Effect of N-glycosylation on the transport activity of the peptide transporter PEPT1

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Stelzl T, Baranov T, Geillinger KE, Köttra G, Daniel H. Effect of N-glycosylation on the transport activity of the peptide transporter PEPT1. Am J Physiol Gastrointest Liver Physiol 310: G128–G141, 2016. First published November 19, 2015; doi:10.1152/ajpgi.00350.2015.—The intestinal peptide transporter PEPT1 provides bulk quantities of amino acids to epithelial cells. PEPT1 is a high-capacity and low-affinity solute carrier of the SLC15 family found in apical membranes of enterocytes in small intestine and distal colon. Surprisingly, murine PEPT1 (mPEPT1) has an apparent molecular mass of ~95 kDa in the small intestine but ~105 kDa in the large intestine. Here we describe studies on mPEPT1 protein glycosylation and how glycans affect transport function. Putative N-glycosylation sites of mPEPT1 were altered by site-directed mutagenesis followed by expression in Xenopus laevis oocytes. Replacement of six asparagine residues (N) at positions N50, N406, N439, N510, N515, and N532 by glutamine (Q) resulted in a decrease of the mPEPT1 mass by around 35 kDa. Electrophysiology revealed all glycosylation-deficient transporters to be functional with comparable expression levels in oocyte membranes. Strikingly, the mutant protein with N50Q exhibited a twofold decreased affinity for Gly-Sar but a 2.5-fold rise in the maximal substrate turnover rate.

MATERIALS AND METHODS

Site-directed mutagenesis of the mPEPT1 gene. Putative N-glycosylation sites in mPEPT1 (UniProt AC: Q9JIP7) were identified with the NetNGlyc 1.0 server and mutated by site-directed mutagenesis. Asparagines in positions 50, 406, 439, 510, 515, and 532 were converted into glutamine (N50Q, N406Q, N439Q, N510Q, N515Q, and N532Q) or glycine (N50G) residues (Table 1). To this end, two mutation-specific megaprimers, carrying specific restriction sites (EcoRV and XhoI), were amplified and subsequently coupled with mutation-specific megaprimers, carrying specific restriction sites (EcoRV and XhoI), were amplified and subsequently coupled with...
digested with XhoI and EcoRV (Thermo Scientific, Waltham, MA) and cloned into pCRII-TOPO-3’ end vector (Invitrogen, San Diego, CA). Mutations were confirmed by sequencing (GATC Biotech, Constance, Germany).

Table 1. Sequence positions of mPEPT1 asparagine (N) residues modified by mutagenesis

<table>
<thead>
<tr>
<th>Putative N-Glycosylation Site</th>
<th>Protein Sequence</th>
<th>Amino Acid Exchange</th>
</tr>
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<tbody>
<tr>
<td>N50</td>
<td>-42 RNPGLGNLSTAIYHT 58-</td>
<td>AAT → CAA/GGC</td>
</tr>
<tr>
<td>N406</td>
<td>-398 KVLIQDNIMTVWFCQ 414-</td>
<td>AAC → CAG</td>
</tr>
<tr>
<td>N439</td>
<td>-431 DIKGLTSINWSSGEGP 447-</td>
<td>AAC → CAA</td>
</tr>
<tr>
<td>N510</td>
<td>-502 KNSGKVYENTSHNASG 518-</td>
<td>AAC → CAA</td>
</tr>
<tr>
<td>N515</td>
<td>-507 YVENYTSNASGGQFPF 523-</td>
<td>AAC → CAA</td>
</tr>
<tr>
<td>N532</td>
<td>-524 SQGQRTIINNTAYAPTC 540-</td>
<td>AAC → CAA</td>
</tr>
</tbody>
</table>

Numbers indicate amino acid positions within the protein sequence.

Fig. 1. Topology model of murine PEPT1 (mPEPT1). The intestinal mPEPT1 transporter consisting of 709 amino acids is assumed to be arranged in 12 transmembrane domains (TMDs). Membrane-spanning helices are connected by intra- and extracellular loops, carrying 6 putative N-glycosylation sites orientated to the luminal site (labeled with a branched tree at corresponding amino acid positions). The compilation of the model is based on domain predictions for the mPEPT1 transporter AC: Q9JJF7 emitted by the UniProtKB/Swiss-Prot platforms.

- Electrophysiological measurements. Two-electrode voltage clamp (TEVC) experiments were conducted according to Amasheh and Kottra et al. (1, 36). Oocytes were placed in an open chamber and continuously superfused with Barth solution in the absence or the presence of 0.3–10.0 mM glycyl-sarcosine (Gly-Sar) or 1–20 mM cefadroxil (Sigma-Aldrich) with a flow rate of 3 ml/min. Current and potential electrodes, backfilled with 0.5 mM KCl and an electrode resistance between 1–3 MΩ, were used to voltage clamp the oocytes at −60 mV. Current flow was calculated from the difference between baseline after rinsing oocytes with Barth solution and reaching a plateau phase in the presence of substrate. Current-voltage (I-V) relations were recorded with a Tec-03 amplifier (Npi Electronic, Tamm, Germany) for the duration of 100 ms in the potential range of +80 to –160 mV. Transport currents were normalized to recording of currents generated by 1 mM α-methyl-D-glucopyranoside (pH 6.5) as substrate for msGLT1 expressed as a reference transporter. After normalization of transport currents, kinetic constants $K_m$ and $I_{max}$ as maximal transport current were determined by submitting data to a Michaelis-Menten equation with approximation by the least-square method.

- Protein preparation from Xenopus laevis oocytes. Three to four days after cRNA injection, 20–30 oocytes expressing the different transporter genes were transferred into 200 μl lysis buffer (20.0 mM HEPES, 10.0 mM KCl, 1.5 mM MgCl2, 1.0 mM dithiothreitol) and mechanically homogenized in the presence of 1 mM protease inhibitor phenylmethanesulfonyl-fluoride (PMSF). Following centrifugation for 1 min at 4°C, supernatants were collected and protein contents determined by Bradford assay (Bio-Rad, München, Germany). Unless otherwise specified, 15 μg total protein was loaded per lane of a 10% SDS-acrylamide gel.

- Enzymatic protein deglycosylation. Enzymatic deglycosylation experiments from mouse intestinal tissues were carried out with purified brush-border membrane protein, obtained by the divalent cation precipitation technique (DivCatPre) previously described by Wuensch et al. (59) and Schmitz et al. (49). Treatment of protein extracts with the different glycosidases (New England Biolaboratories) was done following manufacturer’s instructions.
Western blot analysis. SDS-PAGE was performed with a mini-protein 3 system from Bio-Rad. Isolated protein (15 μg) was mixed with 4× Laemmlui-buffer (125.0 mM Tris, pH 6.8, 8.0% SDS, 20.0% glycerol, 0.4% bromphenol blue sodium salt, 20.0% β-mercaptoethanol) and separated on a 10% SDS-acrylamide gel for 1 h and 3 h at 120–160 V. Proteins were then transferred onto nitrocellulose membranes (Whatman, Maidstone, UK) in a wet tank blotting system at 0.36 A for 25 min. Membranes were blocked in 1% bovine albumin (AppliChem, Darmstadt, Germany) for 1 h, succeeding overnight staining at 4°C with antibodies against mPEPT1 (custom-made; Pineda, Berlin, Germany; dilution 1:5,000, polyclonal rabbit IgG against COOH terminus of rat PEPT1: NH2-CVGKENPYS-SLEPVSQTN-MOOH) and β-actin as loading control (dilution 1:2,000, polyclonal goat IgG, C-11 sc-1615; Santa Cruz Biotechnology, Dallas, TX). After three washings with PBS-T (0.137 mM NaCl, 2.700 mM KCl, 10.000 mM Na2HPO4, 1.800 mM KH2PO4, pH 8.0), oocytes were incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS for 15 min. Residual biotinylation reagent was removed by three washings with PBS. After incubation with quenching buffer (100 μM glycine in PBS) for 20 min on ice, oocytes were lysed 30 min with lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.6), containing 0.5 mM protease inhibitor PMSF. Solubilized oocytes were centrifuged for 15 min at 14,000 g and 4°C, and supernatants were collected. Total protein (200 μg) was incubated with 50 μl streptavidin-agarose (Sigma-Aldrich, Taufernken, Germany) overnight at 4°C with gentle agitation. The beads were washed with cold PBS, followed by the addition of Laemmli buffer and heating at 95°C for 5 min to break biotin-streptavidin bonds. Obtained biotinylated proteins were directly transferred to SDS-PAGE.

**Tracer flux studies with Xenopus laevis oocytes.** Oocytes expressing PEPT1 transporters were incubated in solutions of increasing Gly-Sar concentrations (0.3–50.0 mM) containing radio-labeled [3H]-Gly-Sar (56 Ci/mol) custom synthesized by GE Healthcare
Table 3. Predicted results of N-glycosylation sites in mPEPT1 with the NetNGlyc 1.0 platform

<table>
<thead>
<tr>
<th>Asparagine Position (AC: Q9HP7)</th>
<th>Sequon</th>
<th>Predicted PEPT1 Location</th>
<th>NetNGlyc Potential Prediction</th>
<th>NetNGlyc Agreement Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>NLS</td>
<td>extracellular</td>
<td>0.563</td>
<td>+</td>
</tr>
<tr>
<td>112</td>
<td>NGS</td>
<td>extracellular</td>
<td>0.146</td>
<td>+</td>
</tr>
<tr>
<td>354</td>
<td>NFT</td>
<td>intracellular</td>
<td>0.945</td>
<td>+</td>
</tr>
<tr>
<td>406</td>
<td>NMT</td>
<td>extracellular</td>
<td>0.645</td>
<td>+</td>
</tr>
<tr>
<td>439</td>
<td>NIS</td>
<td>extracellular</td>
<td>0.533</td>
<td>+</td>
</tr>
<tr>
<td>510</td>
<td>NVT</td>
<td>extracellular</td>
<td>0.755</td>
<td>+</td>
</tr>
<tr>
<td>515</td>
<td>NAS</td>
<td>extracellular</td>
<td>0.376</td>
<td>+</td>
</tr>
<tr>
<td>532</td>
<td>NTT</td>
<td>extracellular</td>
<td>0.469</td>
<td>+</td>
</tr>
</tbody>
</table>

*This column describes a potential score for predicted N-glycosylation sites that is based on the averaged output of 9 neural networks of the NetNGlyc platform. Predictions indicating positive sequon occupancy are marked with + (+, potential > default threshold of 0.5; ++, potential > 0.75), while probabilities for nonglycosylated sites are indicated with − (−, potential < 0.5; −−−, potential < 0.32).

We found that the molecular mass of PEPT1 derived from small or large intestine of C57BL/6N mice differed by around 10 kDa (59). This variation was also observed in different mouse strains (C57BL/6N, C57BL/6J, AKR/J, A/J, and SV129/S6) in both male and female animals of either 8 or 40 wk of age. Germ-free C57BL/6N mice (male, 8 wk of age on standard diet) also revealed this marked mass difference, which seems therefore independent of the genetic background, sex, age, or bacterial colonization. Figure 2A shows the mass shift exemplarily for C57BL/6N mice. For assessing the contribution of glycans to overall mass, we performed deglycosylation experiments with peptide-N-glycosidase F (PNGaseF), endoglycosidase H (EndoH), and neuraminidase with membrane protein extracts collected from murine small intestine and colon (Fig. 2, B and C). PNGaseF, releasing N-linked oligosaccharides of high mannose, hybrid, and complex type from glycoproteins, reduced the mPEPT1 mass in jejunum from ~95 kDa and in colon from 105 kDa to ~60 kDa. Unmodified mPEPT1 is predicted to have a protein mass of 79 kDa (UniProtKB). EndoH, cleaving the chitobiose core of high mannose and some hybrid oligosaccharides of N-linked glycoproteins, did not alter the PEPT1 protein mass. Release of N-acetyl-neuraminic acids by neuraminidase, used in combination with EndoH to improve EndoH accessibility, did not change the mPEPT1 mass in small intestine or colon. These findings suggest that the PEPT transporter carries predominantly N-linked glycans of a complex type.

**RESULTS**

**PEPT1 mass shift in murine intestinal segments depends on glycosylation.** Our initial observation based on Western blot analysis was that the molecular mass of PEPT1 derived from small or large intestine of C57BL/6N mice differed by around 10 kDa (59). This variation was also observed in different mouse strains (C57BL/6N, C57BL/6J, AKR/J, A/J, and SV129/S6) in both male and female animals of either 8 or 40 wk of age. Germ-free C57BL/6N mice (male, 8 wk of age on standard diet) also revealed this marked mass difference, which seems therefore independent of the genetic background, sex, age, or bacterial colonization. Figure 2A shows the mass shift exemplarily for C57BL/6N mice. For assessing the contribution of glycans to overall mass, we performed deglycosylation experiments with peptide-N-glycosidase F (PNGaseF), endoglycosidase H (EndoH), and neuraminidase with membrane protein extracts collected from murine small intestine and colon (Fig. 2, B and C). PNGaseF, releasing N-linked oligosaccharides of high mannose, hybrid, and complex type from glycoproteins, reduced the mPEPT1 mass in jejunum from ~95 kDa and in colon from 105 kDa to ~60 kDa. Unmodified mPEPT1 is predicted to have a protein mass of 79 kDa (UniProtKB). EndoH, cleaving the chitobiose core of high mannose and some hybrid oligosaccharides of N-linked glycoproteins, did not alter the PEPT1 protein mass. Release of N-acetyl-neuraminic acids by neuraminidase, used in combination with EndoH to improve EndoH accessibility, did not change the mPEPT1 mass in small intestine or colon. These findings suggest that the PEPT transporter carries predominantly N-linked glycans of a complex type.

**Site-directed mutagenesis of putative mPEPT1 N-glycosylation sites.** N-glycosylation sites were predicted using the NetNGlyc 1.0 platform (27). Thereby, six asparagine residues, N50, N406, N439, N510, N515, and N532, were selected according to the following attributes: 1) location within an N-X-S/T sequon, 2) N-X-S/T sequon located in an extracellular PEPT1 domain, 3) high N-glycosylation prediction score (Table 3). By use of site-directed mutagenesis, several mutant proteins lack...
ing single or multiple sequons were generated, and proteins were heterologously expressed in Xenopus laevis oocytes. Transporter mass was thereafter visualized by Western blot analysis (Fig. 3). Imaging of the maximal fluorescence intensity of immunoblots (Fig. 4) performed with the LI-COR Bioscience Imaging software showed that the mutants N50Q, N50G, N406Q, and N439Q had lower mass than the WT PEPT1 protein (H110 kDa), whereas N532Q had a comparable mass (Figs. 3 and 4). Interestingly, the N515Q mutant revealed a mass increase, most likely attributable to a modification of the glycosylation pattern at residual asparagine residues within sequons.

Replacement of all asparagines in sequons by glutamine in mPEPT1 (Fig. 3B) resulted in a molecular mass of H110 kDa comparable with the results obtained by PNGaseF treatment of intestinal membrane proteins (Fig. 2, B and C). A similar mass was also obtained when glycosylation was impaired in oocytes by injection of the N-glycosylation inhibitor tunicamycin (Fig. 8C).

N-glycosylation of PEPT1 does not affect membrane targeting. To elucidate whether N-glycosylation is essential for PEPT1 protein folding and plasma membrane targeting, we biotinylated Xenopus oocytes that expressed the various mPEPT1 constructs with the membrane-impermeable reagent sulfo-NHS-LC-biotin. Biotinylated surface proteins were isolated by streptavidin-agarose before Western blot quantification. None of the mutant proteins lacking individual or all N-glycosylation sites showed impairment in plasma membrane abundance (Fig. 5). This was also confirmed by immunohistochemical staining of oocyte sections (Fig. 6). Oocytes co-injected with tunicamycin showed unaltered fluorescence for the native mPEPT1 protein.

**Effects of N-glycosylation on transport kinetics.** Functional analysis of the WT transporter recorded by TEVC showed growing currents when Gly-Sar concentrations were increased from 0.3 to 10.0 mM (Fig. 7A) with an I_{max} at 10 mM of 330 ±
107 nA at −60 mV (Fig. 7B). Kinetic analysis revealed an apparent $K_m$ of Gly-Sar of 0.66 ± 0.12 mM (Fig. 7, C and D, Table 4) comparable to 0.75 mM in mouse (21, 22) and 1.1 mM in human PEPT1 (10). Exchange of asparagine residues at N50 had diminished transport activity. Oocyte sections were incubated with an anti-mPEPT1 primary antibody (custom-made, dilution 1:1,000; Pineda) and a Cy3-conjugated secondary antibody (AffinityPure donkey anti-rabbit IgG Cy3-conjugated; dilution 1:500; Jackson ImmunoResearch) and visualized with a Leica DMI4000B fluorescence microscope at 40-fold magnification with Leica Application suite LAS AF Lite v. 2.6.3. 

**For tri-L-alanine (Fig. 10A), the N50Q mutant yielded a slightly reduced affinity [apparent $K_m$ (N50Q) = 0.18 ± 0.04 mM] compared with WT [apparent $K_m$ (WT) = 0.14 ± 0.04 mM] but had also substantially increased transport currents amounting to 1.92 ± 0.41 nA compared with WT with 0.81 ± 0.15 nA (Fig. 10B). To demonstrate that the effects of N50 are not a species-specific phenomenon, the same mutation was generated in the human PEPT1 transporter (UniProt AC: P46059) (Fig. 11, A and B). TEVC recordings also revealed a significant reduction in Gly-Sar affinity in the human N50Q mutant from 0.88 ± 0.19 mM to 1.16 ± 0.18 mM (Fig. 11A), whereas maximal currents recorded at −60 mV in the mutant increased more than twice to 3.70 ± 0.65 nA compared with WT with 1.63 ± 0.23 nA as shown in Fig. 11B.**

Amino acid exchange at N50 does not open a proton leak. To assess whether the high maximal currents detected by TEVC are caused by a proton conductance, tracer flux studies with $[^{14}C]$-Gly-Sar were performed (Fig. 12A). Oocytes expressing N50Q showed significantly higher transport activity for Gly-Sar, amounting with 50 mM Gly-Sar to 4.47 ± 0.32 pmol/min per oocyte in N50Q and 2.32 ± 0.30 pmol/min per oocyte in WT. Tunicamycin treatment slightly increased maximal $[^{14}C]$-Gly-Sar uptake rates in oocytes expressing WT PEPT1 amounting to 2.59 ± 0.21 pmol/min per oocyte. Apparent $K_m$ values increased from 0.82 ± 0.19 mM in WT to 2.92 ± 0.37 mM in N50Q and to 1.26 ± 0.12 mM in tunicamycin-treated oocytes. Competition of tracer influx by the presence of a 10-fold excess of glycy-glutamine (Gly-Gln) (Fig. 12B) revealed a reduction of tracer uptake by 82% in N50Q and inhibition of the WT mPEPT1 by 57%.
DISCUSSION

It is assumed that 50–90% of all proteins in higher eukaryotes undergo posttranslational modifications, of which the glycosylation of asparagine residues is the most frequent one (38). Previous mass-spectrometric analysis (58) identified PEPT1 as a cell surface glycoprotein. Targeted screening for asparagine residues in mPEPT1 delivered in total 35 matches, whereas 8 asparagines are positioned within an N-X-S/T con-
sensus motif (Table 3). Acceptor sequences containing proline in center position, known to have a low N-glycosylation probability (7, 46a), as well as sequons located in an intracellular protein domain and motifs with a low N-glycosylation prediction score (10) were neglected. The remaining six asparagines N50, N406, N439, N510, N515, and N532 were exchanged for glutamine. Mutant transporter proteins lacking single or multiple glycosylation sites all showed expression in the plasma membrane of oocytes with no indication of impaired targeting or membrane insertion, allowing the proteins to be studied also at the functional level. Disruption of N50, N406, and N439 was accompanied with a mass reduction of mPEPT1, whereas single mutations at N510Q and N532Q did not appear to change the protein mass. It would be erroneous to conclude that these two sequons are not N-glycosylated. Disruption of single sequons can trigger glycan modification on other sequons. This was shown by Tanaka et al. (55) for the organic anion transporter OAT1, demonstrating that simultaneous replacement of asparagines in sequons leads to oligosaccharide transfer onto previously neglected downstream acceptor sites.

Also the fact that N510 and N532 are both located in sequons where threonine is found at the third position of the N-X-S/T motif, which is known to be preferred glycosylated over serine residues (7, 25, 46), would suggest the presence of N-glycans. Simultaneous disruption of multiple sequons containing N510 and N532, however, showed a decrease in the PEPT1 protein mass of about 3–10 kDa between mutants N50Q/N406Q/N439Q/N515Q/N532Q and N50Q/N406Q/N439Q/N510Q/N515Q/N532Q as well as N406Q/N439Q/N515Q and N406Q/N439Q/N510Q/N532Q, indicating that both sequons of N510 and N515 are occupied by N-glycans. Disruption of all six N-glycosylation sites in the mouse PEPT1 protein caused a mass reduction of around 35 kDa. Moreover,
the sextuple mutant revealed an altered migration pattern with increased mobility and reduced protein band size, probably attributable to altered detergent binding (57). When glycosylation in oocytes was suppressed by tunicamycin, a similar loss of mass was observed, and this matches also with the results obtained by treatment of murine intestinal brush-border membrane samples with PNGaseF.

Protein glycosylation is important for proper protein folding, sorting, membrane targeting, and protein structure and function (30). Disruption of N-glycosylation can thus cause protein misfolding and promotes post-translational degradation (5, 57) or can change turnover rates by affecting the transporter membrane density (18). However, for the mPEPT1 and hPEPT1 mutants described here, neither immunofluorescence analysis nor oocyte surface protein biotinylation revealed any evidence for alterations in membrane protein density.

Kinetic characteristics of rhodogenic transporters can most easily be characterized by electrophysiology (3, 12, 37). With the TEVC technique, we demonstrate kinetic changes in peptide transporters lacking N-glycosylation. The most impressive changes were obtained for position N50, where the exchange N50Q and N50G caused a reduction of substrate affinity accompanied by a gain of velocity. The kinetic changes were found with three different substrates. An increase in transport currents can be caused by 1) increased substrate transport, 2) increased ion conductance induced by the mutation, or 3) increased transporter density. However, quantification of protein density in oocyte plasma membranes via biotinylation proved that unaltered protein levels and tracer flux analyses revealed any evidence for alterations in membrane protein density.

That glycosylation affects transporter function has been shown for the glucose transporter GLUT1 (2) and the ammonium transporter MEP2 (41), in which tunicamycin causes a loss in substrate affinity. Treatment of mPEPT1-expressing oocytes with tunicamycin led to a protein mass comparable to that of the sextuple mutant. However, there were no significant effects on cell-surface expression in oocytes but robust changes in affinity and transport rate assessed by both current measurements and tracer flux analyses. Substrate affinities declined by 50–72% with Gly-Sar as substrate, whereas transport rates increased between 52 and 57%.

In understanding how glycans attached to N50 affect the transport characteristics with a gain of maximal transport rate when removed, protein structure and membrane topology need to be inspected. However, structural information on mammalian peptide transporters is very limited and relies on homologies with crystal structures of other members of the major facilitator superfamily, like the prokaryotic homologue of PEPT1 from Shewanella oneidensis (43). On the basis of modeling data from prokaryotic SLC15 members (19, 44), N50 is placed between TMD 1 and 2, just above the membrane projecting into the extracellular space. Glycans attached to N50 would thus be in a loop comprising about 32 amino acids, close to the membrane surface, and this may constrain necessary movements of amino-terminal TMDs in the transport cycle (Fig. 13). Removal of the glycans could thus increase mobility of the protein. Membrane domains 1 and 2 are known to contribute to the substrate binding and translocation pore, and changes in substrate affinity, as shown for all N50 variants, are therefore not unexpected. Assessing the precise glycan mass and architecture requires more sophisticated approaches, but immunoblots reveal a
slight decrease of around 1–5 kDa when glycosylation of N50 is prevented.

Taking a total of around 35-kDa mass shift for an expected glycan-free protein, the majority of glycans are therefore attached to asparagine residues in the large extracellular loop between TMD 9 and 10, predominantly N406 and N439. Recent crystallization of this loop domain from the mouse and rat PEPT1 transporter disclosed two immunoglobulin-like domains.

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Fig. 12. $^{14}$C-Gly-Sar flux studies. A: oocytes expressing the mPEPT1 WT, WT coinjected with tunicamycin and the N50 mutant transporter were incubated in $^{14}$C-Gly-Sar (0.3–50.0 mM) for 10 min. Posttreatment, intracellular radioactivity was determined using a liquid scintillation counter. Values are depicted as means ± SE of 5 oocytes. Data are corrected for basal radioactivity observed in water-injected control oocytes (data not shown). One-way ANOVA with Dunnett’s multiple comparison (*$P < 0.05$, **$P < 0.01$) vs. mPEPT1 WT transporter. B: specificity of $^{14}$C-Gly-Sar transport was proven by coincubation of the competitive inhibitor Glycyl-Glutamine (Gly-Gln) in 10-fold excess. Gly-Gln (pH 6.5) significantly reduced the uptake of 10 mM $^{14}$C-Gly-Sar in oocytes expressing mPEPT1 WT, WT coinjected with tunicamycin and the N50Q mutant transporter. Data are expressed as means ± SD of 5 oocytes. Statistical analysis was performed by 2-tailed unpaired $t$-test (*$P < 0.05$, ***$P < 0.001$).

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Fig. 13. Hypothesized mechanism for the substrate turnover in PEPT1 mutant transporter N50Q. The mammalian PEPT1 transporter is predicted to consist of 12 transmembrane helices (TM) that are arranged in 2 bundles (TM 1–6, TM 7–12), thereby forming a central binding cavity (53). In the presence of a low extracellular pH ($\Delta$H) and/or a decreasing membrane potential ($V_m$), the transporter orientates to an outward open conformation. In this state, protons and peptides from the gut lumen have access to the PEPT1 binding pocket. Following binding (occluded state), PEPT1 undergoes a conformational change (inwardly open state) to release peptides and protons into the cytosol. The formation of the inward open conformation is thereby induced by the reduction of the intracellular pH and/or the increase in $V_m$. N-glycans linked to the $N$-glycosylation site N50 appear to limit the mobility of the NH2-terminal PEPT1 domain during the transport cycle. The covalent attachment of N-linked glycans to sequon N50 appears to attenuate the PEPT1 rotation mobility. In absence of an inert N50 N-glycan mass, the PEPT1 turnover rate is accelerated, resulting in considerably higher intracellular substrate concentrations.
structures, transiently interacting with trypsin for luminal concentration of peptides rich in arginine and lysine (4). Peptide transporter proteins from prokaryotes are lacking this big extracellular loop but have very similar functional characteristics (17, 29, 56), arguing against a role of the loop in the transport process.

On closer examination of N50, N406, N439, N510, N515, and N532, it appears that there exists a strong conservation within species (Fig. 14). Protein sequence alignment of 50 different vertebrate species showed strongest conservation of N50 and N439 within N-X#P-S/T sequons, amounting to 58–60%. Whereas N50 is mainly found in mammals, birds, and reptiles, N439 is primarily preserved along mammalian species. With 44–46%, N510 and N515 both are highly conserved in mammals, exhibiting 30% cooccurrence. N406 and N532 have a minor prevalence of 10–20% in mammals. In consideration of these findings, it appears that some sequons are more conserved between species than others, implying a more important function. Although N50 and N439 are both located in the two most conserved sequons, our study demonstrates that only disruption of N-glycosylation of N50 alters PEPT1 transport kinetics, whereas N439 has no obvious effects. Therefore, in silico analysis of sequon conservation can be a good tool to specify and predict the most important N-glycan acceptor sites in proteins although they do not provide information on sequon occupancy. Moreover, protein

Fig. 14. Conservation of putative PEPT1 N-glycosylation sites within species. Protein alignment of mPEPT1 with 50 individual vertebrate species was performed to visualize asparagine residue conservation within N-X#P-S/T sequons (marked with a gray box). Asparagines N50, N439, N510, and N515 revealed highest conservation rates. With 58–60%, N50 and N439 were highly abundant in mammals and birds. N510 and N515, with 30% cooccurrence, were mainly conserved in mammals. N406 and N532 had minor conservation, amounting to 10 and 20%. Protein alignment was implemented with ClustalO provided by the UniProt platform. Protein sequences chosen for alignment were selected according to the following criteria: 1) assignment to the group of peptide transporter, 2) protein sequence >600 bp, and 3) sequence identity to mPEPT1 >50%.
sequence alignment alone is not potent enough to completely replace functional protein studies.

Western blot analysis disclosed differences in the PEPT1 mass between small and large intestine of mouse attributable to altered protein N-glycosylation. It is not unusual that a glycoprotein appears in different glycoforms under physiological conditions (45). Variations in PEPT1 glycosylation patterns could arise from changes in the N-glycosylation site occupancy or by modification of surface glycan structures catalyzed by cellular glycosidases and glycosyltransferases (33). Intestinal glycosyltransferases have been shown to be developmentally regulated, region specific, and susceptible for changes by the endogenous microflora (15). Because of the observation that the molecular mass of PEPT1 resembles gut segments of germ-free and conventionally raised mice, the microbiota might play a minor role in modulating glycosyltransferase activities or in the degradation of glycoconjugates (23).

To verify these issues, further glycosyltransferase expression and activity studies, combined with a structural glycan analysis, need to be performed. Besides, in vitro experiments have shown that environmental factors can significantly change the glycan profile of a protein. Low glucose levels, for instance, have been found to reduce sequo occupancy in murine myeloma cells (52). At low cellular oxygen and pH concentrations, glycosyltransferase activities are known to alter significantly (40, 47). As the gastrointestinal tract is a highly complex organ system, varying PEPT1 glycoforms might result from the interplay of various environmental factors.

Besides, there is evidence for the existence of organ-related protein glycoforms (6). To verify this, the glycosylation status of PEPT1 was examined in different mouse organs. Beyond the gut, highest PEPT1 expression levels were reported for bile duct epithelial cells and the proximal tubule of kidney. However, the majority of available data are solely based on mRNA expression studies. Although immunostaining of the extrahepatic biliary tract localized PEPT1 in the apical membrane of cholangiocytes in mice (34), there is currently no information on the transporter protein mass. Because of its low abundance, all our efforts to determine the PEPT1 mass in mouse liver were unsuccessful so far.

In contrast, expression studies in dogs showed a PEPT1 mass of about 78 kDa in liver (28). However, it is currently unclear whether species-specific differences exist in PEPT1 glycosylation. For murine kidney, a PEPT1 mass of 75 kDa was reported (48). Compared with the gut, exhibiting a PEPT1 mass of ~95 kDa in small intestine and 105 kDa in colon, the PEPT1 mass in liver and kidney rather corresponds to the predicted protein core mass of 79 kDa (UniProtKB). PEPT1 expressed in human pancreatic cancer cells was detected in multiple glycoforms varying from 90–120 kDa (26). However, in a diseased state such as cancer, N-glycosylation of proteins is known to be dysregulated (13). Under this aspect, tumor cells are of limited suitability to predict the glycosylation status of a healthy cell. Nonetheless, immunobLOTS performed with the human colon carcinoma cell line Caco-2, exhibiting post-differentiation characteristics of small intestinal cells (20), resulted in a similar PEPT1 mass as observed in the small intestine of mouse (data not shown). As PEPT1 transport significantly differs between kidney and gut (9), the individual PEPT1 glycoforms might also have altered functional and kinetic properties. To confirm this, additional transport studies need to be performed.

In summary, our study demonstrates that glycosylation of intestinal PEPT1 amounts to ~1/3 of its overall mass and that it differs between small intestine and distal colon. This phenomenon of varying protein glycosylation of an intestinal membrane transporter within the same organ is hitherto a unique observation and raises new questions on the biological significance of glycosylation. We identified six asparagine residues used for N-glycosylation with five residues located in the large extracellular loop between TMD 9 and 10. Removal of N-glycans by site-directed mutagenesis or by preventing glycosylation with tunicamycin did not alter membrane protein abundance but changed the transport kinetics. The phenomenon of reduced substrate affinity but an increased maximal transport rate was found upon an exchange of N50 to glutamine or glycine. This gain of function in velocity may be attributed to sterical interference of surface glycans with the substrate binding site of PEPT1.

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