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REGIONAL EXPRESSION AND DIETARY REGULATION OF RAT SMALL INTESTINAL PEPTIDE AND AMINO ACID TRANSPORTER mRNAs

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Summary. RT-PCR was used to obtain rat small intestinal cDNAs for two peptide transporters, showing conclusively for the first time that both are present in normal intestinal mucosa. Sequencing of these cDNAs showed them to be highly homologous and similar to two different types of peptide transport proteins from either colorectal carcinoma cells (Caco-2) or human and rabbit intestine. An even distribution profile of steady state levels of mRNA for both peptide transporters was observed along the longitudinal axis of small intestine. Both were upregulated in the distal regions of intestine by a high protein diet. Also, high levels of the rat high affinity glutamate transporter EAAC1 were observed in the distal intestine. These results suggest that the distal regions of small intestine play an important role in the absorption of some amino acids and peptides. Furthermore this area appears to be a primary site where dietary-induced changes in peptide and amino acid transport occurs.

Under normal conditions, dietary protein undergoes a series of degradative steps carried out by the hydrolytic enzymes originating from the stomach, pancreas and small intestine. This proteolytic activity results in a mixture of free amino acids and small peptides which are efficiently absorbed by the small intestinal enterocyte. Thus cellular transport of amino acids and small peptides is a key final step in their assimilation by the intestine and is mediated by carrier-transporters of varying specificities. Two distinct types of brush border membrane associated transport systems are involved in this process: 1. amino acid transport which may or may not be sodium dependent, 2. transport of small peptides (2-3 amino acids) coupled to a H^+-gradient.

Intestinal peptide transport is of major nutritional significance in that the intraluminal products of protein digestion are predominantly small peptides (1,2). Also many amino acids are more rapidly and efficiently absorbed in peptide form (3). Peptide transport is also associated with the intestinal uptake of peptidomimetic drugs such as ß-lactam antibiotics (4) and several drugs currently used as inhibitors of angiotensin-converting enzyme (5). At the present time the number and types of membrane transport proteins involved in the translocation of amino acids and peptides

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has not been determined and until recently detailed knowledge regarding their molecular structure has been lacking.

During the past several years a number of groups have reported the cloning and sequencing of amino acid/peptide transporters from a variety of sources (6-11). These studies indicate that a number of these transporters are present in the mammalian small intestine, however little is known regarding their intestinal distribution, expression and regulation. Current evidence suggests that there may be several types of peptide transporters energized by a H^+-gradient (12,13). Two have been recently cloned and sequenced and shown to be structurally very different from one another. One isolated from Caco-2 cells (9) is related to the cadherin family of adhesion proteins while the second type from rabbit and human intestine (10,11) contains multiple membrane spanning domains typical of other transporters (14). To date their respective roles in the cellular transport of peptides and other macromolecules remains unknown.

Therefore in an effort to begin to understand the role and function of these two peptide transporters in the intestinal absorption of nutrients and drugs, we isolated, cloned and partially sequenced both of them from rat small intestine. Using rat cDNA probes for these two transporters and probes for two recently cloned membrane proteins associated with transport of acidic (8) and neutral amino acids (7), we looked at the distribution of their respective mRNAs along the longitudinal axis of rat small intestine and examined the effect of various protein diets on their levels of expression.

Materials and Methods

Wistar rats (240-260g, Simonsen Labs, Gilroy, CA) were maintained on a low protein (4% casein) diet for 7 days and then switched to an isocaloric high protein (50% gelatin) diet for 14 days. Control rats were fed an isocaloric diet consisting of 17.5% casein for 7 days. The rational for the diets used has been previously described (15). Rats were sacrificed from each diet group and the entire small intestine removed. The intestine was divided into three equal length segments from which total RNA was prepared by the procedure of Chirgwin (16).

Northern analysis. Total RNA (10 µg) was electrophoresed in formaldehyde-agarose (1%) gels, then transferred and fixed to a nylon membrane. The membranes were hybridized using either of the following dCTP[^{32}P] cDNA probes: 1. mouse angiotensin-converting enzyme (ACE, EC 3.4.15.1) (17), 2. rat dipeptidyl peptidase 1V (DPPIV, EC 3.4.14.5) (18), 3. rat glutamate transporter EAAC1, clone CX5-1 (8), 4. rat D_2 neutral/dibasic amino acid transporter (7), 5. the two rat oligopeptide transport proteins described in this work. The ACE probe was kindly provided by Dr. Kenneth Bernstein (Emory University, Atlanta, GA) and the DPPIV probe was the generous gift of Dr. Yukio Ikehara (Fukuoka University, Fukuoka, Japan). The glutamate (EAAC1) and neutral/dibasic (D_2) amino acid transporter probes were the kind gift of Dr. Matthias Hediger (Harvard Medical School, Boston, MA). After autoradiography, the amount of transporter/enzyme RNA was quantified by densitometry and RNA was stained on the membrane with methylene blue to assess the integrity and loading of RNA (19).

Isolation of peptide transporter cDNAs. Oligonucleotide primers (20-24 bp) (Table 1) were chosen that spanned various portions of the cDNA squences of two previously published peptide transporter proteins (9,10). The oligonucleotides were synthesized by the UCSF Biomolecular Resource Center (San Francisco, CA). Total RNA (1 ug) from rat small intestine was reverse transcribed with random primers using a SuperScript II RNase H$^-$ Reverse Transcription Kit from Life Technologies (Gaithersburg, MD). A PCR reaction was performed with the reverse transcriptase product, dNTP's, 0.5 µM specific primers and Taq polymerase. Amplification was carried out for 35 cycles consisting of denaturation (94 °C, 40 s), annealing (45 °C, 1 min) and extension (72 °C, 3 min). PCR products were electrophoresed in agarose gels containing ethidium bromide, DNA standards and then photographed. PCR products chosen for further study were first amplified, then ligated into a vector and transformed into competent cells

using a TA Cloning Kit from Invitrogen (San Diego, CA). Positive colonies containing inserts were cultured, plasmids isolated and the cDNA insert prepared following excision with the appropriate restriction enzymes.

Sequencing of rat peptide transporter cDNAs. The cloned PCR products were sequenced at the UCSF Biomolecular Resource Center using an Applied Biosystems model 373A fluorescence sequencer. The sequence gap between the two rat PepT 1 clones (Table 1) was determined using PCR (primers: 5'-GCTGTTGCCCTAATTGTGTTTGTC-3' and 5'-ATTTTCCCAGTCATGGTCGTTG-3' and cDNA generated from random primed rat intestinal RNA) followed by cycle sequencing using SequiTherm reagents and protocols (Epicentre Technologies). The nucleotide sequences were submitted to GenBank with accension numbers L46873 and L46874. Compilation and analysis of sequences was performed using IBI MacVector and Intelligenetics Suite software.

Results

The sequences of the PCR products used as molecular probes were compared to homologous proteins from other species (Fig. 1A). Rat Pept 1 (rPepT 1) allowed the deduction of 438 amino acid residues, exhibiting 86% similarity to the homologous human protein (11) and 82% similarity to the rabbit protein (10). Hydrophobicity analysis indicated that the protein encoded by the rat cDNA contained 9 potential membrane spanning domains located in similar positions as found in the human and rabbit proteins (Fig. 2). Thus, the overall structures of the proteins is very similar. The potential N-glycosylation site present between the first and second putative membrane spanning domains of the human and rabbit proteins is absent at residue 21 of the rat protein. An extra N-glycosylation sit is present however, in the postulated large extracellular domain between segments 9 and 10 (Fig. 1A). Similar to its homologues, the rat protein has a protein kinase C site present at residue 328. A cyclic AMP dependent protein kinase A site is present at residue 333, similar to the rabbit protein but unlike the human which lacks this site.

The PCR product rat pt-1 (rpt-1) gave the sequence encoding 578 residues of the rat protein. The rat protein had 77% similarity to the human protein (Fig. 1B). In spite of this relatively low similarity between the two proteins, 100% identity was observed in the 5 cadherin repeated elements originally identified by Dantzig et al. (9), (Fig. 1B). Hydrophobicity analysis of rpt-1 and its human homologue indicates these proteins to be much more hydrophilic than those in the first group with multiple membrane spanning domains (Fig. 2). Similar to its human

Table 1. Oligonucleotide Primers used in RT-PCR

cDNA	Position	Sequence	Product
PepT 1[a]	96-117	5'-TGAGTTCTGCGAAAGGTTCTCC-3'	743 bp
	838-816	5'-CGTATTTCTCCTTAGCCCAGTCC-3'	
PepT 1[a]	855-877	5'-GCAGATCAAGATGGTTACGAGGG-3'	593 bp
	1447-1425	5'-TGACCACTCGGTAGTTATTGGGG-3'	
hpt-1[b]	245-268	5'-CCAATCCTCCTGCTGTGACTTTTG-3'	1783 bp
	2027-2004	5'-AAGGAAGACCCCCCTACTTCTGTG-3'	

[a] From reference 10.
[b] From reference 9.

homologue, rpt-1 encoded multiple potential glycosylation sites suggesting that the protein contains a major, hydrophilic extracellular domain. (Fig. 1B).

Northern analysis of rat peptide transporter mRNAs from intestinal segments of rats maintained on a normal (control) diet is shown in Fig.3A. For both rPepT 1 and rpt-1 a relatively even distribution of mRNA along the longitudinal axis of small intestine was apparent. Also a single major species of mRNA (3.0 & 2.7 kb respectively) was observed for both of the peptide transporters. Following the switch from a low protein to a high protein diet (Fig.3B), a 1.5-2 fold increase in rPepT 1 mRNA was observed in the middle and distal intestine. Interestingly there was a corresponding increase in rpt-1 mRNA in these two segments.

Northern analysis of mRNA for the two putative amino acid transport proteins D_2/rBAT and EAAC1 are also shown in Figs.3A, 3B. The neutral/dibasic transporter D_2/rBAT displayed a even intestinal distribution and two species of mRNA (2.7 & 3.6 kb) were present. Also the different protein diets had no apparent effect on the steady state levels of intestinal D_2/rBAT mRNA. In contrast, the glutamate transporter (EAAC1) showed a more pronounced gradient of mRNA along the proximal-distal intestinal axis with the highest levels observed distally. After the change in protein diets there was a 2-3 fold increase in EAAC1 mRNA observed in the middle intestine. Little change was observed in the proximal and distal regions of the intestine.

Previously we have shown that steady state levels of mRNA for two intestinal brush border membrane peptidases, angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPPIV), are influenced by diets of varying protein composition, particularly those containing high (50%) gelatin (14). In the present study Northern analysis of intestinal mRNA for these two proteins were included as positive controls. As seen in Figs. 3A and 3B, ACE typically has a pronounced gradient of mRNA along the intestinal axis with the highest levels found in the proximal-middle regions. After the switch in protein diets, ACE mRNA levels were increased in all areas of the intestine, especially the proximal region (3-5 fold). On the other hand, DPPIV mRNA was somewhat more evenly distributed along the small intestinal axis with elevated levels found distally (Fig. 3A and 3B). Rats maintained on the high protein diet had elevated levels of DPPIV mRNA particularly in the distal intestine (1.3-1.5 fold increase).

Discussion

This study presents the first description of the distribution, expression and dietary regulation at the mRNA level of two recently identified peptide transporters along the longitudinal axis of rat small intestine. The rat peptide transporter cDNA clones that were isolated and used in this study show a high degree of homology and structural similarity to their counterparts originally

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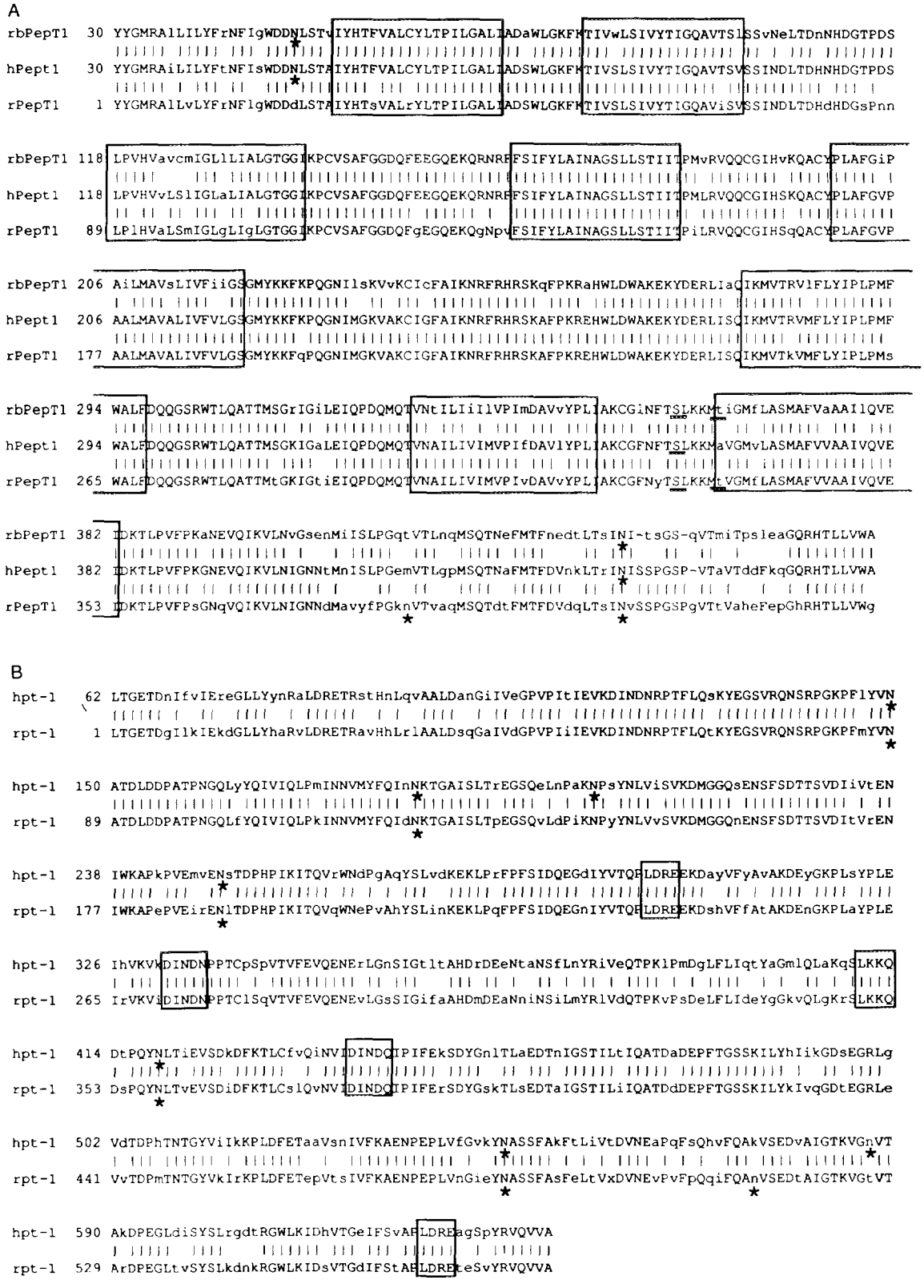
Figure 1. Comparison of rat peptide transporter sequences to known mammalian homologues. Alignments were performed using the Intelligenetics GENALIGN program. A. Comparison of the rabbit (rbPepT 1) (10), human (hPepT 1) (11), and rat (rPepT 1) proton coupled transporters. Boxed sequences indicate potential N-glycosylation sites, and underlined residues are potential protein kinase sites as described in the text. B. Comparison of the rat cDNA (rpt-1) to the human sequence (hpt-1) determined by Dantzig et al. (9). Boxed sequences represent conserved regions with similarity to cadherins, asterisks denote potential N-glycosylation sites.


A
rbPepT1 30 YYGMRAlLILYFrNFIgWDDNLSTvIYHTFVALCYLTPILGALIADaWLGKFKTIVwLSIVYTIGQAVTSLSSvNeLTDnNHDGTPDS
hPept1 30 YYGMRAiLILYFtNFIsWDDNLSTAIYHTFVALCYLTPILGALIADSWLGKFKTIVSLSIVYTIGQAVTSVSSINDLTDHNHDGTPDS
rPepT1 1 YYGMRAlLvLYFrNFlgWDDdLSTAIYHTsVALrYLTPILGALIADSWLGKFKTIVSLSIVYTIGQAViSVSSINDLTDHdHDGsPnn
rbPepT1 118 LPVHVavcmIGLlLIALGTGGIKPCVSAFGGDQFEEGQEKQRNRFFSIFYLAINAGSLLSTIITPMvRVQQCGIHvKQACYPLAFGiP
hPept1 118 LPVHVvLSlIGLaLIALGTGGIKPCVSAFGGDQFEEGQEKQRNRFFSIFYLAINAGSLLSTIITPMLRVQQCGIHSKQACYPLAFGVP
rPepT1 89 LPlHVaLSmIGLgLIgLGTGGIKPCVSAFGGDQFgEGQEKQgNpVFSIFYLAINAGSLLSTIITPiLRVQQCGIHSqQACYPLAFGVP
rbPepT1 206 AiLMAVsLIVFiiGSGMYKKFKPQGNIlsKVvKCIcFAIKNRFRHRSKqFPKRaHWLDWAKEKYDERLIaQIKMVTRVlFLYIPLPMF
hPept1 206 AALMAVALIVFVLGSGMYKKFKPQGNIMGKVAKCIGFAIKNRFRHRSKAFPKREHWLDWAKEKYDERLISQIKMVTRVMFLYIPLPMF
rPepT1 177 AALMAVALIVFVLGSGMYKKFqPQGNIMGKVAKCIGFAIKNRFRHRSKAFPKREHWLDWAKEKYDERLISQIKMVTkVMFLYIPLPMs
rbPepT1 294 WALFDQQGSRWTLQATTMSGrIGiLEIQPDQMQTVNtILIiILVPImDAVvYPLIAKCGiNFTSLKKMtiGMfLASMAFVaAAIlQVE
hPept1 294 WALFDQQGSRWTLQATTMSGKIGaLEIQPDQMQTVNAILIVIMVPIfDAVlYPLIAKCGFNFTSLKKMaVGMvLASMAFVVAAIVQVE
rPepT1 265 WALFDQQGSRWTLQATTMtGKIGtiEIQPDQMQTVNAILIVIMVPIvDAVvYPLIAKCGFNyTSLKKMtVGMfLASMAFVVAAIVQVE
rbPepT1 382 IDKTLPVFPKaNEVQIKVLNvGsenMiISLPGqtVTLnqMSQTNeFMTFnedtLTsINI-tsGS-qVTmiTpsleaGQRHTLLVWA
hPept1 382 IDKTLPVFPKGNEVQIKVLNIGNNtMnISLPGemVTLgpMSQTNaFMTFDVnkLTrINISSPGSP-VTaVTddFkqGQRHTLLVWA
rPepT1 353 IDKTLPVFPsGNqVQIKVLNIGNNdMavyfPGknVTvaqMSQTdtFMTFDVdqLTsINvSSPGSPgVTtVaheFepGhRHTLLVWg
B
hpt-1 62 LTGETDnIfvIEreGLLYynRaLDRETRstHnLqvAALDanGiIVeGPVPItIEVKDINDNRPTFLQsKYEGSVRQNSRPGKPFlYVN
rpt-1 1 LTGETDgIlkIEkdGLLYhaRvLDRETRavHhLrlAALDsqGaIVdGPVPIiIEVKDINDNRPTFLQtKYEGSVRQNSRPGKPFmYVN
hpt-1 150 ATDLDDPATPNGQLyYQIVIQLPmINNVMYFQInNKTGAISLTrEGSQeLnPaKNPsYNLViSVKDMGGQsENSFSDTTSVDIiVtEN
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rpt-1 177 IWKAPePVEirENlTDPHPIKITQVqWNePvAhYSLinKEKLPqFPFSIDQEGnIYVTQPLDREEKDshVFfAtAKDEnGKPLaYPLE
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rpt-1 265 IrVKViDINDNPPTClSqVTVFEVQENEvLGsSIGifaAHDmDEaNniNSiLmYRlVdQTPKvPsDeLFLIdeYgGkvQLgKrSLKKQ
hpt-1 414 DtPQYNLTiEVSDkDFKTLCfvQiNVIDINDQIPIFEkSDYGnlTLaEDTnIGSTILtIQATDaDEPFTGSSKILYhIikGDsEGRLg
rpt-1 353 DsPQYNLTvEVSDiDFKTLCslQvNVIDINDQIPIFErSDYGskTLsEDTaIGSTILiIQATDdDEPFTGSSKILYkIvqGDtEGRLe
hpt-1 502 VdTDPhTNTGYViIkKPLDFETaaVsnIVFKAENPEPLVfGvkYNASSFAkFtLiVtDVNEaPqFsQhvFQAkVSEDvAIGTKVGnVT
rpt-1 441 VvTDPmTNTGYVkIrKPLDFETepVtsIVFKAENPEPLVnGieYNASSFAsFeLtVxDVNEvPvFpQqiFQAnVSEDtAIGTKVGtVT
hpt-1 590 AkDPEGLdiSYSLrgdtRGWLKIDhVTGeIFSvAPLDREagSpYRVQVVA
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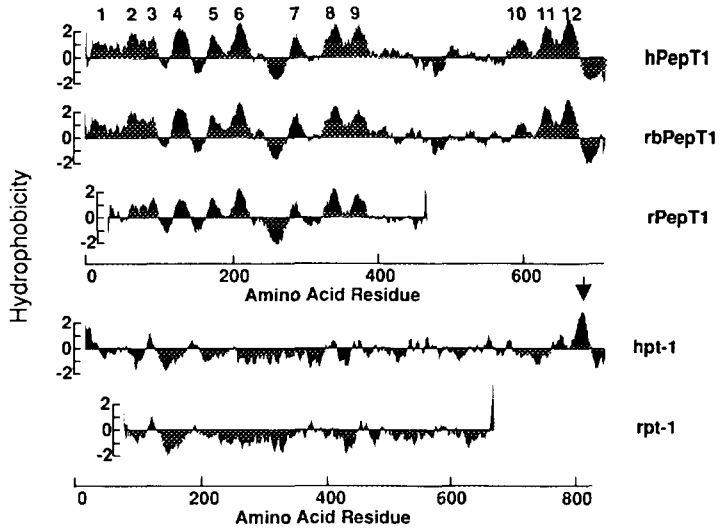

Figure 2. Hydrophobicity analysis of the two types of peptide transporters. Clones are designated as in Figure 1. Hydrophobicity was determined using the Kyte and Doolittle scale (27) with a 21 residue window. The numbers above the top graph indicate putative membrane spanning domains of the proton-coupled transporter. The arrow indicates a putative membrane spanning domain.

isolated from Caco-2 cells (9) (hpt-1) and rabbit (10) and human (11) small intestine (PepT 1). In the present study we have found that the steady state levels of mRNA for both types of transporter had a relatively even distribution along the rat small intestinal axis. In the rabbit the highest levels were observed in the jejunum (10). Interestingly, levels of their respective mRNAs were both increased some 1.5-2 fold by a high protein diet showing that diet plays a role in modulating levels of these two proteins. These changes occurred primarily in the middle-distal segments which is consistent with observations regarding other intestinal proteins such as ACE and DPPIV that tend to be differentially regulated along the intestinal axis. Regulation of peptide transport is likely to be a complex phenomenon influenced by a number of factors as suggested by the presence of protein kinase C and cAMP dependent phosphorylation sites in the rPepT 1 protein. Recent studies have indicated that protein kinase C (20) and calmodulin-dependent processes (21) are important in peptide transport regulation.

The structure of the rPepT 1 protein is similar to that described for other transport proteins in that it has multiple membrane spanning domains (10,11). However the rpt-1 transport protein with a single membrane domain and calcium binding cadherin elements suggests possible functional similarity to the rat D_2/rBAT protein which is involved in the transport of amino acids (6,7,22). The D_2/rBAT protein has sequence similarity to glycosidases, potential calcium binding ligands and may be part of a multimeric amino acid transport complex in which it serves a regulatory role (6,7,21,23). In this regard it is of interest to note that we observed parallel segmental changes in rPept 1 and rpt-1 mRNA levels in response to diet. This may suggest that these two proteins have some as yet undefined functional association in peptide transport.

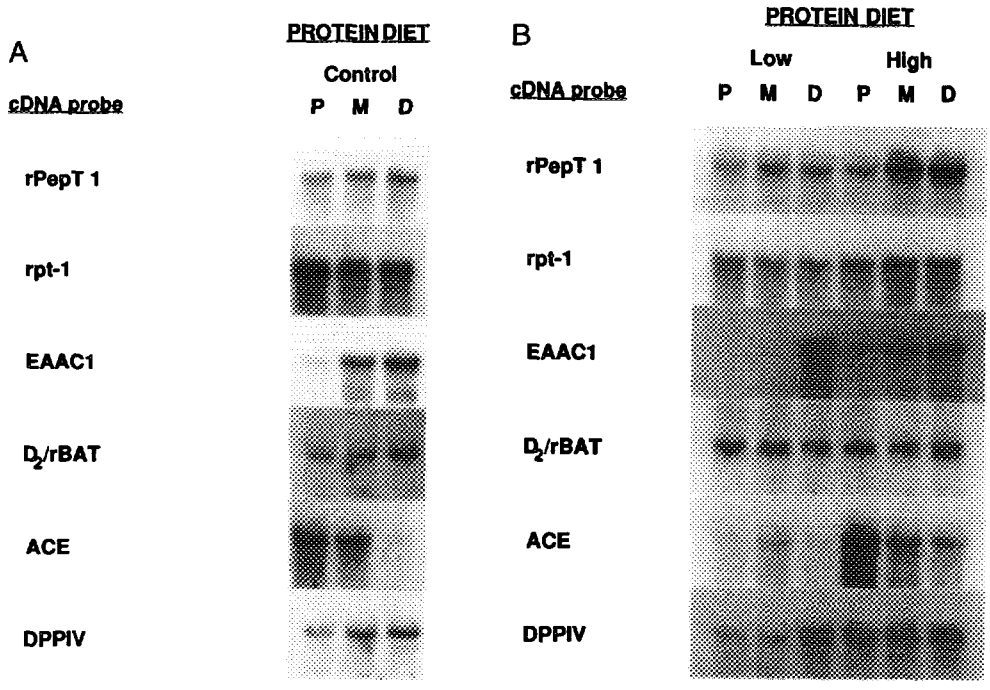

Figure 3. Northern analysis of rat small intestinal peptide/amino acid transporter and enzyme mRNAs. A. Rats maintained on a control protein diet (17% casein) for 7 days. Total RNA was isloated from proximal, middle and distal (P,M,D) intestinal segments and used in the analysis. B. Rats initially maintained on a low protein diet (4% casein) for 7 days and then switched to a high protein diet (50% gelatin) for 14 days. Northern analysis was performed on RNA samples from three animals in each of the three diet groups. Representative data from a single animal is shown in each case. Equal loading and transfer of RNA was monitored by methylene blue staining (19) of membranes after autoradiography (not shown). cDNA probes used: rPepT 1, rat peptide transporter (743 bp, Table 1); rpt-1, rat peptide transporter (1783 bp, Table 1); EAAC1, rat high affinity glutamate transporter (8); D_2/rBAT, rat neutral/dibasic amino acid transporter (7); ACE, angiotensin-converting enzyme (17); DPPIV, dipeptidyl peptidase IV (18).

The two amino acid transport proteins D_2/rBAT and EAAC1 had different distribution patterns along the small intestine. Surprisingly mRNA levels for the high affinity glutamate transporter (EAAC1) were high in the distal region, suggesting that this is an important area for transport of acidic amino acids. Also of interest was the effect of the high protein diet on the levels of the two amino acid transporter mRNAs. EAAC1 mRNA levels were increased while D_2/rBAT levels were unchanged. These observations are consistent with the idea proposed earlier that the small intestine has the ability to upregulate its absorptive capacity for nonessential amino acids (glutamic and aspartic acid) whereas transporters for essential amino acids may or may not be upregulated (24).

Little is currently known about the distribution and peptide transport capacity of various regions of the intestine although it is currently believed that the bulk of peptides and amino acids are transported in the proximal regions of the small intestine (25). However the results of this study show that for some types of amino acids and peptides the primary site for absorption may be

distal intestine. Also, the distal intestine is apparently the site where the primary adaptive response of these transporters to changes in the diet occurs. Finally it is of interest to note that previous studies from our laboratory showed that nearly 50% of the tetrapeptide Leu-Gly-Gly-Gly was transported intact by the rat small intestine (26). Similarly, in the recent description of the cloned peptide transporter from rabbit intestine, intact transport of tetraglycine was reported (10). Thus cloning of the peptide transporters from an experimental animal model such as the rat will afford us the unique opportunity to assess their specificities, regulation and role in the transport of various types of peptides and clinically relevant drugs.

Acknowledgments

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