptake in the choroid plexus of null mice, but it is small (0.042 μM/g in Pept2−/− compared with a total uptake of 1.02 ± 0.05 μM/g in Pept2+/+ at 37 °C). Whether or not this process reflects the basolateral low affinity transporter is unclear. Some of the residual activity might also be explained by other factors. Integrity of the radiolabel was considered, but GlySar had a radiochemical purity of 98–99%.

This dipeptide has also been shown to be stable in rat choroid plexus (20, 22). Although tissue binding might occur, this effect
Targeted Disruption of the PEPT2 Gene

would be minimal, at best, based on our GlySar uptake time profiles in whole tissue rat choroid plexus (22). Finally, a non-specific, non-temperature-dependent process (i.e. diffusion) may be operational to a minor extent in the absence of PEPT2 protein.

Brain homeostasis depends upon the composition of both brain interstitial fluid and cerebrospinal fluid (28–30). Whereas the former is largely controlled by the blood-brain barrier, the latter is regulated by a highly specialized blood-CSF interface, the choroid plexus epithelium. Similar to other epithelia, the choroid plexus is polar with distinct brush border (CSF-facing) and basolateral (blood-facing) surfaces. The polarity of the cell has distinct characteristics, and in particular, transporters are uniquely distributed among the two membrane surfaces. More recent studies (31, 32) have shown that choroid plexuses participate in a variety of functions in addition to their traditional roles of controlling the volume and ionic composition of cerebrospinal fluid. In this regard, they have specialized transport systems that allow blood to brain influx of micronutrients or brain to blood efflux of harmful neurotransmitter metabolites. The choroidal epithelium is also a source of and a target for hormones and other neuroactive or neuronal metabolites. The choroidal epithelium is also a source of and a target for hormones and other neuroactive or neuro-modulating compounds. Moreover, the presence of drug-metabolizing enzymes in the cerebral capillaries, choroidal epithelium, and nervous tissue suggests a concerted mechanism of brain protection by which substrates are inactivated and then cleared from the cerebrospinal fluid (28, 33). Peptide transporters may, therefore, serve a nutritive function (as in the intestine and kidney) by supplying small peptides from the blood circulation to choroid plexus tissue and cerebrospinal fluid. Alternatively, they may serve as a clearance mechanism to remove unwanted neuropeptides (and peptide fragments) from the CSF. Finally, they may also affect the disposition of peptidomimetic drugs and toxic agents in the CSF and brain.

The lack of an observable pathological phenotype in PEPT2 null mice suggests that other transporter and/or metabolic systems can compensate for this loss of function. It may also mean that PEPT2 plays an insignificant role in the disposition of neuropeptides (and/or fragments) in choroid plexus and that its main role is in protecting the organism from toxic xenobiotics. This last scenario is analogous to P-glycoprotein-deficient null mice were without obvious visible abnormalities, further testing for biological phenotypes is being performed. Notwithstanding this surprising lack of physiological and/or pathological change, the development of transgenic PEPT2-deficient mice provides a unique opportunity for probing its in vivo role and relative importance in the brain, kidney, and other tissues of interest.

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