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Review

Structure and function of eukaryotic peptide transporters

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Abstract. The cotransport of protons and peptides is now recognised as a major route by which dietary nitrogen is absorbed from the intestine, and filtered protein reabsorbed in the kidney. Recently, molecular biology has had a very substantial impact on the study of peptide transport, and here we review the molecular and functional information available within the framework of physiology. To this end we consider not only the mammalian peptide transporters and their tissue distribution and regulation but also those from other species (including *Caenorhabditis elegans*) which make up the proton-dependent oligopeptide transport superfamily. In addition, understanding the binding requirements for transported substrates may allow future design and targeted tissue delivery of peptide and peptidomimetic drugs. Finally, we aim to highlight some of the less well understood areas of peptide transport, in the hope that it will stimulate further research into this challenging yet exciting topic.

Key words. PepT1; PepT2; epithelia; β-lactam antibiotics; PTR2; oligopeptide transport; apical membrane; basolateral membrane.

Introduction

What is the purpose of a new review on proton-coupled peptide transport, especially when there are already some excellent recent ones available in the published literature (e.g. [1–10]) as well as a scholarly earlier study [11]? Since the field remains highly active experimentally, an obvious reason is simply the continuing growth in the body of new experimental material. A second reason relates to parallel activities in other areas of biology that shed light on peptide transport either directly or indirectly. A third purpose is to highlight areas of continuing ignorance.

Here we have confined our focus to studies that have been published since an earlier survey [12]. This critique is therefore firmly embedded in the post-cloning epoch, but it also attempts to emphasise the significance of studies in which molecular and physiological problems inform each other. Because of the role of the transporters discussed here in oral absorption of a wide variety of drugs, this review may also be useful to the pharmacological and pharmaceutical community.

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Comparison of sequences

The POT/PTR superfamily

All peptide transporters from species as diverse as yeast to humans, including those from *Caenorhabditis elegans*, have been grouped into a superfamily known as the POT (proton oligopeptide transporter) superfamily, originally proposed by Paulsen and Skurray [13]. In a

recent review [9], Fei et al. gave a comprehensive overview of the sequence homologies of the members of the POT superfamily and how they could be distinguished from the ATP-binding cassette (ABC) transporter superfamily with which they share some substrate specificity. Of the dozen POT family members cloned at that date (listed as the first part of table 1), all but CHL1 (the *Arabidopsis thaliana* proton-coupled nitrate transporter) had been shown to have peptide transport activity. The number of members of the POT superfamily has now increased to 40 (second part of table 1). It is important to note that many of the extra 28 proteins are hypothetical and/or have not been shown to transport peptides; however, they have two characteristic protein 'signatures' assigned by Steiner and Becker in the Pfam database, which can be accessed at the Sanger Centre website (URLhttp://www.sanger.ac.uk/Software/Pfam/), known as PTR2 family signatures [14]. The first of these signature sequences includes the end of transmembrane span 2 (TM2), the intracellular loop and TM3, and the consensus sequence is as follows: [GA]-[GAS]-[LIVMFYWA]-[LIVM]-[GAS]-D-x-[LIVMFYWT]-

Table 1. Sequences that contain the PTR-2 signature domain—see text for details.

Transporter name	Species	Length (amino acids)	Consensus site		Accession number
			PKC	PKA	
Rabbit PepT1	*O. cuniculus*	707	Yes	Yes	P36836
hPepT1	*H. sapiens*	708	Yes	No	P46059
hPepT1	*H. sapiens*	708*			O43641
Rat PepT1	*R. norvegicus*	710	Yes	Yes	P51574
Rat PepT1	*R. norvegicus*	453†			Q63422
hPepT2	*H. sapiens*	729	Yes	Yes	Q16348
Rat PepT2	*R. norvegicus*	729	Yes	No	Q63424
Rabbit PepT2	*O. cuniculus*	729	Yes	No	P46029
			Transporter of		
CHL1	*A. thaliana*	590	H^+/nitrate		Q05085
PTR2B/NTR1	*A. thaliana*	586	peptide/histidine		P46032
PTR2(A)	*A. thaliana*	610	peptide		P46031
PTR2	*S. cerevisiae*	601	peptide		P32901
PTR2	*C. albicans*	623	peptide		P46030
DTPT	*L. lactis*	463	peptide		P36574
			Transporter of		
PHT1	*R. norvegicus*	572	peptide/histidine		O09014
CPTA	*C. elegans*	785	peptide (high affinity)		O76185
CPTB	*C. elegans*	796	peptide (low affinity)		O76186
OPT1	*C. elegans*	786	putative peptide		Q17758
OPT2	*C. elegans*	701	putative peptide		O01840
OPT3	*C. elegans*	783	putative peptide		Q21219
OPT1/YIN	*D. melanogaster*	737	putative peptide		P91679
	D. melanogaster	743			O77281
	D. melanogaster	734			O77282
PTR	*H. vulgare*	579	peptide		048542
NITR1	*C. sativus*	484	nitrite		Q96400
RCH2	*B. napus*	589	putative nitrate		Q43390
LjNOD65	*L. japonica*	574	peptide		O22305
PTR2-B	*A. thaliana*	583	putative peptide		O22889
	A. thaliana	521	putative peptide		O80436
	A. thaliana	586	putative nitrate		O48784
	A. thaliana	568	putative peptide		O04585
	A. thaliana	614	putative transporter		O04557
	A. thaliana	563	putative transporter		O04553
NTL1	*A. thaliana*	585	nitrate (low affinity)		O81393
DTPT	*L. helveticus*	497	peptide		O07380
YBGH	*E. coli*	493	hypothetical protein		P75742
YCLF	*E. coli*	492	hypothetical protein		P94408
YDGR	*E. coli*	500	hypothetical protein		P77304
YHIP	*E. coli*	489	hypothetical protein		P36837
YJDL	*E. coli*	485	hypothetical protein		P39276

* Cloned from Caco-2 cell line.
† Fragment.

[LIVMFYW]-G-x(3)-[TAV]-[IV]-x(3)-[GSTAV]-x-[LIVMF]-x(3)-[GA]. The second PTR2 signature corresponds to the core of TM5: [FYT]-x(2)-[LMFY]-[FYV]-[LIVMFYWA]-x-[IVG]-N-[LIVMAG]-G-[GSA]-[LIMF]. A third consensus sequence proposed [9], GTGGIKPXV, is well conserved between mammalian and the *C. elegans* peptide transporters CPTA and CPTB (but not OPT2), but is not specific to or well conserved in other members of the POT superfamily. The presence of these genes in both prokaryotes and eukaryotes, including bacteria, yeast, plants, nematodes and animals up to and including humans, suggests that the POT superfamily has been well conserved during evolution.

Exploring the *C. elegans* genome

Along with the fly *Drosophila melanogaster*, the nematode *C. elegans* has been a leading genetic model system for exploring developmental biology over the last 2 decades. Now that the genome of this multicellular organism has been sequenced in its entirety, the challenge is to learn about protein function from this unique genetic knowledge. In relation to peptide transport, these studies have already begun [15], with the cloning of two oligopeptide transporters from *C. elegans*, CPTA and CPTB. These were isolated by preparation of a complementary DNA (cDNA) library from homogenised nematodes, which was screened with probes prepared by reverse-transcriptase polymerase chain reaction (RT-PCR) of the messenger RNA (mRNA). Full-length clones were then isolated from the cDNA library to gain the full sequences. The resulting clones were expressed in *Xenopus laevis* oocytes, and transport assessed by measurement of [^{14}C]-glycylsarcosine (Gly-Sar) uptake and by determining peptide-induced currents electrophysiologically.

The 796-amino acid peptide transport protein CPTA was predicted to have only 11 transmembrane spanning regions (TMs) (compared with the 12 seen with other members of the oligopeptide transporter family, but see below). Interestingly the sequence was already in the database under the designation OPT1 [16], which had been predicted to be an oligopeptide transporter by sequence homology. This was indeed proved to be the case, and functional studies [15] showed that CPTA/OPT1 had a relatively high affinity (K_m of ~ 0.3 mM at -50 mV) but low capacity ($I_{max} \sim 30$ nA) for Gly-Sar. The higher-affinity mammalian peptide transporter isoform is PepT2; however, CPTA showed less similarity and identity for hPepT2 at the amino acid level (60.3 and 36.0%, respectively) than it did for the lower-affinity hPepT1 (61.7 and 40.1). Intriguingly, it was also able to transport *N*-acetyl-aspartylglutamate (NAAG, more properly N-Ac-DE), an endogenous peptide found in mammalian brain which displays high affinity and specificity for a subsite on the *N*-methyl-D-aspartate receptor [17] and is a PepT2 substrate [18] (see below).

Similar expression studies with CPTB (796 amino acids, again, 11 predicted TMs) showed that this, too, encoded a functional peptide transporter but with lower affinity and higher capacity (K_m of ~ 4 mM at -50 mV, $I_{max} \sim 2000$ nA for Gly-Sar). Unlike CPTA, CPTB did not transport NAAG (N-Ac-DE), but the carboxyl-terminal-blocked tetrapeptide Phe-Met-Arg-Phe-amide (FMRF-amide) was a good substrate, generating an inward current (I_{max}) about 24% that seen with Gly-Sar. Since the Arg-Phe-amide motif is required for biological activity in numerous FMRF-amide-related peptides isolated from nematodes, this observation may be physiologically important. Again, despite the kinetic profile, the CPTB primary structure was no more closely related to hPepT1 than to hPepT2 (62.8 and 62.1% similarity, and 36.9 and 37.5% identity, respectively). Searching the *C. elegans* genome database revealed a highly similar protein, the hypothetical peptide transporter OPT3 [16], which is identical to CPTB except for the N-terminus (see fig. 1). Like CPTB, OPT3 is predicted to have only 11 TMs. The N-terminus of OPT3 shares strong homology with the mammalian PepT family. Study of the genomic DNA sequence shows what we believe to be an exon (previously missed) that is also strongly conserved in both PepT1 and PepT2 and is also (excluding the sequence in parentheses) in the CPTB clone. This sequence is WPKTTLCIVSNEFCERFSYYGMR(SELLYLL), which might allow for another transmembrane span, bringing the total to the more common 12 TMs for a transport protein (the sequence of the predicted TM1 in PepT1 is SLSCFGYPLSIFFIVVNEFC [19]). Interestingly, this sequence has the correct periodicity to give an amphiphilic α-helix, with one hydrophobic face. While the topology of the N-terminus of CPTA and CPTB is not clear from the hydropathy plots, there are other reasons for suspecting that there may be 12 TMs:

1) All other members of the POT superfamily are predicted to have 12 TMs.

2) It does not contain a predicted eukaryote signal sequence for the externalisation of the N-terminal, which would be necessary if the large loop between TMs 8 and 9 was to be extracellular (as in other peptide transport proteins) [20].

3) The conserved essential histidine (His_{57} in PepT1) corresponds to His_{75} in CPTA. In the 11-TM model this residue would lie at the intracellular end of TM1, which would make it unlikely to be accessible to external

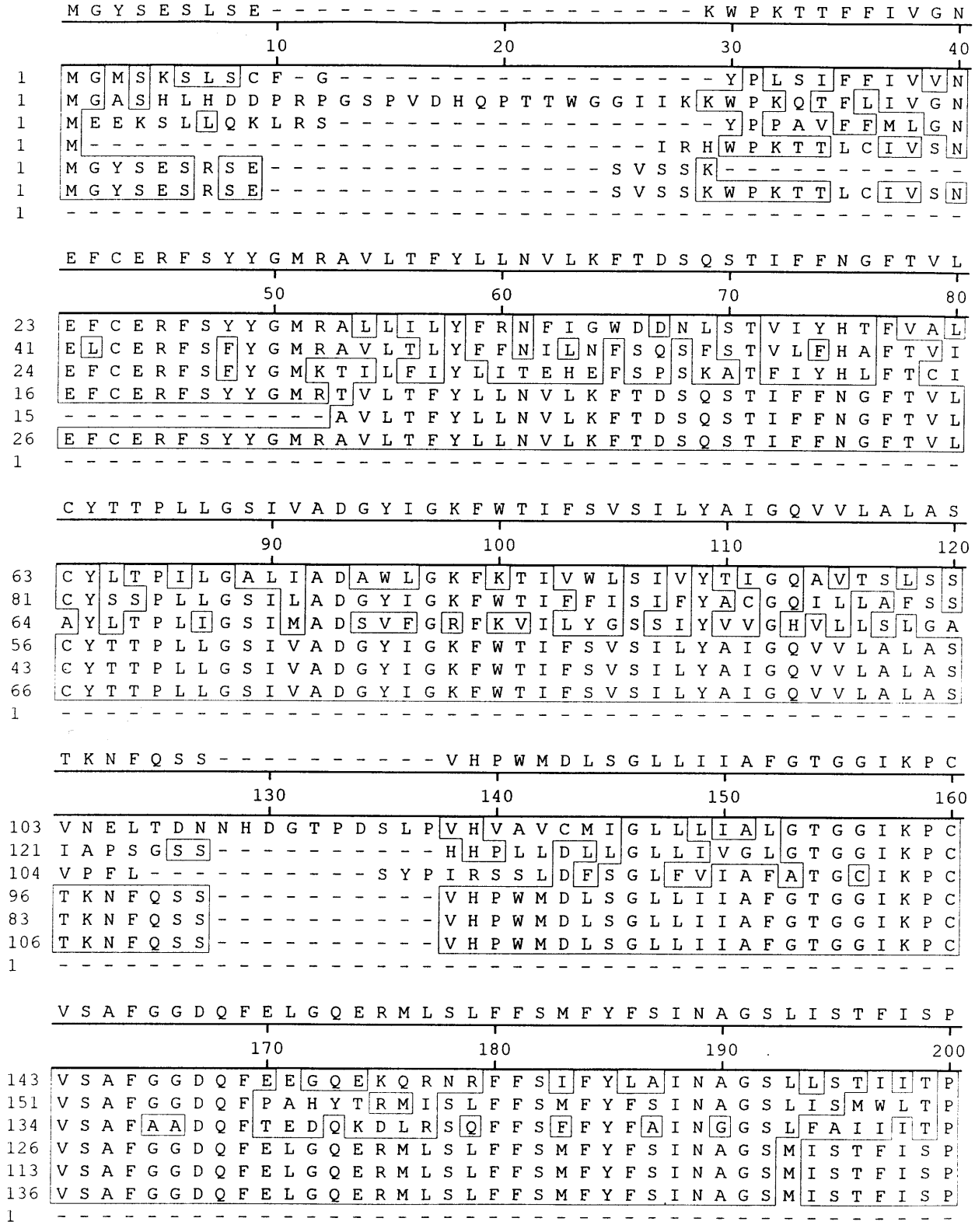
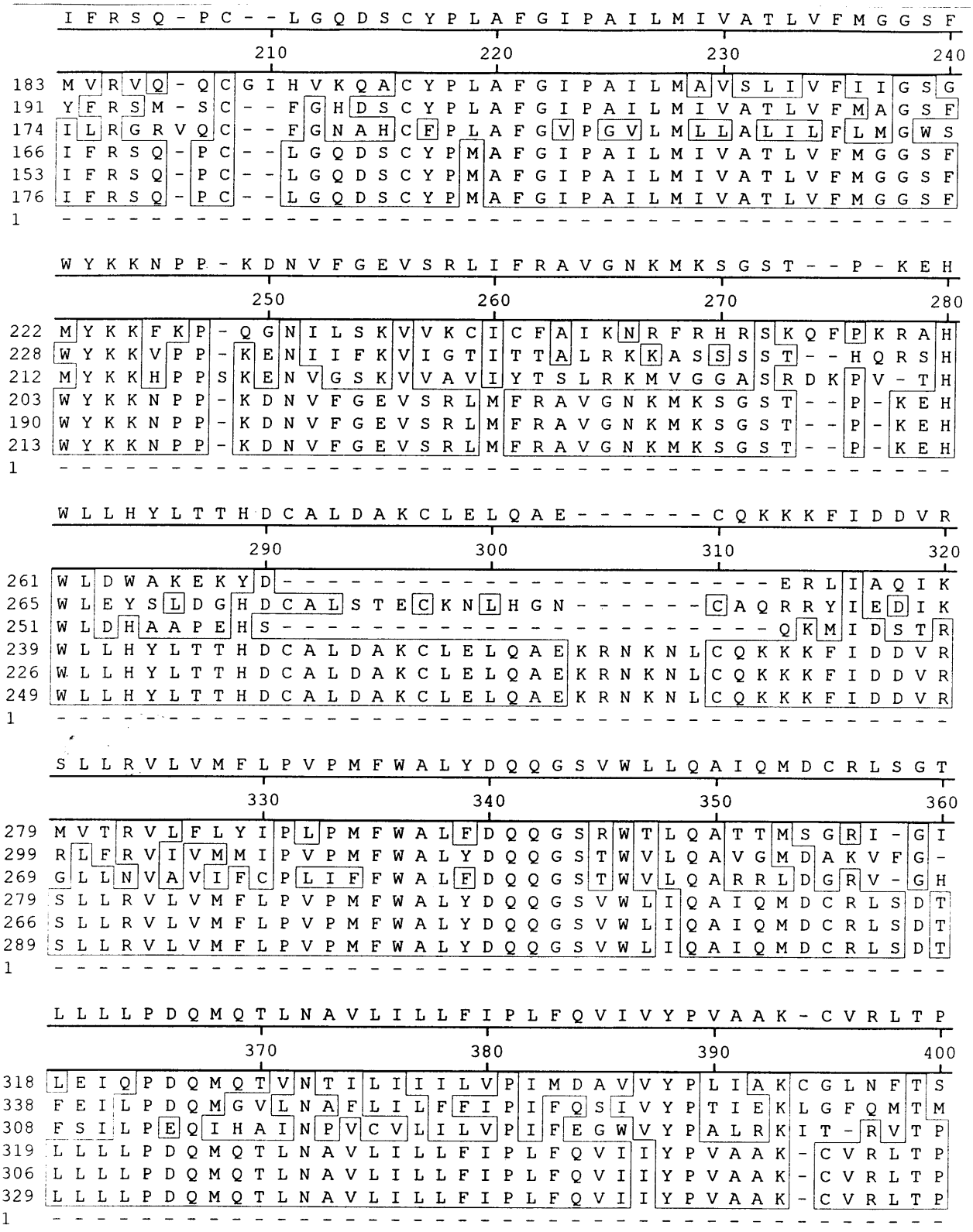


Fig. 1.



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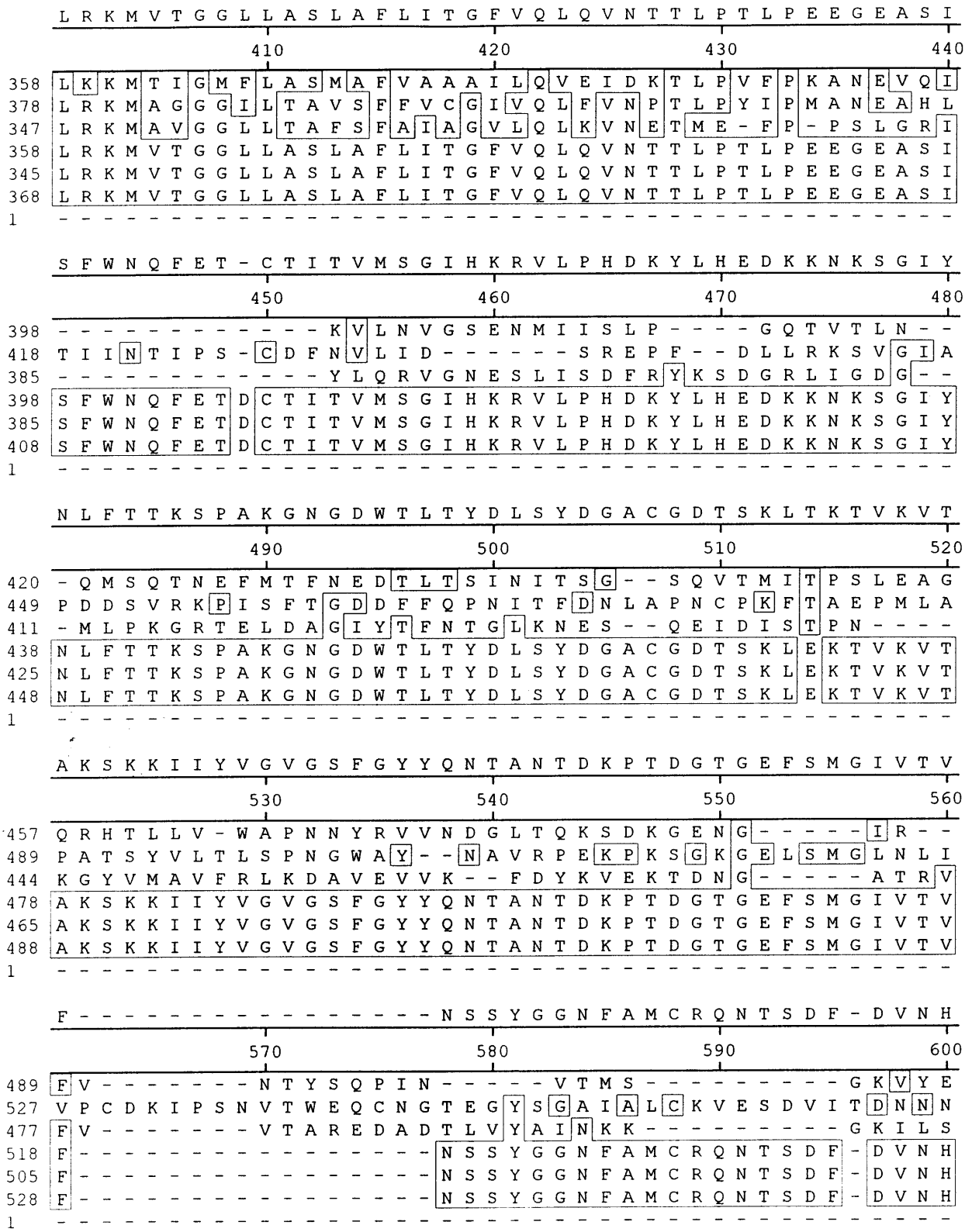

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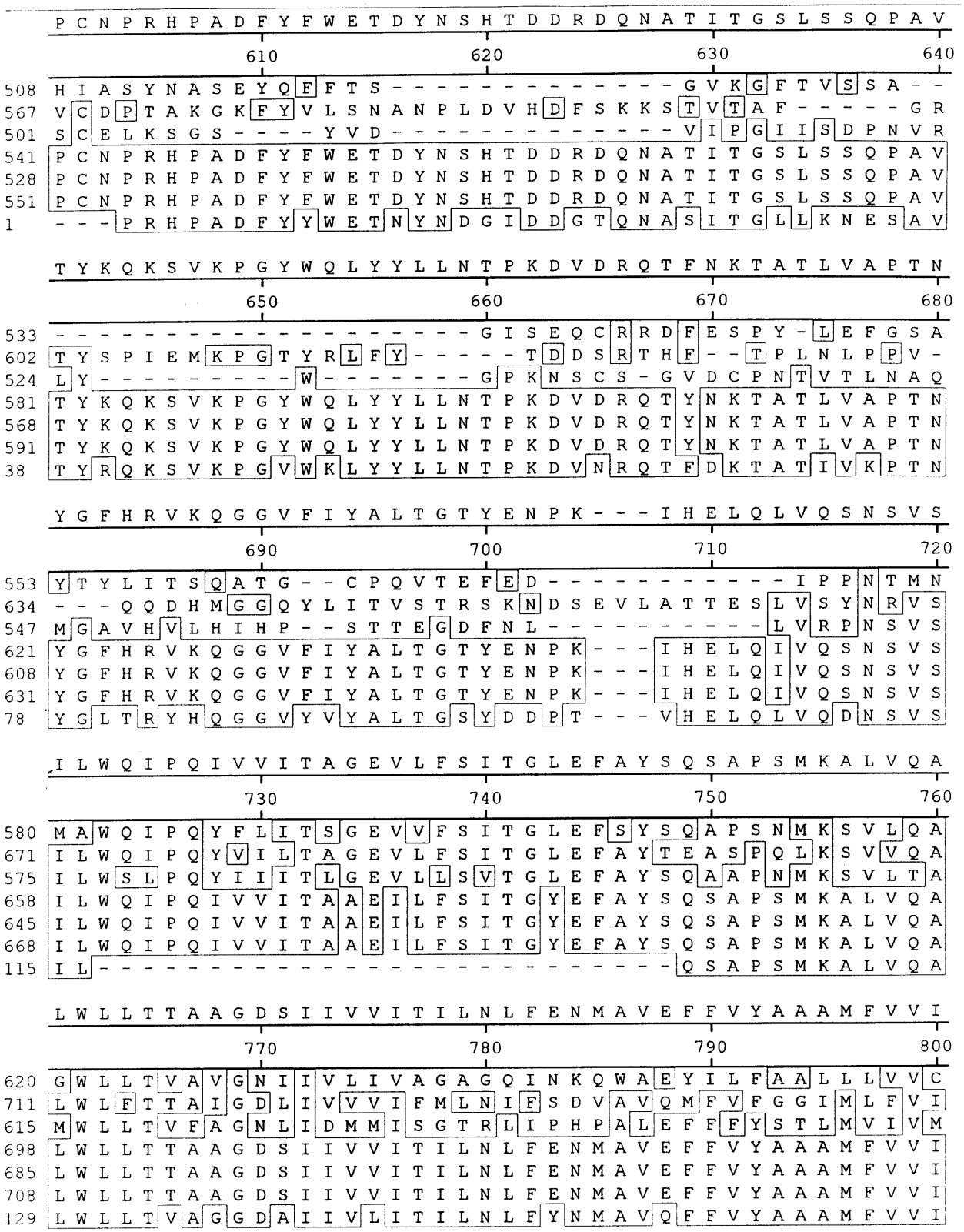


Fig. 1.

Table 2. Tissue distribution of PepT1 and PepT2.

Tissue	Peptide transport	mRNA	Isoform	Protein present
Small intestine	✓	✓	PepT1	✓
Kidney (pars convoluta)	✓	✓	PepT1	✓
Kidney (pars recta)	✓	✓	PepT2	✓
Pancreas			PepT1	✓
Pancreatic carcinoma cell lines AsPc-1 and Capan-2	✓	✓	PepT1	✓
Fibroblast carcinoma cell line HT1080	✓		PepT1-like	
Liver (lysosomes)	✓		PepT1-like	
Lung	✓	✓	PepT2	
Central nervous system	✓	✓	PepT2	
Mammary gland	×	✓	PepT2	
Placenta	✓	✓	PepT1	

protons [interestingly, the corresponding residue in CPTB is an asparagine (Asn_{50})].

In further support of the 12-TM model, there is another putative peptide transporter in the *C. elegans* database, OPT2 [16], which is predicted to have 12 TMs, and also has the conserved histidine residue (His_{58}), which would be at the extracellular end of TM2 (as with His_{57} in PepT1, the topology of which has been experimentally confirmed [21]. It is related similarly to CPTA/OPT1, CPTB, OPT3 and PepT1, with an amino acid identity of $\sim$30% to all these four proteins (31.5, 30.5, 28.8 and 29.8, respectively, see fig. 1).

Finally, homology searching the *C. elegans* genome database has revealed that there might be an additional peptide transporter, which we will call OPT4. Found in unfinished chromosome IV sequence (Y46C8 contig 91), OPT4 to date is 227 amino acids long, and has a 71.9% identity with the N-terminus of OPT3/CPTB but a much lower identity to CPTA/OPT1. OPT2 and PepT1 (23.1, 20.8 and 17.6%, respectively). (See Note added in proof at the end of the article.)

Tissue distribution of PepT1 and PepT2 (table 2)

Intestine

The original reports of the cloning of PepT1 and PepT2 used Northern blots and RT-PCR, respectively, to indicate tissue distribution of the two isoforms [19, 22]; however, since mRNA from whole tissue was used, transporter expression at a cellular level was not shown. Freeman et al. [23] used in situ hybridisation to investigate PepT1 expression along the length of rabbit intes-

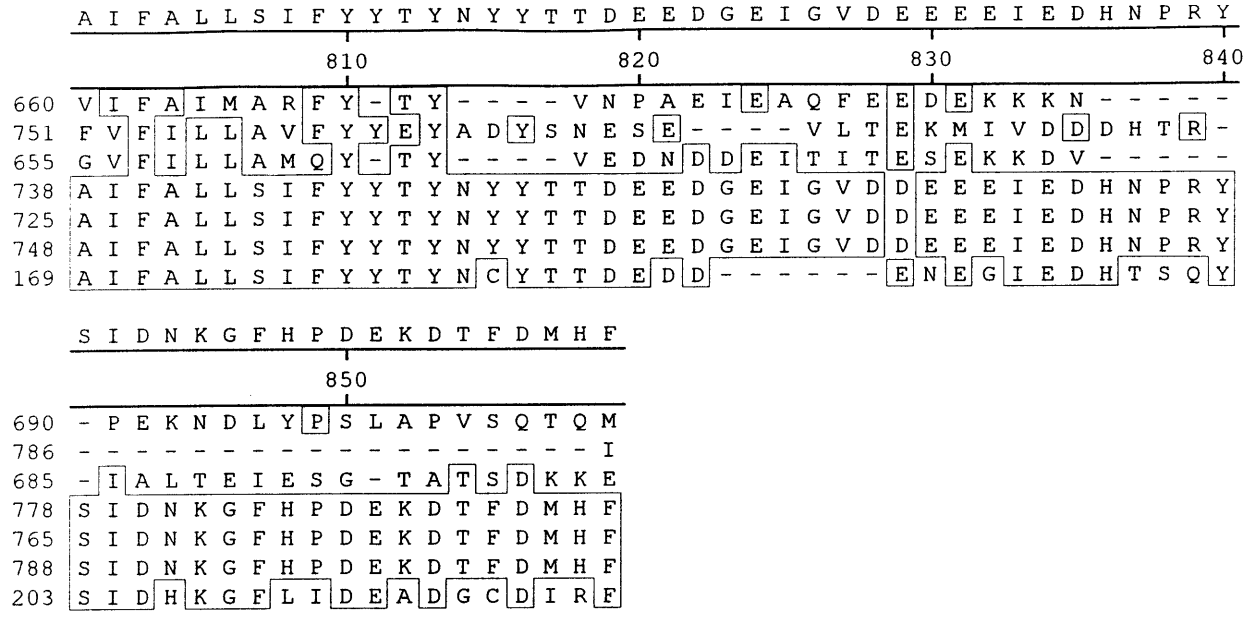


Figure 1. Sequence alignment of known and putative peptide transport proteins from the *C. elegans* genome compared with that of rabbit PepT1. In order from the top: rabbit PepT1, OPT1/CPTA, OPT2, CPTB, OPT3, OPT3 with the putative extra exon, and putative OPT4 (see text for details). Above the sequence line up is the majority sequence. Alignment was by the Clustal method with PAM250 residue weight table using MegAlign, DNASTAR, Madison WI, USA. Residues that match the consensus exactly are enclosed in boxes.

tine, and found that expression was most abundant in duodenum and jejunum, lower in ileum and at a very low level in colon. No expression was detected in stomach, sacculus rotundus or caecum. At the cellular level, PepT1 expression was restricted to epithelial cells, and only to those on the villus: PepT1 mRNA became detectable at the villus/crypt junction and reached a maximum 100–200 μm from this point. Antibody studies (using anti-rat PepT1 [24–26]) confirmed this, showing that expression of the PepT1 protein is regulated, and that only as cells leave the crypt and migrate towards the tip of the villus (i.e. when they will perform a role in nutrient absorption) do they express the peptide transport protein. Miyamoto et al. [27] used Northern blots to look at the distribution and developmental changes in rat PepT1, the signal for which they found in intestine and kidney. The highest levels of intestinal mRNA were found in 4-day-old rats, and levels decreased to the adult values by day 28 after birth.

Kidney

While it is widely accepted that only PepT1 is expressed in the intestine, the kidney expresses both PepT1 and the higher-affinity PepT2. Smith et al. [28] used RT-PCR on rat microdissected renal tubules to investigate the pattern of expression of the two isoforms: whereas PepT1 was specific for the early regions of proximal tubule (pars convoluta), PepT2 was overwhelmingly (but not exclusively) expressed in the later regions of the proximal tubule (pars recta). All other nephron segments were negative for both isoforms. This distribution was confirmed by Northern blot and by immunohistochemistry [29]. Thus, the reabsorption of peptides from the nephron appears to be a sequential, two-stage process, with the low-affinity, high-capacity PepT1 accounting for the majority of uptake, and the high-affinity, low-capacity PepT2 scavenging remaining peptide from the ultrafiltrate. This is similar to the process responsible for glucose reabsorption, and with the kidney continuously filtering the blood, it is obvious that failure to reabsorb even a small fraction of such filtered solutes would result in a large loss over time.

Lung

Previous studies have shown that isolated type II pneumocytes and brush-border membrane vesicles prepared from rat lung were able to transport the hydrolysis-resistant dipeptide D-Phe-L-Ala [30, 31]. Such small peptides can cross-culture monolayers of alveolar cells [32] and have also been shown to be removed from the alveolar (luminal) air spaces of the in situ perfused rat lung [33]. Recently, lung has been shown to express the high-affinity peptide transporter isoform, PepT2 [34], although the previous functional studies had suggested a lower-affinity transporter might be responsible for the transport measured. Work is still continuing on investigating the potential of the respiratory tract for peptidomimetic drug delivery [35].

Placenta

Dipeptide transport in human placental brush-border membrane vesicles was first shown almost 15 years ago [36] using Gly-Sar, when it was concluded that transport was probably occurred down a concentration gradient, i.e. was facilitated diffusion. Similar studies [37] using a series of differently charged, hydrolysis-resistant dipeptides suggested the presence of very low affinity transporter(s) which were not sensitive to the histidine-modifying reagent diethylpyrocarbonate (DEPC). Despite the presence of a weak signal for PepT1 in Northern blots of whole human placental mRNA [38], the proton-coupling and electrogenicity of transport were very different to those seen for PepT1, and intriguingly the transport had some of the features reported for the basolateral transporter (see below). Curiously, Adibi et al. [39], failed to find uptake of the dipeptide [^{3}H]Gly-Gln by placental brush-border membrane vesicles.

That there is transplacental peptide transport was demonstrated by studies that showed that angiotensin-converting enzyme (ACE) inhibitors temocapril and enalapril crossed a perfused placenta model system [40]. Adibi et al. [39] also showed that Gly-Sar could cross a human placenta intact (although the authors concluded it was due to passive diffusion because of their finding with Gly-Gln above). While the peptide transport system of placenta is not clearly understood, it may be an important factor to consider when prescribing drugs carried by the PepT isoforms to patients during pregnancy.

Mammary gland

Recently the presence of PepT2 mRNA in rabbit mammary gland has been shown by RT-PCR [34]; however, in recent studies, the low peptide uptake observed (of D-Phe-L-Gln and D-Phe-L-Glu) in perfused mammary gland was not inhibitable with high concentrations of L-Leu-L-Ala (50 mM) [41]. This suggested that any transport system present was of very low affinity, and not via PepT1 or PepT2. As with the placenta, the presence of a peptide transport system in the mammary gland could result in peptidomimetic compounds being present in the milk, a fact that would need to be borne in mind when prescribing such drugs to mothers who are breast-feeding.

Pancreas

Recent studies have revealed unexpected and interesting locations for peptide transporter expression. One sur-

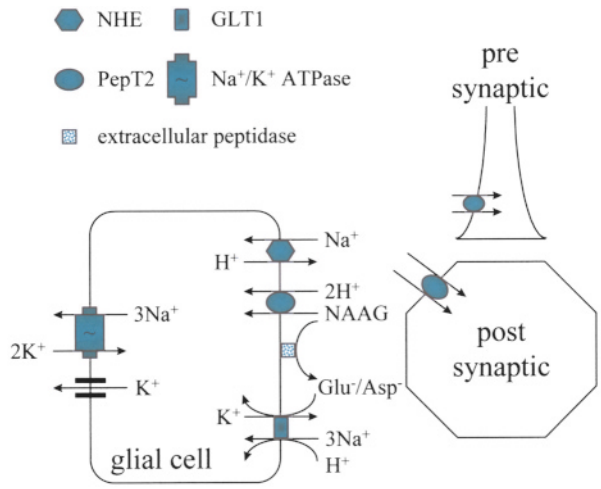


Figure 2. Schematic diagram of the clearance of NAAG (N-Ac-DE) from the synaptic cleft. NAAG (N-Ac-DE) released as a neurotransmitter presynaptically could be removed by a number of routes: first by uptake intact by PepT2 at the pre- or postsynaptic termini or by the glial cell, or second by uptake via GLT1 after hydrolysis by extracellular peptidases (see text for more details).

prising finding was that in the pancreas, where immunohistochemistry with PepT1 antibodies showed two subcellular structures to be positive: lysosomes in acinar cells, and nuclei of both smooth muscle cells of arteriole walls and of Schwann cells in unmyelinated nerves [42]. It was postulated that the lysosomal location of the peptide transporter could be for the removal of small peptides formed by intralysosomal protein degradation, and dipeptide transport in liver lysosomes has recently been demonstrated [43]. However, the function of PepT1 in nuclei is not clear, although it could be involved in putative peptide signals moving between the nucleus and the cytoplasm.

Another potentially important observation has been the finding that fibroblast and pancreatic carcinoma cell lines express peptide transporter. Nakanishi et al. [44] compared the uptake of Gly-Sar into the fibroblast carcinoma cell line HT1080 with that of the nonmalignant fibroblast cell line IMR-90. While the nonmalignant cell line did not transport Gly-Sar, the malignant HT1080 cells showed low-affinity peptide transport, similar but not identical to that of PepT1. Gonzalez et al. [45] used RT-PCR, Northern and Western blotting to show that two pancreatic carcinoma cell lines, AsPc-1 and Capan-2, both express PepT1, and that uptake of Gly-Sar showed similar parameters to Caco-2 and CHO-PepT1 cells. Immunochemistry with a PepT1 antibody showed staining at the plasma membrane and intracellular vesicular structures, again similar to that found with Caco-2 and CHO-PepT1 cells. These findings could have useful clinical implications, since if malignant cells express a peptide transporter not present in the normal cell, it may be possible to use the presence of these transporters to deliver antineoplastic peptidomimetic agents such as bestatin.

Central nervous system

The final area where peptide transporters have been investigated in detail is in the central nervous system (CNS). Wang et al. [18] cloned PepT2 from rat brain (identical sequence to renal isoform), and showed by electrophysiology that *N*-acetyl-L-aspartyl-L-glutamate [NAAG (N-Ac-DE)] is a substrate. NAAG (N-Ac-DE) is known to have the highest concentrations of any peptide present in the CNS (0.2–4.5 mM, [46]) and is also a neurotransmitter [47, 48]. Doring et al. [34] have also confirmed PepT2 expression in the rabbit brain. More recently these studies in the CNS have been extended by showing that the cellular site of PepT2 expression is in glial (specifically astroglial) cells in the substance of the brain, in Muller cells in the retina and in satellite cells in dorsal root ganglia [49, 50]. The uptake of the glutathione precursor Cys-Gly has been demonstrated in astroglia [51]; transport was inhibited by known PepT2 substrates, and the message for PepT2 was detected by RT-PCR. Thus PepT2 is also implicated in antioxidant mechanisms in the CNS.

NAAG (N-Ac-DE) is known to be hydrolysed by cell surface peptidases. This would produce the breakdown products (*N*-acetyl-)Asp and Glu, which would be removed from the cleft by the well-characterised glutamate transporter GLT1 [52]. However, another potential mechanism for its removal from the synaptic cleft would be uptake into glia via PepT2 (fig. 2), which would both circumvent generation of the excitotoxic hydrolysis products and be energetically less expensive (by a calculated factor of 4). This proposed combination of removal of the intact peptide and surface hydrolysis is obviously reminiscent of the situation in the intestine and kidney.

The colocalisation of PepT2 with the areas of high NAAG (N-Ac-DE) concentration in the CNS needs further investigation, although it has been shown that levels of PepT2 mRNA are relatively homogeneous throughout the rat brain with the exception of low levels in ventral forebrain [50].

Although functional studies have investigated the nature of peptide transport across the blood-brain barrier (e.g. reviewed in [53]), it is only recently that molecular studies have contributed to this field. Berger and Hediger [50] have shown that PepT2 is expressed in cells contributing to the blood-brain barrier, i.e. those of the choroid plexus, ependyma and subependyma in rat brain, and suggest that these findings implicate PepT2 in clearance of neuropeptide fragments from brain ex-

tracellular fluid. Torok et al. [54] looked at peptide transport in freshly isolated and in cultured porcine brain capillary endothelial cells, and showed that Gly-Sar was not able to penetrate these cells. Thus it is still not clear to what extent circulating substrates for PepT isoforms will have access to, or be removed from, the interstitial fluid of the CNS.

Structure function studies

Changing the binding site

In an earlier review, we proposed that 'the effects of external proton concentration on the kinetics imply that among the residues which contribute to the substrate binding site will be ones that are titrated by changes in extracellular pH over the range 5.5 to 7.4', adding that two likely candidate amino acid residues in PepT1 were His_{57} and His_{121} [12]. The suggestion that histidine residues would be involved was based not only on the suitable pK_a of this amino acid but also on the observation [55] in renal brush-border membrane vesicles that pretreatment with the histidine-modifying reagent DEPC completely inhibited peptide transport. This experimental observation was repeated a decade later in PepT1-expressing oocytes [56]. The essential nature of the His_{57} residue for PepT1 (and the conserved His_{87} residue of PepT2) has been shown by the technique of site-directed mutagensis, with the mutated transporters expressed in *Xenopus* oocytes [57–59], where mutation of His_{57} to either an alanine, asparagine or glutamine totally abolished peptide uptake. The case is less clear for the other conserved histidine (i.e. His_{121} in PepT1, His_{142} in PepT2). Terada et al. [58] mutated His_{121} to glutamine, and showed that when expressed in oocytes, H121Q-PepT1 did not transport Gly-Sar or the anionic oral β-lactam antibiotic ceftibuten. Fei et al. [59] performed a similar experiment on PepT1, but found that H121Q and H121N transported Gly-Sar at rates comparable to the wild-type protein. For PepT2, the H142N mutant had reduced but not absent transport, and thus the His_{142} residue was deemed to play a significant, but not essential, role. In both cases the mutant PepT proteins were shown by immunohistochemistry to be expressed at the oocyte membrane at levels similar to those found in the wild type. Fei et al. [59] also identified a third conserved histidine, His_{260}, in PepT1 (His_{278} in PepT2), but mutation of this residue did not affect peptide transport for either PepT isoform.

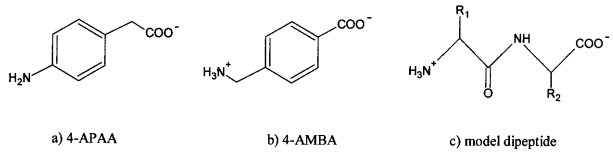
Histidine residues are not the only ones that have been investigated by point mutation studies. Computer modelling studies of the TM domains of hPepT1 suggested that Tyr_{167}, situated in TM5, might be important in transport function [60]. The mutant Y167A-hPepT1 was transiently expressed in HEK293 cells, and unlike the wild type was unable to transport Gly-Sar, despite being expressed at the cell surface at a similar level. Other mutant proteins (Y167F, Y167S or Y167H) were also unable to transport peptide, showing that Tyr_{167} does indeed play an essential role perhaps due to the unique chemistry of its phenolic side chain [since phenylalanine (lacking only the phenolic hydroxyl group) and serine (having an aliphatic hydroxyl group) were unable to substitute for tyrosine]. The same group [61] again used computer modelling of the human PepT1 molecule to reveal a putative 'transport channel' and the approximate locations of several charged or aromatic amino acid residues, which were mutated to assess their role in transport function. The first two, Tyr_{12} and Arg_{282}, when separately mutated to Ala, had only a modest effect on Gly-Sar transport into transfected HEK293 cells. However, mutation of Trp_{294} or Glu_{595} into alanine reduced peptide uptake by 80 and 95%, respectively. Thus the approach of computer modelling and site-directed mutagenesis has revealed three extra amino acids that are essential for efficient peptide transport through hPepT1, i.e. Tyr_{167}, Trp_{294} and Glu_{595}. This type of approach, along with the determination of the structural properties required for a compound to be a good substrate, are resulting in the mapping of the substrate binding site of the peptide transporter, a structure which will be of great interest to the pharmaceutical industry.

Another approach that will help towards the elucidation of the amino acid residues involved in substrate binding is that of making chimeric proteins of PepT1 and PepT2, which can be tested by exploiting the different affinities and substrate specificities of the two. Doring et al. [62] were the first to use this technique, constructing a chimera of residues 1–401 of PepT2 and 402–707 of PepT1 (i.e. the end of TM9 onwards of PepT1). The peptide transport characteristics of the resulting chimeric protein, CH1Pep, were studied in *Xenopus* oocytes by electrophysiology and radiolabelled dipeptide uptake, and shown to have the characterisitics of PepT2. This suggests that the phenotypical characteristics of the high-affinity isoform PepT2 are determined by its amino-terminal region (residues 1–401). A similar approach was used to narrow down the region responsible for conveying the kinetic phenotype of the transporter to TMs 7, 8 and 9 and the loops in between [63]. It is interesting that the essential residues identified in the point mutation studies, i.e. Tyr_{167}, Trp_{294}, Glu_{595} and His_{57}, do not all lie in the region of TMs 7 to 9, suggesting that the binding site for peptide substrates is made up of residues from the length of the protein, brought into a suitable geometry by the three-dimensional (3D) structure of the protein. Without a crystal structure, the elucidation of the detailed structure of these peptide transporters seems very unlikely.

Changing the substrate

The dogma that the epithelial peptide transporters were membrane proteins that interacted solely with substrates that contained a peptide bond (most obviously with di- and tripeptides) was implicit in the nomenclature used, initially by physiologists and subsequently by biochemists, when defining such proteins as 'peptide transporters'. Clearly a very wide range of peptides are readily transported (see e.g. [11, 2, 64]) by these membrane proteins, whereas amino acids are not: therefore, logically it followed that, since a peptide bond was the only feature distinguishing a dipeptide from its two constituent amino acids, a peptide bond was a sufficient requirement to make oligomers of amino acids become substrates able to bind to the transporter. But until very recently it had been assumed that it was also an essential feature, i.e. that molecules could not be substrates unless they possessed a peptide bond. A number of studies have recently shown that this is incorrect. Temple et al. [65] showed that (i) 4-aminophenylacetic acid (4-APAA, fig. 3) was transported across the intact intestinal epithelium; (ii) this transport was proton-coupled, causing an intracellular acidification in isolated small intestinal enterocytes; and (iii) that 4-APAA influx into PepT1-expressing oocytes was substantially greater than it was into water-injected control oocytes. Moreover there was transstimulation of efflux of labelled nonhydrolysable peptide from PepT1-expressing (but not water-injected control) oocytes by this nonpeptide. Taken together, these results show unambiguously that 4-APAA is translocated as a PepT1 substrate.

Further studies [66] showed that the chemically closely-related molecule 4-aminomethylbenzoic acid (4-AMBA, fig. 3) was also able to bind to the substrate binding site of PepT1 in *Xenopus* oocytes, but that this molecule (in contrast to 4-APAA) was not transported (and hence that it was unable to stimulate efflux of labelled peptide from oocytes). However 4-AMBA did competitively inhibit the transstimulation of efflux produced by addition of unlabelled peptide to the external medium, proving that it was a competitive nontranslocated inhibitor of PepT1. [Although this makes 4-AMBA the first such inhibitor of peptide transport to be described, the affinity of this molecule for PepT1 is low, with a K_i for PepT1 expressed in oocytes of approximately 3 mM. Subsequently lysine dipeptide conjugates of fluorescein isothiocyanate, such as Val-Lys-fluorescein, were developed that are also competitive nontranslocated inhibitors of PepT1-mediated transport [67]; these larger compounds are inhibitors with very high affinity (5 μM)].



a) 4-APAA b) 4-AMBA c) model dipeptide

Figure 3. Chemical structures of a) 4-aminophenylacetic acid (4-APAA), b) 4-aminomethylbenzoic acid (4-AMBA) and c) a model dipeptide, shown at neutral pH. The pK_a for the amino group will be approximately 10 for 4-AMBA and the model dipeptide, but for 4-APAA it will be not be ionised at neutral pH due to the electron delocalisation effect of the aromatic ring. In all three molecules the carboxyl group will have a pK_a of approximately 2.5.

The conclusion (initially surprising) of these studies, that nonpeptides can be substrates for PepT transporters, is now strongly supported by experiments from other laboratories. Doring et al. [68] showed that ω-amino fatty acids are substrates transported by PepT1, although they do not possess any peptide bonds. For such an amino fatty acid to be a PepT1 substrate, it had to have the carboxyl and amino groups at opposite ends of an aliphatic chain with a minimum of four methylene groups separating these charged moieties. PepT1-mediated amino fatty acid transport was electrogenic, and substrate affinity was higher when the number of methylene groups in the fatty acid backbone was increased. The same group [34] also showed that δ-aminolevulinic acid (i.e. 4-oxo-5-aminopentoic acid) was a substrate for expressed PepT1 and PepT2, a finding which further confirms the ability of these transporters to translocate substrates lacking a peptide bond. As discussed below (see below), recent observations showing that antiviral prodrug valacyclovir is a good substrate for peptide transport [69] leads to the same conclusion.

In a natural peptide, other than the peptide bond, the presence of an amino and carboxyl terminal is a consistent structural feature. To what extent (given the conclusions discussed above) might these two groups be prerequisites? Borner et al. [70] showed, in an important paper, that there was transport of amino acid aryl amides by PepT1 both endogenously in Caco-2 cells and when expressed in *Xenopus* oocytes. This not only confirms yet again that a peptide bond is not a prerequisite but also shows that an amide group can replace an amino group without loss of transport; indeed, Ala-4-nitroanilide, Phe-4-nitroanilide and Ala-4-phenylanilide were accepted as substrates with higher affinity than natural alanyl-dipeptides. These authors found that Phe-amide was not a substrate; recently the original studies have extended by showing that the *N*-acetyl-Phe and the peptide Phe-Tyr-amide were excellent competitive inhibitors of labelled peptide (D-Phe-L-Gln) transport into PepT1-expressing oocytes, whereas all other derivatives of Phe and Phe-Tyr did not bind ([71] and reference therein). Taken together, these recent

studies show that it is mistaken to assume that any single feature of a substrate defines its ability to interact with the binding site on PepT isoforms. It is rather the sum of a number of discrete interactions that will determine the overall binding affinity, and very recently molecular modelling has suggested a 3D substrate template that explains all of the binding and transport properties of PepT1 substrates [71]. This is a finding that must be of relevance to targeting intestinal drug absorption using these transporters.

Stoichiometry of proton-peptide cotransport

The stoichiometry of PepT1-mediated symport of protons with a neutral peptide had been shown by studies in a variety of systems to be 1:1, i.e. 1 mol of protons is cotransported with 1 mol of peptide [4, 72–75]; thus, net transport will be electrogenic and driven by the inside-negative membrane potential of the cell. Interesting recent studies on rabbit PepT2 expressed in *Xenopus* oocytes show that, in contrast to PepT1, transport of 1 mol of neutral peptide (Phe-Ala) appears to be coupled to the symport of 2 mol of protons [76]. The 2:1 stoichiometry of PepT2 fits with the physiological demands placed on this high-affinity transporter, which is expressed specifically in the more distal part of proximal tubule (S3) (see above). Here it will be required to reabsorb the final fraction of filtered substrates (some of which may not even be peptides, see above) from a very low concentration against a very substantial electrochemical gradient. In contrast, the more proximal distribution of PepT1 in the nephron is in keeping with its observed 1:1 stoichiometry, allowing for rapid reabsorption of the bulk of filtered peptide in an economic and efficient way.

Although this stoichiometry of neutral peptide transport is accepted, there is continuing interest in the effect of peptide substrate charge on the stoichiometry of proton-peptide coupling. It is generally agreed that for anionic substrates (such as Phe-Glu), translocation of substrate through PepT1 is associated with a flux of two protons per single catalytic cycle [4, 72–75]. Recent work suggests that for PepT2 the equivalent figure is three per cycle [76] (although these studies on rabbit PepT2 differ from previous findings [77] and from those on rat PepT2 [78]; the reasons for this are as yet unclear). The consequence of this stoichiometry is in each case that the transport of anionic substrate is electrogenic; thus the membrane potential across the apical membrane of the epithelium is important both for neutral and anionic substrates. For cationic peptides (such as Phe-Lys) where transport has to be electrogenic, peptide flux must depend on membrane potential. Here there is some discrepancy between studies using membrane vesicles (which suggest uncoupled Phe-Lys transport via PepT1 [72]) and electrophysiological studies in oocytes, which suggest a coupling stoichiometry of 1:1 for PepT1 [74] and 1:1.4 for PepT2 [76].

While His_{57} (His_{87}) has been implicated in the movement of one proton through PepT1 (PepT2) (see above), the precise mechanism of multiple proton coupling to peptide movement remains unclear, i.e. whether it is the substrate (when in the substrate binding site) or the transporter itself that is titrated by external protons [7, 72, 78–80].

Regulation

The regulation of peptide transport is a relatively little studied area, and involves a complex interplay between different levels of regulatory mechanisms. For example, within a single epithelial cell there must be regulation at the level of posttranslational modification by second messengers of previously synthesised transport proteins, at the level of transport protein insertion into and retrieval from the plasma membrane, at the level of gene transcription and at the level of mRNA stability. Within a tissue there may be regulation by hormonal or neuronal signals, and, for example, in small intestine there may be additional regulation dependent on diet. In vivo it is difficult to separate these different levels of regulation. Here we will consider their individual effects on peptide uptake.

Regulation of PepT1/2 by second messengers

In the cloning paper of rabbit PepT1 [19] it was noted that there were consensus sequences for protein kinase A (PKA) and C (PKC) modification of the protein, immediately implying that regulation by these second messengers may be of physiological importance. The human PepT1 (hPepT1) sequence which was cloned shortly afterwards revealed that there was a PKC site, but not one for PKA [38]. Brandsch et al. [81] investigated the regulation of transport of the peptide Gly-Sar in the Caco-2 cell line, which expresses hPepT1, and showed that the activation of PKC by phorbol esters inhibited transport. This effect was attributed by the authors to be entirely due to a reduction of V_{max}, with the K_m remaining unchanged, and not an effect on the driving force for peptide uptake via a change in the pH_i. More recently, Wenzel et al. [82] looked directly at the effect of activating PKC by increasing the intracellular Ca^{2+} concentration in LLC-PK1 cells, which express PepT2. Whereas again uptake of peptide (D-Phe-L-Ala) was decreased by PKC activation (reversible with staurosporine), they reported it was purely due to a change in K_m, with V_{max} unaffected.

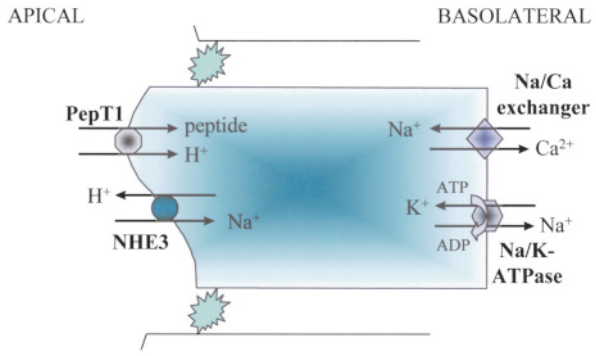


Figure 4. Schematic diagram of an epithelial cell. Anticlockwise from top left: peptides enter the cell with protons via PepT1; the protons are exchanged for sodium via NHE3; the sodium is removed from the cell by the basolateral sodium pump (Na/K-APTase); if the rate of sodium entry exceeds the capacity of the sodium pump to remove it, the sodium/calcium exchanger ability to keep intracellular calcium concentrations low will be compromised. The rise in calcium concentration would act to reduce the rate of peptide entry through PepT1 (see text for details) and open calcium-dependent potassium channels, allowing the cell to repolarise and to restore its normal ionic balance.

Both transporters thus respond to a rise in $[Ca^{2+}]_i$ in such a way as to reduce peptide influx: this may be interpreted as representing the cellular response to the challenge of a rapid rate of solute influx (see fig. 4). For example, peptide influx, accompanied by cotransport of protons, will activate pH_i regulation through the sodium-hydrogen exchanger (NHE, in this case the apically located isoform NHE3 [83]). This will increase the rate of sodium entry into the cell, activating extrusion via the sodium pump. When the capacity of the sodium pump is exceeded, $[Na^+]_i$ will begin to rise, slowing Ca^{2+} extrusion through Na^+/Ca^{2+} exchange; the resulting rise in $[Ca^{2+}]_i$ by reducing the rate of peptide influx will allow the cell's interior milieu to be protected. In addition, the increase in $[Ca^{2+}]_i$ will open Ca^{2+}-sensitive potassium channels, leading to repolarisation of the cell membrane. (The central role of NHE in this response is critical in HCO_3^--free media; however, recent evidence [84] suggests that in a physiological (HCO_3^--containing) environment, protons entering enterocytes at the apical membrane are rapidly equilibrated throughout the cell by the actions of carbonic anhydrase, preventing the local fall in pH underneath the apical membrane.)

However, Brandsch et al. [85] report that calmodulin antagonists decrease the uptake of peptide (Gly-Sar) into PepT1-expressing MDCK cells, an effect associated with a fall in V_{max}. Since these inhibitors are expected to block the response of the cell to a rise in $[Ca^{2+}]_i$, this result appears paradoxical; it may reflect the relatively poor pharmacological specificity of this class of agents. As mentioned above, the human PepT1 sequence, unlike that of rabbit, does not contain a PKA consensus site. Thus, the finding that treatment of hPepT1-expressing Caco-2 cells with cholera toxin inhibits peptide (Gly-Sar) uptake via an increase in the intracellular cyclic AMP (cAMP) levels due to a decrease in V_{max} was unexpected [86]. Since PKC (as well as PKA) inhibitors had similar effects, it was suggested that the consequence of exposure to cholera toxin was cAMP-dependent phosphorylation of the PKC site on hPepT1. However, recent evidence [87] suggests that increases in cAMP (via forskolin application) in Caco-2 cells lead to the reduction of Gly-Sar uptake indirectly, via an inactivation of the apical sodium-proton exchanger NHE3 (which does have a PKA site). This conclusion was based on the observation that the inhibition of peptide transport by such reagents was Na^+- as well as pH-dependent. However, regulation of NHE3 by cAMP also requires the regulatory cofactor NHE-RF [88], which itself also has a PKA phosphorylation site; it has been reported that Caco-2 cells do not express this protein (nor the closely related E3KARP) and that transfected NHE3 in Caco-2 cells was not inhibitable by cAMP [89]. To this extent the mechanism of cAMP action on peptide transport into Caco-2 cells remains unresolved. Physiologically, hormones such as VIP, glucagon and adrenaline act to increase cAMP concentrations, hence activating fluid secretion and inhibiting absorption. However, diseases such as cholera cause secretory diarrhoea by activating adenylate cyclase, raising cAMP and stimulating inappropriate levels of intestinal secretion, which can cause severe dehydration.

Hormonal regulation

The effects of hormones on peptide absorption have not been the subject of systematic study. Very recently it was reported that exposure to physiological levels of insulin (5 nM) for more than 1 h stimulates peptide uptake, approximately doubling the rate of Gly-Gln influx into cultured Caco-2 cells [90]. This effect, which was blocked by the tyrosine kinase inhibitor genistein, was due to an apparent increase in the insertion of the transport protein from a cytoplasmic pool. This hypothesis is supported by several observations: the V_{max} of hPepT1 was almost doubled, without a change in K_m; the effect occurred with a time scale of 30–60 min; and the effect was abolished by disruption of microtubules, but unaffected by dismantling of the Golgi apparatus.

The existence and regulated insertion of a cytoplasmic pool of peptide transporters may account for an observation that was made by C. A. R. Boyd and M. Ward in the early 1980s (unpublished, see figure 6 in [12]), whereby the repeated application of saturating concen-

trations of dipeptide (either L-Leu-L-Leu or L-carnosine) to *Necturus* intestine lead to an increase in the I_{max}, as measured by microelectrodes.

It is well accepted that in addition to glucose, amino acids can cause the release of insulin from pancreatic β-cells. The plasma level of amino acids would reflect the protein load of the intestine during absorption of a meal; thus, a high level of amino acids could signal the need for more peptide transporters via a rise in plasma insulin. The concept of hormones being able to affect the rate of solute absorption in the intestine is not novel: it has been shown that glucagon-like peptide 2 (GLP-2) increases sodium-dependent glucose uptake across the intestinal brush-border membrane by increasing the abundance of the transport protein SGLT-1 [91].

Interactions between diet and peptide transporter expression and function

Upregulation of transporter expression is a common physiological response to increased demand, and the uptake mechanism for peptides is no exception. Thus a number of studies in which high-protein diets have been imposed have shown that the response is an increased capacity of the system, and the mechanism of this increase has been investigated. An early molecular study used RT-PCR to investigate mRNA levels in rat small intestine [92]. A message expressed by two different genes was detected, corresponding to the rat isoforms of PepT1 and HPT1 (see below), which showed an even distribution profile of steady-state mRNA levels along the longitudinal axis of the small intestine. In response to a high-protein diet, the levels of both mRNAs were increased only in the distal portion of the small intestine, suggesting that this is the region of the small intestine where dietary-induced changes in absorption occur. Functional studies in Caco-2 cells showed that addition of 4 mM Gly-Gln to the medium resulted in the capacity for peptide uptake being almost doubled (V_{max} for Gly-Sar influx increased $\sim$1.6-fold) [93]. At the molecular level, this could be accounted for by an increase in hPepT1 message levels by $\sim$1.9-fold, due partly to a longer mRNA half-life, with the rest of the increase being presumed to be due to increased transcription rates. The increase in mRNA levels was mirrored by an increase of $\sim$1.7-fold in the peptide transporter protein at the membrane, as measured with a hPepT1-specific antibody. In a similar study, supplementation of the medium of Caco-2 cells with Gly-Sar increased the uptake of the peptide Gly-Gln by twofold via a doubling of the hPepT1 protein in the membrane [94]. In these studies the increase in peptide transport was brefeldin-sensitive, whereas the threefold increase in hPepT1 mRNA level was not. Since brefeldin disrupts the Golgi, this experiment shows that the increased rate of transport is due to de novo protein synthesis.

While the studies mentioned above clearly show an increase in capacity for peptide uptake (via synthesis of more peptide transport protein) in the presence of a high-protein diet, they do not suggest a mechanism by which the cells sense the concentration of dietary protein in the medium. However, in the case of cultured cells it is clear that the effects must be mediated directly, as no other (neural or endocrine) inputs are possible. A recent study showed that dietary amino acids (Phe, Arg and Lys) and peptides (Gly-Sar, Gly-Phe, Lys-Phe and Asp-Lys) could upregulate the expression of the rat PepT1 gene (as assessed by a promoter-luciferase construct) [95]. The PepT1 gene promoter region has several potentially interesting regulatory sequences [95]: an AP-1 binding site, TGACTCAG, at -295 nucleotides relative to the transcription start; and a region with high homology to an amino-acid-responsive element (AARE). The transcription factor AP-1 is known to be associated with regulating gene expression during amino acid deprivation, by binding to the cis elements on genes such as those for the oncogenes c-myc, c-jun, junB and c-fos, and that for the enzyme ornithine decarboxylase, resulting in increases in their mRNA levels [96]. Thus it is possible that AP-1 levels in Caco-2 cells could be increased by high concentrations of specific amino acid and peptides, and act to upregulate PepT1 expression. The AARE was identified as a 7-bp sequence (5′-CATGATG-3′) that is a part of the promoter region of the asparagine synthetase gene necessary for transcription to be upregulated in HeLa cells deprived of essential amino acids [97]. The rat PepT1 promoter has a sequence with high homology to an AARE at -277 nucleotides relative to the transcription site, with the sequence 5′-CATGGTG-3′, but it has not been shown as yet that it can function as an AARE [95].

However, despite the presence of these amino acid/peptide response elements in the PepT1 promoter region, it is still not clear whether in vivo amino acids and peptides have to enter the cell to exert their effects, or whether they can act indirectly via an unknown signalling pathway. Ford et al. [98] attempted to answer this question by comparing the effects of adding amino acid, natural peptides or nonhydrolysable peptides to the medium of Caco-2 cells. Using the peptides (all at 4 mM) Gly-Sar, L-Ala-L-Phe and D-Ala-D-Phe, or the amino acids (2 mM) L-Ala together with L-Phe to supplement the medium of Caco-2 cells, only L-Ala-L-Phe could increase the levels of hPepT1 mRNA above those seen under control conditions (6 mM L-Gln). The transported but hydrolysis-resistant peptide Gly-Sar failed to increase hPepT1 mRNA levels, whereas L-Ala-L-Phe (well transported and readily hydrolysed) did so;

this was taken to suggest that not only transport but also the hydrolysis of peptide was important. (D-Ala-D-Phe, which is neither hydrolysed nor transported, also failed to elicit any increase in gene expression). The same authors had shown previously that Gly-L-Gln also upregulated hPepT1 mRNA, protein and function in Caco-2 cells [93]; however, in contrast, in whole animal studies, Shiraga et al. showed Gly-L-Gln to be without effect, whereas free L-Phe readily increased hPepT1 mRNA, protein and function [95]. Thus, the mechanism by which dietary components act to regulate transporter expression is still not completely clear.

As well as peptide uptake being stimulated by a high-protein diet, under conditions of fasting it is essential that the maximal fraction of the limited protein ingested be absorbed. It is thus unsurprising to find that under fasting conditions there is an increase in peptide uptake by small intestine [99]. After 1 day of fasting, the uptake of Gly-Gln into brush-border membrane vesicles prepared from rat intestine was doubled; this increase in transport capacity was matched by a threefold increase in PepT1 protein and also in PepT1 mRNA. Interestingly, the hormonal signal for fasting is glucagon, which acts by activating adenylate cyclase via a G-protein linked receptor: as mentioned earlier, a rise in cAMP has an immediate effect of inhibiting peptide uptake. However, in the case of fasting, it looks as though here cAMP acts via transcription to increase peptide uptake by increasing the level of PepT1 protein.

Response to injury of the intestinal epithelium

In the case of injury to the intestinal epithelium by the chemotherapeutic agent 5-fluorouracil in rats, whereas the mRNA level of transport proteins such as those for amino acids, glucose and the phosphate transporter were depressed [100], the mRNA levels for PepT1 actually rose. At the protein level, PepT1 was largely unaffected, whereas the levels of the enzyme sucrase and the sodium-dependent glucose transporter in brush-border membranes were markedly decreased. These findings suggest that the cells remaining after the 5-fluorouracil treatment increase their synthesis of PepT1, rather than there being a change in the kinetics of transport. While the physiological mechanism behind these adaptations is not known, nutritionally it may be useful to exploit this ability of patients on chemotherapy to absorb peptides well.

σ_1 (nonopiate, nonphencyclidine) receptor ligand-induced regulation

Fujita et al. [101] have reported that a selective σ_1 ligand, (+)pentazocine, upregulated Gly-Sar transport in the Caco-2 cell line after 24-h pretreatment via an increase in V_{max}. Semiquantitative RT-PCR revealed an increase in PepT1 mRNA, and although the relative level of PepT1 protein was not assayed, their conclusion that the increased uptake is caused by an increase in the amount of transporter protein in the cell membrane appears reasonable. Caco-2 cells have been shown to express the σ_1 receptor [102]; however, the intracellular signalling pathway between σ_1 receptor stimulation and PepT1 gene expression is not known. Progesterone is a putative endogenous σ_1 receptor ligand, and with a K_d of ~ 80 nM is also physiologically relevant. It has been suggested that during pregnancy intestinal absorption of peptide will need to be increased to match the demands of the developing conceptus, including both foetus and placenta [101].

Neural regulation

An interesting report showed that perfusion of rat small intestine with nifedipine (a Ca^{2+} antagonist) increased the rate of absorption of the antibiotic cefixime [103]. This effect was not due to a change in intestinal blood flow or motility. These authors concluded that 'nifedipine could affect cefixime absorption, directly or indirectly, via a neural mechanism by increasing the activity of carrier-mediated transport systems within the intestinal epithelium'. Alternatively, a direct effect is possible, since nifedipine (see above) could cause a decrease in $[Ca^{2+}]_i$ by blocking pathways for Ca^{2+} entry, hence reducing the activity of PKC, resulting in increased peptide transport.

Usefulness of substrate information for improving drug delivery

A major problem with oral delivery of potential therapeutic agents is poor bioavailability; this can be the result of chemical instability within the lumen of the gastrointestinal tract (for example as a result of intestinal/pancreatic enzyme activity) or minimal/absent intestinal absorption. It has been known for more than 15 years that those antibiotics that are orally available (e.g. those with a β-lactam ring) have sufficient structural similarity to tripeptides to be substrates for the peptide transporter. Other well-established substrates are the angiotensin-converting enzyme (ACE) inhibitors (very widely used both to treat hypertension and failing circulatory output) and the antineoplastic agent bestatin (see [10] for a review). Cloning of the isoforms of the PepT family and their subsequent expression in cell lines, both endogenously (e.g. Caco-2, MDCK cells) and following transfection (e.g. LLC-PK1, CHO), will undoubtedly provide an alternative to animal studies for investigating the intestinal absorption of such drugs e.g. [104]. Table 3 shows a selection of these compounds that are transported by PepT1/2.

Table 3. Table of β-lactam antibiotics, ACE inhibitor, antitumour agents and other compounds transported by PepT1/2.

Compound	Features	Assay system	Uptake	Inhibition	Reference
Antibiotics					
Cephalexin	zwitterionic β-lactam	PepT1-XLO	✓		19
		PepT1-XLO	✓		105
		hPepT1-HeLa		✓	38
		CHO-PepT1		✓	106
		Caco-2	✓	✓	107
		Caco-2	✓		108
		bovine SI BBMV		✓	109
		Caco-2	✓		110
		rat renal BBMV	✓		111
		rat renal BBMV	✓		112
		in vivo rat intestine		✓	113
		SKPT (PepT2)	✓		114
Penicillin G		human SI BBMV	✓		115
Cyclacillin	amino-penicillin	PepT1-XLO	✓		19
		hPepT1-HeLa		✓	38
		LLC-PK1		✓	116
		Caco-2		✓	114
		SKPT (PepT2)	✓	✓	114
		rat renal BBMV		✓	112
Ceftibuten	anionic β-lactam, no α-amino group	PepT1-XLO	✓		117
		PepT1-XLO	✓		26
		PepT1-XLO	✓		105
		LLC-PK1		✓	58, 116
		Caco-2		✓	118
		SKPT (PepT2)		✓	118
		LLC-rPepT1	✓		119
		Caco-2	✓		108
		rat SI BBMV	✓		120
		rat renal BBMV	✓		121
		Caco-2	✓		122
		rat SI BBMV	✓		123
		rat renal BBMV	✓		123
		rat renal BBMV	✓		124
		rat renal BBMV		✓	112
		Caco-2	✓		125
Loracarbef	β-lactam	Caco-2	✓		126
		Caco-2	✓	✓	127
Cefixime	anionic β-lactam	PepT1-XLO	✓		105
		Caco-2	✓		126
		Caco-2	✓	✓	127
		Caco-2		✓	118
		SKPT (PepT2)		✓	118
		Caco-2	✓		108
		in situ rat intestine	✓		
Cefadroxil	zwitterionic β-lactam	PepT1-XLO	✓		19
		PepT1-XLO	✓		26
		PepT1-XLO	✓		105
		hPepT1-HeLa		✓	38
		Caco-2		✓	114
		SKPT (PepT2)		✓	114
		Caco-2	✓	✓	127
		PepT2-XLO	✓		128, 129
		rat renal BBMV	✓		130
		rat renal BBMV	✓	✓	130
		PepT1-yeast		✓	131
Cefdinir	anionic β-lactam	Caco-2		✓	118
		SKPT (PepT2)		✓	118
		rabbit SI BBMV	✓*		132
Cephradine	zwitterionic β-lactam	PepT1-XLO	✓		19
		PepT1-XLO	✓		117
		PepT1-XLO	✓		105
		LLC-rPepT1	✓		58, 119
		Caco-2	✓		108
		rabbit SI BBMV		✓	133
		rabbit enterocytes	✓		134
		rat renal BBMV	✓		112

Table 3. (Continued)

Compound	Features	Assay system	Uptake	Inhibition	Reference
β-lactam antibiotics		rabbit conjuctiva		✓	135
Cefroxadine		rabbit SI BBMV	✓		133
S-1090	cephalosporin	rat SI BBMV	✓		136
ACE inhibitors					
Enalapril		CHO-PepT1	✓		104
		rabbit renal bbmv		✓	138
		rat ileum	✓		139
		rabbit SI BBMV		✓	133
		Caco-2 cells	✓*		140
		rat intestinal rings	✓*		140
		in vivo rat intestine	✓	✓	113
Captopril		rabbit renal bbmv		✓	138
		rabbit SI BBMV		✓	133
		PepT1-yeast		✓	131
Quinapril		rabbit renal bbmv		✓	141
		rabbit renal bbmv		✓	138
		rabbit SI BBMV		✓	133
Zefenopril		rabbit renal bbmv		✓	138
Fosinopril		rabbit renal bbmv		✓	138
Enalaprilat		rabbit renal bbmv		✓	138
		in vivo rat intestine	✓*	✓	113
Lisinopril		rabbit renal bbmv		✓	138
		in vivo rat intestine	✓	✓	113
Quinaprilat		rabbit renal bbmv		✓	138
Ramipril		rabbit renal bbmv		✓	138
ACE inhibitors		rabbit conjunctiva		✓	135
Renin inhibitors					
CH3-18		rat SI BBMV	✓		142
Antitumour					
Bestatin		PepT1-XLO		✓	117
		LLC-rPepT1		✓	119, 143
		LLC-rPepT2		✓	143
		rabbit conjunctiva		✓	135
Others					
Ochratoxin A	nephrotoxin	MDCK-C7/C11	✓	✓	144
fMLP	chemotactic peptide	Caco-2	✓		145
		hPepT1-XLO	✓	✓	145

* Passive.

As well as being central to the process of intestinal absorption of such drugs, peptide transporters are important following absorption in determining the half-life of many drugs within the body. The way in which the kidney, as a major organ of drug excretion, handles peptidic molecules may greatly influence the rate of drug clearance. The kidney has both the low-affinity, high-capacity PepT1 and the high-affinity, low-capacity PepT2 isoforms of the peptide transporter, which are found in different parts of the kidney (see above). It is noteworthy that the two PepT isoforms do have different specificity; for example, the ACE inhibitors enalopril and captopril are substrates for PepT1 [137], and not for PepT2 [129], whereas quinapril is a noncompetitive inhibitor of PepT2 [141]. Also, the intestine and the kidney are not the only tissues in the body which express PepT1/2, and so any differences in transport characteristics between the transporters will contribute to the pattern of distribution of the drug in the body. For example, Doring et al. [34] looked at the transport of δ-aminolevulinic acid (ALA, a precursor of phorphyrin synthesis which is being tested in clinical trials as a photosensitiser for tumour therapy) by expressed PepT1 and PepT2, and found that it is carried by both. This is of interest as it not only accounts for ALA's rapid intestinal and renal (re-)absorption but also causes this substrate to be accumulated in the brain, lung and mammary gland, all of which express PepT2. This accumulation, which has been demonstrated experimentally, may improve the response of these tissues to photodynamic therapy.

One recent and fascinating development in the targeting of drugs through the peptide transporters has been the use of prodrugs, based in part on the finding that a peptide bond is not an essential prerequisite to be a PepT1 substrate [65] (see above). Acyclovir (ACV) is an antiviral agent that is widely used in the treatment of herpes simplex and the AIDS opportunistic cy-

tomegalovirus, and is a nonpeptide with poor oral bioavailability. Its prodrug form, valacyclovir (VACV), has a valine joined by an ester linkage, i.e. it still has no peptide bond (fig. 5); however, now it is a substrate for both PepT1 and PepT2 [69], as shown by inhibition of expressed transporter in both *Xenopus* oocytes and HELA cells.

Once taken up into the intestinal epithelium it can be converted to its active form by intracellular nonspecific esterases; in mammals, including humans, VACV has a bioavailability that is three- to fivefold higher than that for ACV, and is converted to its active form by first-pass intestinal and hepatic metabolism [146]. That VACV is transported has been confirmed using radiolabelled compounds [147, 148]. Intriguingly, transport has been demonstrated not to be proton-coupled in hPepT1-expressing oocytes [149]. Uptake was maximal at an extracellular pH of 7.5; transport was reduced approximately twofold above and below this pH. The three pK_a's of VACV are 1.90, 7.47 and 9.43; Guo et al. [149] propose that it is the neutral form that is the favoured substrate for hPepT1, with the instability of VACV at pH 8.0 accounting for the decreased uptake at this pH. The 5′-amino acid esters of another therapeutic nucleoside, the anti-HIV drug AZT in the prodrug form L-Val-AZT, has also been shown to be a substrate of PepT1 [148].

Rather than addition of amino acid groups via ester bonds, peptidyl derivation of compounds has been demonstrated to be another successful way to increase bioavailability. Thus, the anti-Parkinsonian drug L-dopa is absorbed more efficiently in the form L-dopa-L-Phe; when this peptide is added apically to Caco-2 cell monolayers L-dopa/dopamine appears more rapidly in the basolateral medium than it does from apical L-dopa alone [150]. This shows both that L-dopa-L-Phe is taken up more effectively by the peptide transporter than is the free L-dopa by amino acid transporter(s), and that the peptide is rapidly hydrolysed by intracellular peptidases. A similar approach was used to improve the oral bioavailability of a series of novel broad-spectrum antibacterials [aminomethyl tetrahydrofuranyl (THF)-1 β-methylcarbapenems] [151]. These have a 3- to 10-fold higher efficacy after oral administration than the peptidyl prodrug (with the L-stereoisomer of Ala, Val, Ile or Phe added at the aminomethyl THF side chain of the active drug molecule).

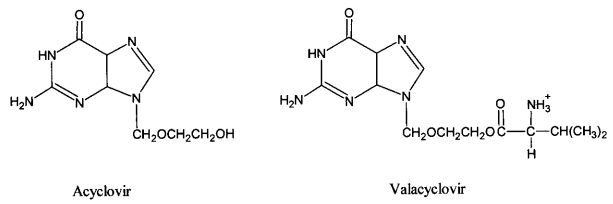


Figure 5. Chemical structures of acyclovir (ACV) and valacyclovir (VACV), shown at neutral pH. The pK_a of the amino group of the ester-linked valine group of VACV will be approximately 10, whereas that of the enol form of the intracyclic NH-CO group will be similar to that of guanine (9.2).

The finding that the absorption of the prodrugs mentioned above is greater than that of the free form shows that the peptide transporter is an efficient drug delivery pathway. The recent realisation that a peptide bond is not a prerequisite for a compound to be a PepT substrate will not only simplify the chemistry required to synthesise such prodrugs (for example, it is usually easier to add an amino acid via an ester than a peptide bond) but also opens up a wider range of potentially useful synthetic pathways.

Basolateral membrane transport

One area of epithelial peptide transport that is relatively little studied is how peptides cross the basolateral membrane of the cell, despite the obvious importance of this step to the overall transepithelial transport of substrate from lumen to systemic circulation. It is now becoming apparent that the transporter present in the basal membrane must differ from that present apically; the arguments for this assertion are both empirical and theoretical (energetic). For example, in Caco-2 monolayers the affinities for transport of different β-lactam antibiotics differed at the apical and basolateral membranes, despite the fact that there was uptake at both sides of the epithelium [108]. The authors concluded that these drugs were transported at the basolateral membrane via 'the dipeptide transport system in an H^+ gradient-independent manner' and that this step was 'rate-limiting for their transcellular transport'. This was supported by studies showing that basal membrane transport of the β-lactam antibiotic loracarbef was neither proton-dependent nor saturable, unlike that at the apical membrane [152]. However, this does not fit with earlier evidence (e.g. [153]) that basolateral transport of the β-lactam antibiotic cephalexin in Caco-2 cells produces intracellular acidification. In addition, more recent studies have shown that basolateral membrane vesicles from the teleost *Oreochromis mossambicus* take up the dipeptide Gly-Sar by an electroneutral, Na^+-independent, DEPC-sensitive low-affinity transport system [154]. Most recently, Terada et al. [155] studied the transport of Gly-Sar at the basolateral membrane of Caco-2 cells and showed that it is less sensitive to changes in external pH than is transport via apically located hPepT1. (Uptake across the basal membrane

increased approximately 1.7-fold on acidification from pH 7.4 to 6.0; across the apical membrane an identical acidification stimulated peptide uptake more than threefold). Basal transport was, however, sensitive to inhibition by DEPC. The basolateral transporter was also, in contrast to apical transport, unable to concentrate intracellular Gly-Sar markedly (the accumulation ratio across the basolateral membrane was only 1.25, compared with 12.9 for the apical membrane). These results led the authors to support their group's previous findings [108] that the basolateral peptide transport is mediated by a single, low-affinity, H^+-independent facilitated transport system, although they could not account for the differences between their results and those of other workers. A very similar study [156] produced similar results; however, a fall of intracellular pH associated with basal membrane exposure to Gly-Sar was observed, and it was thus concluded that this transport process was proton-coupled.

Many studies support the proposal that it is solute movement across the basolateral membrane that is rate-limiting to transepithelial peptide transport. Lister et al. [157] demonstrated that in isolated loops of rat jejunum there was accumulation of transported but hydrolysis-resistant dipeptides (D-Phe-L-Ala and D-Ala-L-Phe) within the epithelium, showing that it is indeed the exit step that is rate-limiting.

Other potential peptide transport proteins

PepT1 and Pept2 are not alone in being the only reported mammalian peptide transporters. At the same time as the cloning of PepT1 [19], the 832-amino acid intestinal peptide transport protein HPT1 was reported, with a very different proposed structure of only 1 TM and homology to the cadherin superfamily of calcium-dependent, cell-cell adhesion proteins [158]. This protein was identified from a monoclonal antibody which prevented the uptake of the β-lactam antibiotic cephalexin, and the cloned HPT1 restored peptide transport activity when expressed in transport-deficient CHO cells: the sequence has been patented for its potential drug delivery interest. Although it has been suggested that HPT1 may act as a regulatory element of another peptide transport protein [12, 158], this has not as yet been shown experimentally. However, it is noteworthy that coregulation of HPT1 mRNA with that of PepT1 in response to a high-protein diet has been reported [92] (see above).

Another potential peptide transporter has been cloned which induced high-affinity proton-dependent histidine and carnosine transport when expressed in *Xenopus* oocytes, termed the peptide/histidine transporter or PHT1 [159]. PHT1 is a 572-amino acid protein, with 12 putative TMs, and a weak similarity with PepT1 and PepT2 (17% identity and 32% similarity, and 12% identity and 27% similarity, respectively) [although it shows a 27.5% identity (40.8% similarity) with rat PepT1 over PHT1 residues 31–422, and a 25.9% identity (38.7% similarity) with PepT2 over PHT1 residues 31–447]. Most interestingly it does have the PTR2 consensus domain (see above). PHT1-mediated histidine transport was inhibited by di- and tripeptides, but not by free amino acids such as Glu, Gly, Leu, Met or Asp. In situ hybridisation revealed PHT1 mRNA was widely distributed throughout the whole brain, with especially intense signal in the hippocampus, choroid plexus, cerebellum and pontine nucleus. Signals were located in both neuronal and small nonneuronal cells in these areas, and the authors suggested that PHT1 could contribute to the uptake of neuromodulatory peptides and the clearance of degraded neuropeptides.

Finally, a 208-amino acid regulatory factor for hPepT1, hPepT1-RF, was cloned and shown by RT-PCR and Northern blots to be expressed in Caco-2 cells [160]. Amino acids 18–195 of hPepT1 are identical to those of hPepT1, whereas the N-terminal (residues 1–17) and the C-terminal (residues 196–208) are unique. In oocytes, hPepT1-RF expressed alone showed no Gly-Sar transport activity; however, when coexpressed with hPepT1, the pH optimum for transport was shifted to a higher pH (similar to that seen in Caco-2 cells). Thus, the role of this protein may be to adapt the pH optimum of hPepT1 towards the physiological pH seen in the intestinal lumen, although the way in which it does this is not known.

Conclusions

In this review we have sought to highlight specific recent studies that have shed light on mechanism. It is apparent that there is still much to do, particularly in the area of structure. As with many other transporters, it is a weakness that we know nothing of the secondary or tertiary structure of the PepT family of membrane proteins. Very recent structural studies on tripeptide binding to the oligopeptide binding protein OppA of the Gram-negative bacterial peptide transporter [161] show the power of X-ray studies to obtain useful structural data (in this case performed using 20 different crystals of substrate-ligand complexes, each with a different amino acid at position 2 of the bound tripeptide ligand). From this have emerged some interesting initial insights into the principles by which a protein can bind a wide range of different peptide substrates. At least in these bacterial binding proteins the conclusion is that water molecules act as flexible adapters, matching the hydrogen-bonding requirements of the protein to its

peptide ligand, thus shielding charges on the buried ligand. This use of water by OppA to broaden the repertoire of its binding site contrasts sharply with other proteins which use water to help bind ligands highly selectively. In a companion paper [162] the authors also point to some other important chemical principles of peptide binding, commenting that 'the tripeptides fall into three series of ligands, which have been designed to examine the effects of small changes to the central side chain. The results show a definite preference for the binding of hydrophobic residues over the positively charged side chains, the latter binding only weakly due to unfavourable enthalpic effects. Within the series of positively charged groups, a point of lowest affinity has been identified and this is proposed to arise from unfavourable electrostatic interactions in the pocket, including the disruption of a key salt bridge. Marked entropy-enthalpy compensation is found across the series, and some of the difficulties in designing tightly binding ligands are thus explained'. It is clear that as soon as a structural approach is feasible, similar studies will have to be carried out for mammalian peptide transporters to see if these principles have been retained in evolution; indirect functional evidence would suggest they may well be since there are obvious functional similarities with respect to for example the contribution of hydrophobic residues.

More generally, the broad substrate specificity of the PepT family of transporters raises questions for other families of transporters that have not yet been studied with this new knowledge. For example, are nonpeptides potential substrates for the ATP-dependent peptide transporters (TAPs) found in the endoplasmic reticulum of antigen-presenting cells? It is interesting that recent studies show that modified peptides are translocated substrates for these transporters [163]. Investigation of substrate-binding properties of different classes of peptide transporter may be mutually beneficial if novel strategies for therapeutic interactions can be discovered through the building of bridges between different fields.

Note added in proof:

A third oligopeptide transporter from *Caenorhabditis elegans* has recently been described, which interestingly is shown to act both as a H^+-coupled peptide transporter and as a H^+ channel (Fei Y. J., Romero M. F., Krause M., Liu J. C., Huang W., Ganapathy V. et al. (2000) A novel H^+-coupled oligopeptide transporter (OPT3) from *C. elegans* with a predominant function as a H^+ channel and an exclusive expression in neurons. J. Biol. Chem. **275:** 9563–9571). The authors named this protein OPT3 (Genbank accession number AF142441); however it is identical at the amino acid sequence level to the predicted *C. elegans* protein OPT2 (accession number O01840) reported by Wilson et al. [16], although there is a discrepancy to the chromosomal location of the coding gene F56F4.5 (I versus III respectively). In this latest paper of Fei et al. [15] CPTA is correctly renamed OPT1 (to which it is identical [16]), while CPTB [15] is renamed OPT2, although it is much more closely related to the predicted protein OPT3 [16] (see section 'Exploring the *C. elegans* genome' for details and table 1 for accession numbers).

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