Membrane Topology of the Human Dipeptide Transporter, hPEPT1, Determined by Epitope Insertions[†]

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ABSTRACT: We have used epitope insertion to analyze the transmembrane topology of the human H^+−dipeptide symporter hPEPT1. An epitope tag, EYMPME (EE), was inserted into different locations at amino acids 39, 78, 106, 412, and 708 of hPEPT1 by site-directed mutagenesis. The functional integrity of the tagged protein was tested by measuring its dipeptide transport activity in transfected Cos7 cells. Further, cells expressing hPEPT1 or EE-tagged hPEPT1 derivatives were labeled with an anti-EE-monoclonal antibody (anti-EE-mAb) or an antiserum raised against the carboxyl terminus of hPEPT1 (anti-hPEPT1) and examined by immunofluorescence confocal microscopy. EE106-, 412-, and 708-hPEPT1 transported the dipeptide tracer as well as wild-type hPEPT1. Tags at position 106 and 412 were shown to be extracellular because they were accessible to anti-epitope antibody in nonpermeabilized cells. In contrast, the carboxyl-terminal domain and EE708 were shown to be intracellular since they were only accessible to the antibodies in permeabilized cells. These results are consistent with a 12-transmembrane domain (TMD) topological model of PEPT1. Epitope insertions at regions linking the putative TMD1 and TMD2, and TMD2 and TMD3 (EE39- and EE78-hPEPT1), abolished the dipeptide transport into the cells. In transfected Cos7 cells, these tagged proteins remained largely intracellular rather than at the plasma membrane. These results suggest that the integrity of these regions is essential for transporter trafficking and/or function. Thus, the topology of the amino-terminal portion, including putative TMD1 and -2, remains to be clarified.

Intestinal transporters play important roles in nutrient uptake and also mediate drug absorption. The human intestinal transporter hPEPT1 mediates absorption of dipeptides and tripeptides, and of peptidomimetic drugs such as β-lactam antibiotics (*1*−*5*). Transport is driven by a transmembrane proton gradient (*2*, *3*). A second dipeptide transporter expressed mainly in human kidney, hPEPT2, has a sequence very similar to that of PEPT1 (*6*). Recently, PHT1 from rat brain (*7*) was identified as a third member of the mammalian dipeptide transporter family. These are part of a peptide transporter superfamily, PTR, which is distributed over distant species from bacteria to mammals (*8*, *9*), but little is known about their structure.

The hydropathy plot of hPEPT1 (Figure 1) suggests the presence of 12 putative TMDs. A membrane model of rabbitt PEPT1 has been proposed by Fei et al. (*10*) to contain 12 TMDs. However, the hydropathy profile fails to establish the number and locations of the TMDs with any certainty. Figure 1 also shows the relative membrane orientation of hPEPT1, with both the C and N termini localized inside the cell, as predicted on the basis of criteria described by Kyte and Doolittle (*11*) and Claros and von Heijne (*12*). This study was designed to test the topology of hPEPT1 by inserting epitope tags into predicted intra- or extracellular loops, and determining their accessibility to an anti-epitope antibody.

EXPERIMENTAL PROCEDURES

Materials. Glycyl[^{3}H]sarcosine (specific radioactivity of 39 Ci/mmol) was synthesized by Amershan (Arlington Heights, IL). Glycylsarcosine and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Bacterial strains, ung- and DH5α, were purchased from Invitrogen (San Diego, CA).

Cell Culture. Monkey Cos7 cells were obtained from American Type Culture Collection and grown in DMEM H21 medium containing 10% fetal bovine serum and a penicillin (100 units/mL)/streptomycin (100 μg/mL) mixture at 37 °C in a humidified atmosphere with 5% CO_2. Chinese hamster ovary cells (CHO) and CHO cells stably transfected with hPEPT1 were grown as described previously (*4*).

Polyclonal Anti-PEPT1 Antiserum and EE-Monoclonal Antibody. Polyclonal antiserum was raised in rabbits against 15 amino acids of the carboxyl terminus of hPEPT1 (KSNPYFMSGANSQKQ). One cysteine residue was added to the amino terminus to allow oligopeptide coupling to the carrier protein keyhole limpet hemocyanin (KLH). Affinity-purified anti-PEPT1 rabbit antiserum was used for Western

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Table 1: Oligonucleotides Used for Site-Directed Mutagenesis[a]

tagged hPEPT1	oligonucleotide sequence	digestion site
EE39-hPEPT1	5′-CAGCTGATGAAATTTGTGAAGTA**CTCCATTGGCATGTACTC**GAGAATCAGGATTGCTCGC-3′	*Xho*I
EE78-hPEPT1	5′-CAATGGTCTTGAACTTTCC**CTCCATTGGCATGTACTC**GAGCCACGAGTCGGCG 3′	*Xho*I
EE106-hPEPT1	5′ CATGGTTGTGGTCTGT**CTCCATTGGCATGTACTC**GAGGTCATTAATGGAGC-3′	*Xho*I
EE412-hPEPT1	5′-GTTTGAGACATTGGGCC**TTCCATTGGCATGTACTC**GAGTGTCACCATCTCTCC-3′	*Xho*I
EE708-hPEPT1	5′-CCACTTGCCTCCTGAATTCA**TTCCATTGGCATGTATTCCC**CATCTGTTTCTGTGAATTG-3′	*Xmn*I

[a] The EE tag sequence is bold, and the unique restriction site is underlined.

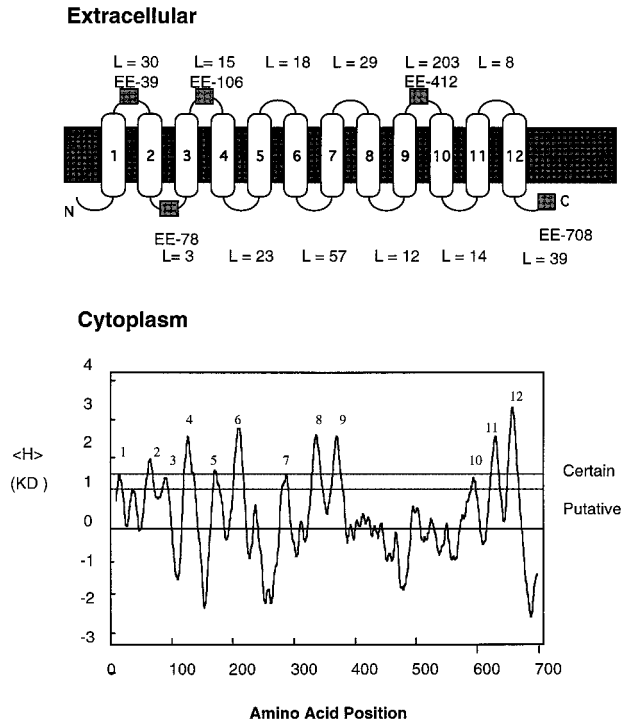

FIGURE 1: Predicted topology (top) and hydropathy plot (bottom) of hPEPT1. The orientation and number of TMDs are predicted using the method of Claros and von Heijne (*11*). Hydropathy was calculated using a 12-amino acid moving window and the Kyte−Doolittle scale. A hydropathy value of >1 was taken to suggest a putative TMD, whereas a value of >1.5 was considered certain.

blotting and immunofluorescense confocal microscopy. The antiserum is described in ref *13*.

The monoclonal mouse antibody directed against the EE epitope (EYMPME) was obtained from Onyx (South San Francisco, CA). It was also affinity purified before being used (*14*).

Site-Directed Mutagenesis. The EE tag (EYMPME) was inserted at different locations of PEPT1 in pBluescripts by designing 50−60 bp oligonucleotides encoding the tag sequence and flanking sequences from hPEPT1. The oligonucleotides used are listed in Table 1. Oligonucleotide-directed mutagenesis was carried out as described by Kunkel (*15*). The tag was inserted at Leu residues 39, 78, 106, 412, and 708. By using an alternative codon for Leu (CTG or CTT → CTC), a unique *Xho*I site was created in conjunction with first three bases from the tag, to facilitate analysis of the mutated sequences. The tag inserted at the carboxyl tail of PEPT1 (EE708-hPEPT1) was constructed differently, by adding an additional Gly residue before the EE tag to ensure flexibility of the epitope before the TGA stop codon. Moreover, the sequence 3′ of TGA was changed from AGGT to ATT to create a unique *Xmn*I site. The tagged PEPT1 derivatives were obtained by screening the mutants with the restriction enzymes *Xho*I or *Xmn*I. The hPEPT1 gene was excised with *Eco*RV and *Not*I, and the generated DNA fragment was subcloned into the mammalian expression vector, pCDNA3. All constructs were then sequenced to ensure proper insertion of the tag.

Transient Transfection of PEPT1 in Cos7 Cells by Electroporation. Cos7 cells were harvested with trypsin, washed with PBS, and finally resuspended in RPMI 1640, without fetal calf serum. DNA (7 μg) was added to 10^7 Cos7 cells and the mixture electroporated in a 0.4 cm cuvette at 0.3 kV and 960 μF for 33−36 ms. Cells were allowed to recover in growth media and plated onto 12-well plates. The transport assay was performed 48 h after electroporation. CHO cells stably expressing hPEPT1 and EE412-hPEPT1 were selected as described previously (*4*).

Membrane Preparation and Western Blotting. Membrane proteins from stably transfected CHO cells were prepared as described previously (*16*). The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Forty micrograms of protein was loaded into each lane of an 8% SDS−PAGE gel. Proteins were electroblotted to PVDF membranes, followed by blocking with a TBST [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 40] solution containing 5% nonfat milk at room temperature for 1 h. The blot was incubated with anti-PEPT1 antiserum (Quality Control Biochemical, Hopkinton, MA) at room temperature for 2−3 h. After the solution had been washed four times with TBST, the blot was incubated with secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2500 dilution, Bio-Rad) at room temperature for 1 h. The Western blot was developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad).

Transport Assay. Transport assays were performed with a [^{3}H]Gly-Sar tracer as described previously (*4*).

Laser Scanning Immunofluorescence Confocal Microscopy. After electroporation with plasmid DNAs, Cos7 cells were grown on poly-L-lysine-coated glass slides for 48 h and fixed with 3% paraformaldehyde, at room temperature for 10 min. Cells were then simultaneously blocked and permeabilized with a phosphate-buffered saline (PBS) solution containing 0.25% cold-water fish gelatin and 0.04% saponin, at 4 °C, overnight. For nonpermeabilizing conditions, cells were blocked similarly without saponin. Subsequently, cells were incubated with the primary antibodies (anti-EE-mAb and anti-hPEPT1 antiserum, either alone or in combination), in blocking buffer for 2 h. After three washes, the coverslip was stained for 30 min with fluorescently labeled secondary antibodies, goat anti-mouse IgG conjugated with Cy3 or goat anti-rabbit IgG conjugated with Cy5 (Amersham) at a 1:400 dilution in blocking buffer. The coverslips were rinsed three times with PBS and then with

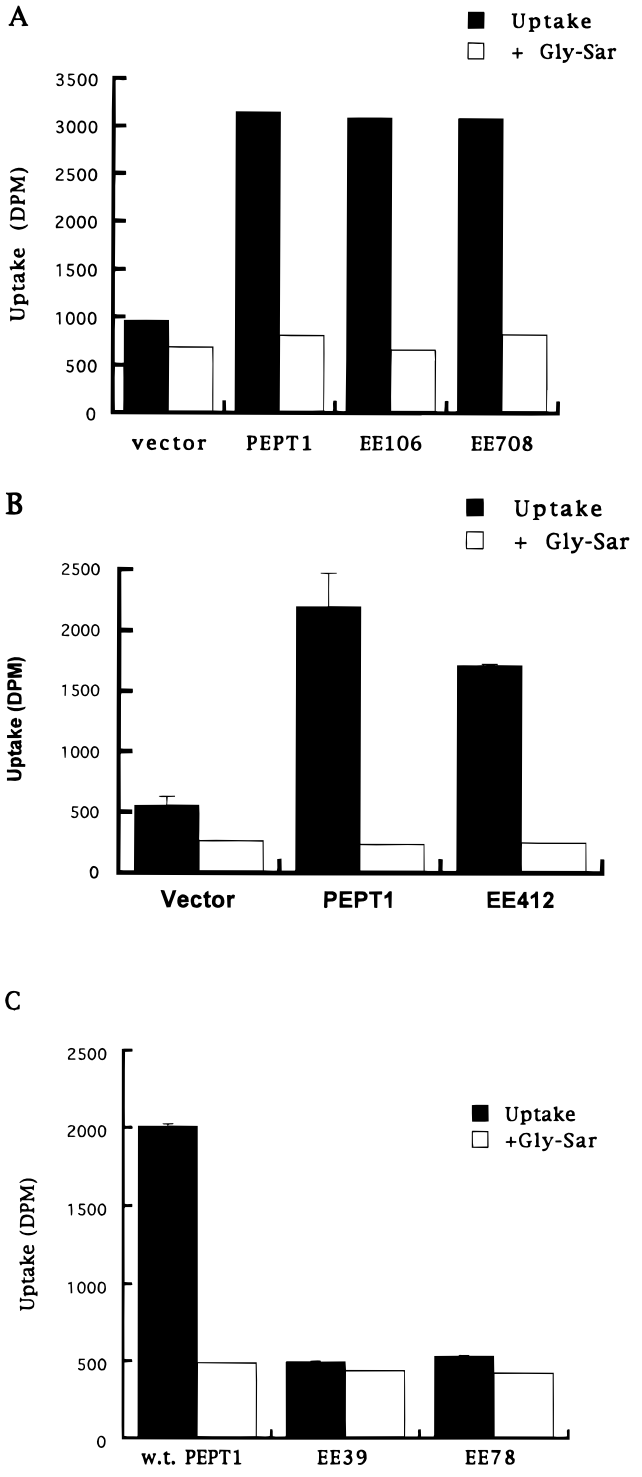

FIGURE 2: Dipeptide transport activities of hPEPT1 and EE-tagged hPEPT1. Cos7 cells were transfected with EE-hPEPT1 as indicated. The uptake of ^{3}H-labeled Gly-Sar in these cells was measured in the presence (white bars) or absence (black bars) of unlabeled Gly-Sar. Error bars represent the standard deviation from three measurements. Each experiment was repeated at least twice, with similar results.

water, and mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL). Cells were examined using a Bio-Rad MRC-600 Laser Scanning Confocal Microscope coupled with krypton−argon (568 nm) and helium−neon (633 nm) lasers. Confocal images were collected from a single channel with only one laser light source activated at a time.

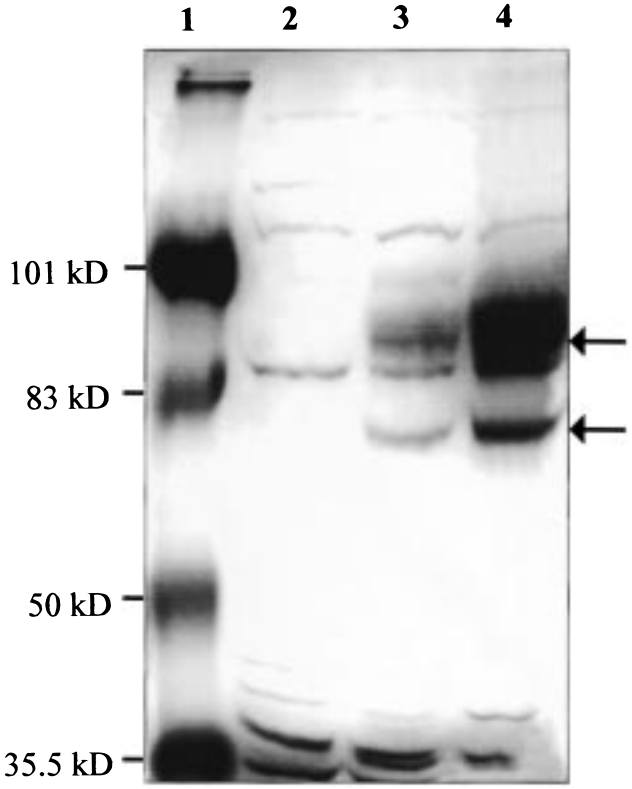

FIGURE 3: Western blot analysis of membrane proteins with anti-hPEPT1 antiserum directed against the C terminus. Membrane proteins were made from untransfected CHO cells (lane 2) or CHO cells transfected with wild-type hPEPT1 (lane 3) or EE412-hPEPT1 (lane 4). Proteins were run on an 8% SDS−PAGE gel; molecular mass markers are as indicated (lane 1).

RESULTS

Characterization of Tagged hPEPT1 Constructs. The EE epitope was inserted into five different locations of hPEPT1, and insertion of the tag was confirmed by sequencing. To test whether the tagged PEPT1 constructs mediate dipeptide transport, the extent of [^{3}H]Gly-Sar uptake was measured in Cos7 cells transiently transfected with wild-type hPEPT1 and epitope-tagged hPEPT1. As shown in Figure 2A, the extent of tracer uptake was low in vector-transfected cells, and it was only marginally reduced further in the presence of excess unlabeled Gly-Sar. In contrast, the extent of tracer uptake was high in Cos7 cells transfected with hPEPT1, and the extent of transport was reduced upon adding unlabeled Gly-Sar. Transfections with EE106-hPEPT1, EE708-hPEPT1 (Figure 2A), and EE412-hPEPT1 (Figure 2B) resulted in transport activities comparable to that of wild-type hPEPT1. This result suggests that insertion of the epitope tag at these locations did not alter hPEPT1-mediated transport activity substantially.

Insertion of the epitope tag close to amino-terminal domains of hPEPT1 did affect transport function. As shown in Figure 2C, the extent of tracer uptake in Cos7 cells transfected with EE39-hPEPT1 and EE78-hPEPT1 was much lower than that observed with wild-type hPEPT1, and comparable to levels observed with nontransfected cells.

To test whether tagged hPEPT1 is as stable as wild-type hPEPT1 at the protein level, crude membrane preparations were made from Chinese hamster ovary (CHO) cells stably

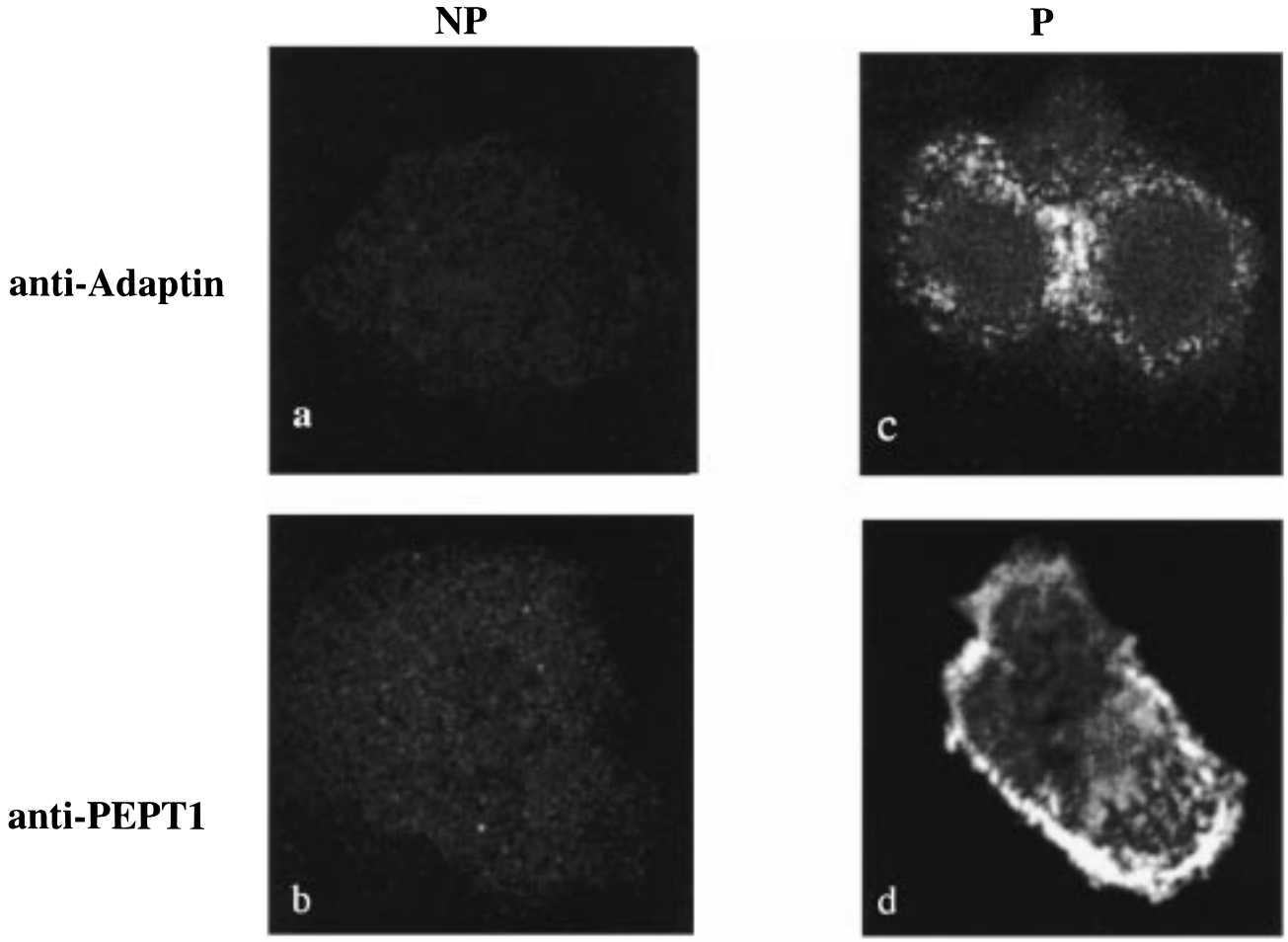

FIGURE 4: Extent of immunofluorescence staining as a function of the permeabilizing condition. Nontransfected Cos7 cells were used to examine the cellular localization of the α-adaptin protein by labeling with α-adaptin antibody under nonpermeabilizing conditions (NP, panel a) or under permeabilizing conditions (P, panel c). Cos7 cells were transfected with wild-type hPEPT1 and labeled with the anti-hPEPT1 antiserum under nonpermeabilizing (panel b) or permeabilizing conditions (panel d).

transfected with hPEPT1 or EE412-hPEPT1. Figure 3 shows a Western blot probed with anti-PEPT1 antiserum (directed against the carboxyl terminus). Two immunoreactive bands corresponding to 90 and 70 kDa PEPT1 proteins were detected in transfected cells (Figure 3, lanes 3 and 4), but not in untransfected CHO cells (Figure 3, lane 2). The apparent molecular mass of 90 kDa suggests the presence of glycosylated forms of hPEPT1 which carries seven putative glycosylation sites in the putative extracellular loop between predicted TMD9 and -10. The lower band could represent a deglycosylated form or a degradation product of PEPT1. The same protein band pattern was found in cells transfected with EE412-hPEPT1 (Figure 3, lane 4). This result suggests that EE412-hPEPT1 is as stable as wild-type hPEPT1. We further note that the extent of protein expression is much higher for EE412-hPEPT1 than for the wild type. However, the extent of tracer transport was similar (data not shown), suggesting that much of the EE412-hPEPT1 protein resides intracellularly.

Immunofluorescence Confocal Microscopy of Permeabilized and Nonpermeabilized Cells. Because this topology study is based on antibody accessibility to the epitope in saponin-permeabilized and nonpermeablilized cells, we needed to establish the fact that antibody is not able to cross the plasma membrane under intact, nonpermeabilizing conditions. Since α-adaptin is localized in the cytoplasm and peripherally associated with the plasma membrane (*17*), we tested whether this protein is labeled under the nonpermeabilizing condition used in this study. As shown in Figure 4, immunostaining appears only in permeabilized cells (c), but not in nonpermeabilized cells (a). This result shows that under the nonpermeabilizing condition, the antibody had no access to a peripheral protein localized at the cytoplasmic side of membrane, or that access was so limited as to be undetectable.

Localization of the Carboxyl Tail of hPEPT1. According to the predicted topology of hPEPT1 (see Figure 1), the carboxy tail of PEPT1 localizes to the cytoplasm. To test this, Cos7 cells were transfected with wild-type hPEPT1, followed by labeling the cells with anti-PEPT1 antiserum directed against the carboxyl terminus. As shown in Figure 4, intense immunostaining occurred only in permeabilized cells (d), but not in nonpermeabilized cells (b). This result indicates that the carboxyl tail of hPEPT1 indeed resides in the cytoplasm.

Orientation of hPEPT1 with Epitope Tags Inserted in Putative Intracellular and Extracellular Locations. To further test the location of the carboxyl terminus of hPEPT1, the EE tag was introduced after the last amino acid (EE708). Cos7 cells transfected with this tagged PEPT1 were examined with confocal microscopy after they were labeled with EE-mAb. As shown in Figure 5, the epitope was accessible to

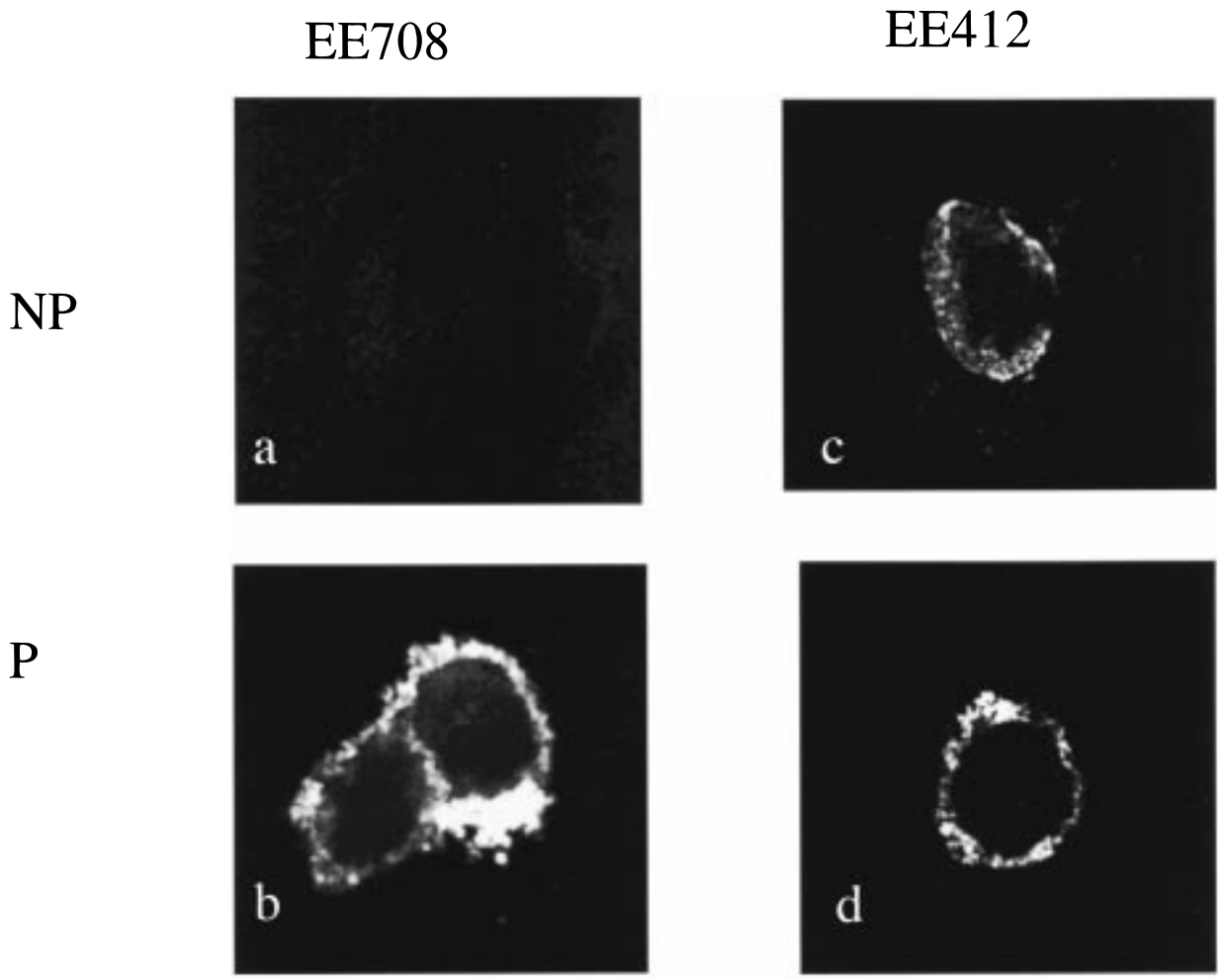

FIGURE 5: Orientation of EE708- and EE412-hPEPT1 in the plasma membrane. The tagged hPEPT1 constructs were transfected into Cos7 cells and labeled with EE-mAb under nonpermeabilizing (NP, panels a and c) or permeabilizing conditions (P, panels b and d).

the antibody only in permeabilized cells (b), but not in nonpermeabilized cells (a). Identical results obtained with the antiserum against the carboxyl tail of wild-type hPEPT1 and with EE-mAb using EE708-hPEPT1 establish an intracellular location of the carboxyl terminus, and it validates the epitope tagging approach in this case.

To test the orientation of the putative extracellular loop between TMD9 and -10, an epitope was also inserted at this domain, at Leu-412. Cos7 cells transfected with EE412-hPEPT1 were examined with confocal microscopy after labeling with EE-mAb. As shown in Figure 5, the epitope was accessible to the antibody in both nonpermeabilized cells (c) and permeabilized cells (d). This result indicates that the epitope is located extracellularly.

Double Labeling of hPEPT1 To Distinguish Intra- and Extracellular Orientations in the Same Cells. In the tagged EE106-hPEPT1 construct, the epitope was inserted in the proposed extracellular loop between TMD3 and -4. To obtain an internal control for the orientation of hPEPT1, Cos7 cells transfected with EE106-hPEPT1 were simultaneously labeled with anti-EE-mAb and the antiserum against the carboxyl tail. As shown in Figure 6, in nonpermeabilized cells, only the EE106 epitope was accessible to its antibody (b), while in the same cell, the carboxyl tail of PEPT1 was not (a). However, in permeabilized cells, both the epitope and the carboxyl tail of PEPT1 were accessible to their respective antibodies (c and 6). This result suggests that the loop between TMD3 and -4 is on the opposite side of the cytoplamic carboxyl tail of PEPT1. These results validate the extracellular orientation of EE106.

hPEPT1 with Epitope Tags Inserted into Putative Intracellular and Extracellular Locations Near the Amino Terminus. To determine topology near the amino terminus of PEPT1, two tags were inserted between putative TMD1 and -2 (EE 39) and TMD2 and -3 (EE78). As shown above, introduction of these tags suppressed dipeptide transport activity. To determine whether the tagged PEPT1 constructs were expressed and localized at the cell membrane, Cos7 cells were transfected with tagged hPEPT1 (EE39-hPEPT1 or EE78-hPEPT1) and examined with confocal microscopy after labeling with EE-mAb and antiserum against the carboxyl tail of hPEPT1. As shown in Figure 7, use of the carboxyl-tail antiserum under permeabilizing conditions revealed that the hPEPT1 protein was expressed, but localized mostly inside the cells (a and c). However, the EE epitopes were not detectable in these cells (b and d). These results suggest that the PEPT1 proteins were made, but the EE epitope was occluded within the hPEPT1 structure, or metabolized. This indicates that the regions between TMDs1 and -2 and TMD2 and -3 are sensitive to distortion by epitope insertion, affecting cellular trafficking, transport activity, or metabolic stability.

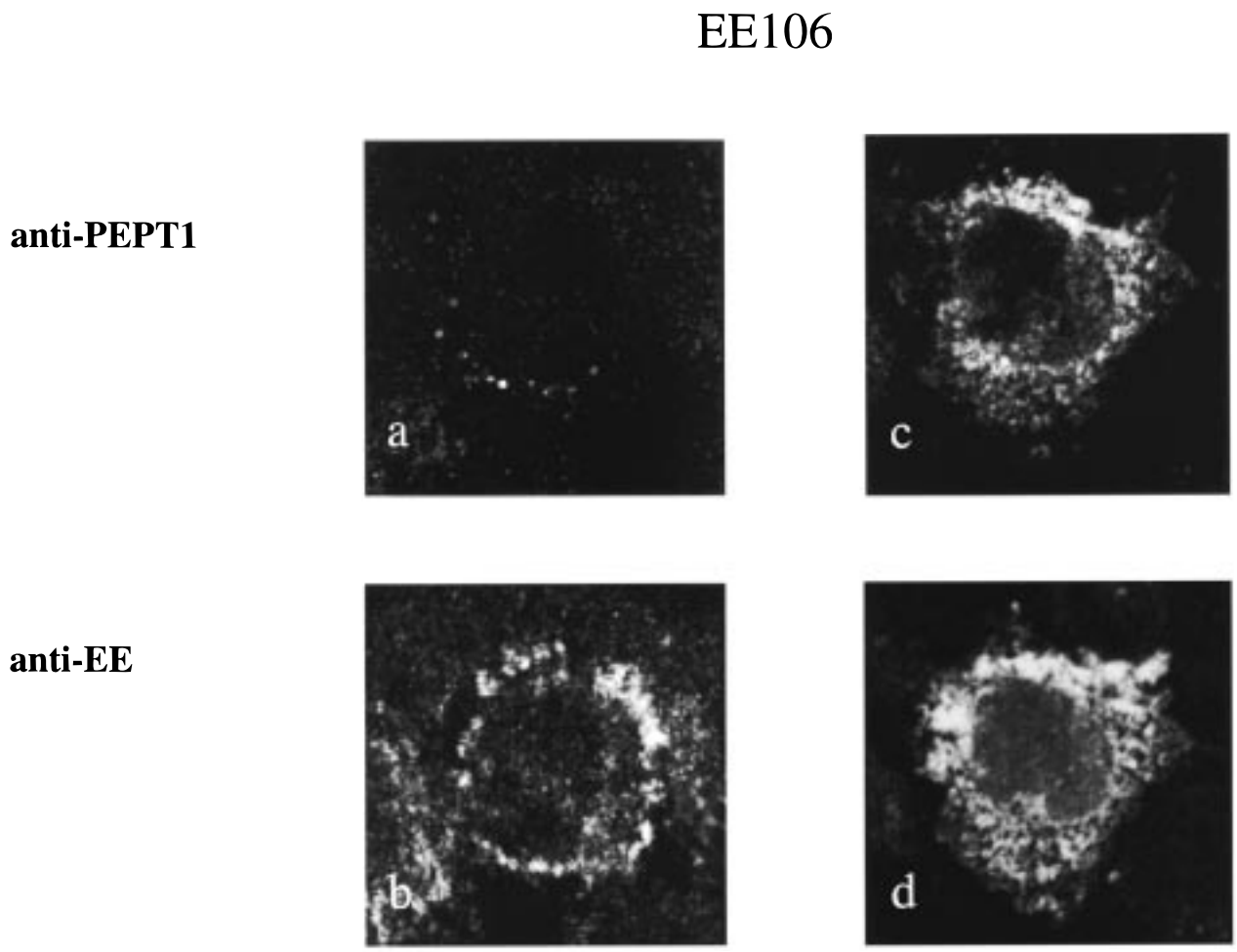

FIGURE 6: Dual labeling of EE106-hPEPT1 transfected into Cos7 cells with anti-PEPT1 antiserum (panels a and c) and EE-mAb (anti-EE; panels b and d). Labeling was performed either under nonpermeabilizing conditions (panels a and b) or under permeabilizing conditions (panels c and d). The same cells are shown in panels a and b, and c and d. Each experiment was performed at least three times with similar results.

DISCUSSION

Because integral membrane proteins are difficult to crystallize, few three-dimensional structures are available for polytopic proteins consisting of multiple TMDs. Establishing the number and relative orientation of the TMDs is a first step in understanding the structure of a membrane protein. Topological studies have relied on fusion of the gene encoding a polytopic membrane protein, or portions thereof, to *Lac Z* or *Pho A* reporter genes (*18*, *19*). However, the bulkiness of the reporter protein may affect the orientation or membrane insertion of the protein. Alternatively, one can raise antisera against various domains of the protein and determine its accessibility to the antibody (*16*). A variation of this approach involves the insertion of epitope tags against which monoclonal antibodies are available. This can be more readily accomplished than raising antisera against domains of the protein of interest. This approach has been applied to a topology study of Na,K-ATPase (*20*). Furthermore, the topology of a bacterial protein, LamB, derived from epitope tagging experiments has been confirmed by comparison to an X-ray structure of the LamB protein (*21*). Our study shows that insertion of a small epitope (EYMPME) into several locations of the polytopic protein, hPEPT1, in combination with immunofluorescence confocal microscopy, provides a suitable approach for testing the local topology of the protein. Further, dual labeling of permeabilized and nonpermeabilized cells with a monoclonal antibody raised against the inserted epitope tag and antiserum raised against a native sequence from the wild-type hPEPT1 confirms the relative membrane orientation of the epitopes and provides an internal control within the same cell.

Our epitope tagging experiments indicated that the carboxyl tail of hPEPT1 is oriented toward the cytoplasm. Similarly, for the distantly related PTR dipeptide transporter, DTPT from *Lactobacillus*, both the amino and carboxyl termini were proposed to be located on the cytoplasmic side of the membrane (*22*). However, a fragment of two putative TMD segments is inserted into DTPT which is absent in, and is not homologous to, any region of hPEPT1 (*9*). Therefore, extrapolation of the topology from DTPT to hPEPT1 is not readily possible.

The EE epitopes inserted into two predicted extracellular loops between TMD3 and -4 and TMD9 and -10 (EE106 and EE412) were accessible to antibody in nonpermeabilized cells, indicating an extracellular orientation. Moreover, the transfected EE-PEPT1 proteins were properly translocated to the plasma membrane and capable of transporting the dipeptide tracer into the cell under a proton gradient. These results are consistent with the predicted topology of PEPT1 shown in Figure 1. Specifically, this result confirms the

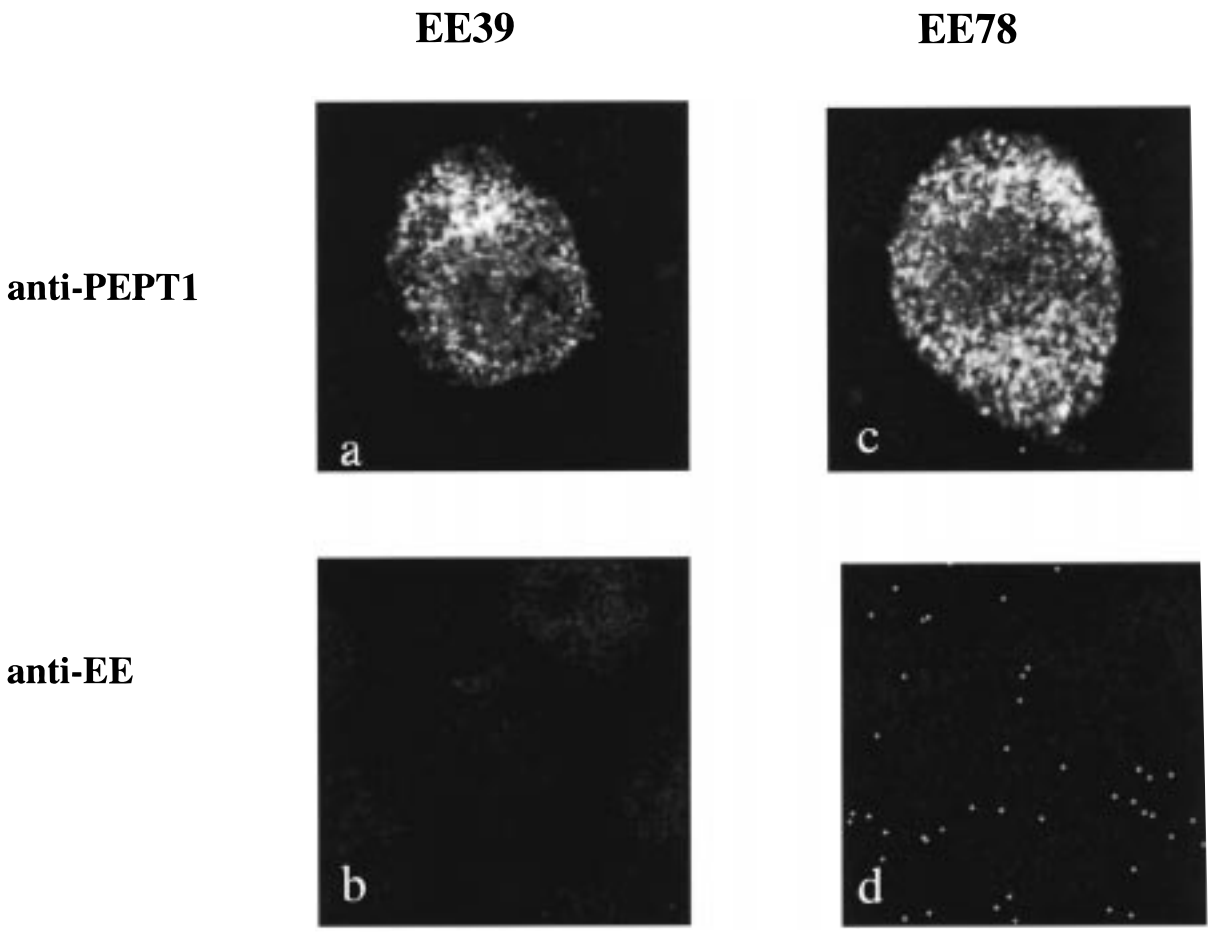

FIGURE 7: Dual labeling of EE39- and EE78-hPEPT1 transfected into Cos7 cells with anti-PEPT1 antiserum (panels a and c) and EE-mAb (anti-EE; panels b and d). Labeling occurred in each case under permeabilizing conditions.

extracellular location of the large hydrophilic loop between TMD9 and -10, which also contains the consensus domains for glycosylation (*5*). We cannot exclude the possibility that insertion of EE106 could have affected the membrane orientation of the rather small loop between predicted TMD3 and -4. However, the dual labeling experiment with EE-mAb and C terminus antiserum confirmed that at least the C terminus remains intracellular in the EE106-hPEPT1 construct. Moreover, this construct transported the dipeptide tracer as well as the wild-type hPEPT1. Any gross distortion of the membrane topology likely would have abrogated transporter function.

Assuming then that the loops between TMD3 and -4 and TMD9 and -10 are extracellular, and the carboxyl tail is intracellular, we can address the question of whether the number and orientation of predicted TMDs between TMD4 and TMD12 are consistent with our results. The hydropathy profile in Figure 1B indicates that TMD assignments are least ambiguous for the following domains: TMD4−6, -8, -9, -11, and -12. Some ambiguity remains with TMD7 and -10. However, our results dictate that both TMD7 and TMD10 domains indeed traverse the membrane, for the following reasons. If only one of the two domains were to transverse the membrane, the C terminus would have to be on the external side of the membrane, which is not the case. If both domains fail to cross the membrane, the large loop between predicted TMD9 and -10 would be located intracellularly. Again, this is not the case. Therefore, our results experimentally confirm all predictions about the numbers and orientation of TMDs and loops from TMD4 to the C terminus.

In contrast, two epitope insertions at the predicted loops between TMD1 and -2 and TMD2 and -3 interfere with hPEPT1 function, either by perturbing translocation to the plasma membrane, by abrogating transporter function, or both. Therefore, we were unable to verify the number and orientation of TMDs within the amino-terminal domains, using epitope insertion. This result also suggests that these regions are essential for hPEPT1 folding and/or function.

Expressed in the intestines, hPEPT1 is important for drug absorption (*23*); moreover, extensive hPEPT1 expression has been detected in colon carcinoma cells and in pancreatic carcinoma cells (*13*). Though the role that PEPT1 plays in these cells is not known, one might consider hPEPT1 as a means of delivering anticancer drugs. Better understanding of hPEPT1 structure will assist in the design of drugs with better absorption and targeting as a result of enhanced interaction with this transporter.

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