

**Cloning, Expression, and Developmental and Dietary Regulations of a
Chicken Intestinal Peptide Transporter and Characterization and
Regulation of an Ovine Gastrointestinal Peptide Transporter Expressed
in a Mammalian Cell Line**

by

Hong Chen

Dissertation submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Animal Science

Dr. E. A. Wong, co-chair
Dr. K. E. Webb, Jr., co-chair
Dr. J. R. Bloomquist
Dr. D. M. Denbow
Dr. H. Jiang

September 28, 2001

Blacksburg, Virginia

Key Words: Peptide Transport, PepT1, Northern blot, Dietary protein, Development,
Protein Kinase.

Copyright 2001, Hong Chen

Cloning, Expression, and Developmental and Dietary Regulations of a Chicken Intestinal Peptide Transporter and Characterization and Regulation of an Ovine Gastrointestinal Peptide Transporter Expressed in a Mammalian Cell Line

Hong Chen

(ABSTRACT)

To study peptide absorption in chickens, an intestinal peptide transporter cDNA (cPepT1) was isolated from a chicken cDNA library. The cDNA was 2,914-bp and encoded a protein of 714 amino acid residues. Twenty-three di-, tri-, and tetra-peptides were used for functional analysis of cPepT1 in *Xenopus* oocytes and Chinese hamster ovary (CHO) cells. For most di- and tripeptides tested, the K_t was in the micromolar range, except Lys-Lys and Lys-Trp-Lys. Northern analysis demonstrated that cPepT1 is expressed strongly in the small intestine, and at lower levels in kidney and cecum. These results demonstrated the presence and functions of a peptide transporter in chickens.

cPepT1 mRNA abundance was evaluated in response to developmental and dietary regulations. In Experiment 1, eggs at incubation day 18 (E18) and Cobb chicks after hatch (d 0) were sampled before treatments. Three groups of chicks were fed diets containing 12, 18, or 24% crude protein (CP). Feed intake of chicks fed the 18 or 24% CP diets was restricted to that of chicks fed the 12% CP diet. In Experiment 2, a fourth group with free access to the 24% CP diet was added. cPepT1 mRNA abundance was quantified from northern blots. By d 0, there was a 50-fold increase in cPepT1 mRNA abundance compared with E 18. In chicks fed the 12% CP diet, cPepT1 mRNA abundance decreased throughout the 35 d. Chicks fed 18 or 24% CP diets showed an increase in cPepT1 mRNA abundance with time. In chicks with free access to the 24% CP diet, cPepT1 mRNA decreased until d 14 but returned to an intermediate level at d 35. Our results indicate that cPepT1 mRNA is regulated by both dietary protein and developmental stage.

To investigate the kinetics of an ovine peptide transporter (oPepT1), CHO cells were transfected with oPepT1 cDNA. Uptake of Gly-Sar by transfected cells was pH-dependent, concentration-dependent, and saturable. Competition studies showed that all di-, tri-, and tetra-peptides inhibited uptake of Gly-Sar. Pretreatment of the cells with staurosporine resulted in an increase in peptide transport. This increase was blocked by pretreatment with PMA. The results indicate that protein kinase plays a role in oPepT1 function.

Key Words: Peptide Transport, PepT1, Northern blot, Dietary protein, Development, Protein kinase

Acknowledgements

I thank Drs. E. A. Wong and K. E. Webb, Jr., my committee chairmen, for their guidance, inspiration and assistance during my graduate training and in the preparation of this manuscript. Without their efforts and support, I would never have continued my academic development at Virginia Tech.

I am indebted to Dr. J R. Bloomquist for his guidance, comments and discussions of my data throughout my training at Virginia Tech. I am grateful to Drs. D. M. Denbow and H. Jiang for their interest, encouragement, and willingness to serve as my committee members.

I thank the John Lee Pratt Fellowship for providing the opportunity for me to study animal nutrition and pursue advanced education here at Virginia Tech.

I am grateful to Drs. Dongmin Liu and Xiaobo Lin for their assistance, friendship, and many valuable discussions that facilitated my research.

I especially thank Donald Shaw, Kristin Lee, Julia Pugh, Martha McCollum, Lisa Gazillo, Alice Kuo, Brian Weng, and Larry Kuehn for their friendship, technical discussions and support during my research.

I give my deepest appreciation to my dear husband, Dr. YuanXiang Pan, for his support, understanding, and suggestions under all circumstances, and for his love and friendship.

Table of Contents

Abstract	ii
Acknowledgments	iii
List of Tables	vi
List of Figures	vii
Chapter I. Introduction	1
Chapter II. Review of Literature	3
<i>Introduction</i>	3
<i>Structural Requirements for Substrate Recognition by PepT1 and PepT2</i>	4
<i>Identification of Inhibitors for PepT1 and PepT2</i>	9
<i>Genomic Analysis of Peptide Transporter Genes</i>	11
<i>Regulation of Peptide Transporter Expression and Activity</i>	13
<i>Dietary Factors</i>	15
<i>Nervous System</i>	18
<i>Hormonal Regulation</i>	20
<i>Growth Factor-Mediated Regulation</i>	22
<i>Developmental Regulation of PepT1</i>	24
<i>Regulatory Effects of PepT1 in the Small Intestine</i>	25
<i>Effect of PepT1-mediated Uptake on Amino Acid Transporter</i>	25
<i>Effect of PepT1-mediated Uptake on Neutrophil-Epithelial Interactions</i>	28
<i>Novel Peptide Transporters</i>	30
<i>Tetra- and Pentapeptide Transporters</i>	30
<i>PepT2 Expression in Lung</i>	31
<i>Peptide Transporters in Intestinal and Renal Basolateral Membranes</i>	32
<i>Summary</i>	34
Chapter III. Cloning and Functional Expression of a Chicken Intestinal Peptide Transporter (cPepT1) in <i>Xenopus</i> Oocytes and Chinese Hamster Ovary Cells	36
<i>Abstract</i>	36
<i>Introduction</i>	37
<i>Materials and Methods</i>	38
<i>Construction and Screening of a Chicken Intestinal cDNA Library</i>	38
<i>Northern Blot Analysis</i>	39
<i>Functional Expression in <i>Xenopus</i> Oocytes</i>	40
<i>Expression in CHO Cells</i>	41
<i>Results</i>	43
<i>Sequence and Structure of the Chicken Intestinal PepT1 cDNA</i>	43

<i>Tissue Distributions of cPepT1 mRNA</i>	44
<i>Functional Features of cPepT1</i>	45
<i>Discussions</i>	47
Chapter IV. Dietary Protein Level and Stage of Development Affect Expression of the Intestinal Peptide Transporter (cPepT1) in Chickens	60
<i>Abstract</i>	60
<i>Introduction</i>	61
<i>Materials and Methods</i>	62
<i>Animals and Tissue Sampling</i>	62
<i>Experiment 1</i>	63
<i>Experiment 2</i>	64
<i>Northern Blot Analysis</i>	64
<i>Results</i>	65
<i>Feed Intake and Growth Rate</i>	65
<i>cPepT1 mRNA Abundance in Experiment 1</i>	66
<i>cPepT1 and Beta-Actin mRNA Abundance in Experiment 2</i>	67
<i>Discussions</i>	68
Chapter V. Characterization and Regulation of a Cloned Ovine Gastrointestinal Peptide Transporter (oPepT1) Expressed in a Mammalian Cell Line	82
<i>Abstract</i>	82
<i>Introduction</i>	84
<i>Materials and Methods</i>	85
<i>Cell Culture</i>	85
<i>Transfections</i>	85
<i>Transport Assay</i>	86
<i>In Vitro Regulation Assay</i>	87
<i>Calculations and Statistics</i>	87
<i>Results</i>	87
<i>Functional Expression of oPepT1 cDNA in CHO Cells</i>	87
<i>pH and Concentration Effects on Gly-Sar Uptake</i>	88
<i>Inhibition Effects of Peptides on Gly-Sar Uptake</i>	89
<i>Regulation of oPepT1 Activity by Protein Kinase C in CHO Cells</i>	90
<i>Discussions</i>	91
Chapter VI. Epilogue	101
Literature Cited	107
Vita	126

List of Tables

Table

3.1. Kinetics of peptide transport in oocytes and CHO cells expressing cPepT1-Leu70353
3.2. Comparison of kinetic constants of cPepT1-Leu703 and cPepT1-Ser703 in transfected CHO cells54
4.1. Composition of the experimental diets (% , as fed)74
5.1. Kinetics of oPepT1 in CHO cells transfected with oPepT1 cDNA95

List of Figures

Figure

3.1. Alignment of predicted amino acid sequences of human, rat, mouse, rabbit, sheep and chicken PepT1	55
3.2. Putative membrane spanning model of cPepT1	56
3.3. Northern blot analysis of cPepT1 mRNA in chicken tissues	57
3.4. pH and ion-dependency of cPepT1 in cRNA-injected oocytes and cPepT1-transfected CHO cells	58
3.5. Kinetic parameters of Gly-Sar uptake by cPepT1 measured by recording current amplitudes in oocytes injected with cPepT1 cRNA and [³ H]-Gly-Sar uptake in CHO cells transfected with cPepT1 cDNA	59
4.1. Body weight at sampling time from Experiment 1 and 2	75
4.2. Northern blot analysis of cPepT1 mRNA expression during embryonic day 18 (E 18) and day of hatch (d 0)	76
4.3. Northern blot analysis of cPepT1 mRNA expression from Experiment 1	77
4.4. Densitometric analysis of cPepT1 mRNA abundance from Experiment 1	78
4.5. Northern blot analysis of cPepT1 mRNA expression from Experiment 2	79
4.6. Densitometric analysis of cPepT1 mRNA abundance from Experiment 2	80
4.7. Densitometric measurement of beta-actin mRNA expression from experiment 2	81
5.1. Time course for oPepT1 expression and uptake incubation	96
5.2. pH dependency in oPepT1-transfected CHO cells	97
5.3. Kinetic parameters of Gly-Sar measured in CHO cells transfected with pBK-oPepT1 expression vector	98
5.4. Inhibition of Gly-Sar uptake by different classes of peptides	99
5.5. Influence of a protein kinase C activator or inhibitor on Gly-Sar uptake in pBK-oPepT1 transfected CHO cells	100

Chapter I

Introduction

Peptide transporters were identified by molecular cloning in several mammalian species in the past decade. The two major peptide transporters characterized, the intestinal PepT1 and the renal PepT2, have opened a new era in research of peptide transport. PepT1 and PepT2 have been shown to direct proton-coupled absorption of small peptides and some clinically important drugs such as beta-lactam antibiotics. Therefore, recent research has been focused on both nutritional and pharmaceutical aspects of peptide transport.

Reviewed in this dissertation are some recent studies on basic mechanisms of peptide transport, nutritional and physiological impacts of peptide transport, and regulation of peptide transport for nutritional and clinical applications. Studies of interactions between peptide transporters and their substrates or inhibitors provide tools for further research to elucidate the mechanisms of peptide transport and to design suitable drugs for transport by peptide transporters. Analysis of genomic structures of PepT1 and PepT2 opens the possibility for generating targeted gene knockout mice. Studies on regulation of PepT1 and PepT2 expression facilitate the future discovery of pathways of nutritional and physiological controls of peptide transport.

Although peptide transport is found to be important in organisms from bacteria to mammals, little information is available on peptide absorption in avian species. The studies in this dissertation were to first identify peptide transporters in chickens. The cloned chicken peptide transporter, cPepT1, was then characterized in *Xenopus* oocytes and

Chinese hamster ovary cells. Tissue distribution of cPepT1 in chicken was investigated. Effects of developmental and nutritional changes on cPepT1 abundance were then studied in chickens fed different dietary protein levels from embryonic stage to 5 wk of age. The transport characteristics of an ovine PepT1 that was cloned in our laboratory was also studied by in vitro expression in Chinese hamster ovary cells. Results from these studies provide information on nutritional and physiological relevance of peptide transport in these food-producing animals. Knowledge gained from these studies will facilitate further research on mechanisms of peptide transport and control of the transporter expression and transport activity. All the information will enhance our ability to manage animals for better production.

Chapter II

Review of Literature

Introduction

A transport system for small peptides is widely present in single cell organisms as well as plants and animals. One of the unique features of peptide transport is the dependence on a proton gradient as the driving force instead of a Na^+ gradient (Leibach and Ganapathy, 1996). The specific but broad range of substrates for transport also is of particular interest. In mammals, two peptide transporters, PepT1 and PepT2, have been cloned and characterized intensively. PepT1 and PepT2 are distinct in their tissue distributions, substrate kinetics, and possible specific roles in different tissues. PepT1, which is predominantly expressed in the small intestine, has been shown to have nutritional importance and potential clinical and pharmaceutical applications (Daniel, 1996; Leibach and Ganapathy, 1996; Adibi, 1997). PepT2, found in the kidney, the central nervous system, and several other peripheral tissues, is believed to have complicated functions in various tissues and is very important for pharmaceutical applications as a transporter of certain drugs (Daniel and Adibi, 1994; Daniel and Herget, 1997; Wang et al., 1998; Lin et al., 1999).

To date, studies of peptide transporters have been focused largely in four general areas. The first is the study of the basic mechanisms of peptide transporters. This area is always of great interest because the fundamental functions of PepT1 and PepT2 are binding

and translocating substrates, mostly small peptides and some important peptidomimetic drugs. A second area is the study of nutritional and physiological impacts of peptide transport. The existence of such peptide transporters, especially the abundant expression of PepT1 in the small intestine of various species, leads to investigations on the roles of peptide transporters in nutrient absorption and balance, nutrient-gene interaction, and physiological balance of the local environment. Pharmaceutical and clinical applications of peptide transporters, especially of the cloned PepT1 and PepT2, are yet another area of investigation. One of the main driving forces in peptide transport research is the application of the peptide transporter systems as a better or an alternate drug delivery method. This research has focused on designing suitable drug molecules for targeted or more efficient drug delivery via peptide transporters. Finally, regulation of peptide transporters has become an area of investigation. To achieve a better understanding of peptide transport systems, the mechanisms that control the expression of the peptide transporter genes and the activity of the transporter proteins need to be elucidated.

This chapter focuses on summarizing the recent findings about the intestinal peptide transporter PepT1, its nutritional and physiological roles, its regulatory mechanisms, and possible substrates. Studies on the related PepT2 are included for comparisons between PepT1 and PepT2.

Structural Requirements for Substrate Recognition by PepT1 and PepT2

After identification of the two major peptide transporters, PepT1 and PepT2, many studies have been carried out to clarify the characteristics of both transporters. Despite the significant similarities in the primary and secondary structures of the two transporter

proteins, transport characteristics (i.e., proton gradient driving force, electrogenicity, and substrate range), and cellular locations, PepT1 and PepT2 differ markedly in their tissue distribution with PepT1 predominantly found in the small intestine with low levels in kidney and PepT2 found in various tissues such as kidney, brain, and lung (Leibach and Ganapathy, 1996). In addition to differences in their tissue distribution, remarkable differences in PepT1/PepT2 substrate affinity have been found. Generally, PepT2 shows higher affinity for chemically diverse peptides as well as amino beta-lactam antibiotics than PepT1 (Amasheh et al., 1997a; b; Terada et al., 1997). Nonpeptidic compounds such as valacyclovir, an oral prodrug of the antiherpetic agent acyclovir, are preferentially recognized by PepT2 rather than PepT1 (Ganapathy et al., 1998; Sawada et al., 1999a). These results suggest that PepT1 and PepT2 have low and high affinity properties, respectively. It is generally accepted that the differences in substrate transport by PepT1 and PepT2 may lie in their different affinity for, rather than in their specificity for substrates.

A recent study shows that anionic beta-lactam antibiotics without an alpha-amino group appear to have a higher affinity for PepT1 than for PepT2 (Terada et al., 1997). To investigate the functional role that the α -amino group of substrates has on affinity to both PepT1 and PepT2, inhibition of radiolabeled Gly-Sar uptake was measured in the presence of a number of di- and tripeptides along with some nonpeptide substrates (Terada et al., 2000b). In both LLC-rPepT1 and LLC-rPepT2 cells, Gly-Sar uptake was greatly inhibited by di- and triglycine, but not by Gly or tetraglycine. Most cyclic dipeptides tested, which lack free amino and carboxyl groups, showed no inhibitory effect on Gly-Sar uptake in

either PepT1 or PepT2 transfectants. This finding supports the hypothesis that the free amino and/or carboxyl groups play an important role in substrate interaction with the peptide transporters (Döring et al., 1998a; b; Ganapathy et al., 1998; Sawada et al., 1999a), although further studies are needed to examine cyclic peptides other than cyclic dipeptides.

Inhibition studies were performed in both PepT1 and PepT2 transfected cells using various di- and tripeptides (Terada et al., 2000b). PepT2, as compared to PepT1, had a higher affinity for all dipeptides and tripeptides tested. Carnosine and the anticancer agent bestatin, which has a beta-amino group instead of an alpha-amino group, also showed higher affinity for PepT2. Dipeptides with a modified alpha-amino group such as N-methyl-glycylglycine and N-formyl-methionylalanine, showed much lower affinity for both PepT1 and PepT2 than their original counterparts (Gly-Gly for N-methyl-glycylglycine and Met-Ala for N-formyl-methionylalanine). Nevertheless, these peptides still had higher affinity for PepT2 than for PepT1. Nonpeptide drugs, including valacyclovir, δ -aminolevulinic acid (δ -ALA), and alanine-4-nitroanilide, all had potent inhibitory effects on PepT2 rather than PepT1, whereas 8-amino-octanoic acid (8-AOA) did not.

Valacyclovir and δ -ALA, which both have a free amino group, have higher affinity for PepT2 than PepT1 (Döring et al., 1998a; Ganapathy et al., 1998; Sawada et al., 1999a). Furthermore, alanine-4-nitroanilide tested in this study, with an amide bond and a free amino group, showed more inhibition of uptake by PepT2 than PepT1. On the other hand, 8-AOA without an α -amino group preferentially bound PepT1 to PepT2. This confirms previous results showing that the anionic beta-lactam antibiotics, such as ceftibuten, have higher affinity for PepT1 than PepT2 (Terada et al., 1997). Together these data show that,

PepT1 has lower affinity for various substrates when compared to PepT2 and that the α - or beta-amino carbonyl group may be the key structure in determining the differences in affinity between PepT1 and PepT2.

Structural requirements for the recognition and binding of substrates to the transporter protein remain inconclusive. In an attempt to define the structural domains that influence the functional characteristics of PepT1 and PepT2, Terada et al (2000a) constructed chimeric peptide transporters consisting of an N-terminal half (transmembrane domains 1-6) of rat PepT1 and a C-terminal half (transmembrane domains 7-12) of rat PepT2 (PEPT-N1C2) and the reciprocal (PEPT-N2C1). Uptake studies on these two chimeric molecules showed that Gly-Sar and amino beta-lactam antibiotics had higher affinity for PEPT-N2C1 than for PEPT-N1C2. On the other hand, ceftibuten and cefixime were preferentially transported by PEPT-N1C2. These findings indicate that PEPT-N1C2 and PEPT-N2C1 possess substrate recognition characteristics of PepT1 and PepT2, respectively. Moreover, Gly-Sar uptake was maximal at pH 5.5 in both PepT1- and PEPT-N1C2-expressing cells, whereas maximal uptake was seen at pH 6.5-7.0 in PepT2- and PEPT-N2C1-expressing cells. The pH profiles of Gly-Sar uptake should be closely related to the amino acid residue that serves as the H^+ -binding site. Therefore, results from this study suggest that the H^+ -binding site of both PepT1 and PepT2 is located in the N-terminal half. The substrate recognition sites of both PepT1 and PepT2 are also located in the N-terminal halves. This is in contradiction to the previous study suggesting that transmembrane domains 7, 8, and 9 may be responsible for substrate recognition (Fei et al.,

1998). Further studies are needed to confirm the proposed regions on the transporters responsible for contacting substrates and proton molecules.

A more detailed study was conducted focusing on the N-terminal half of PepT1 (Chen et al., 2000b). Mutations were generated for histidine residues at amino acid positions 57 (H57), 111 (H111), and 121 (H121) of rabbit PepT1 that are predicted to be in the transmembrane segments, as well as tyrosine residues adjacent to H57. The H57 mutation did not show much transport activity. Mutations of Y56 and Y64 from tyrosines to phenylalanines caused a slight decrease in transport activity, whereas mutations of these tyrosine residues into alanine exhibit no measurable transport activity. Therefore, comparisons between functions of wildtype and mutant PepT1 revealed that not only the H57 but also the aromatic residues near it were essential for the normal function of PepT1. The results support the concept (Fei et al., 1997) that the histidine participates in H^+ -binding and the flanking aromatic residues stabilize the charge on H^+ when interacting with the histidine. Mutations of H111 did not cause substantial changes. In contrast, the mutation at H121 (H121R, histidine to arginine) decreased the substrate affinity for Gly-Leu, Gly-Glu, and Gly-Lys by five-, 22-, and 13-fold, respectively. The affinity of mutation H121C (histidine to cysteine) decreased by 1.5-, seven-, and four-fold for Gly-Leu, Gly-Glu, and Gly-Lys, respectively. The negatively charged Gly-Glu requires protonation to be transported. The above result then suggests that H121 is involved in the protonation of acidic substrates. The 13-fold decrease in affinity for Gly-Lys in mutation H121R was probably due to electrostatic repulsion between R121 and Gly-Lys. When H121 was replaced by the neutral residue cysteine (H121C), the change in affinity appeared

to be less dramatic for Gly-Lys. Taken together, H57 and H121 are closely associated with the coupling of the ion H^+ and the recognition of transportable substrates, respectively.

In conclusion, these results from investigations indicate that the free amino and/or carboxyl groups of substrates play an important role in substrate interaction with the peptide transporters; the alpha- or beta-amino carbonyl group may be the key structure in determining the differences in affinity between PepT1 and PepT2; and the H^+ -binding site of both PepT1 and PepT2 is located in the N-terminal half of the transporters and is associated with histidine residues at amino acid positions 57 and 121.

Identification of Inhibitors for PepT1 and PepT2

Although the above mentioned studies provide some information on the location and structure of the substrate binding domain(s), available data are limited to results from experiments using chimeric mammalian peptide transporters generated from PepT1 and PepT2 or site-directed mutagenesis. The lack of specific high affinity inhibitors for the peptide transporters is the main limitation in studying substrate binding. Previous studies have identified a couple of potential inhibitors (Taub et al., 1997; Meredith et al., 1998; Abe et al., 1999). But the results need to be confirmed as to whether the agents are actually translocated. Also, the affinities of the inhibitors are too low as compared to regular substrates for PepT1 and PepT2.

A novel inhibitor for PepT1 has been identified (Knütter et al., 2001). The Lys-Pro derivative, Lys[Z(NO₂)]-Pro (Z, benzyloxycarbonyl), was tested for competition with regular dipeptides for uptake. Lys[Z(NO₂)]-Pro showed a concentration-dependent inhibition of Gly-Sar uptake with a K_i of $10 \pm 1 \mu\text{M}$ in Caco-2 cells. Compared to a K_i of

$350 \pm 90 \mu\text{M}$ for Lys-Pro, Lys[Z(NO₂)]-Pro has much higher affinity for PepT1. A very similar interaction with the substrate-binding site of PepT1 was also observed when PepT1 was heterologously expressed in *P. pastoris* cells. Uptake of Phe-Ala was inhibited in a dose-dependent manner by Lys[Z(NO₂)]-Pro with an apparent K_i of $5.1 \pm 0.4 \mu\text{M}$. Kinetic analysis of Gly-Sar uptake in the absence or presence of the inhibitor showed that the inhibitor increased the K_t for Gly-Sar by ~2-fold without significantly changing its V_{max} . This suggests that Lys[Z(NO₂)]-Pro inhibits PepT1-mediated Gly-Sar uptake in a competitive manner. In PepT1-expressing *Xenopus* oocytes, while regular Lys-Pro induced inward currents by electrogenic transport via PepT1, Lys[Z(NO₂)]-Pro did not show any measurable response. The presence of Lys[Z(NO₂)]-Pro reduced inward currents evoked by Gly-Gln and the inhibition was reversible as Gly-Gln induced currents returned to initial levels when Lys[Z(NO₂)]-Pro was washed out. HPLC analysis further confirmed the absence of Lys[Z(NO₂)]-Pro in cell extracts after incubation. Therefore, modification of the ϵ -amino group of the Lys residue turns Lys-Pro into a nontransportable molecule that binds to PepT1 with a significantly higher affinity. The modification (adding the 4-nitrobenzyloxycarbonyl) prevents the conformational change needed for translocation of the loaded carrier after binding, making this molecule a competitive inhibitor for PepT1 transport.

In the process of searching for substrates for peptide transporters, several groups of researchers discovered another class of inhibitors. Although they are peptide-like hypoglycemic agents, nateglinide and glibenclamide are not transported by PepT1 or PepT2 (Sawada et al., 1999b; Terada et al., 2000d). These two agents are observed to be

able to inhibit Gly-Sar uptake in both Caco-2 cells and PepT1- or PepT2-transfectants. The inhibition appears to be noncompetitive since the K_t of Gly-Sar uptake remains unchanged while the V_{max} is greatly reduced. The inhibitory effect is not due to nonspecific interaction because the other transport systems tested, the Na^+ -coupled alanine and α -methyl-D-glucoside transport, are not affected. Further studies on the effects of these hypoglycemic agents on peptide transporters may provide information regarding long-term administration of these drugs. For the purpose of studying peptide transporters, more studies of PepT1 employing these inhibitors along with the competitive inhibitor, Lys[Z(NO₂)]-Pro should provide new insights into the mechanisms of substrate recognition and binding to PepT1.

Genomic Analysis of Peptide Transporter Genes

While functions of PepT1 and PepT2 are well characterized and potential applications of the peptide transporters have been intensively studied, the relative importance of peptide transport in either the digestive tract or renal tubules remains unclear. From a nutritional perspective, it is generally assumed that peptide transport is more important than amino acid transport based on the fact that the end products of protein digestion in the lumen of the intestine are mainly in small peptide form (Leibach and Ganapathy, 1996; Ganapathy and Leibach, 1999). To date, the only reports of genetic defects in amino acid transport in intestinal epithelial cells are associated with defects in certain transporters of free amino acids. Since there are multiple amino acid transporters in the apical membrane of epithelial cells and their substrate specificities overlap each other, these defects rarely cause malnutrition. There are no reports of genetic defects of intestinal

peptide transport to directly address the importance of the peptide transport on protein nutrition. Targeted disruption of the *pept1* gene in an animal such as the mouse would provide a model with a defective intestinal peptide transporter. Use of this animal model would reveal the relative importance of intestinal peptide transport versus amino acid transporters. The same holds true for renal peptide transport. Functional studies show that the primary role of PepT2 protein may be the reabsorption of amino acids in small peptide form in the kidney. But the cell type-specific expression of PepT2 in other tissues suggests more specialized roles. All these need to be verified by specific animal knockout models.

Gene structures of *pept1* and *pept2* need to be clarified first in order to get targeted gene disruption. The cloning of PepT1 and PepT2 cDNAs from various species provided a first step towards identifying mouse PepT1 and PepT2 genes. The mouse PepT1 cDNA was cloned by screening a mouse kidney cDNA library using rat PepT1 cDNA as the probe (Fei et al., 2000). The mouse PepT2 cDNA was cloned by the Rapid Amplification of cDNA Ends (RACE) method based on the sequence information from rabbit PepT2 cDNA (Rubio-Aliaga et al., 2000). After confirmation by sequencing and functional characterization, mouse PepT1 cDNA was used to screen a bacterial artificial chromosome library of mouse genomic DNA, and mouse PepT2 cDNA was used to screen a mouse P1 artificial chromosome library to identify the *pept1* and *pept2* genes, respectively. Sequence analysis following screening revealed the gene structures of mouse PepT1 and PepT2. The *pept1* gene is approximately 38 kb long and has 23 exons and 22 introns. The promoter of the *pept1* gene does not have a typical TATA box. Promoter analysis showed that essential elements are present within 1,140 bp upstream of the transcription start site. The promoter

region contains regulatory elements such as GC-rich boxes and also binding sites for activators/regulators of transcription AP-1, Jun-B, c-Myb, c-Myc, GATA-1, and nuclear factor NF-E1. The potentially important regulatory elements for the expression of the *pept1* gene in the small intestine are the amino acid-responsive element (AARE) at -431 to -437 bp upstream and the transcription factor *cdx-2* binding site at -795 to -812 bp upstream (Fei et al., 2000). The *pept2* gene is 34 kb long and organized in 22 exons and 21 introns. The *pept2* gene was mapped on mouse chromosome 16. Interestingly, like the *pept1* gene, the *pept2* gene also has a TATA-less promoter with two major transcription start sites (35 and 235 bp upstream of the translation start site). Results also showed the presence of elements for the regulator AP-1, the CAAT-enhancer binding protein CEBP/B, IRF-2 and GRE-C, and the repressor E4BP4 (Rubio-Aliaga et al., 2000). The actual regulatory effects of all the identified elements and factors on the expression of the *pept1* and *pept2* genes remain to be investigated.

In conclusion, the cloning of the mouse *pept1* and *pept2* genes shows that the *pept1* gene is 38 kb long with 23 exons and 22 introns, while the *pept2* gene is 34 kb long with 22 exons and 21 introns. The analysis of their promoter regions reveals several potential regulatory elements and provides the basis for further investigation on the regulation of gene expression. The availability of the information on the structure of the genes opens the door to the targeted disruption of the peptide transporter genes. The creation of peptide transporter-specific knockout mouse models would directly address the importance and the specific roles of peptide transporters.

Regulation of Peptide Transporter Expression and Activity

Regulations of membrane-bound transporter proteins involve transcriptional factors at the gene level and also translational and protein processing controls. Regulation at the transcriptional level results in changes in gene expression expressed as variations in mRNA abundance. Regulation of protein translation and processing changes the number of functional proteins on the membrane. These regulations result in changes in overall transporter protein function.

Intestinal digestive and absorptive activities are influenced by multiple factors. Digestive enzymes, transporters, and ion channels are all reported to be regulated (Freeman et al., 1993; 1995b). Substrate availability appears to affect reprogramming of intestinal glucose transport (Jiang and Ferraris, 2001). Aging plays an important role in gene expression and activity of many proteins such as apolipoprotein B and intestinal fatty acid-binding protein (Lee et al., 2001). Food intake and glucocorticoids influence cholecystinin gene expression in the intestine (Hansen, 2001). While the EAAT1 amino acid transporter is upregulated during intestinal differentiation, another member of the same amino acid transporter group, EAAT3, does not respond to the changes during differentiation (Mordrelle et al., 2000). The cloning of PepT1 from various species has led to more detailed studies of the mechanisms involved with the regulation of intestinal peptide transporter expression and activity. Earlier studies showed that expression patterns of PepT1 were different in different sections of the small intestine as well as in different segments along the crypt-villus axis (Freeman et al., 1995a). Further research will likely confirm these findings and lead to the discovery of factors and mechanisms that influence the overall peptide transport activity.

Dietary Factors. It is well known that nutrient substrates are among the most significant factors regulating intestinal digestive enzyme and transporter activity. Earlier research showed that the activity of most digestive enzymes and transporters, such as hydrolases and monosaccharide and amino acid transporters, are all closely related to dietary intakes, although little is known about the mechanisms of dietary regulation (Buddington et al., 1990; Ferraris et al., 1992; Tolza and Diamond, 1992; Biviano et al., 1993).

Before the cloning of PepT1, there was clinical and physiological evidence showing that intestinal peptide uptake was regulated by dietary nutrients (Ferraris et al., 1988; Buddington and Diamond, 1989; Ferraris and Diamond, 1989; Erickson et al., 1995). PepT1 is the only transporter protein identified so far to be responsible for the intestinal uptake of small peptides and some peptidomimetic drugs. With the cloning of PepT1 from various species came considerable effort to investigate the mechanisms for dietary regulation of peptide transport.

Shiraga et al. (1999) studied the effects of dietary protein on rat PepT1 by using brush border membrane vesicles (BBMV). After rats were fed protein-free, 5%, 20%, and 50% casein diets, BBMV were prepared from the ileal section of the small intestine and Gly-Sar uptake was measured. Four days after the start of dietary treatment, the Gly-Sar transport activity in the ileum was decreased in the protein-free diet group. On the contrary, Gly-Sar uptake increased in BBMV from rats fed 50% casein. The Gly-Sar transport activity was about two-fold greater in rats fed 50% casein compared with those fed the protein-free diet. Kinetic data of Gly-Sar transport showed that the increased

transport activity was due to an elevation of V_{\max} but not K_m . Accordingly, northern blot and immunoblot analysis revealed that both PepT1 mRNA and protein levels increased about two-fold in rats fed 50% casein compared with rats fed the protein-free diet. The researchers also tested the effects of the dietary dipeptide, Gly-Phe, or the constituent amino acids, Gly or Phe, on Gly-Sar transport activity. In rats fed the Gly-Phe diet, Gly-Sar transport activity was about 2.6-fold higher than when rats were fed the protein-free diet. Interestingly, the Phe diet also induced a 2.7-fold increase in Gly-Sar transport activity compared to the protein-free diet. In contrast, the Gly diet did not change the level of the transport activity. Kinetic analysis showed that the increase in transport activity in rats fed Gly-Phe or Phe diet was due to an increase of V_{\max} but not K_m . Similar to results from the previous study with casein, feeding either Gly-Phe or Phe caused the PepT1 mRNA and protein levels to be elevated. These results suggest that PepT1 transport activity is induced by selective amino acids and peptides through the stimulation of its gene expression.

A more recent study on the rat *pept1* gene also showed upregulation of PepT1 under special dietary conditions (Ihara et al., 2000). Rats were given starving, semi-starving diets (50% of control), or total parenteral nutrition (TPN) for 4, 10, and 10 days, respectively. Total RNA from small intestinal sections was isolated and northern blot analysis was performed to study PepT1 and SGLT1 mRNA abundance. Results showed that the PepT1 mRNA in the starved group increased to 179% of the control value in the proximal portion of the small intestine. The PepT1 mRNA level in the TPN and semi-starved groups also increased to 161% and 164% of control, respectively. In contrast,

SGLT1 mRNA in all of the treatment groups did not change significantly. Returning to a normal diet and feeding pattern by the starved group resulted in a drastic decrease in PepT1 mRNA level. Western blot analysis of PepT1 protein expression confirmed the same pattern as mRNA abundance in all treatment groups. The study demonstrated that, under various malnourished conditions, PepT1 expression was greatly enhanced. The possible stimulatory mechanisms are currently unknown.

To further elucidate the molecular mechanisms of dietary regulation on rat PepT1, Shiraga et al. (1999) were the first to identify the promoter region of the rat PepT1 gene. A promoter of 1,620 bp flanking the 5' region was identified. The promoter region included typical TATA and GC box sequences. Regulatory elements for the octamer-binding protein, AP-1, AP-2, Cdx-2, hepatocyte nuclear factor 1, and an amino acid-responsive element were also found. To map the transcriptional regulatory elements of the PepT1 promoter, a series of 5'-deletion fragments were cloned in front of a luciferase reporter gene and the constructs were transfected into Caco-2, HepG2, and opossum kidney cells. The constructs containing proximal elements of the promoter (-351 to -1) displayed the most activity, suggesting that elements upstream of -351 bp may have a negative regulatory function. Deletions of -351 to -171 bp and -50 to -27 bp resulted in a 2.5-fold and a four-fold drop in activity, respectively, suggesting that these regions may contain important elements that regulate PepT1 transcription. Response of various amino acids and dipeptides were tested with the -351 bp construct. It showed that the region contains response elements for all the dipeptides tested. Asp-Lys strongly induced the reporter luciferase activity, whereas Gly-Sar, Gly-Phe, Phe-Val, and Lys-Phe slightly increased the

activity. Amino acids Lys, Arg, and especially Phe induced luciferase activities. The other amino acids tested, Gly, Asp, Gln, Val, and Ala did not change the activity. Since there are no data available to verify the stimulation from dipeptides, it is possible that the response was the result of amino acids hydrolyzed from the peptides absorbed inside the cell.

From these reports, it seems very likely that some amino acids and dipeptides directly stimulate the activation function of the PepT1 gene promoter. Transport activity thus is induced through elevated PepT1 gene expression. Further studies are needed to clarify the responsive mechanisms of the PepT1 promoter to amino acids and dipeptides.

Nervous System. Beta-lactam antibiotics are among the most important drugs that can be absorbed via PepT1. Many beta-lactam drug molecules have been tested for transport efficiency via PepT1. The goal is to achieve maximum absorption efficiency in order to use as little antibiotic as possible. Increased bioavailability of cefixime (a beta-lactam drug) was observed with the co-administration of nifedipine (NFP), a calcium channel blocker (Harcouët et al., 1997). The result is not due to increased local blood flow, morphological changes of the small intestine, or passive diffusion. Rather the neural blockage by either hexamethonium (HXM) or tetrodotoxin abolishes the interaction. Therefore, the authors hypothesize the involvement of neural networks.

To further investigate the possible neural mediation of transport by PepT1, several agents that act as agonists or antagonists at receptors on nerves or epithelial cells were tested (Berlioz et al., 1999). The effect of NFP on cephalexin (CFX) absorption was completely suppressed by addition of dipeptides confirming the total dependence of the CFX transport on PepT1. HXM, a nicotinic receptor antagonist, and lidocaine, a local

anesthetic, suppressed the effect of NFP without changing basal CFX absorption. On the other hand, atropine, a muscarinic receptor antagonist, capsaicin, an agent affecting extrinsic sensory neurons, clonidine, an alpha-2 adrenoceptor agonist, and isoproterenol, a beta adrenoceptor agonist, enhanced CFX absorption by the same magnitude as NFP. All these results suggest that complex neural networks, including nicotinic synapses, intestinal sensory neurons, and sympathetic noradrenergic fibers, are involved in modulating the function of PepT1.

The effects of the alpha-2 agonist, clonidine, which induce a two-fold increase in the intestinal absorption of CFX, was studied using a clone of Caco-2 cells stably expressing alpha_{2A}-adrenergic receptors (Caco-2 3B, Berlioz et al., 2000). The CFX uptake in Caco-2 3B cells responded to clonidine and resulted in a two-fold increase in CFX uptake compared to control Caco-2 cells. The stimulatory effect of clonidine was abolished by Gly-Sar. In Caco-2 3B cells, clonidine increased V_{max} of CFX transport without changing K_m . The clonidine effect was inhibited by colchicine treatment but was not altered by amiloride, indicating that microtubule integrity but not Na^+/H^+ exchanger activity was necessary. Therefore, direct activation by clonidine of alpha₂-adrenergic receptors located on epithelial cells resulted in increased PepT1 activity. The increased activity may have been induced by increased numbers of PepT1 molecules on the apical membrane that were translocated from an intracellular pool through microtubules. The molecular mechanisms by which the local neural networks modulate PepT1 activity remain to be investigated.

Hormonal Regulation. Insulin is an important hormone in metabolic regulation. The regulatory effects of insulin on tissues and cells are mediated through its receptor. Since insulin receptors have been found on both apical and basolateral membranes of intestinal epithelial cells (MacDonald et al. 1993; Baron-Delage et al. 1994), investigation into the possible regulatory effect of insulin on PepT1 was initiated (Thamotharan et al., 1999). Thamotharan et al. (1999) used Caco-2 cells as the in vitro model of small intestinal epithelium and the dipeptide, Gly-Gln, as the model substrate for PepT1. In the Caco-2 model system, previous studies showed that most Gly-Gln is transported into Caco-2 cells as intact dipeptide and that the transport of Gly-Gln was mediated by a peptide transporter similar to PepT1 (Adibi, 1997; Thamotharan et al., 1998). Gly-Gln uptake was stimulated two-fold after 60 min of preincubation with insulin (5 nM). Any longer preincubation or increase in the insulin concentration did not further affect Gly-Gln uptake. Binding of insulin to the insulin receptor was required because genistein, a tyrosine kinase inhibitor completely blocked the stimulatory effect of insulin without affecting the uptake of Gly-Gln by the Caco-2 cells.

Thamotharan et al. (1999) also investigated the mechanisms of the insulin effect. Kinetic data of Gly-Gln uptake from the insulin treated Caco-2 cells showed the presence of a single transport system, the same as in the control. Furthermore, data showed that insulin increased the V_{max} of Gly-Gln uptake (from 3.53 ± 0.61 to 6.31 ± 0.5 nmol/mg protein/5 min.) but did not affect the K_m . The authors concluded that there was only one system responsible for Gly-Gln uptake in insulin-treated Caco-2 cells and that insulin treatment did not alter the affinity of the transporter for Gly-Gln. The increased V_{max}

indicated that insulin increased the number of transporters in the apical membrane of the Caco-2 cells. This was confirmed by western blot analysis showing increased amounts of PepT1 protein in insulin-treated cells. On the other hand, quantitative northern blot analysis did not show any difference in PepT1 mRNA abundance between control and insulin-treated cells. To determine whether insulin has any direct effect on PepT1 translation, Caco-2 cells were treated with brefeldin. Brefeldin selectively disassociates the Golgi apparatus which is required for the processing of newly synthesized PepT1 protein. Result showed that brefeldin treatment did not have any effect on Gly-Gln uptake either in control or in insulin-treated Caco-2 cells. Finally, Caco-2 cells were treated with colchicines to depolymerize microtubules, which results in disruption of the translocation of proteins from internal pools to designated membranes. Results showed that colchicines did not have any effect on Gly-Gln uptake in the control cells but completely abolished the stimulatory effect of insulin in the treated cells.

The study by Thamotharan et al (1999) provided not only the first evidence of stimulation effects of a hormone on peptide transport but also a possible post-translational regulatory mechanism of PepT1. Results from another study showed that insulin treatment did not affect glucose transport in Caco-2 cells (MacDonald et al., 1993). Therefore, at least in Caco-2 cells, the stimulatory effect of insulin appears to be specific to PepT1 protein. Further studies are necessary to discover the possible location of the cytoplasmic pool of PepT1 protein before they are moved to the surface of the cell and the physiological reasons for having such a storage mechanism. Studies comparing the regulatory effects of insulin on PepT1 and the glucose transporter in Caco-2 cells will further elucidate the

reasons for the differential regulation of these two transporters. Whether the lack of response to insulin of the glucose transporter is due to the lack of an internal protein pool or whether other protein recruitment mechanisms are involved in the insulin regulation of PepT1 is yet to be determined.

Growth Factor-Mediated Regulation. Epidermal growth factor (EGF) is a small peptide growth factor consisting of 53 amino acid residues. It has stimulatory effects for the proliferation of epidermal cells in a variety of in vivo and in vitro biological systems (Fisher and Lakshmanan, 1990). Little is known about the role of EGF in tissue development and proliferation. Generally, EGF binds and activates its receptor (EGFR). The activation of EGFR results in changes in the steady-state mRNA concentration of a number of genes (Haley, 1990). In Caco-2 cells, for example, long-term treatment of EGF resulted in the down-regulation of sucrase-isomaltase (Cross and Quaroni, 1991). EGF acts mainly on the pretranslational level of sucrase-isomaltase expression by affecting its gene transcription and/or mRNA stability.

Nielsen et al. (2001) investigated the effects of EGF treatment on peptide transport and hPepT1 expression. In EGF-treated Caco-2 cells, EGF caused a decrease in both transepithelial transport and apical uptake of [^{14}C]-Gly-Sar. The inhibition values were as much as 50.2% of the control for transepithelial transport and 80% for apical uptake of [^{14}C]-Gly-Sar. Incubation of Gly-Sar on both apical and basolateral sides of Caco-2 cells revealed that EGF treatment reduced the V_{\max} of transepithelial transport and apical uptake of [^{14}C]-Gly-Sar but did not affect basolateral uptake. The V_{\max} of transepithelial transport of [^{14}C]-Gly-Sar decreased from 3.00 ± 0.17 in control cells to $0.50 \pm 0.07 \text{ nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$

in the EGF-treated cells. The difference observed between the apparent K_m in control cells (2.71 ± 0.31 mM) and in the EGF-treated cells (1.89 ± 0.28 mM) was not significant. For the apical uptake, V_{max} changed from 2.61 ± 0.4 in control cells to 1.06 ± 0.1 $\text{nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in EGF-treated cells. Meanwhile, the apparent K_m remained unchanged (0.66 ± 0.3 mM in control and 0.57 ± 0.2 mM in EGF-treated cells). RT-PCR analysis of hPepT1 mRNA showed that in EGF-treated cells, the level of hPepT1 mRNA was reduced to only 65% of that in control cells. Western blot analysis using anti-hPepT1 antibody showed a 35% decrease in band intensity of hPepT1 protein in EGF-treated cells compared with control cells. Therefore, EGF does have regulatory effects on hPepT1 activity by decreasing its mRNA transcription and protein expression.

Previous studies have shown that the EGF receptor is present on both apical and basolateral membranes of Caco-2 cells (Bishop and Wen, 1994). To test the source of EGF stimulation, Caco-2 cells were treated with EGF from either the apical or basolateral side (Nielsen et al., 2001). Results showed that cells treated with EGF on the apical side displayed the same Gly-Sar flux as in control cells without EGF treatment. In cells treated with EGF on the basolateral side, the flux was significantly lower than in control untreated cells.

Based on the investigation of EGF regulation, it was concluded that epithelial peptide transport activity is regulated by growth factors acting through their receptors located on the basolateral side of the enterocytes (Nielsen et al., 2001). This study showed that regulation of hPepT1 mRNA transcription and protein expression was most likely the mechanism for controlling peptide transport activity.

Developmental Regulation of PepT1 Expression. Kinetically it has been shown that amino acids are much more effectively absorbed from the intestine in peptide form in newborns than in adults (Himukai et al., 1980; Guandalini and Rubino, 1982). This developmental change was related to a difference in maximal transport capacity (V_{\max} of Gly-Gly, $51 \text{ nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in suckling guinea pigs versus $20 \text{ nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in adults) instead of substrate affinity (K_m , 2.4 and 2.1 mM in sucklings and adults, respectively). Furthermore, Gly-Gly uptake was substantially higher than that of Gly in fetal, newborn, and suckling rabbits (Guandalini and Rubino, 1982). Based on these results, some researchers have suggested the use of small peptides to substitute for free amino acids in enteral and parenteral solutions (Vazquez et al., 1993). After the cloning of the intestinal peptide transporter PepT1, further studies have indicated marked changes during development (Ganapathy et al., 1994; Leibach and Ganapathy 1996).

Shen et al (2001) investigated the molecular basis for age-related changes in peptide transport activity in both small intestine and kidney. Results show that rat PepT1 mRNA level and protein expression follow the same pattern throughout the developmental stages examined. At d 20 of fetal life, both PepT1 mRNA and protein were evident, but at low levels in all sections of the small intestine. Both PepT1 mRNA and protein reached peak levels by d 3 to 5. PepT1 mRNA levels then declined rapidly to 11 to 13% of the maximum by d 14. PepT1 protein expression also decreased rapidly. Both PepT1 mRNA and protein levels then rose so that by d 24, about the time of weaning, PepT1 mRNA and protein levels were 23 to 58% and 59 to 88% of the maximum, respectively. At postpartum d 75 (adult), PepT1 mRNA levels were approximately 25% and PepT1 protein

levels were 70% of that observed at d 3 to 5. These findings show that the expression pattern of PepT1 is parallel to its activity changes found in previous studies in guinea pigs and rabbits. Interestingly, significant PepT1 expression was observed in the colon at d 1 to 5 and then it rapidly decreased to undetectable level at d 14 and subsequently.

The results from these studies indicate that there is developmental regulation of PepT1 mRNA and protein. Expression of PepT1 before birth indicates a possible early gene response. Expression of PepT1 is induced postpartum, probably by suckling and weaning. The colon may take part in peptide transport in early life. Changes in PepT1 expression level may be responsible for different levels of transporter activity in young and adult lives.

Regulatory Effects of PepT1 in the Small Intestine

Effect of PepT1-Mediated Uptake on Amino Acid Transport Systems. The digestive products of proteins in the small intestine are mainly small peptides and free amino acids. The peptide and amino acid transport systems mediate amino acid uptake into the small intestine. There are several distinct transport systems available in the apical membrane of enterocytes that mediate the uptake of free amino acids (Malandro and Kilberg, 1996; Palacin et al., 1998), whereas at present, only one transport system has been identified for uptake of peptides. It is not clear to what extent the different systems contribute to overall amino acid absorption. It has been shown that amino acids do not interact with peptide transport by PepT1 (Brandsch et al., 1994; Liang et al., 1995). But little is known about the effects of peptide uptake on amino acid transport activities of the epithelial cells.

To elucidate the interactions between peptide transport and free amino acid influx, Caco-2 cells were used (Wenzel et al., 2001). Besides their confirmed PepT1 expression, Caco-2 cells have been shown to express a variety of transporters including those for cationic, anionic, and neutral amino acids (Ferruzza et al., 1995; Nicklin et al., 1995; Kekuda et al., 1997). Therefore, it is a good cell model to study the interactions between peptide transport and amino acid absorption.

Using Caco-2 cells, Wenzel et al. (2001) demonstrated the interaction of dipeptide transport with the apical influx of cationic amino acids. They first confirmed the extent to which the different amino acid transport systems contribute to L-Arg uptake. In their model, system $b^{0,+}$ (Na^+ -independent cationic and neutral amino acid transporter) mediated the majority of L-Arg influx (about 85%) and system y^+ (Na^+ -independent cationic amino acid transporter) accounted for only 15% of the influx. On the other hand, transport of L-Ala, as a neutral amino acid, into Caco-2 cells was mediated mainly by systems other than those responsible for L-Arg uptake. Therefore, the comparison of uptake of these two amino acids could be considered to show the specific effects on the $b^{0,+}$ system. The Caco-2 cells were preloaded with either dipeptides, the constituent free amino acids or the combination, then the uptake of L-Arg or L-Ala was quantified (Wenzel et al. 2001). Results showed that preincubation of Caco-2 cells with either neutral, mono- or dibasic dipeptides or the constituent amino acids (except Gly in free or dipeptide form) all led to an increase in the rate of L-Arg uptake compared to cells preincubated with buffer only. The stimulation was higher in preincubations with dipeptides than with free amino acids and was highest when a combination of dipeptides and free amino acids was used. A mixture

of Gly-Arg, Gly and Arg or Ala-Ala and Ala induced L-Arg transport up to 4.6-fold. Conversely, L-Ala transport was not significantly induced by any of the preincubation treatments. Therefore, the stimulation from the preincubation appears to be specific to $b^{0,+}$. Another interesting observation was that, when a hydrolysis-resistant dipeptide such as D-Phe-L-Ala or an aminopeptidase inhibitor amastatin was preloaded to prevent intracellular hydrolysis, the stimulatory effect of the dipeptides on the L-Arg influx was not observed. Moreover, amastatin did not have any effect on the stimulation caused by free amino acids. The half maximal stimulation of L-Arg influx occurred at about 489.7 μM with Gly-Arg and 534.7 μM with Lys-Lys. These EC_{50} values for the stimulation of L-Arg transport are very similar to the affinities of these peptides for transport by PepT1. Therefore, it appears that the transport of dipeptides into the cell rather than the loading of amino acids inside the cell causes the stimulation of amino acid influx by the $b^{0,+}$ system. The rate-limiting step for the stimulation would be the transport of dipeptides into the cell.

This study demonstrated for the first time the functional interaction between uptake of amino acids and peptides at the cellular level. The study provided evidence for PepT1 as a modifier of amino acid transport. By its high capacity, PepT1 may quickly transport large amounts of amino acids in the form of peptides into the cell and this may stimulate the uptake of limiting or essential amino acids such as Arg and Lys through the induced amino acid transporters. Therefore, PepT1 may help to increase uptake efficiency of limiting or essential amino acids by transporting peptides containing these amino acids and by stimulating uptake of these amino acids through their amino acid transporters. This information may lead to a new direction for improvement of balanced amino acid nutrition.

Effect of PepT1-Mediated Uptake on Neutrophil-Epithelial Interactions.

Intestinal epithelia have long been believed to act only as barriers to passive diffusion and selective transporters for nutrients, ions, and water. Recently it was shown that epithelial apical membranes recognize intestinal pathogens and induce secretion of chemoattractants from epithelia. The secreted chemoattractants participate in orchestration of neutrophil movement through the underlying matrix and also across the epithelial monolayer (McCormick et al., 1995; 1998). An important question to be answered is whether or not the major functions of epithelia such as transport of nutrients may also have direct effects on immune responses of intestinal epithelia.

Bacteria such as *Escherichia coli* release potent neutrophil chemotactic substances. It is known that at least a portion of such chemoattractant activity is related to small peptides with blocked NH₂ groups. One of the so-called n-formyl peptides, formyl-Met-Leu-Phe, is the major peptide neutrophil chemotactic factor produced by *E. coli* (Marasco et al., 1984), and thus has been widely studied as a model of n-formyl peptides. These types of n-formyl peptides are recognized by specific G protein-coupled integral membrane proteins on neutrophils (Bommakanti et al., 1992). Thus, n-formyl peptides likely influence the migration of neutrophils in the intestinal tract and affect neutrophil-enterocyte interactions. If peptides are involved, how are they delivered to neutrophils that are normally located in the subepithelial region?

As the only peptide transporter identified in the intestine, PepT1 is a potential transporter for these n-formyl peptides. Results from recent studies indicate that, in chronic disease states, hPepT1 is abnormally expressed in colonic epithelia, the site where

high concentrations of n-formyl peptides exist (Ziegler et al., 1998). Therefore, the possibility of the association of PepT1 with intestinal inflammation associated with n-formyl peptides induced neutrophil movement was examined (Merlin et al., 1998). The Caco-2 cell line was used as a model because of its well-known expression of PepT1 and also for its ability to form monolayers in culture dishes that mimic intestinal epithelium. The authors first showed that n-formylated tripeptide formyl-Met-Leu-Phe (fMLP) was transported into *Xenopus* oocytes expressing hPepT1. In addition, fMLP competitively inhibited uptake of a known hPepT1 substrate, Gly-Sar. In Caco-2 cells, apical fMLP uptake was also inhibited by several hPepT1 substrates such as Gly-Pro (78% inhibition), Gly-Leu (93% inhibition), and carnosine (93% inhibition). Therefore, PepT1 recognized bacterially derived peptide, fMLP, and was responsible for its uptake by PepT1-expressing enterocytes. The presence of fMLP actively directed migration of neutrophils across epithelial monolayers from the basolateral to apical side (19.9×10^4 versus 4.43×10^4 with and without fMLP gradient, respectively). Furthermore, transepithelial neutrophil migration induced by fMLP was significantly attenuated in the presence of some peptides such as Gly-Pro (18.1×10^4 versus 9.3×10^4) or Gly-Leu (18.1×10^4 versus 9.3×10^4). In a cell line that does not express hPepT1, the peptides did not affect neutrophil transmigration. Increasing the inwardly directed H^+ gradient across the apical membrane enhanced hPepT1-mediated absorption of fMLP. This resulted in a 70% increase in neutrophil transmigration (18.2×10^4 at luminal pH of 7.2 versus 30.9×10^4 at luminal pH of 5.2). Therefore, the results show that both negative and positive changes in hPepT1-

mediated fMLP absorption exhibit corresponding alterations in fMLP-driven migration of neutrophils across Caco-2 monolayers.

This study demonstrated for the first time that PepT1 could mediate uptake of a bacteria-derived peptide, fMLP, and that this event may play a role in neutrophil-epithelial interactions. Under normal conditions, PepT1 is rarely exposed to these chemotactic peptides. In the case of bacterial overgrowth, PepT1 expression is upregulated (Ziegler et al., 1998). The transepithelial transport of chemotactic peptides by PepT1 may lead to an inflammatory state resulting from movement of neutrophils. Further studies are needed to clarify the factors that upregulate PepT1 expression. This may help elucidate the pathways from chemoattractant recognition to transcriptional upregulation of PepT1 and subsequent effects of this transporter on epithelial-neutrophil interactions.

Novel Peptide Transporters

Tetra- and Pentapeptide Transporters. In microbes and plants, there is a class of peptide transporters named the OPT family that is responsible for uptake of small peptides of four to five amino acid residues (Lubkowitz et al., 1997; 1998). Among peptides of this size, enkephalins are endogenous pentapeptides involved in analgesia in the central nervous system. Existence of an enkephalin transporter in brain has been reported, but no protein has been identified in eukaryotes as the enkephalin carrier. Results from a recent study indicate that yeast cells expressing ORF YJL212C (open reading frame YJL 212C in *S. cerevisiae* genome) can sequester Met-enkephalin and Leu-enkephalin (Hauser et al., 2000). The transport of radiolabeled Leu-enkephalin showed saturable kinetics with a K_m of 310 μ M. Transport activity was optimal at an acidic pH suggesting an energy

dependence on a proton gradient. Chromatographic analysis indicates that the peptide was translocated intact across the cell membrane. The system was specific to tetra- and pentapeptides since the Leu-enkephalin transport was not inhibited by the presence of free amino acids and di- or tripeptides. But the Leu-enkephalin transport was inhibited by the addition of the opioid receptor antagonists such as naloxone and naltrexone. Naloxone and naltrexone are synthetic receptor antagonists used to reverse opiate overdose. This is the first report for a genetically defined eukaryotic transport protein responsible for transport of tetra- to pentapeptides such as enkephalin. The identification of this transporter in *Saccharomyces* opens the possibility for the existence of a homologue in higher species and may lead to discovery of such a transporter associated with the pain control system of the brain.

PepT2 Expression in Lung. The purpose of including lung PepT2 expression in this novel peptide transporter section is to state that continuous research on the identified PepT1 or PepT2 has led to discovery of novel functions of peptide transporters. This is important in terms of thoroughly understanding peptide transport networks in the whole animal.

Both mRNA abundance and protein expression of PepT2 were tested in lung (Groneberg et al., 2001). RT-PCR was used to detect PepT2 mRNA in lung extracts. In situ hybridization and immunohistochemistry techniques helped to locate the PepT2 mRNA in pneumocytes, bronchial epithelium, and endothelium of small arteries. Using murine whole-organ preparations, transport studies revealed that Ala-Lys-AMCA, a fluorophore-conjugated dipeptide derivative, was transported into bronchial epithelial cells

and pneumocytes. The transport was competitively inhibited by Gly-Gln and cefadroxil. Although the physiological role of peptide transport in the lung remains to be clarified, the finding of PepT2 activity in the lung provides the basis for pulmonary drug delivery through a peptide transporter. This may lead to the development of novel therapeutic strategies utilizing alternative drug delivery methods.

Peptide Transporters in Intestinal and Renal Basolateral Membranes. Both intestinal and renal epithelial cells have brush-border and basolateral membranes. The well-studied transporter systems such as the glucose transport system have asymmetric distribution of at least two distinct transporters (Baldwin, 1993; Hediger and Rhoads, 1994). The two groups of transporters contribute to the overall transepithelial uptake of glucose or other nutrients.

In the small intestine, there are reports indicating circulation of peptide-bound amino acids in portal blood from intestinal epithelial cells (Koeln and Webb, 1982; Seal and Parker, 1991). In addition, orally active beta-lactam antibiotics, which are absorbed via peptide transporters and are not broken down in the enterocytes, are released efficiently into the blood stream. These reports lead to the hypothesis that a peptide transporter(s) exists in basolateral membranes that is different from the known apical peptide transporters and is responsible for efflux of peptides and peptidomimetic drugs from the cell and into the blood. Although intensive studies have been conducted on the apical peptide transporters, little information is available on basolateral peptide transporters.

Based on the above information and hypothesis, the basolateral membranes of two epithelial cell lines were studied for possible peptide transport (Terada et al., 1999, 2000c).

Functional comparison of Gly-Sar uptake between apical peptide transport (PepT1) and basolateral peptide transport in Caco-2 cells showed that Gly-Sar uptake via basolateral peptide transporter was less sensitive to pH than PepT1 transport (Terada et al., 1999). The Gly-Sar uptake was mediated by a single peptide transporter as shown by kinetic analysis. The K_m of Gly-Sar was 0.65 mM for PepT1 mediated transport and 2.1 mM for basolateral peptide transporter mediated uptake. Tests of other peptides and peptide-like drugs showed that the basolateral peptide transporter has much lower affinity for all of the substrates than does PepT1. Based on these findings, it is proposed that the transport of peptides and peptide-like drugs across the basolateral membrane of the intestine is facilitated by a single basolateral peptide transporter that is different from the apical peptide transporter PepT1.

The basolateral peptide transport in the kidney epithelial cells has also been carried out on MDCK kidney cell line (Terada et al., 2000c). The renal basolateral peptide transport has a distinct pH profile from the intestinal basolateral transporter with more pH dependence. The kinetic analysis showed that the renal basolateral peptide transporter also differs from the intestinal peptide transporter, PepT1, and PepT2 in that it has a much lower K_m for Gly-Sar (71 μ M in MDCK cells compared to 2.1 mM for intestinal basolateral transporter, 650 μ M for PepT1, and 440 μ M for PepT2). Therefore, the low affinity of the intestinal basolateral peptide transporter may be advantageous for the function of facilitating the efficient efflux of accumulated peptides into the blood, whereas the higher affinity of the renal basolateral peptide transporter may contribute to the uptake of small peptides from the circulation. These studies confirm the presence of basolateral

peptide transporters both in intestinal and renal epithelial cells. Further research based on these results will lead to more knowledge on the entire peptide transport network.

Summary

Peptide transporters were identified in various species almost a decade ago. The cloning of PepT1 and PepT2 facilitated further study of the mechanisms of peptide transport, the relevance of the peptide transport in the whole animal, and the possible pharmaceutical and clinical application of peptide transport.

The studies on the interactions of peptide transporters with their substrates have identified possible structural requirements for substrate recognition and the regions on the transporters that are responsible for recognition and binding. Results from studies of novel inhibitors and substrates for peptide transporters provide tools for further research to elucidate the mechanisms of peptide transport and to design suitable drugs that can be transported efficiently by peptide transporters.

Identification of genomic structures of PepT1 and PepT2 opens the possibility for generating mice with targeted gene disruptions. Creation of knockout animals will provide a powerful tool to further study the physiological and nutritional roles of peptide transport in the whole animal.

The regulation of peptide transporters occurs at the level of gene expression as well as at the post-translational level. Dietary factors, developmental stages, and epidermal growth factors all have long-term effects on gene transcription and translation of PepT1. Hormones like insulin and local neural networks mainly influence the cytoplasmic

translocation of PepT1 from its cellular pools. All of these factors have profound impacts on the transport activity of PepT1.

As a major transporter expressed in the small intestine, PepT1 also has some regulatory effects on other systems. The transport of small peptides and peptidomimetics by PepT1 greatly affects free amino acid transporters and interacts with immune responsive elements in the small intestine.

Peptide transport in animals is much more complicated than in a single cell. Therefore, PepT1 and PepT2 may be only the first components in the complete transport system for peptide transport. There has been continuous discovery of novel peptide transporters as well as novel functions of identified proteins. Further efforts to find new members of the peptide uptake network will provide information on the contribution of peptide transport to whole-animal physiology and nutrition.

Chapter III

Molecular Cloning and Functional Expression of a Chicken Intestinal Peptide Transporter (cPepT1) in *Xenopus* Oocytes and Chinese Hamster Ovary Cells¹

ABSTRACT

To study peptide absorption in chickens, a chicken intestinal peptide transporter cDNA (cPepT1) was isolated from a chicken duodenal cDNA library. The cDNA was 2,914-bp long and encoded a protein of 714 amino acid residues with an estimated molecular size of 79.3 kDa and a pI of 7.48. cPepT1 protein is approximately 60% identical to PepT1 from rabbit, human, mouse, rat and sheep. Sixteen dipeptides, three tripeptides, and four tetrapeptides that contained the essential amino acids Met, Lys, and (or) Trp were used for functional analysis of cPepT1 in *Xenopus* oocytes and Chinese hamster ovary cells. For most di- and tripeptides tested, the substrate affinities were in the micromolar range, indicating that cPepT1 has high affinity for these peptides. Lys-Lys and Lys-Trp-Lys were exceptions with substrate affinities in the millimolar range. Neither free amino acids nor tetrapeptides were transported by cPepT1. Northern blot analysis using a full-length cPepT1 cDNA as the probe demonstrated that cPepT1 is expressed strongly in the duodenum, jejunum and ileum, and at lower levels in kidney and ceca. The present study demonstrated for the first time the presence and functional characteristics of a peptide transport system from an avian species.

Key Words: Peptide, Transport, Chicken, cPepT1

¹ All functional assays done in *Xenopus* oocytes were conducted by Dr. YuanXiang Pan

INTRODUCTION

Protein is a costly nutrient in poultry diets. Optimizing or improving the efficiency of protein or amino acid utilization will not only result in reduction of production costs, but also minimize excessive nitrogen pollution of the environment. Traditionally, it was believed that proteins had to be digested to single amino acids in order to be absorbed into cells in animals. However, it is now known that peptide transport is an important physiological process that occurs in tissues of animals (Matthews, 1991). The cloning and characterization of two structurally similar peptide transporters, PepT1 and PepT2, has provided valuable information about peptide transport in mammalian species (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995; Miyamoto et al., 1996; Pan et al., 2001). These peptide transporters recognize di- and tripeptide substrates, as well as pharmacologically active compounds (Leibach and Ganapathy, 1996).

Although peptide transporters have been cloned from the rabbit (Fei et al., 1994), human (Liang et al., 1995), rat (Saito et al., 1995), and mouse (Fei et al., 2000), nearly all functional analysis has focused on model peptide substrates or pharmacologically useful compounds. Less research has been conducted to examine the contributions of peptide transporters from a nutritional perspective. Recent reports from our laboratory demonstrate the existence and tissue distribution of a peptide transporter(s) in sheep, cows, pigs, and chickens (Matthews et al., 1996; Pan et al., 1997; Chen et al., 1999). These results suggest that peptide absorption may be nutritionally important in all domestic animals. The cloning and expression of an ovine peptide transporter (oPepT1) in our laboratory provided information for the first time on the molecular structure and basic functions of a peptide

transporter in food-producing animals (Pan et al., 2001). The purpose of this study was to analyze the peptide transport system in an avian species by cloning the chicken peptide transporter (cPepT1) and characterize the function of this transporter in vitro using both *Xenopus* oocytes and Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Materials. All chemicals, substrates, and reagents were of either molecular biology or cell culture grade. The ZAP Express cDNA synthesis system and Gigapack III were purchased from Stratagene (La Jolla, CA). Magna nylon transfer membranes were purchased from Osmonics, Inc. (Westborough, MA). Restriction enzymes were from New England BioLabs (Beverly, MA). The RNA transcription kit, mMMESSAGE mMACHINE was obtained from Ambion (Austin, TX). *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Amino acids and peptides (dipeptides to tetrapeptides) were purchased from Sigma Chemical Co. (St. Louis, MO). [³H]-Gly-Sar (specific radioactivity, 110 mCi/mmol) was purchased from Moravек Biochemical (Brea, CA).

Construction and screening of a chicken intestinal cDNA library. Poly(A)⁺ RNA was isolated from a pool of duodenal tissues collected from 15 adult Cobb broilers. A cDNA library was constructed using the ZAP Express cDNA synthesis system. Only cDNA of a size greater than 400 bp were used for library construction. Phage DNA containing cDNA were then packaged with Gigapack III Gold packaging extract and introduced into the XL1-Blue MRF['] *Escherichia coli* cell line. The phage library was plated out immediately on a series of large, 150-mm NZY agar plates (50,000 plaques/plate) for screening.

Positive clones were identified by plaque hybridization of the cDNA library transferred to Magna nylon transfer membranes. The cDNA probe used for screening was a cloned ovine peptide transporter (oPepT1; Pan et al. 2001). Hybridization was carried out for 16 h at 42 °C in a solution containing 50% formamide, 5 × Denhardt's solution, 6 × SSPE (1 × SSPE = 0.15 mmol/L NaCl, 10 mmol/L NaH₂PO₄, and 1 mmol/L EDTA), 0.5% SDS and 10 mg/L yeast tRNA. Post-hybridization washing was done under medium-stringency conditions, which involved washing twice with 5 × SSPE, 0.5% SDS at room temperature for 15 min, and twice with 1 × SSPE, 0.5% SDS at 42 °C for 15 min. Positive clones were subjected to three more rounds of screening using the same conditions. After the quaternary screening, 100% of the plaques showed positive hybridization by autoradiography.

The positive plaques identified after screening of the cDNA library were used to generate the excised pBK-CMV phagemid containing the cDNA insert. Sequencing by the dideoxynucleotide chain termination method was performed manually using a Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp., Cleveland, OH). Analysis of nucleotide and amino acid sequence was performed using the sequence analysis software Lasergene (DNASar, Inc, Madison, WI).

Northern blot analysis. Tissue distribution of cPepT1 mRNA transcripts was determined by northern blot. Poly (A)⁺ enriched RNA samples (10 µg) from different tissues were denatured and size-fractionated on a 1% agarose gel containing 2.2 mol/L formaldehyde. The 18s rRNA was visualized by ethidium bromide staining and used as an estimate of the amount of RNA loaded per lane. The size-fractionated RNA was then

transferred onto a nylon membrane and probed with the full-length cPepT1 cDNA. The probe was labeled with [α - 32 P]dATP (ICN Pharmaceutical, Costa Mesa, CA) by nick translation using DNA polymerase I/DNase I (Life Technologies, Gaithersburg, MD). Blots were hybridized overnight at 42 °C for 16 to 18 h and post-hybridization washing was done under medium stringency conditions. The blot was then exposed to Kodak XAR-5 film with an intensifying screen at -80 °C.

Functional expression in *Xenopus* oocytes. cRNA was synthesized using the RNA transcription kit mMESSAGE mMACHINE. For sense cRNA synthesis, phagemid containing the cDNA insert was transcribed in vitro by T₃ RNA polymerase in the presence of an RNA cap analog. For antisense cRNA synthesis, phagemid containing the cDNA insert was transcribed in vitro by T₇ RNA polymerase in the presence of an RNA cap analog. The resultant cRNA was purified and resuspended in nuclease-free water at a concentration of 1 g/L and stored frozen at -80 °C in aliquots.

Healthy *Xenopus* oocytes at stage V were defolliculated and the resting membrane potential was sampled (Goldin, 1992). Only oocytes with a resting membrane potential more negative than -30 mV one day after defolliculation were used for injection. Using a microinjection system, 25 ng of either sense cRNA or antisense cRNA were injected into the vegetal pole of each oocyte, near the polar interface. Antisense cRNA was used as a control. The injected oocytes were incubated in culture solution at 18 °C for 1 to 7 d.

The two-electrode voltage-clamp technique was used to characterize the induced peptide transport activity in oocytes injected with sense cRNA or antisense cRNA. All responses were monitored by a voltage-clamp amplifier (TEV-200, Dagan Corporation,

Minneapolis, MN), and analyzed by a MacLab (AD Instruments, Milford, MA), which is an analog-digital converter and software system that uses an Apple Macintosh computer for performing data acquisition (Soderlund et al., 1989). Normally, electrophysiological measurements in sense-cRNA- or antisense-cRNA-injected oocytes were carried out 3 to 6 d after injection. Only oocytes with a resting membrane potential more negative than -30 mV were used for recordings. An oocyte was perfused continuously with measurement buffer with or without peptide at a rate of 1.2 mL/min using a gravity feed perfusion system (Model BPS4, Ala Scientific Instruments, Inc., Westburg, NY). All peptide substrate solutions were prepared by dissolving the peptides in measurement buffer (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, 5 mmol/L HEPES, pH 6.0). All experiments were performed at room temperature (~21 °C).

Expression in CHO cells. CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% nonessential amino acids, and penicillin (100, 000 U/L)/ streptomycin (100 g/L). All cells were maintained in an atmosphere of 5% CO₂ at a relative humidity of 90% at 37 °C. One day before transfection, cells were trypsinized and plated onto 12-well plates at a density of 2.4 x 10⁵/well. For each well, 0.8 µg plasmid (with or without insert) were mixed with 2.4 µl Lipofectamine (2 g/L, Life Technologies, Gaithersburg, MD) in 40 µl OPTI-MEM (Life Technologies) and incubated at room temperature for 30 min. The DNA-lipid complex was then added to each well and cells were transfected for 5 hr at 37 °C. For each assay, at least two transfections were performed.

Uptake measurements were done 16 to 18 hr after transfection. The cells were washed three times with uptake buffer containing 25 mmol/L MES/Tris, pH 6.0, 5 mmol/L glucose, 0.8 mmol/L MgSO₄, 1.8 mmol/L CaCl₂, 5.4 mmol/L KCl, and 140 mmol/L NaCl. Gly-Sar solution was prepared at six concentrations (0.02 to 10 mmol/L, with 1 µCi/ml [³H]-Gly-Sar). Uptake solutions were added to each well and incubated for 20 min at room temperature. Uptake was stopped by washing cells with ice-cold uptake buffer. Cells were lysed by adding 0.5 ml 0.1% SDS and incubating at room temperature for 10 min. Uptake of [³H]-Gly-Sar was quantified by scintillation counting and the amount of protein present in each well was quantified using Bio-Rad DC Protein kit (Bio-Rad, Hercules, CA). Inhibition studies were performed under the same condition as described above, except that 20 µM [³H]-Gly-Sar (50 mCi/mmol) was used for radiolabeled substrate and five concentrations (0.001 to 10 mmol/L) of inhibitor peptides were added for inhibition studies.

Calculations and statistics. Kinetic parameters including the transport constant (K_t), the maximal current (I_{max}), and the concentration at 50% inhibition (IC_{50}) and all other calculations (linear and nonlinear regression analysis) were performed using PRISM (GraphPad, San Diego, CA). The experiments were carried out with five or six replicates, and results are presented as the mean \pm SEM. Data were evaluated using one way analysis of variance and the least significant difference test was used for post hoc comparisons (SAS Institute Inc. 1989).

RESULTS

Sequence and structure of the chicken intestinal PepT1 cDNA. The screening of a chicken duodenal cDNA library using the cloned ovine intestinal peptide transporter cDNA as the probe resulted in the isolation of a chicken intestinal peptide transporter cDNA (cPepT1). The cPepT1 cDNA was 2,914-bp long with an open reading frame of 2,142-bp. There was a typical Kozak consensus sequence, GCCGCC(A/G)CATGG (Kozak, 1987) flanking the initiation codon. A 69 bp 5'UTR and a 703 bp 3' UTR flanked the open reading frame. At the 3' end, the cDNA had a polyadenylation signal (AATAAA) 14 nt preceding the polyA tail. The encoded protein was predicted to have 714 amino acids with a molecular mass of 79.3 kDa and an isoelectric point (pI) of 7.48. Alignment results showed that the first fifteen amino acids of cPepT1 at the N-terminus were completely different from mammalian PepT1 (Figure 3.1). The predicted amino acid sequence of cPepT1 protein is 62.4, 62.5, 63.8, 64.8 and 65.1 percent identical to PepT1 from rabbit, human, mouse, rat and sheep, respectively. Hydrophobicity analysis (Kyte and Doolittle, 1982) indicated that cPepT1, like the mammalian PepT1, has 12 putative transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 (Figure 3.2). The model also predicts that both the amino terminus and the carboxyl terminus are on the cytoplasmic side of the membrane. About 84% identity is found in the transmembrane domain regions, whereas the large extracellular loop shows only 21% identity with PepT1 from other species. Sequence analysis also showed the presence of the PTR2 family signatures, which are the signature sequences of members of the proton-dependent oligopeptide transporter (POT) superfamily (Hofmann et al., 1999). The PTR2

sequences in the cPepT1 are the sequences spanning the second and the third transmembrane domains (GALIADSWLGKFKTIVSLSIVYTI) and the sequences in the core of the fifth transmembrane domain (FSIFYLAINAGSL, Figure 3.1).

The large extracellular loop contained four potential N-linked glycosylation sites at positions Asn 414 (N-F-S), Asn 423 (N-V-T), Asn 485 (N-F-T), and Asn 508 (N-I-T). An additional putative N-linked glycosylation site at position Asn 56 (N-F-S) in the extracellular loop between transmembrane domains 1 and 2 was observed. The predicted protein also contained one potential site for PKC-dependent phosphorylation in the intracellular loop between transmembrane domains 6 and 7 (Ser 272, S-E-K) and one potential site for PKA-dependent phosphorylation in the intracellular loop between transmembrane domains 8 and 9 (Thr 365, R-R-I-T).

Our screening identified two cPepT1 cDNA variants from the same cDNA library. The cDNA differed by a single amino acid alteration from Leucine to Serine at amino acid 703 (TTG→TCG). The clones were confirmed by sequencing the RT-PCR products from RNA pools from chicken intestine. These cPepT1 were designated as cPepT1- Leu 703 and cPepT1- Ser 703.

Tissue distribution of cPepT1 mRNA. A 2.9 kb mRNA was detected in the duodenum, jejunum, ileum, kidney, and cecum (Figure 3.3). mRNA from duodenal, jejunal, and ileal tissues showed the strongest hybridization while the kidney and cecum mRNA showed less hybridization. Poly(A)⁺ RNA from other tissues, including the liver, crop, proventriculus, pectoralis and fibularis longus muscles, showed no detectable hybridization.

Functional features of cPepT1. Two systems were used to investigate the function of cPepT1, the *Xenopus* oocyte expression system and a mammalian cell culture system. Two-electrode voltage-clamp analysis demonstrated that control oocytes (antisense cRNA injected) did not evoke any current with the substrates in all experiments. Inward currents are indicative of peptide transport into oocytes. In standard recording buffer, inward currents were detected in sense cPepT1 cRNA-injected oocytes after perfusion of 1 mmol/L Gly-Sar. Currents were greater ($P < 0.05$) at pH 6.0 and 6.5 compared to pH 5.0, 5.5 and 7.0 (Figure 3.4A). Therefore, the transport process was pH dependent with an optimal pH at 6.0 to 6.5. Substitution of Na^+ , K^+ , or Ca^{2+} did not have any effect on peptide transport activity of cPepT1 at any pH tested (Figure 3.4A). The inward currents in the standard measurement buffer induced by cPepT1 at all pH levels were similar to the values obtained in the absence of Na^+ , K^+ , or Ca^{2+} .

Expression of cPepT1 was also studied by measuring the uptake of [^3H]-Gly-Sar into transfected CHO cells. Uptake studies confirmed that, in cPepT1-transfected CHO cells, uptake was greater at pH 6.0 and 6.5 than at 5.0, 5.5, 7.0, and 7.5 (Figure 3.4B). Therefore, cPepT1 mediated Gly-Sar uptake had an optimal pH between 6.0 and 6.5.

Transport kinetics of cPepT1 was measured using the hydrolysis-resistant dipeptide Gly-Sar as a model peptide. Currents evoked when Gly-Sar was perfused at concentrations ranging from 0.01 to 10 mmol/L demonstrated that transport of Gly-Sar was saturable (Figure 3.5A). The K_t for Gly-Sar was 0.47 mmol/L. Uptake of Gly-Sar by cPepT1-transfected CHO cells showed a K_t of 2.6 ± 0.3 mmol/L and a V_{\max} of 34.6 ± 1.2 nmol/mg protein \cdot 20 min $^{-1}$ (Figure 3.5B). Therefore, the uptake of Gly-Sar in oocytes and CHO

cells expressing cPepT1 was concentration dependent and saturable. Transformation of the data from uptake of Gly-Sar resulted in a straight line in the Eadie-Hofstee plot ($r = 0.98$; Figure 3.5, insets), which indicated the presence of a single transport system responsible for the uptake of Gly-Sar in both oPepT1-injected oocytes and oPepT1-transfected CHO cells.

The substrate specificity of cPepT1 was investigated using both the *Xenopus* oocyte system and a cell culture system. In *Xenopus* oocytes injected with cPepT1 cRNA, all sixteen dipeptides and three tripeptides examined were able to evoke inward currents when they were added to the recording chamber (Table 3.1). Both neutral and charged di- and tripeptides were transported. All dipeptides and tripeptides examined evoked inward currents in a saturable manner, with an affinity constant (K_t) ranging from 30 $\mu\text{mol/L}$ to 6.9 mmol/L and a maximum current (I_{max}) ranging from 61 to 331 nA. With one exception, the dipeptides tested had substrate affinities in the micromolar range (30 $\mu\text{mol/L}$ to 170 $\mu\text{mol/L}$), indicating that cPepT1 has high affinity for these dipeptides. Lys-Lys and Lys-Trp-Lys appeared to be the least favorable substrates of all di- and tripeptides tested (K_t of 6.3 and 6.9 mmol/L for Lys-Lys and Lys-Trp-Lys, respectively). In contrast to peptide substrates, perfusion of 1 mmol/L of the amino acids, Gly, Met, Glu, or Lys, did not show any response (data not shown). Neither were responses detected from four tetrapeptides tested in this perfusion study. The results indicated that the optimal length of peptides preferred by cPepT1 was two to three amino acids.

In cPepT1-transfected CHO cells, inhibition of [^3H]-Gly-Sar uptake by 14 dipeptides, three tripeptides, three tetrapeptides, and two amino acids was tested. The IC_{50}

of 13 dipeptides and two tripeptides for inhibition of Gly-Sar uptake ranged from 0.02 to 0.25 mmol/L (Table 3.1). Lys-Lys and Lys-Trp-Lys were the exceptions and showed weak inhibition of Gly-Sar uptake (IC_{50} of 7.9 mmol/L for Lys-Lys and 5.9 mmol/L for Lys-Trp-Lys), which was consistent with the large K_t observed for these two peptides in the *Xenopus* oocyte assay. Overall in both the oocyte and the mammalian cell system, Lys-Lys and Lys-Trp-Lys were poor substrates for cPepT1. No inhibition of Gly-Sar uptake by free amino acids (Met and Lys) was observed (data not shown). The three tetrapeptides tested had very large IC_{50} values in cPepT1-transfected CHO cells. The peptides examined constitute a variety of substrates differing in their molecular weight, composition, electrical charge, and hydrophobicity. For all peptides, no correlation was found between current evoked or affinity to cPepT1 and molecular weight, net charge, or hydrophobicity.

Both cPepT1-Leu 703 and cPepT1-Ser 703 were present in our chicken RNA pools. A comparison of kinetic features between the two cPepT1 is shown in Table 3.2. From this study, we found that change in this amino acid of cPepT1 has no statistically significant effect on transport ability of the cPepT1 to the substrates we tested, with the possible exceptions of Trp-Ala and Trp-Leu.

DISCUSSION

The identification of peptide transporters, PepT1 and PepT2, provided a major advancement toward the understanding of the peptide transport systems that are responsible for absorption of dietary protein. Comparison of sequences of these mammalian peptide transporters and peptide transporters of other species has provided insight into the basic characteristics of the peptide transport system. All peptide transporters, from those present

in humans to those in yeast, form a family known as the POT superfamily (Paulsen and Skurray, 1994). This suggests that the peptide transporter genes have been well conserved. The cloned chicken peptide transporter conserved the signature sequences of the POT superfamily. Along with its peptide transport activity and tissue distribution pattern, we concluded that the chicken intestinal peptide transporter cPepT1 also belongs to the POT family.

However, the amino acid identity of cPepT1 to mammalian PepT1 is relatively low. Comparison of the predicted amino acid sequences of cPepT1 to mammalian PepT1 shows approximately 60% identity, whereas mammalian PepT1 show 80% identity among themselves. How much these sequence differences affect the function of cPepT1 remains unknown. Studies using PepT1 and PepT2 chimeras have shown that the putative substrate binding site in PepT1 and PepT2 is comprised of transmembrane domains 7, 8, and 9 and the loops in between (Döring et al., 1997; Fei et al., 1998). Using site-directed mutagenesis to identify amino acid residues essential for peptide transport showed that histidyl residues His-57 and His-121 in PepT1 were essential for the transport function of these proteins (Terada et al., 1996; Fei et al., 1997). Sequence alignment indicated that these regions were well conserved in cPepT1. Compared with PepT1 from other species, cPepT1 has lower identity at its C-terminus. Functional analysis of a chimeric peptide transporter derived from PepT1 and PepT2 showed that the N-terminal sequences determined the substrate affinity constant of the peptide transporter (Döring et al., 1997). No information is available as to how the amino acid sequence at the C-terminal end of the peptide transporter affects the functions of the transport system. The substrate specificity

experiment in the present study indicates that the change from a hydrophobic amino acid (Leu) to a hydrophilic amino acid (Ser) at position 703 has little if any effect on substrate affinity.

The cPepT1 mRNA transcript appeared to be expressed predominantly in the small intestine. The expression pattern of poly (A)⁺ RNA in these tissues was consistent with those from a previous study in our laboratory (Chen et al., 1999), indicating that the small intestine appeared to be the primary site of expression of cPepT1 mRNA transcripts. However, the present study using the cloned cPepT1 as the probe only detected a 2.9 kb mRNA, whereas our previous study detected a 1.9 kb mRNA using a short sheep PepT1 probe and low stringency conditions (Chen et al., 1999). The difference in mRNA size detected in the two studies indicates the presence of a 1.9 kb transcript in chickens that has structural homology to the sheep peptide transporter. Although previous reports from our lab showed that rat myogenic cells (C₂C₁₂) and ovine myogenic satellite cells could utilize exogenous peptides as sources for protein synthesis (Pan et al., 1996; Pan and Webb, 1998), we did not detect any hybridization in mRNA from pectoralis or fibularis longus muscle. Other studies reported the presence of PepT1 mRNA in the liver and kidney of the rabbit, rat, and human (Fei et al., 1994; Liang et al., 1995; Miyamoto et al., 1996). In the present study, cPepT1 mRNA was barely detectable in kidney and cecum, but not detectable in liver. Therefore the expression pattern of cPepT1 mRNA is slightly different from other PepT1.

The influence of different ions on peptide transport in cPepT1-injected oocytes agrees with other reports that the proton is critical for the transport process, but Na⁺, Cl⁻,

and K^+ are not (Fei et al., 1994; Mackenzie et al., 1996). However, the transport activity of a PepT1-like transporter in the canine renal cell line (MDCK cells) was affected by Ca^{2+} and a calmodulin-dependent pathway (Brandsch et al., 1995). Transport of dipeptides by PepT2 in the porcine cell line (LLC-PK1 cells) was also shown to be altered by Ca^{2+} , but not through a calmodulin-specific pathway (Wenzel et al., 1999). In the present study, peptide transport activity of cPepT1 in *Xenopus* oocytes was independent of Na^+ , K^+ , or Ca^{2+} . Overall, in both the oocyte and mammalian cell systems, cPepT1 mediated Gly-Sar uptake was pH dependent with an optimal pH between 6.0 and 6.5.

Most efforts to date that have characterized PepT1 and PepT2 in other species (rabbit, human, rat, and mouse) have focused on model peptides and drugs. There has been less attention directed toward the nutritional implications of this process although a few peptides have been examined. We evaluated the transport characteristics of cPepT1 using peptides containing essential amino acids (mainly methionine and lysine). The maximum transport rate and transport affinity of different peptides varied. In general, functional expression in both *Xenopus* oocytes and CHO cells indicated that cPepT1 has high affinity for most of the dipeptides and tripeptides examined, which is considerably different from published data of PepT1 from mammalian species. The nutritional implications of our data are not clear at the moment. The wide range of peptides transported by cPepT1 indicates that peptide transport in the chicken could be quite important. The very large K_t and IC_{50} of Lys-Lys and Lys-Trp-Lys represent two peptides with low affinity for cPepT1. This may indicate that these two peptides are less favorable substrates for cPepT1 under the pH used for the experiment. In deed, it was reported that in oPepT1-expressing *Xenopus* oocytes

Lys-Lys was transported much more rapidly at pH 7.0 than pH 5.0, which is the optimum pH for zwitterionic and anionic peptides (Pan et al., 2001).

Comparison of the two expression systems showed similar results for substrate affinity. The CHO cell is a suitable system to study competition between substrates whereas the *Xenopus* oocyte system monitors the cotransport of protons with the peptides. In the present study, for example, tetrapeptides showed weak inhibition of Gly-Sar uptake in cPepT1-expressing CHO cells. In the *Xenopus* oocyte system, these tetrapeptides did not evoke any detectable inward current at all. Therefore, we conclude that, in the CHO cell system, these tetrapeptides may be degraded by membrane peptidases and compete for Gly-Sar uptake as smaller di- or tripeptides. They alternatively might interact with the transporter itself as competitive inhibitors causing a change in the transporter conformation that indirectly affects Gly-Sar uptake. Therefore, it seems that cPepT1 uses mainly di- and tripeptides as its substrates.

In summary, this paper describes the cloning and characterization of a novel member of the POT family, cPepT1, with the ability to transport oligopeptides. The cPepT1 has lower amino acid identity compared with PepT1 from other species. Northern blot analysis demonstrated that cPepT1 is strongly expressed in the small intestine, and at lower levels in cecum and kidney, but not in liver, crop, proventriculus, or pectoralis and fibularis longus muscles. The transport function of cPepT1 in *Xenopus* oocytes and CHO cells indicated that cPepT1 has high affinity for most of the dipeptides and tripeptides tested. The transport process is electrogenic and independent of Na^+ , K^+ , and Ca^{2+} . Neither tetrapeptides nor free amino acids are substrates for cPepT1. Our studies have

characterized the substrate specificities of cPepT1, which provides new information for better understanding of protein absorption in chickens and ultimately may lead to the formulation of new chicken diets to enhance growth performance.

Table 3.1. Kinetics of peptide transport in oocytes and CHO cells expressing cPepT1-Leu703. The two-electrode voltage-clamp technique was used to characterize peptide transport activity in oocytes injected with cPepT1 cRNA. Oocytes were treated with various concentrations of substrates and inward currents induced were measured to calculate K_t and I_{max} . In CHO cells expressing cPepT1, inhibition studies were performed using 20 μ M [3 H]-Gly-Sar (50 mCi/ mmol) as the radiolabeled substrate and five concentrations (0.001 mM to 10 mM) of peptides as inhibitors to calculate the concentration at 50% inhibition (IC_{50}).

Substrate	pI	cPepT1		
		IC_{50} in CHO cells (mM)	K_t in oocytes (mM)	I_{max} (nA)
Dipeptide				
Gly-Met	5.5		0.10	126
Gly-Sar			0.47	206
Leu-Trp	5.5	0.02	0.06	110
Lys-Lys	10.0	7.9	6.3	183
Lys-Met	8.7	0.11	0.16	304
Lys-Phe	8.7	0.11	0.15	70
Lys-Trp	8.7	0.02	0.03	61
Met-Glu	4.6	0.02	0.06	94
Met-Gly	5.3	0.25	0.08	166
Met-Leu	5.3	0.04	0.13	271
Met-Lys	8.5	0.08	0.17	160
Met-Met	5.3	0.02	0.08	113
Trp-Ala	5.5	0.04	0.07	119
Trp-Gly	5.5	0.06	0.06	88
Trp-Phe	5.5	0.02	0.03	62
Trp-Leu	5.5	0.02	0.12	64
Tripeptide				
Leu-Gly-Gly	5.5	0.08	0.25	179
Lys-Trp-Lys	10.0	5.9	6.9	124
Met-Leu-Phe	5.3	0.04	0.55	331
TetraPeptide				
Met-Gly-Met-Met	5.3	3.9	No response	
Pro-Phe-Gly-Lys	9.2	4.3	No response	
Val-Gly-Asp-Glu	4.1	27	No response	
Val-Gly-Ser-Glu	4.6		No response	

Table 3.2. Comparison of kinetic constants of cPepT1-Leu703 and cPepT1-Ser703 in transfected CHO cells. The transport constants (K_t) for Gly-Sar uptake are 2.6 mM for cPepT1-Leu703 and 2.9 mM for cPepT1-Ser703. In CHO cells expressing either cPepT1-Leu703 or cPepT1-Ser703, inhibition studies were performed using 20 μ M [3 H]-Gly-Sar (specific activity 50 mCi/ mmol) as the radiolabeled substrate and five concentrations (0.001 mM to 10 mM) of peptides as inhibitors. IC_{50} of each inhibitor for cPepT1-Leu703 and cPepT1-Ser703 was compared using t-test. Values with an asterisk are different ($P < 0.05$).

IC₅₀ (mM)	cPepT1- Leu703	cPepT1- Ser703
Lys-Lys	6.7	7.0
Met-Gly	0.28	0.51
Met-Met	0.02	0.03
Trp-Ala	0.05 *	0.01 *
Trp-Gly	0.06	0.03
Trp-Leu	0.02 *	0.03 *
Trp-Phe	0.02	0.04
Lys-Trp-Lys	5.0	5.8
Leu-Gly-Gly	0.15	0.32
Met-Gly-Met-Met	3.9	4.0
Pro-Phe-Gly-Lys	5.5	10.5

CHAPTER III CLONING AND EXPRESSION OF CHICKEN PEPTIDE TRANSPORTER

humanMGM	SKSHSFFGY	LSIFFIVVNE	FCERFSYYGM	RAILILYFTN	FISWDDNLST	AIYHTFVALC	<u>YLTPILGALI</u>	<u>ADSWLGKFKT</u>	<u>IVSLSIVYTI</u>	GQAVTSVSSI
rat---	--RGC---	-----	-----	--L-V--R-	-LG--D---	-----	-----	-----	-----	--I-----
mouse---	--RGC---	-----	-----	--L-V--R-	-LG--D---	-----	-----	-----	-----	--I-----
rabbit---	-L-C---	-----	-----	--L--R-	-G-----	V-----	-----	-A-----	-W-----	--L--V-
sheep---	-VPK-C---	-----	-----	--L--QR	-LG-N--G-	-----	-----	-----	-----	--V-IA---
chicken	MAAKSKSKGR	-VP.NC---	-----	-I-----	--V-V--KY	-LR--F---	-----	-----	-----	-----	--MA---
human	NDLTDHNDHG	TPDSLPHVHV	LSLIGLALIA	LGTGGIKPCV	SAFGGDQFEE	GQEKQRNRF	<u>SIFYLAINAG</u>	<u>SLLSTIITPM</u>	LRVQCCGIHS	KQACYPLAFG	VPAALMAVAL
rat	-----D-	S-NN--L--A	--M-----	-----	-----	-----	-----	-----I	-----	Q-----	-----
mouse	-----D-N-	S-----A	--MV-----	-----	-----	-----	-----	-----G-	-----I	Q-----	-----
rabbit	-E--N-----	-----A	VCM--L---	-----	-----	-----	-----	-----	V-----V	-----	I--I---S-
sheep	-----F---	--NNIS--A	--M--V---	-----	-----	-----	-----	-----	-----V	-----	-----S-
chicken	--M--Q-R--	N--NIA--IA	--MT--I---	-----	-----	H-----S---	-----	--I-----I	--A-E-----	R-Q-----	-----S-
human	IVFVLGSGMY	KKFKPQGNIM	GKVAKCIGFA	IKNRFRRHSK	APFKREHWLD	WAKEKYDERL	ISQIKMVTRV	MFLYIPLPMF	WALFDQQGSR	WTLQATTMSG	KIGALEIQPD
rat	-----Q-----	-----R-----	-----	-----	-----N-----	-----	-----IM-K-	-----	-----	-----T-----	-----TI-----
mouse	-----Q-----	-----	-----	-----	-----Y-----	-----	-----K-	-----F-----	-----G-----	-----N-----	-----N-----
rabbit	---II-----	-----L	S--V--C--	-----	-----Q--A--	-----	-----A-----	-----L-----	-----	-----	R--I-----
sheep	-----I-----	--VQ-----	S--R-----	-----IS-----	-----K-----	-----S-----	-----	-----	-----	-----	-----II-----
chicken	V--IA-----	--VQ-----	VR-C-----	-----	-----EY-----	-----S-----K--	A-T--LK-	L-----	-----	-----D-----	DF--MQ---
human	QMOTVNAILI	VIMVPIFDAV	LYPLIAKCGF	NFTSLKMAV	GMVLASMAFV	VAAIVQVEID	KTLVPVFKGN	EVQIKVLNIG	NNTMNISL.P	GEMVTLGPM	QTNAFMTFDV
rat	-----V-----	V-----	-----T-----	--F-----	-----	-----	-----S--	Q-----	--D-AVYF-	-KN--VAQ--	--DT-----
mouse	-----N	--NNG-NV--	V-RS-----	-----T-----	--F-----	-----	-----G--	Q-----	--N-TVHF-	-NS--AQ--	--DT-----I
rabbit	-----T--	I-L--M--	V-----L	-----TI-----	--F-----	A--L-----	-----A-----	-----V-----	SEN-I---	--QT--NQ--	--E-----NE
sheep	-----V-----	V-----L	-----T-----	--F-----	A--D-----	-----	-----	-----	--S-TV-F-	--TT--CDQ--	--G-L--N-
chicken	-----P--	I-----VV--	I-----Q--KI	--P-RRIT-	--F--GL--	A--LL--Q--	-----AAG	QA---II-L-	DSNA-VTFL-	NLQNVIVLPM	ESTGYRMFES
human	NKLTRINISS	PGSP.VTAVT	DDFKQGQRHT	LLVWAPNHVQ	VVK.DGLNQK	PEKGENGIRF	VNTFNELITI	TMSGKVYANI	SSYNASTYQF	FPSGKIGPTI	SSTEIPPCQ
rat	DQ--S--V--	---G--T-A	HE-EP-H---	---G--L-R	-----	-----	--S-L-M--	K-----E-V	T-HS-N--	---Q-DY--	NT--A-N-S
mouse	D--S-----	S--G--T-A	H--E--H--N	---E-SQ-R	---P---	-----	--L--MV-N	K-----EKF	T-H--G-K-	L--E-QY--	NT-AVA-T-L
rabbit	DT--S--T-	GSQ..--MI-	PSLEA-----	---N--R--	--N--T--	SD-----	---YSQP-NV	-----EH-	A-----E--	--T--V--V	--AG-SE--R
sheep	DN-.S-----	T-T-.--P--	HN-ES-H---	---SN---	-----	---R-----	--APG-SFGV	--D-E--N-V	-GH--E-L-	-S--V-S--	N-P--SQ--E
chicken	SQ-KSVMVNF	GSESRSENI	SISSNTHTV	IKNA-AGIVS	SLRS-NFTS-	--E-K-LV--	--NLPQTVN-	--GDTTFGIL	EETSI-N-SP	-SG-.RTYD-	VI-AGSTN-K
human	PNFNIFYLEF	GSAYTYIVQ.	RKNDSCPEVK	VFEDISANTV	NMALQIPQYF	LLTCGEVVFV	VTGLEFSYSQ	APSNMKSVLQ	AGWLLTVAVG	NIIVLIVAGA	GQFSKQWAEY
rat	SD-KSSN-D-	-----VIRS	-AS-G-L--	E---PP---	-----	-----	-----	-----	-----I-	-----E-	H-D-----
mouse	TD-KSSN-D-	-----VIR.	-AS-G-L--	E---PP---	-----	-----	-----	-----	-----	-----	H-P-----
rabbit	RD-ESP---	-----LIT.	SQATG--QVT	E---PP--M	--W-----	-I-S-----	I-----	-----	-----	-----	--IN-----
sheep	KQ-KTS---	---F--VIS.	--S-G--P-	I---P---	S-----	-----	I-----	-----	-----	-----	-----E-----
chicken	-TSEK..-GY	-G--IVIN.	ECSGDVTQLR	YI--QP--	H--W-----	I-----	-----	-----	-----	-----	SKL-E-----
human	ILFAALLLVV	CVIFAIMARF	YTYINPAEIE	AQFDEDEKKN	RLEKSNPYFM	SGANSQKQM	-----	-----	-----	-----	-----
rat	V--S-----	-I-----	-----	-----K	GVG-E--SS	LEPV--TN-	-----	-----	-----	-----	-----
mouse	---S-----	-----	-----	-----K	GIG-E--SS	LEPV--TN-	-----	-----	-----	-----	-----
rabbit	-----	-----	---V-----	---E-----	K NP--NDL-PS	LAPV--T-	-----	-----	-----	-----	-----
sheep	V-----	-I-----	---V-----	---D-ED	D-----AK	LDV--T-	-----	-----	-----	-----	-----
chicken	V-----FA-	-I--V--Y-	---TD-N-V-	--L--E--K	QIKQDPDLHG	KESEAVS--	-----	-----	-----	-----	-----

Figure 3.1. Alignment of predicted amino acid sequences of human, rat, mouse, rabbit, sheep and chicken PepT1. Amino acids identical to the human sequence are indicated by dashes (-). The underlined sequences represent the PTR2 signature sequences of the POT superfamily. The cPepT1 nucleotide and amino acid sequences reported in this paper have been submitted to GenBank under accession number AY029615.

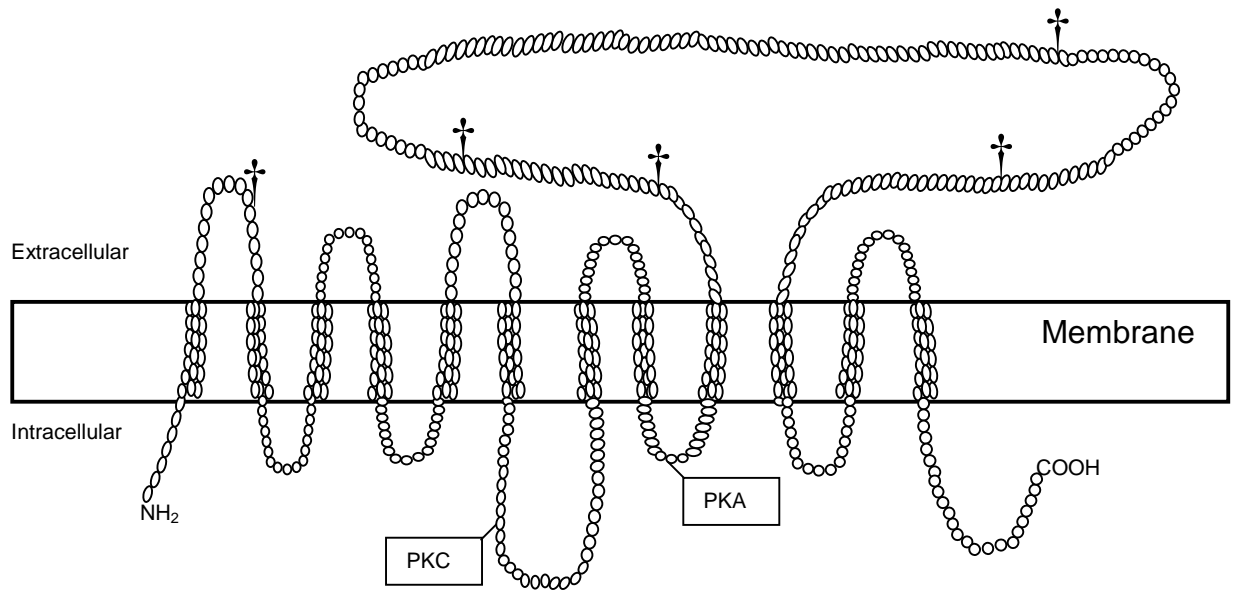


Figure 3.2. Putative membrane spanning model of cPepT1. Hydrophobicity analysis indicated that cPepT1 has 12 putative transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10. Potential N-linked glycosylation sites are indicated by the symbol (†). Potential protein kinase C phosphorylation sites (PKC) and protein kinase A phosphorylation sites (PKA) are also indicated.

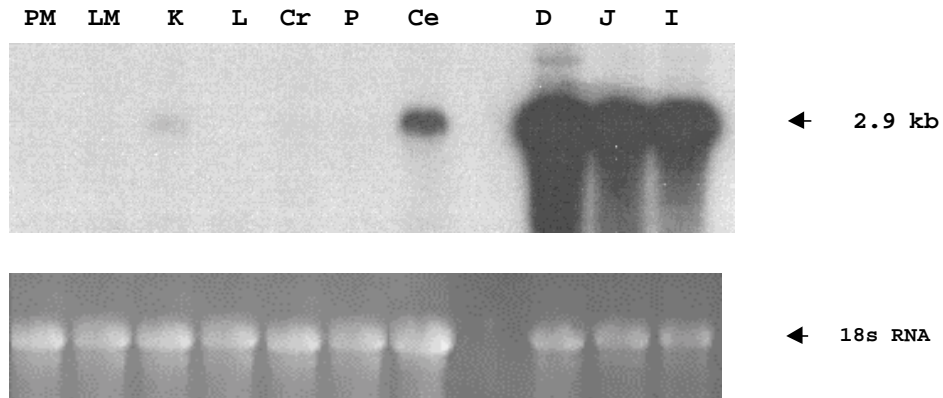


Figure 3.3. Northern blot analysis of cPepT1 mRNA in chicken tissues. Ten micrograms of poly (A)⁺ enriched RNA were loaded per lane and the blot was hybridized with a full-length cPepT1 cDNA as the probe. The 18s rRNA was used as an internal control for loading. Lanes represent the pectoralis muscle (PM), fibularis longus muscle (LM), kidney (K), liver (L), crop (Cr), proventriculus (P), cecum (Ce), duodenum (D), jejunum (J), and ileum (I).

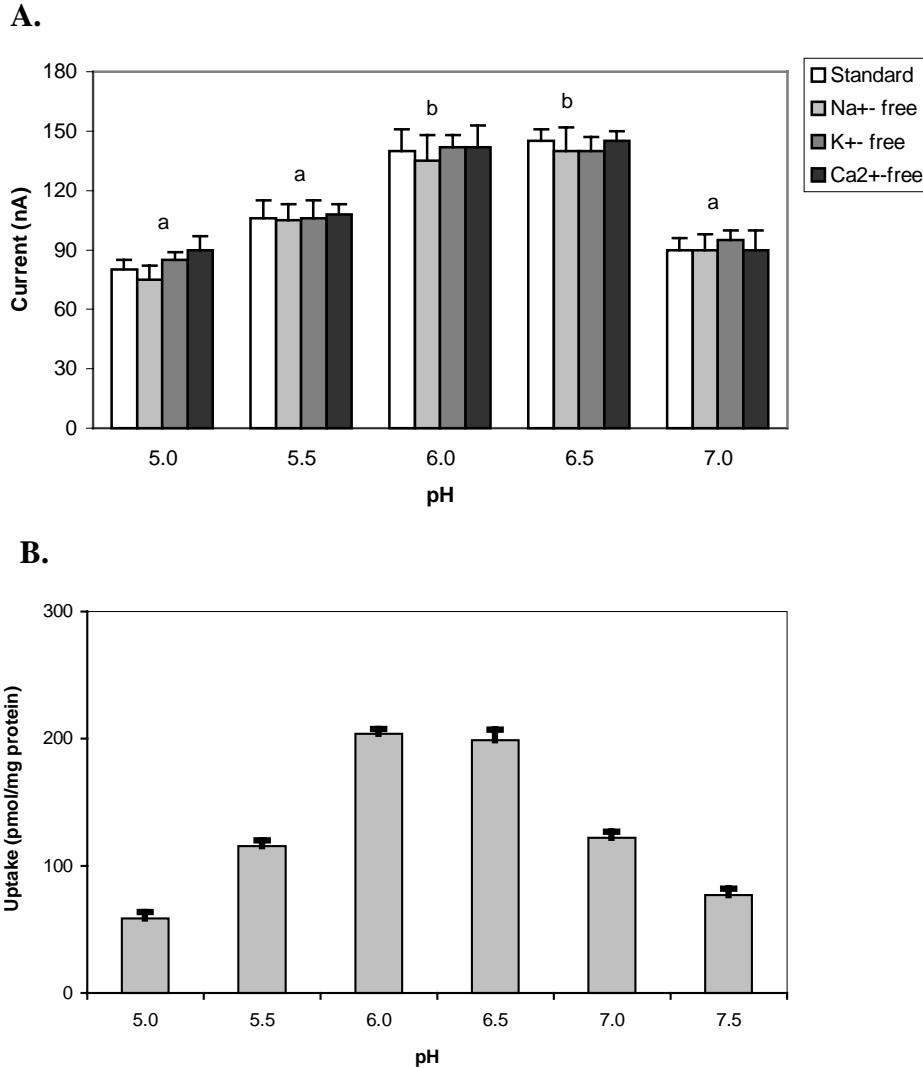


Figure 3.4. pH and ion-dependency of cPepT1 in cRNA-injected oocytes and cPepT1-ransfected CHO cells. **A:** Oocytes were clamped at -60 mV and perfused with 1 mM dipeptide Gly-Sar in standard measurement buffer at pH 5.0, pH 5.5, pH 6.0, pH 6.5, and pH 7.0. To determine if this transport process was dependent upon the presence of Na^+ , K^+ , or Ca^{2+} ions, transport was measured in Na^+ -free, K^+ -free, and Ca^{2+} -free buffers. Na^+ -free and K^+ -free buffers were prepared by replacing NaCl or KCl with choline chloride. Ca^{2+} -free buffer was prepared by replacing CaCl_2 with choline chloride in standard measurement buffer. Values are means \pm SEM, $n = 5$. Means without a common letter differ, $P < 0.05$. **B:** CHO cells were transfected with cPepT1 cDNA using Lipofectamine. Transfected cells were then incubated with uptake buffer containing $[^3\text{H}]$ -Gly-Sar at pH 5.0-7.5. Values are the means of maximum uptake \pm SEM, $n = 6$ total wells in two transfections.

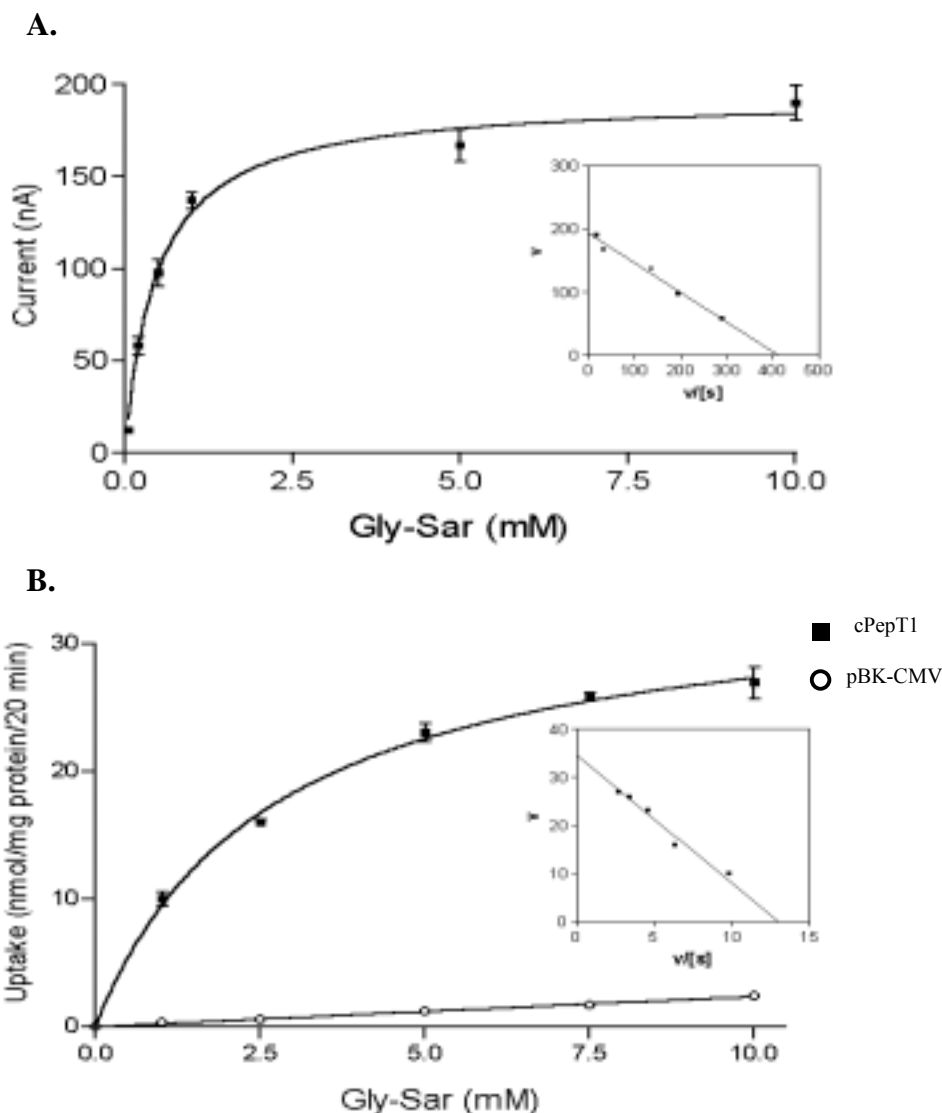


Figure 3.5. Kinetic parameters of Gly-Sar uptake by cPepT1 measured by recording current amplitudes in oocytes injected with cPepT1 cRNA and [3 H]-Gly-Sar uptake in CHO cells transfected with cPepT1 cDNA. **A:** Oocytes were clamped at -60 mV and perfused with 1.2 mL/min at Gly-Sar concentrations ranging from 10 μ M to 10 mM in standard measurement buffer at pH 6.0 . Values are means \pm SEM, $n = 5$. Inset: Eadie-Hofstee plot of Gly-Sar uptake. **B:** cPepT1-transfected CHO cells were incubated with six Gly-Sar concentrations (0.02 to 10 mM, with 1 μ Ci/ml [3 H]-Gly-Sar). Vector pBK-CMV without insert was transfected into CHO cells as a control. Values are means \pm SEM, $n = 6$ total wells in two transfections. Inset: Eadie-Hofstee plot of Gly-Sar uptake in cPepT1-transfected CHO cells.

Chapter IV

Dietary Protein Level and Stage of Development Affect Expression of an Intestinal Peptide Transporter (cPepT1) in Chickens

ABSTRACT

The objective of this study was to evaluate the effect of dietary protein intake and developmental changes on chicken intestinal peptide transporter (cPepT1) mRNA abundance. In Experiment 1, ten eggs were sampled at incubation day 18 (E 18). Diets containing 12, 18, or 24% crude protein (CP) were randomly assigned to mixed sex Cobb chicks in a pen. Feed intake of chickens fed diets containing 18 or 24% CP was restricted to that of chickens fed the diet containing 12% CP. Ten to 12 chickens were sampled on d 0, 1, 3, 5, 7, 10, 14, 21, 28, and 35. In Experiment 2, a fourth group with free access to the 24% CP diet was added. Eleven to 14 chickens were sampled at d 0, 1, 3, 5, 7, 10, 14, and 35. Total RNA was extracted from the duodenum, jejunum, and ileum and cPepT1 mRNA abundance was quantified from northern blots. At E 18, cPepT1 mRNA was barely detectable. By d 0, there was a 50-fold increase in cPepT1 mRNA abundance. In chickens fed the 12% CP diet, cPepT1 mRNA abundance decreased throughout the experiment. Chickens fed 18 or 24% CP diets showed an increase in cPepT1 mRNA abundance with time. Most of the increase occurred during the first 2 wk. In chickens with free access to the 24% CP diet, cPepT1 mRNA decreased until d 14 but returned to an intermediate level at d 35. Our results indicate that expression of cPepT1 mRNA is regulated by both dietary protein intake and developmental stage.

Keywords: cPepT1, Beta-actin, Northern blot.

INTRODUCTION

The cloning and characterization of peptide transporters (PepT1 and PepT2) has provided valuable information on the mechanisms of peptide transport in mammalian species. PepT1 and PepT2 are distinct in their tissue distributions, substrate kinetics, and specific roles in different tissues. PepT1 is predominantly expressed in the small intestine and has been shown to have nutritional, clinical, and pharmaceutical importance (Daniel, 1996; Leibach and Ganapathy, 1996; Adibi, 1997). PepT2 is expressed in kidney, the central nervous system, and several other peripheral tissues and is believed to have multiple functions in various tissues. Results from earlier studies indicate that expression patterns of PepT1 in the small intestine vary in different sections of the small intestine as well as in different areas along the crypt-villus axis (Freeman et al., 1995a). Selective amino acids and peptides stimulate PepT1 gene expression in vitro (Shiraga et al., 1999). Under various conditions of malnourishment, PepT1 expression is reported to be greatly enhanced in vivo (Ihara et al., 2000). In addition, intestinal expression of PepT1 in the rat is induced postpartum (Shen et al., 2001).

A cDNA coding for a chicken intestinal peptide transporter (cPepT1) was recently cloned using an ovine peptide transporter (oPepT1) as a probe to screen a chicken intestinal cDNA library (Chen et al., 2000a). The identified cPepT1 cDNA was 2.9 kb long and encoded a protein of 714 amino acid residues. Results from uptake studies using *Xenopus* oocytes and Chinese hamster ovary cells (CHO) showed that oocytes and CHO cells expressing cPepT1 were capable of uptake of di- and tripeptides. Kinetic analysis of the hydrolysis-resistant dipeptide Gly-Sar showed that the transport process was proton-

dependent, concentration-dependent, and saturable. Affinity constants varied among the peptides tested indicating apparent preference of cPepT1 for different peptides. Free amino acids and tetrapeptides tested were not transported by cPepT1. Results from northern blot analysis showed that cPepT1 mRNA was expressed mainly in the small intestine, and at lower levels in the kidney and cecum, but not in the liver, crop, proventriculus, or pectoralis and fibularis longus muscles (Chen et al., 1999, 2000a). There is evidence from several studies that PepT1 expression in mammals is regulated at the transcriptional level by dietary factors (Shiraga et al., 1999; Ihara et al., 2000), growth factors (Nielsen et al., 2001), and developmental stage (Shen et al., 2001). The objective of the present study was to determine developmental and nutritional changes in PepT1 mRNA abundance in broilers.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise stated. TriReagent was purchased from Molecular Research Center (Cincinnati, OH). Transferring Nylon membranes were purchased from Osmonics, Inc. (Westborough, MA). [α - 32 P]dATP was purchased from ICN Pharmaceutical (Costa Mesa, CA). DNA polymerase I/DNase I was purchased from Life Technologies (Gaithersburg, MD).

Animals and tissue sampling. Cobb-Cobb eggs at d 18 (E 18) of incubation (Experiment 1) and unsexed Cobb-Cobb chicks on the day of hatch (Experiment 1 and 2) were obtained from a commercial hatchery. Ten eggs were sampled at E 18 (Experiment 1). After hatch but before feeding, ten chicks were sampled (d 0; Experiments 1 and 2).

The rest of the chicks were randomly assigned to heated floor pens with wood shavings (Experiments 1 and 2). All pens were provided 24 h lighting and the chicks had free access to water. For a given sampling day, ten to fourteen chicks from each group were sacrificed by cervical dislocation and tissues were sampled immediately thereafter. The sex of the chicks was determined and the intestine was removed from each bird. Except for the E 18 embryos when the entire intestine was used, the duodenum, jejunum, and ileum were separated, washed with ice-cold saline, frozen in liquid nitrogen, and stored at -80°C. For samples collected at E 18 and d 0, tissues from five males and five females were analyzed. For other sampling days, growth data and tissue samples from only the male birds were analyzed in both experiments. All animal procedures were approved by Virginia Tech's Animal Care Committee.

Experiment 1. The chicks (339) were randomly divided among three pens. Diets containing either 12, 18, or 24% crude protein (CP) were randomly assigned to be fed to chicks in a pen. Daily feed intake of the chicks fed diets containing 18 or 24% crude protein was restricted to the intake of chicks fed the 12% crude protein diet. This was accomplished by feeding the chicks receiving 18 and 24% CP diets an amount of feed equivalent to the feed consumed by chicks fed the 12% CP diet the previous day. Composition of the experimental diets is shown in Table 1. Ten to twelve chicks from each group were sacrificed on d 1, 3, 5, 7, 10, 14, 21, 28, and 35 by cervical dislocation and tissues were sampled immediately thereafter. Total feed consumption was monitored daily and body weight was determined for chickens sampled at each time point.

Experiment 2. The protocol of this experiment was the same as that of Experiment 1, except for the addition of one dietary treatment and a slightly different sampling schedule. In Experiment 2, chicks (388) were randomly divided among four pens. The chicks in three pens were fed the same as in Experiment 1. For the fourth treatment, chicks were allowed free access to the diet containing the 24% CP. Eleven to fourteen chicks from each group were sacrificed on d 1, 3, 5, 7, 10, 14, and 35 by cervical dislocation and tissues were sampled immediately thereafter. Total feed consumption was monitored daily and body weight was determined for chickens sampled at each time point.

Northern blot analysis. For both experiments, total RNA from five male chickens from each group was extracted using TriReagent. Absorbance at 260 and 280 nm was used to quantify the RNA extracted and isolated RNA was stored at -80°C . For northern blot analysis, 20 μg of total RNA was denatured and size-fractionated on 1% agarose gels in 2.2 mol/L of formaldehyde, stained with ethidium bromide, and transferred to a Nylon membrane. Five separate gels were run. Each gel comprised one replicate including each of the three small intestinal sections from one of the five chickens of each of the treatment groups from each sampling time. Following prehybridization for 2 h in a solution containing 50% formamide, 5 \times Denhardt's solution, 6 \times SSPE (1 \times SSPE = 0.15 mmol/L NaCl, 10 mmol/L NaH_2PO_4 , and 1 mmol/L EDTA), 0.5% SDS and 10 mg/L yeast tRNA at 42°C , RNA blots were hybridized for 16 h under identical conditions with the addition of cPepT1 cDNA as the probe (Chen et al., 2000a). The probe was labeled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ by nick translation using DNA polymerase I/DNase I and purified by Sephadex G-50 spin column chromatography. Post-hybridization washing was done under high-stringency

conditions, which involved washing twice in $5 \times$ SSPE, 0.5% SDS at room temperature for 15 min, twice in $1 \times$ SSPE, 0.5% SDS at 42°C for 15 min, and twice in $0.1 \times$ SSPE, 1% SDS at 65°C for 15 min. Washed filters were exposed with an intensifying screen to Kodak X-ray film. The density of the signal from cPepT1 was determined by densitometry. In Experiment 2, RNA blots were also hybridized to a chicken β -actin probe labeled with [α - 32 P]dATP under the same conditions as for the cPepT1 probe. To correct for differences in RNA loading onto gels or in RNA transfer to membranes, membranes were stripped of the cPepT1 or β -actin probe and rehybridized to a [32 P]-labeled 18S rRNA probe. The density of hybridization bands was quantified using the Gelwork 1D digital image analysis system (UVP, Upland, CA).

Statistical methods. Data were analyzed with one-way analysis of variance using the General Linear Models procedure (SAS/STAT version 7.12 for Windows, SAS Institute, Cary, NC). For the variables feed intake and body weight, the model included the main effects of diet and time and the diet*time interaction. For hybridization intensity comparisons, the model included the main effect of diet, tissue, and time and all two-way interactions. The main effects for time was further tested for linear and quadratic fit using orthogonal contrast statements in the General Linear Model procedure. Significant difference among diets and tissues was by Tukey's HSD.

RESULTS

Feed intake and growth rate. In Experiment 1, feed intake was equalized among the three treatments. Body weight increased quadratically ($P < 0.0001$) with time (Figure 4.1A). The body weight of the group fed the 12% CP diet was lower ($P < 0.05$) than that of

the groups fed 18% and 24% CP diets throughout the experiment. The interaction ($P < 0.0001$) between crude protein level and time is the result of the slower rate of growth in chicks fed the 12% CP diet. In Experiment 2, chickens fed 18% and 24% CP diets had the same daily intake as that of the group fed the 12% CP diet, while the intake of the chicks with free access to the 24% CP diet was greater than that of the other three groups. Feed intake of the group with free access to the 24% CP diet was 1.4-fold greater than intake by the other three groups at d 14 ($36.4 \text{ g}\cdot\text{bird}^{-1}\cdot\text{d}^{-1}$ for chickens fed 12, 18, or 24% CP diets, and $50 \text{ g}\cdot\text{bird}^{-1}\cdot\text{d}^{-1}$ for chickens with free access to the 24% CP diet) and 2.5-fold by d 28 ($50.5, 50.0,$ and $50.0 \text{ g}\cdot\text{bird}^{-1}\cdot\text{d}^{-1}$ for chickens fed 12, 18, and 24% CP diets, respectively and $129.4 \text{ g}\cdot\text{bird}^{-1}\cdot\text{d}^{-1}$ for chickens with free access to the 24% CP diet). Body weight increased quadratically ($P < 0.0001$) throughout the feeding period, with the chickens with free access to the 24% CP diet growing the fastest, chickens fed the 12% CP diet growing the slowest, and the chickens fed the 18 and 24% CP diets being intermediate ($P < 0.05$; Figure 4.1B). These differing rates of growth account for the interaction between crude protein levels and time ($P < 0.0001$) that was observed.

cPepT1 mRNA abundance in Experiment 1. At E 18, expression of cPepT1 mRNA in the small intestine was barely detectable (Figure 4.2A). By the time of hatch (d 0), there was an approximately 50-fold increase ($P < 0.05$) in cPepT1 mRNA abundance in all three sections of the small intestine (Figure 4.2B). Expression of cPepT1 in the duodenum was the highest in all three sections ($P < 0.05$).

In Experiment 1 there was a linear increase ($P < 0.0001$) in cPepT1 mRNA abundance with time, and there was a protein*time interaction ($P < 0.0001$). The

interaction resulted from cPepT1 mRNA abundance generally decreasing throughout the study in all three sections of the small intestine in chicks fed the 12% CP diet (Figure 4.4A) while cPepT1 mRNA abundance increased with time in all three sections of the small intestine of chicks fed the 18% CP diet (Figure 4.4B) and the 24% CP diet (Figure 4.4C). Data from the three sections were combined to show the main effect of diet (Figure 4.4D). cPepT1 mRNA level was lowest in chickens fed the low protein diet (12%), highest in chickens fed the 24% CP diet, and intermediate for chicks fed the 18% CP diet ($P < 0.05$). Data from the chicks fed the three diets were combined to show the main effect of tissue (Figure 4.4E). cPepT1 mRNA abundance was highest in the jejunum ($P < 0.05$).

cPepT1 and beta-actin mRNA abundance in Experiment 2. In Experiment 2 (Figure 4.5), hybridization density of both cPepT1 and beta-actin was normalized by 18S rRNA. In chickens fed the 12% CP diet, a general decrease in cPepT1 mRNA abundance was observed in the small intestine throughout the duration of the experiment with the exception of an increase in the duodenum at d 14 (Figure 4.6A). Groups fed diets containing 18 and 24% CP showed an increase in cPepT1 mRNA abundance with time in all three sections of the small intestine (Figure 4.6B, 4.6C). Interestingly, in chickens with free access to the 24% CP, a decrease in cPepT1 mRNA abundance was observed in the three sections of the small intestine during the first 14 d (Figure 4.6D). By the end of the feeding period (d 35), cPepT1 mRNA abundance in chickens with free access to the 24% CP increased to near the level of cPepT1 mRNA at d 0. Data from the three sections of the small intestine of chicks in each treatment group were combined to show the main effect of diet (Figure 4.6E). cPepT1 mRNA abundance in chickens fed the 12% CP diet and

chickens with free access to the 24% CP diet was lower ($P < 0.05$) than that of chickens fed 18 or 24% CP diets. cPepT1 mRNA abundance was the highest in chickens with restricted access to the 24% CP diet ($P < 0.05$). Data from the chicks in the four treatment groups were combined to show the main effect of tissue (Figure 4.6F). cPepT1 mRNA abundance was the highest in the duodenum, lowest in the ileum, and intermediate in the jejunum ($P < 0.05$).

In Experiment 2, beta-actin mRNA abundance was less affected than cPepT1 mRNA abundance (Figure 4.7A-D). Data from the three sections of the small intestine of chicks in each treatment group were combined to show the main effect of diet (Figure 4.7E). Beta-actin mRNA abundance was lower ($P < 0.05$) in chickens fed the 12% CP diet, higher ($P < 0.05$) in chickens with free access to the 24% CP diet. Data from the chicks in the four treatment groups were combined to show the mean effect of tissue (Figure 4.7F). Beta-actin mRNA abundance decreased gradually overtime in all three sections of the small intestine ($P = 0.0009$). Beta-actin mRNA abundance was the highest in the jejunum ($P < 0.05$).

DISCUSSION

Growth and development of chicks is dependent on the uptake of nutrients. Appearance and development of intestinal transport proteins are important factors that affect the uptake of nutrients and, therefore, the starting point of growth. We studied cPepT1 mRNA expression at E 18 of the embryonic period and immediately after hatch at d 0. There was significant development that occurred in this 3-d period since cPepT1 mRNA was barely detectable at E 18 and was about 50-fold greater at d 0. A similar

pattern was observed in the rat (Shen et al., 2001). The rPepT1 mRNA was present at d 20 of fetal life and increased rapidly at birth with the maximal expression occurring at d 3 to 5. The authors concluded that rPepT1 was induced postpartum by suckling and later weaning. In chickens, the induction occurred prior to the intake of feed. There should be little nutritional change during late incubation to hatch. Expression of many genes of digestive relevance such as trypsin, amylase, and lipase are stimulated several days after hatch by feed intake (Noy and Sklan, 1995). Obst and Diamond (1992) reported a dramatic increase in glucose transport 2 wk posthatch in chicks. Therefore, cPepT1 may respond to a developmental regulatory factor to be expressed at high levels before or during hatch for immediate availability of peptide substrates when feed intake starts.

We observed a drop in cPepT1 mRNA abundance in chickens with free access to the 24% CP diet during the first 2 wk of age but later the cPepT1 mRNA returned to about the same level as chickens fed diets containing 18% or 24% CP. In rats, rPepT1 mRNA dropped suddenly after d 5 to as low as 11% of the maximal level and then remained at a level of 25% of the maximum at adult age (d 75; Shen et al., 2001). Results from other studies also indicated a decline in small intestinal capacity to transport proline from one to 7 wk of age in chickens (Gonzalez and Vinardell, 1996; Soriano and Planas, 1998) and threonine in ducks (King et al., 2000). Therefore, there may be general developmental factors that control PepT1 mRNA expression in accordance to different developmental needs of the body. Further research is needed to elucidate the detailed timeline of induction of cPepT1 expression.

To improve our understanding of the mechanisms involved in the nutritional regulation of transporter gene expression, we investigated the effects of dietary protein on the abundance of cPepT1 mRNA to determine whether an increase in dietary protein level was associated with a similar increase in cPepT1 mRNA. An increase in cPepT1 mRNA abundance in chickens fed high-protein diets and a decrease in chickens fed a low-protein diet were observed in the present study. mRNA abundance of a housekeeping gene, beta-actin, was analyzed at the same time as cPepT1 mRNA. Results showed that there was less change in beta-actin mRNA expression in all the treatment groups than in cPepT1 mRNA. Therefore, dietary protein may have more effect on cPepT1 mRNA expression than beta-actin mRNA expression. The results are consistent with previous findings that intestinal enzymes, peptide transporters, and amino acid transporters respond to diets with different protein levels (Erikson et al., 1995). Adaptation to a protein diet has been correlated with changes in the activity of enzymes involved in protein digestion (Moundras et al., 1993). A high protein diet enhanced the levels of intestinal peptidases (Jean et al., 2001) and a protein-sufficient diet induced lactase synthesis in protein-starved pigs (Dudley et al., 2001). These are consistent with reports on dietary regulation of PepT1 in rats showing that the rPepT1 mRNA abundance was about two-fold higher in rats fed a 50% casein diet compared with rats fed a protein-free diet (Shiraga et al., 1999). The kinetic analysis from the studies also showed that rPepT1 activity was induced by a 50% casein diet with an increase in V_{max} but not K_m . The comparable patterns of the induced PepT1 mRNA abundance with increased dietary protein level suggested that dietary protein might have a regulatory effect on cPepT1 expression at the transcriptional level. Furthermore, both

PepT1 mRNA abundance and transport rate of Gly-Gln by PepT1 increased when cells were treated with the dipeptide Gly-Sar for 24 hr (Thamotharan et al., 1998), while Gly-Gln treatment of the cells also caused an increase in both PepT1 mRNA abundance and Gly-Sar uptake (Walker et al., 1998). Promoter analysis of rat PepT1 showed that the 5' upstream region of rPepT1 contains elements that respond to peptide substrates such as Asp-Lys, Phe-Val, and free amino acids including Phe, Lys, and Arg (Shiraga et al., 1999). Therefore, end products of protein digestion, small peptides and amino acids, may participate in pathways that control expression of intestinal transporters like PepT1 and eventually cause increased transporter capacity in the intestine. Mechanisms by which dietary protein regulates cPepT1 gene expression may be determined by full analysis of the transcriptional control region of the gene.

Due to the possibility that the increased cPepT1 mRNA abundance in chickens fed 18 or 24% CP diets may be the combination of diet restriction and protein intake rather than increased dietary protein intake alone, we added the fourth group in Experiment 2 of chickens with free access to the 24% CP diet. The major difference in feeding is that the daily feed intake of the groups fed the 18% or 24% CP diet was restricted to the level of the group fed the 12% CP diet, which represented an intake of about 75% of that of the group with free access to the 24% CP diet in the first 2 wk and 40% in the later feeding period. We observed that, in chickens with free access to the 24% CP diet, the overall cPepT1 mRNA level remained lower than that of chickens with restricted access to the 24% CP diet. There was no difference in beta-actin mRNA abundance between these two groups. These results are similar to that from another study of PepT1 expression in rats (Ihara et al.

2000). In that report, the level of rPepT1 mRNA increased to 179%, 164 %, and 161% of control in rats that were starved (no food intake for 4 d), semistarved (50% of control intake), or received total parenteral nutrition, respectively. PepT1 protein expression showed similar changes with mRNA. In contrast, there was no change in SGLT1 mRNA level in these treatment groups. Therefore, the higher cPepT1 mRNA abundance in the present study was possibly also due to the restricted feeding for these two groups. There are reports regarding amino acid transporters showing that gene expression of system A, system L, and system X_{AG}⁻ was up-regulated under amino acid restriction conditions (Nicholson and McGivan, 1996; Palacin et al., 1998). It is suggested that gene expression is upregulated through amino acid sensory pathways at times of amino acid starvation (Fafournoux et al., 2000). Although the regulatory networks have not been fully revealed, a potential amino acid-responsive element has been found in the promoter regions of several genes, including mouse PepT1 (Fei et al., 2000). Currently we do not know whether the upregulatory effect was due to a response to peptide restriction or amino acid restriction derived from dietary protein limitation. Both amino acid-activating and peptide-activating pathways need to be investigated to fully elucidate the regulatory effects of dietary protein limitation. Overall peptide transporters may be one of the essential proteins induced by stress response such as starvation for cell survival.

Further studies are required to establish the mechanism of transcriptional control of the cPepT1 gene, particularly by identification of the factors involved in the regulation of the transporters. Amino acids, peptide substrates, insulin, and growth factors have been reported as factors regulating peptide transport through PepT1 (Nielsen et al. 2001; Shiraga

et al. 1999; Thamocharan et al. 1999). Information on how cPepT1 is regulated at the molecular level will provide the basic mechanisms controlling body N and amino acid homeostasis.

Table 4.1. Composition of the experimental diets (% as fed).

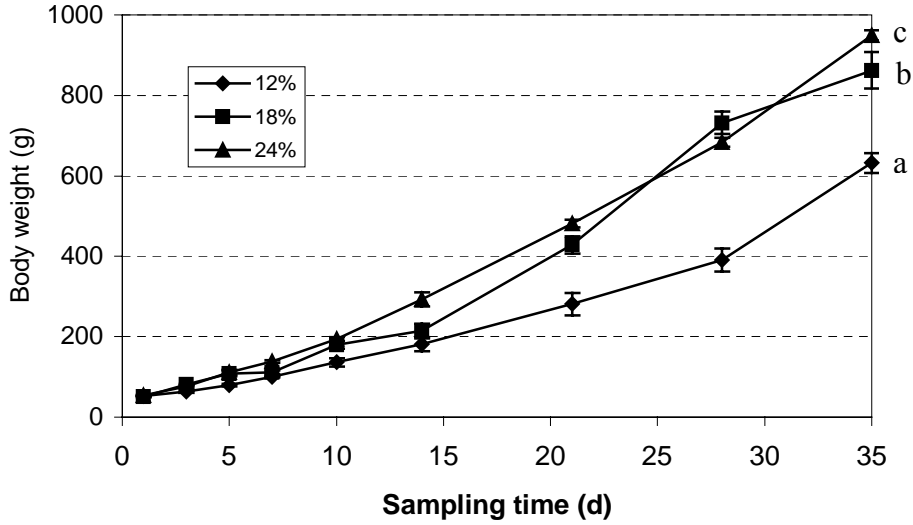
Percentage	12%CP ¹	18%CP	24%CP
Soybean meal	20.41	30.61	40.83
Corn	24.69	37.03	49.39
Soy oil	6.34	6.34	6.34
Dicalcium Phosphate	1.79	1.62	1.45
Limestone	1.17	1.19	1.21
Salt	0.50	0.50	0.50
V-M mix ²	0.16	0.16	0.16
Choline-Cl	0.07	0.07	0.07
BMD-50 ³	0.05	0.05	0.05
Corn starch	44.82	22.43	0

¹. Metabolizable energy was 3,549, 3,300, and 3,051 kcal/kg for diets containing 12, 18, and 24% crude protein, respectively.

². Vitamin-mineral pre-mix was provided by Akey Inc., Lewisburg, OH. The vitamin-mineral pre-mix contained the following (per kg vitamin-mineral mix): calcium, 86.13 g; zinc, 68.76 g; iron, 24.08 g; manganese, 80.07 g; copper, 6.01 g; iodine, 0.636 g; selenium, 0.1875 g; vitamin A, 6908 kIU; vitamin D, 2225 kIU; vitamin E, 13,222 IU; vitamin K, 3.69 g; vitamin B12, 11 mg; menadione, 1.221 g; riboflavin, 5.31 g; pantothenic acid, 8.8 g; niacin, 35.24 g; thiamine, 1.28 g; pyridoxine, 1.89 g; folic acid, 687.6 mg; biotin, 66.86 mg.

³. BMD-50 was provided by ALPHARMA Inc., Fort Lee, NJ.

A.



B.

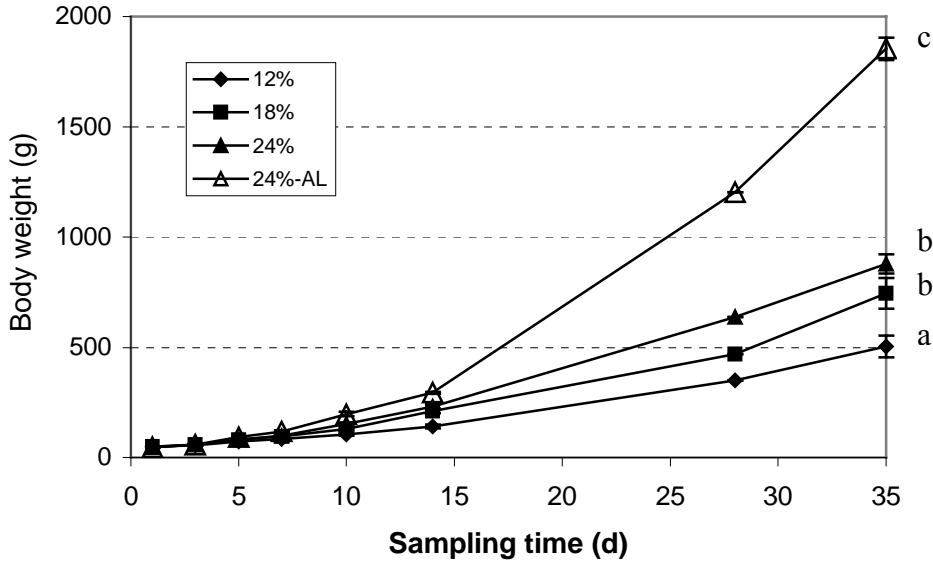


Figure 4.1. Body weights from Experiment 1 and 2. **A:** body weight of birds in Experiment 1. **B:** body weight of birds in Experiment 2. At time of sampling, birds were weighed individually before slaughtering. Each data point represents average body weight of five male chicks sampled at that time. $n = 5$, mean \pm SEM. Diet groups without a common superscript differed ($P < 0.05$). There was a crude protein*time interaction in Experiments 1 and 2 ($P < 0.0001$). There was a quadratic effect of time in Experiments 1 and 2 ($P < 0.0001$).

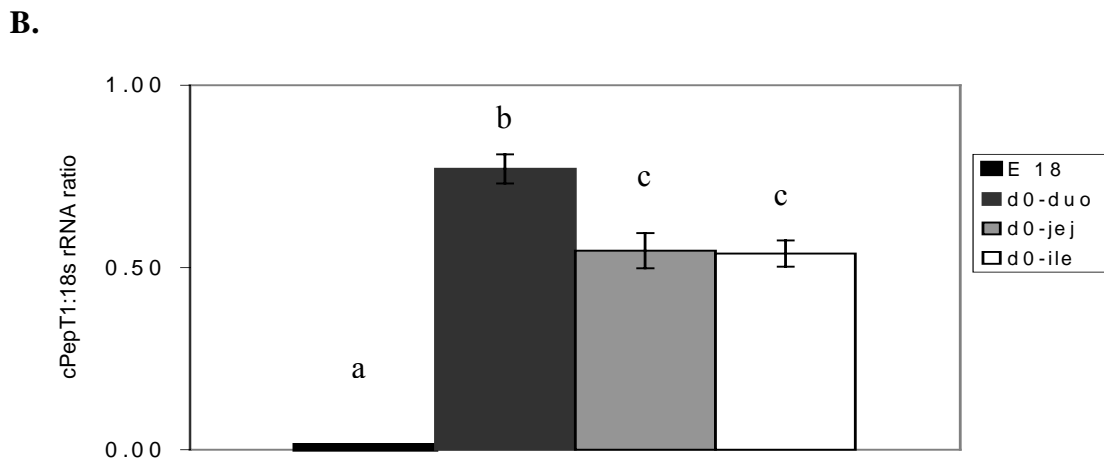
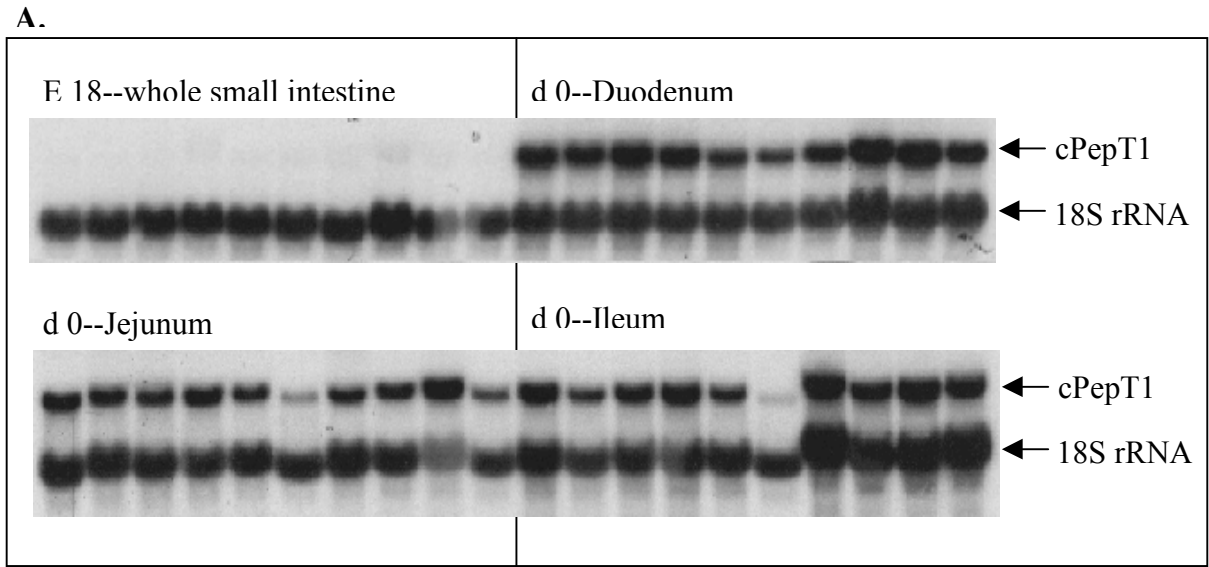


Figure 4.2. Northern blot analysis of cPepT1 mRNA expression during embryonic day 18 (E 18) and day of hatch (d 0). **A:** Northern blot of total RNA from intestinal tissues from E 18 and d 0 probed with cPepT1 cDNA probe. An 18s rRNA was used as internal control for the northern blot. Whole intestine total RNA was used for E 18 and individual intestinal sections were tested for d 0. There were samples from five males and five females in each section. Upper bands represent cPepT1 mRNA (~ 2.9 kb) and lower bands represent the internal control 18s rRNA (~2 kb). **B:** Densitometric data from the northern blot. Each bar represents the average ratio of densitometric measurement of cPepT1 to 18s rRNA from the same section of tissues from ten individual animals (five males and five females). Columns with different letters are different ($P < 0.05$). $n = 10$, mean \pm SEM.

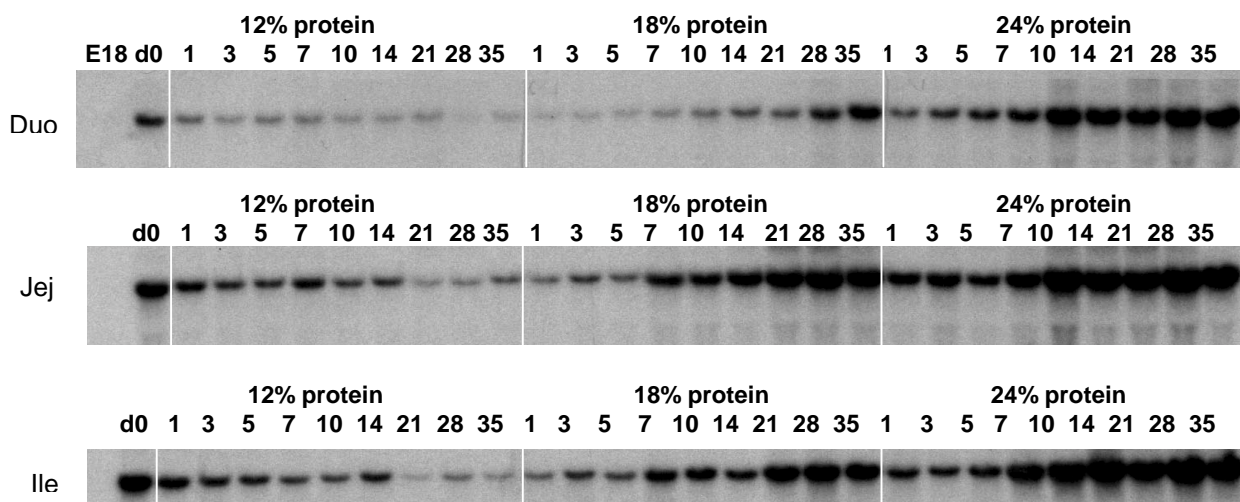


Figure 4.3. Northern blot analysis of cPepT1 mRNA expression from Experiment 1. Total RNA was extracted from individual tissues of chickens fed diets containing 12%, 18%, or 24% crude protein at sampling times of 1, 3, 5, 7, 10, 14, 21, 28, and 35 d. Total RNA was size-fractionated by agarose electrophoresis and transferred onto Nylon membranes. Blots were then hybridized with radiolabelled cPepT1 cDNA probe. The blot represents one of the five replicate gels. Each gel comprised one replicate including each of the three small intestinal sections from one of the five chickens of each of the treatment groups from each sampling time. Each lane represents an individual RNA sample from one section of the small intestine of one chicken sampled at the time point.

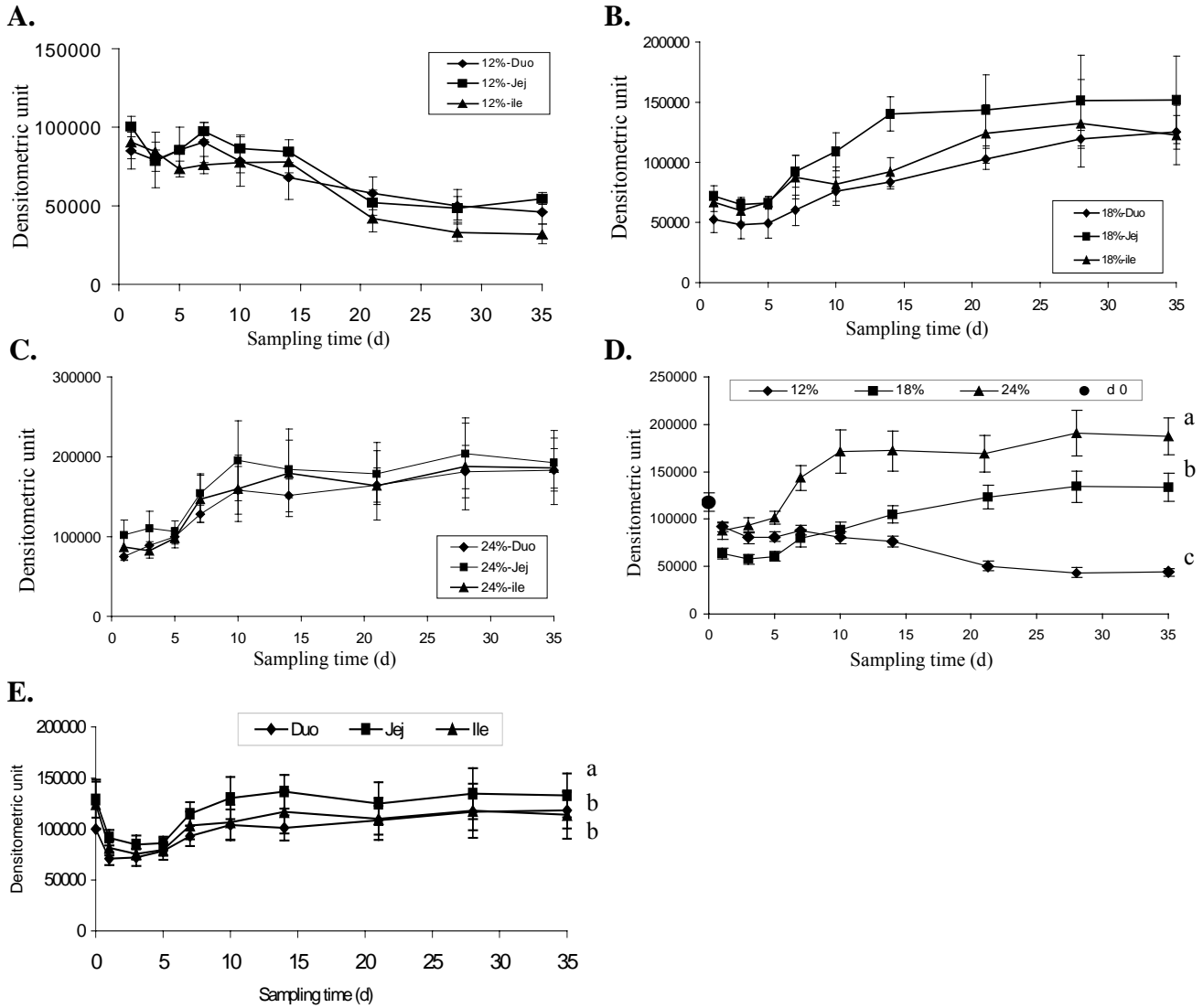


Figure 4.4. Northern blot analysis of cPepT1 mRNA expression from Experiment 1. Densitometric data from the northern blots are presented in **A** (12% CP), **B** (18% CP), **C** (24% CP), **D** (main effect of diet), and **E** (main effect of tissue). Each point in A, B, and C represents average densitometric measurements from one section of the small intestine of five chickens. $n = 5$, mean \pm SEM. In graph D, each point represents average densitometric measurement from three sections of the small intestine from five chickens fed one of the three diets that were sampled at the indicated time point. In graph E, each point represents average densitometric measurement from one section of the small intestine from 15 chickens fed the three diets that were sampled at the indicated time point. In graphs D and E, $n = 15$, mean \pm SEM. Diets or tissues lacking common superscripts differ ($P < 0.05$). There was a linear effect with time ($P < 0.0001$). There was a crude protein*time interaction ($P < 0.0001$).

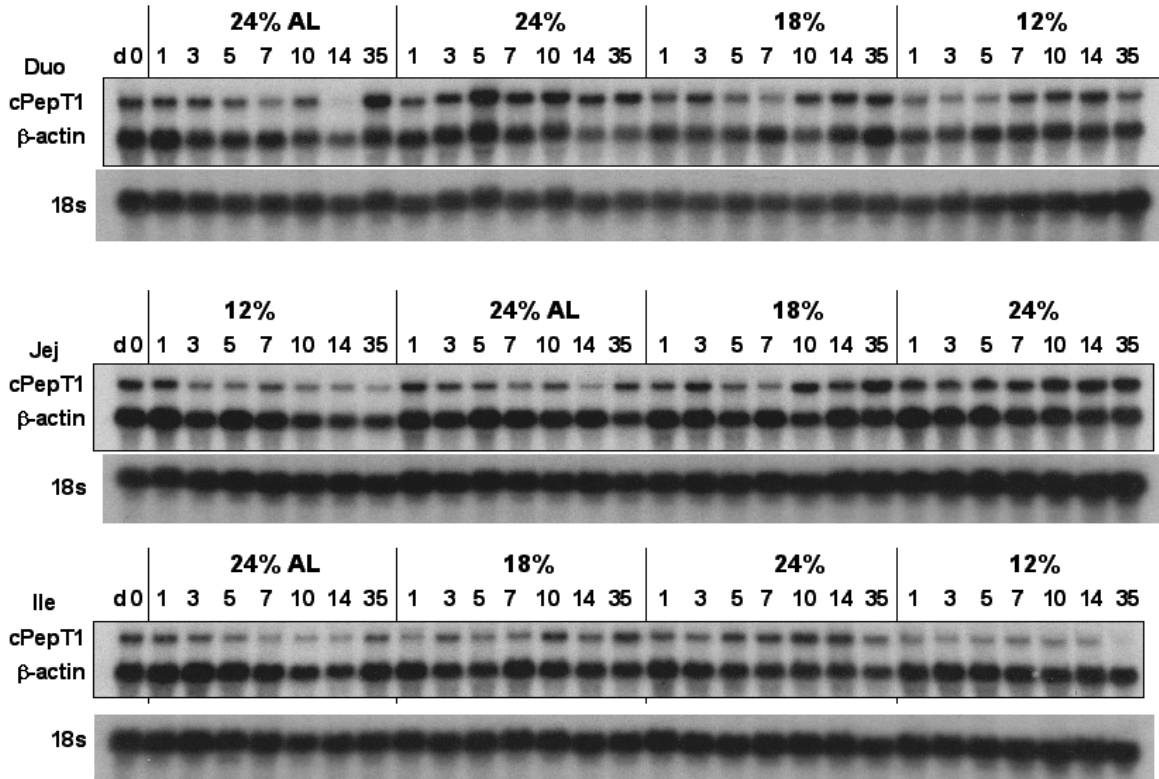


Figure 4.5. Northern blot analysis of cPepT1 mRNA expression from Experiment 2. Total RNA was extracted from individual tissues of chickens fed diets with 12%, 18%, or 24% CP and 24% CP *ad libitum* at sampling times of 1, 3, 5, 7, 10, 14, and 35 d. The blot represents one of the five replicate gels. Each gel comprised one replicate including each of the three small intestinal sections from one of the five chickens of each of the treatment groups from each sampling time. Upper bands represent cPepT1 mRNA, middle bands represent beta-actin, and lower bands represent the internal control 18s rRNA.

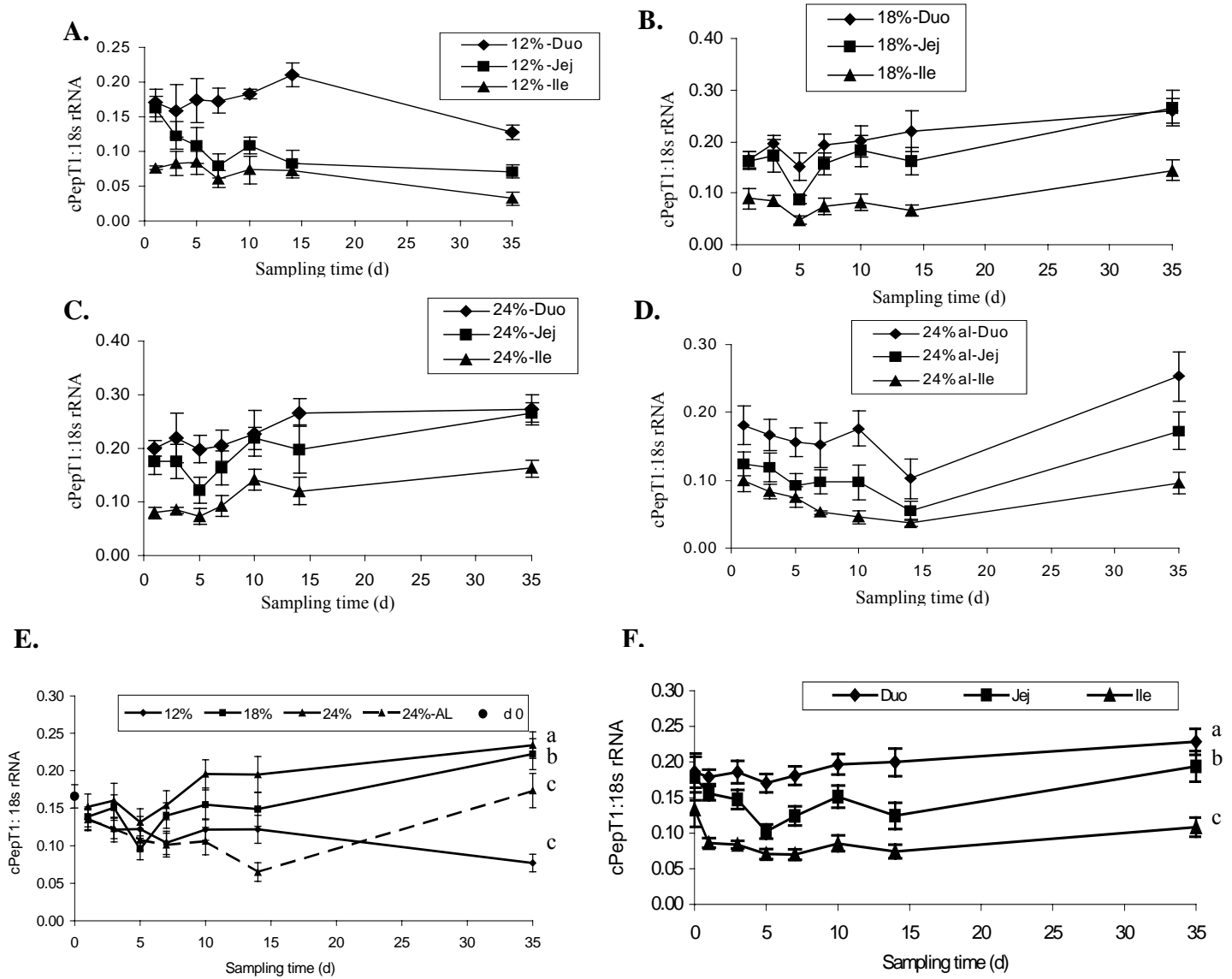


Figure 4.6. Densitometric analysis of cPepT1 mRNA abundance from Experiment 2. Densitometric data from the northern blots are presented in **A** (12% CP), **B** (18% CP) **C** (24% CP), **D** (24% CP *ad libitum*), **E** (main effect of diet), and **F** (main effect of tissue). Data are presented as ratio of densitometric readings of cPepT1 to 18s rRNA. Each point in A, B, C, and D represents mean value from one section of the small intestine of five chickens. $n = 5$, mean \pm SEM. In graph E, each point represents mean value from three sections of the small intestine from five chickens from each treatment group that were sampled at the indicated time point. $n = 15$, mean \pm SEM. In graph F, each point represents mean value from one section of the small intestine from 20 chickens from the four treatment groups that were sampled at the indicated time point. $n = 20$, mean \pm SEM. Diets or tissues lacking common superscripts differ ($P < 0.05$). There was a quadratic effect with time ($P < 0.0001$). There was a crude protein*time interaction ($P < 0.0001$) and a crude protein*tissue interaction ($P = 0.02$).

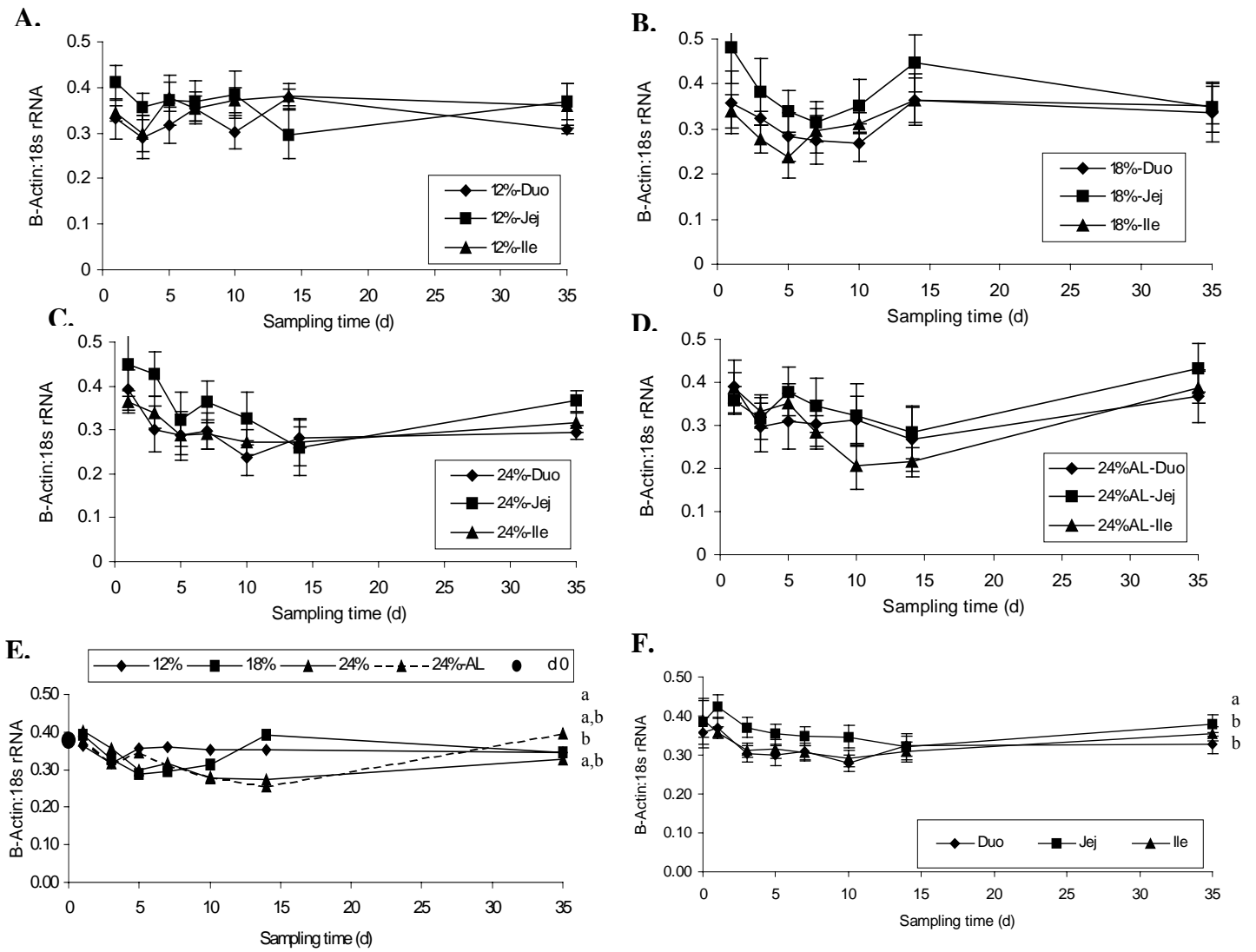


Figure 4.7. Densitometric analysis of beta-actin mRNA abundance from Experiment 2. Densitometric data from the northern blots are presented in **A** (12% CP), **B** (18% CP) **C** (24% CP), **D** (24% CP *ad libitum*), **E** (main effect of diet), and **F** (main effect of tissue). Data are presented as ratio of densitometric readings of beta-actin to 18s rRNA. Each point in A, B, C, and D represents mean value from one section of the small intestine of five chickens. $n = 5$, mean \pm SEM. In graph E, each point represents mean value from three sections of the small intestine from five chickens from each treatment group that were sampled at the indicated time point. $n = 15$, mean \pm SEM. In graph F, each point represents mean value from one section of the small intestine from 20 chickens from the four treatment groups that were sampled at the indicated time point. $n = 20$, mean \pm SEM. Diets or tissues lacking common superscripts differ ($P < 0.05$). There was a linear effect with time ($P = 0.0009$).

Chapter V

Characterization and Regulation of a Cloned Ovine Gastrointestinal Peptide Transporter (oPepT1) Expressed in a Mammalian Cell Line

ABSTRACT

To investigate the kinetics of peptide transport by the peptide transporter, PepT1, Chinese hamster ovary cells (CHO) were transfected with an expression vector containing the cloned ovine PepT1 cDNA. Transport was assessed by uptake studies using the radiolabeled dipeptide, [³H]-Gly-Sar. Expression of oPepT1 was detected at 8 to 24 h post-transfection with an optimal time of 16 to 24 h. Uptake of Gly-Sar by oPepT1 was pH-dependent with an optimal pH of 5.5 to 6.0, concentration-dependent, and saturable with an apparent K_m value of 1.0 ± 0.1 mmol/L and a maximum velocity of 14.3 ± 0.4 nmol·mg protein⁻¹·40min⁻¹. Competition studies with non-radiolabeled peptides and [³H]-Gly-Sar showed that all di- and tripeptides inhibited uptake of [³H]-Gly-Sar. In addition, three tetrapeptides (Met-Gly-Met-Met, Pro-Phe-Gly-Lys, and Val-Gly-Ser-Glu) also showed inhibition of [³H]-Gly-Sar uptake. There was no inhibition of [³H]-Gly-Sar uptake detected in the presence of non-radiolabeled free amino acids. Treatment of the cells with staurosporine, an inhibitor of protein kinase C (PKC), resulted in an increase of the transport system. This increase was specific and could be blocked if treatment was done in the presence of phorbol 12-myristate-13-acetate (PMA), an activator of PKC. The staurosporine- and PMA-induced changes in peptide transport activity were not affected by co-treatment with cycloheximide. These results demonstrate that the transport of peptide

substrates by oPepT1 in transfected mammalian cells is similar to that in microinjected *Xenopus* oocytes, and PKC phosphorylation may play a regulatory role in oPepT1 function.

Key Words: oPepT1, CHO cell, Protein Kinase.

INTRODUCTION

Peptide transport is an important physiological process that occurs in all animals (Matthews, 1991). The cloning and characterization of peptide transporters, PepT1 and PepT2, has provided valuable information about peptide transport in mammalian species (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995; Miyamoto et al., 1996). These peptide transporters recognize di- and tripeptide substrates, as well as pharmacologically active compounds including β -lactam antibiotics, angiotensin-converting enzyme inhibitors, and the antitumor agent, bestatin (Leibach and Ganapathy, 1996). The peptide transporter, PepT1, is mainly expressed in the small intestine with little expression occurring in liver or kidney (Boll et al., 1994; Fei et al., 1994), while PepT2 is mainly expressed in the kidney (Liu et al., 1995; Ramamoorthy et al., 1995; Saito et al., 1996).

Little research has been conducted to identify the system(s) responsible for the absorption of peptides in food-producing animals. The cloning and expression of an ovine peptide transporter (oPepT1) in *Xenopus* oocytes provided information for the first time on the molecular structure and basic functions of a peptide transporter in food-producing animals (Pan et al., 2001). To investigate the function of oPepT1 in mammalian cells, oPepT1 was transiently transfected in CHO cells and expression of oPepT1 was studied by measuring the uptake of [3 H]-Gly-Sar into transfected CHO cells. Our results show that oPepT1 can efficiently transport di- and tripeptides and that activity of oPepT1 can be altered by protein kinase C activity.

MATERIALS AND METHODS

Materials. All chemicals, substrates, and reagents were of either molecular biology or cell culture tested chemical grades. [³H]-Gly-Sar (specific radioactivity, 110 mCi/mmol) was purchased from Moravek Biochemical (Brea, CA). Media, nonessential amino acids, and trypsin were purchased from Fisher Scientific (Pittsburgh, PA). Lipofectamine was purchased from Life Technologies/GIBCO BRL (Gaithersburg, MD). CHO cells were supplied by American Type Culture Collection (Rockville, MD). Penicillin, streptomycin, staurosporine, phorbol 12-myristate-13-acetate (PMA), cycloheximide, and unlabeled peptides (dipeptides to tetrapeptides) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% nonessential amino acids, and penicillin (100 U/mL)/ streptomycin (100 mg/mL). All cells were cultured in an atmosphere of 5% CO₂ and 90% relative humidity at 37°C. Culture medium was changed daily. One day before transfection, cells were trypsinized and plated onto 12-well plates at a density of 2.4×10^5 /well.

Transfection. The construct used in all expression studies contained the oPepT1 cDNA (Pan et al., 2001) cloned into the pBK-CMV expression vector (Stratagene, La Jolla, CA) under the control of the CMV promoter. CHO cells were transfected with the pBK-oPepT1 expression vector or just the pBK-CMV vector as a control for measuring endogenous transport activity. Transfection was done according to the manufacturer's protocol. Briefly, for each well, 0.8 µg plasmid (with or without insert) were mixed with

2.4 μ L Lipofectamine (2 μ g/ μ L) in 40 μ L OPTI-MEM reduced serum medium and incubated at room temperature for 30 min. The DNA-lipid complex was then added to each well and cells were transfected for 5 hr at 37°C in a 5% CO₂, 90% relative humidity incubator. For each assay, at least two transfections were performed.

Transport assay. Transport activity of CHO cells transiently transfected with oPepT1 was examined with [³H]-Gly-Sar, a known hydrolysis-resistant peptide substrate. Transfected cells were washed three times with pH 6.0 uptake buffer containing 25 mmol/L MES/Tris, 5 mmol/L glucose, 0.8 mmol/L MgSO₄, 1.8 mmol/L CaCl₂, 5.4 mmol/L KCl, and 140 mmol/L NaCl. Gly-Sar solution was prepared at 6 concentrations (0.02 to 10 mmol/L, with 1 μ Ci/mL [³H]-Gly-Sar). Uptake solutions of varying pH were added to each well and incubated for 40 min at room temperature. Uptake was stopped by washing cells with ice-cold uptake buffer. Cells were lysed by adding 0.5 mL 0.1% SDS followed by incubation at room temperature for 10 min. The ³H content of the cell lysate was quantified by liquid scintillation counting (LS 500TA scintillation counter; Beckman Instrument, Fullerton, CA) and the protein amount of each cell extract was measured by a modified Lowry assay using the Bio-Rad DC Protein kit (Bio-Rad, Hercules, CA).

Inhibition studies were performed under the same condition as described above, except that 20 μ mol/L [³H]-Gly-Sar (50 mCi/ mmol) was used for the radiolabeled substrate and five concentrations (0.001 to 10 mmol/L) of inhibitor substrates were added to the reaction to measure inhibition of [³H]-Gly-Sar uptake. The concentration of competitive peptide that caused 50% inhibition of Gly-Sar uptake (IC₅₀) was calculated by PRISM (GraphPad, San Diego, CA).

In vitro regulation assay. For studies of protein kinase C inhibition or activation on peptide transport activity, transfected CHO cells were preincubated in the presence of 0.1 and 1 $\mu\text{mol/L}$ staurosporine or 1 and 10 $\mu\text{mol/L}$ PMA. Solutions of staurosporine, PMA, and cycloheximide were prepared in dimethyl sulfoxide. The effects of pretreatment time and concentration of effectors on Gly-Sar uptake were studied at 30 and 60 min. After treatment, cells were washed twice with uptake buffer before initiation of uptake measurements in the absence of effectors. Gly-Sar solution was prepared at 0.02 mmol/L with 1 $\mu\text{Ci/mL}$ [^3H]-Gly-Sar. Uptake solutions were added to each well and incubated for 40 min at room temperature. Uptake was stopped by washing cells with ice-cold uptake buffer. [^3H]-Gly-Sar uptake was determined as described above.

Calculations and statistics. Determination of kinetic parameters and all other calculations (linear as well as nonlinear regression analysis) were performed using PRISM. Results are presented as the mean \pm SEM. Data were evaluated using one-way analysis of variance. The least significant difference test was used for post hoc comparisons. A probability (P) value of less than 0.05 was considered to be significantly different. Data were also analyzed by regression analysis to investigate the form of the relationship between transport affinity constant and peptide characteristics. The REG procedure of SAS (SAS Institute Inc., 1989) was used in this study for regression analysis.

RESULTS

Functional expression of oPepT1 cDNA in CHO cells. The time course for expression of oPepT1 in transfected CHO cells was examined by measuring the uptake of [^3H]-Gly-Sar. Expression of oPepT1 was detectable at 8 h post-transfection and plateaued

between 16 and 21 h (Figure 5.1A). In all subsequent experiments, transport was measured 16 h after transfection. Uptake of [^3H]-Gly-Sar by CHO cells transfected with the pBK-CMV vector alone was only ~1% of that observed in pBK-oPepT1-transfected CHO cells. Thus, there was negligible endogenous Gly-Sar transport activity in CHO cells. Therefore, the uptake values measured in pBK-oPepT1-transfected cells were analyzed directly without being adjusted for the values obtained in pBK-CMV-transfected CHO cells.

The optimal time of incubation for the uptake of Gly-Sar was also determined in transfected CHO cells (Figure 5.1B). Uptake of Gly-Sar by pBK-oPepT1-transfected CHO cells increased as incubation time with [^3H]-Gly-Sar increased. The uptake rate plateaued between 40 and 80 min. There was no increase of the uptake of [^3H]-Gly-Sar in pBK-CMV transfected CHO cells. Therefore, in all subsequent experiments, the time of incubation for transport measurements was 40 min.

pH and concentration effects on Gly-Sar uptake. The effect of extracellular pH on the uptake of [^3H]-Gly-Sar was studied in transfected CHO cells. The results in Figure 5.2 showed that, in pBK-oPepT1-transfected CHO cells, uptake was greater at pH 5.5 and 6.0 than at 5.0, 6.5, 7.0, and 7.5. Thus, optimum uptake was at pH 5.5 and 6.0. Control CHO cells transfected with only the pBK-CMV vector showed negligible transport when measured (data not shown). Therefore, an extracellular pH of 6.0 was used in all subsequent experiments with oPepT1-transfected cells.

Transport kinetics of oPepT1 was measured using Gly-Sar at concentrations ranging from 0.02 to 10 mmol/L at pH 6.0. The uptake of Gly-Sar in CHO cells transfected with oPepT1 was concentration dependent and saturable (Figure 5.3). The

transport constant (K_t) and maximum velocity (V_{max}) for Gly-Sar uptake were determined to be 1.0 ± 0.1 mmol/L and 14.3 ± 0.4 nmol·mg protein⁻¹·40min⁻¹, respectively. When the data from uptake of Gly-Sar were plotted as an Eadie-Hofstee graph (uptake rate/substrate concentration versus uptake rate), a straight line was observed ($r = 0.98$; Figure 5.3, inset). This is indicative of the presence of a single transporter system.

Inhibition effects of peptides on Gly-Sar uptake. The transport of different peptides in transfected CHO cells was determined by inhibition studies. In oPepT1-transfected CHO cells, inhibition of [³H]-Gly-Sar uptake by seven dipeptides, six tripeptides, three tetrapeptides, and two amino acids was examined to calculate the IC₅₀ values of the competitive substrates. Among eighteen peptides used in the inhibition assays, the IC₅₀ of six dipeptides and three tripeptides for inhibition of Gly-Sar uptake ranged from 13.6 to 126.7 μmol/L (Table 5.1). IC₅₀ was defined as the concentration of competitive peptide that showed 50% inhibition of Gly-Sar uptake. Lower IC₅₀ values indicated higher binding affinity of the competitive peptide to the peptide transporter. Some C-terminal Lys-containing peptides, such as Lys-Lys, Lys-Trp-Lys, Lys-Tyr-Lys, and Thr-Ser-Lys showed weak inhibition of Gly-Sar uptake (IC₅₀ from 739.8 to 3732.0 μmol/L). No inhibition of Gly-Sar uptake by free amino acids (Met and Lys) was observed (data not shown). Three tetrapeptides were tested and each had large IC₅₀ values in pBK-oPepT1-transfected CHO cells. Since there was no transport of these tetrapeptides in *Xenopus* oocytes (Pan et al., 2001), the large IC₅₀ observed in transfected CHO cells may be due to membrane peptidases that cleave the tetrapeptides to di- and/or tripeptides, which then compete for transport of Gly-Sar. A summary of the overall inhibition pattern of Gly-

Sar uptake by different classes of peptides is shown in Figure 5.4. Di- and tripeptides are clearly preferred substrates for oPepT1. The peptides examined constitute a variety of substrates differing in their molecular weight, net electrical charge, hydrophobicity, and isoelectric point. However, for all peptides, no correlation was found between IC₅₀ and molecular weight, net charge, hydrophobicity, and isoelectric point. Therefore, oPepT1 transported peptides independent of these physical characteristics.

Regulation of oPepT1 activity by protein kinase C in CHO cells. The effects of a PKC inhibitor or activator on oPepT1 transport activity were investigated. Effect of pre-treatment of the CHO cells with uptake buffer (MES) only as the control, 0.1 and 1 µmol/L staurosporine (an inhibitor of PKC), and 1 and 10 µmol/L PMA (an activator of PKC) for 30 and 60 min is shown in Figure 5.5A. A significant increase (35%) of the transport system occurred when transfected CHO cells were incubated with 1 µmol/L staurosporine for 30 min prior to uptake measurement. A significant decrease (28%) of the transport system occurred when transfected CHO cells were incubated with 10 µmol/L PMA for 30 min before uptake measurement. Treatment of the cells with MES buffer only, staurosporine, or PMA for 60 min before uptake measurement resulted in lower transport activity in all treatment groups compared with the cells treated only 30 min ($P < 0.05$). Therefore, prolonged incubation before uptake measurement was harmful to transfected cells.

To further investigate the action of staurosporine and PMA on PKC, the transfected cells were incubated with staurosporine and PMA before uptake measurement (Figure 5.5B). The increase of transport activity by staurosporine was specific and could be

blocked if treatment was done in the presence of PMA. To evaluate whether the increase or decrease of transport activity by staurosporine or PMA was due to changes in de novo protein synthesis, the transfected cells were co-treated with staurosporine or PMA in the presence of cycloheximide, an inhibitor of cellular protein synthesis, before the uptake measurement. The staurosporine- or PMA induced changes of the peptide-transport system were not affected by co-treatment with cycloheximide (Figure 5.5B). Together these data suggest that PKC phosphorylation plays a regulatory role in oPepT1 function.

DISCUSSION

The gastrointestinal peptide transporter oPepT1 has been shown to transport a broad range of peptide substrates using *Xenopus* oocytes as the expression system (Pan et al., 2001). *Xenopus* oocytes are a robust system for the expression of many different proteins of animal or plant origin (Kay and Peng, 1991). However, one of the unresolved issues regarding evaluation of peptide transport is whether a difference in substrate affinity and transport might exist for PepT1 expressed in *Xenopus* oocytes and mammalian cells. The present study was designed to characterize the activity of oPepT1 expressed in a mammalian cell line, CHO cells.

The present observations with oPepT1-transfected CHO cells are in agreement with previous reports that protons are critical for the transport process with an optimal pH of 5.5 to 6.0 (Steel et al., 1997; Pan et al., 2001). Transport kinetics of Gly-Sar uptake determined in oPepT1-transfected CHO cells were also comparable to those obtained previously (Pan et al., 2001). In general, functional expression in CHO cells indicated that oPepT1 has high affinity (low IC₅₀ values) for most of the dipeptides and tripeptides

examined. A low affinity (high IC_{50} values) was observed for dipeptides and tripeptides containing a C-terminal lysine. The nutritional implications of our data are not clear at the moment. Either these peptides with low affinity are less favorable substrates for oPepT1, or the physiological concentrations of these peptides are higher than other peptides such that they can still be transported by oPepT1.

We evaluated the transport characteristics of oPepT1 using peptides containing essential amino acids (mainly methionine and lysine). A comparison of the IC_{50} data of CHO cells in the present study with the K_t data obtained previously (Pan et al., 2001) revealed that the relationship between IC_{50} and K_t is high ($r = 0.81$, $P < 0.002$). Thus the two in vitro expression systems predict similar transport characteristics for oPepT1. This is also confirmed by other reports using multiple expression systems (Liang et al., 1995; Han et al., 1999). The CHO cell is a suitable system to study competition between substrates whereas the *Xenopus* oocyte system monitors the co-transport of protons with the peptides. Because of the nature of inhibition studies, false transport results might be observed when the competitor binds to the transporter without actually being transported inside the cell. Likewise, membrane peptidases, if present, could result in the hydrolysis of large peptides followed by uptake of the smaller peptides. For example, in the present study, tetrapeptides showed inhibition of Gly-Sar uptake in oPepT1-expressing CHO cells. In the *Xenopus* oocyte system, these tetrapeptides did not evoke any detectable inward current (Pan et al., 2001). Therefore, we conclude that, in the CHO cell system, these tetrapeptides may be degraded by membrane peptidases and absorbed as smaller di- or tripeptides which then compete for Gly-Sar uptake. Alternatively, these tetrapeptides

might just bind to the transporter itself without being actually transported inside, causing a change in the transporter conformation that indirectly affects Gly-Sar uptake.

The translocation of the PepT1 protein from intracellular storage sites to the cell surface, and the signalling pathways that link the regulators to PepT1 translocation remain poorly understood. Results from our previous study have predicted the existence of consensus amino acid sequences for PKC phosphorylation of oPepT1 (Pan et al., 2001). In the present study, the function of oPepT1 was affected by PKC activation or repression, which was also observed in other studies (Brandsch et al., 1994; Wenzel et al., 1999). However, whether the functional regulation of oPepT1 by PKC is due to direct phosphorylation of oPepT1 protein or is achieved in an indirect manner, such as phosphorylation of a mediator protein, remains unknown. Further study using a PKC null mutant, where all putative PKC phosphorylation sites are eliminated by replacement of specific serine/threonine residues, will more directly address this question.

In summary, this paper describes the functional characterization of a peptide transporter oPepT1 in transfected mammalian cells. The transport function of oPepT1 in CHO cells indicated that oPepT1 is capable of transporting all dipeptides and tripeptides examined in a proton dependent manner. Neither tetrapeptides nor free amino acids are proper substrates for oPepT1. The transport of peptide substrates by oPepT1 in transfected mammalian cells is similar to that in microinjected *Xenopus* oocytes. In vitro treatment of CHO cells with PKC effectors indicated that PKC phosphorylation plays a regulatory role in oPepT1 function. Our studies not only demonstrated that the transport of peptide

substrates by oPepT1 is independent of the expression system, but also lay the foundation for further *in vivo* studies to better understand the mechanisms of protein absorption.

Table 5.1. Kinetics of oPepT1 in CHO cells transfected with oPepT1 cDNA.

Substrate	MW	Charge ¹	Hydrophobicity ²	pI ³	IC ₅₀ ⁴ (μM)
Dipeptide					
Lys-Lys	274	positive	-7.8	10.0	739.8
Lys-Met	277	positive	-2.0	8.8	50.6
Lys-Phe	293	positive	-1.1	8.8	23.9
Met-Glu	278	negative	-1.6	4.6	36.6
Met-Gly	206	neutral	1.5	5.3	15.6
Met-Leu	262	neutral	5.7	5.3	20.6
Met-Lys	277	positive	-2.0	8.5	123.0
Met-Met	280	neutral	3.8	5.3	24.0
Tripeptide					
Leu-Gly-Gly	245	neutral	3.0	5.5	126.7
Leu-Ser-Phe	365	neutral	5.8	5.5	70.7
Lys-Trp-Lys	460	positive	-8.7	10.0	3732.0
Lys-Tyr-Lys	437	positive	-9.1	9.7	2028.0
Met-Leu-Phe	409	neutral	8.5	5.3	13.6
Thr-Ser-Lys	334	positive	-5.4	8.4	3012.0
Tetrapeptide					
Met-Gly-Met-Met	468	neutral	5.3	5.3	952.2
Pro-Phe-Gly-Lys	447	positive	-3.1	9.2	2074.0
Val-Gly-Ser-Glu	390	negative	-0.5	4.6	6870.0

¹Charge: calculated at pH 6.0.

²Hydrophobicity: calculated as the average of the value of the constituent amino acids according to Kyte and Doolittle (1982).

³pI: calculated using pK values of the constituent amino acids.

⁴IC₅₀: determined as the unlabeled peptide concentration that showed 50% inhibition of Gly-Sar uptake.

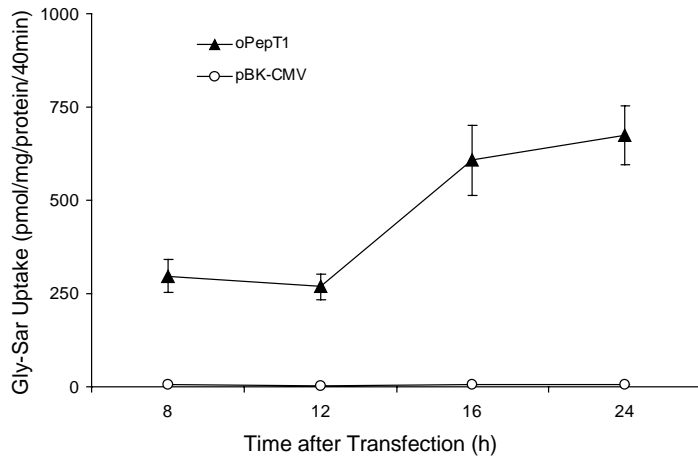
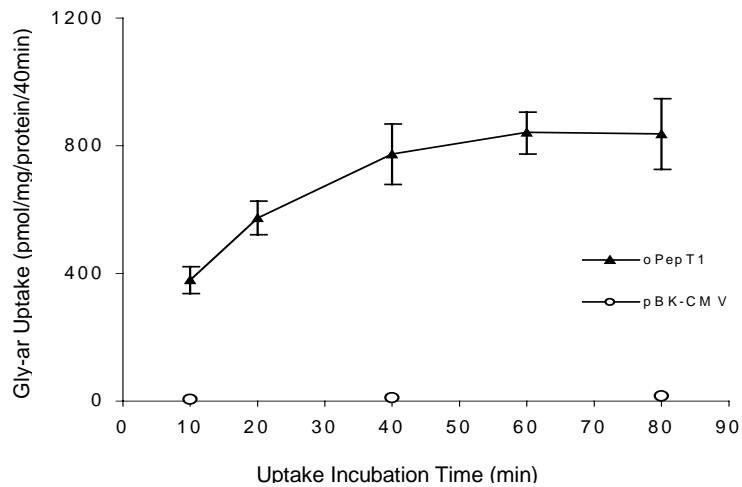
A.**B.**

Figure 5.1. Time course for oPepT1 expression and uptake incubation. Transport activity of CHO cells transiently transfected with pBK-oPepT1 expression vector was examined using [³H]-Gly-Sar. Endogenous transport was determined in parallel experiments by transfection of only the pBK-CMV vector. A. Gly-Sar uptake in transfected CHO cells at 8, 12, 16, and 24 h after transfection. B. Gly-Sar uptake in transfected CHO cells during 10, 20, 40, 60, and 80 min incubation, 16 h after transfection. Values are means \pm SEM, n = 6 total wells in 2 transfections.

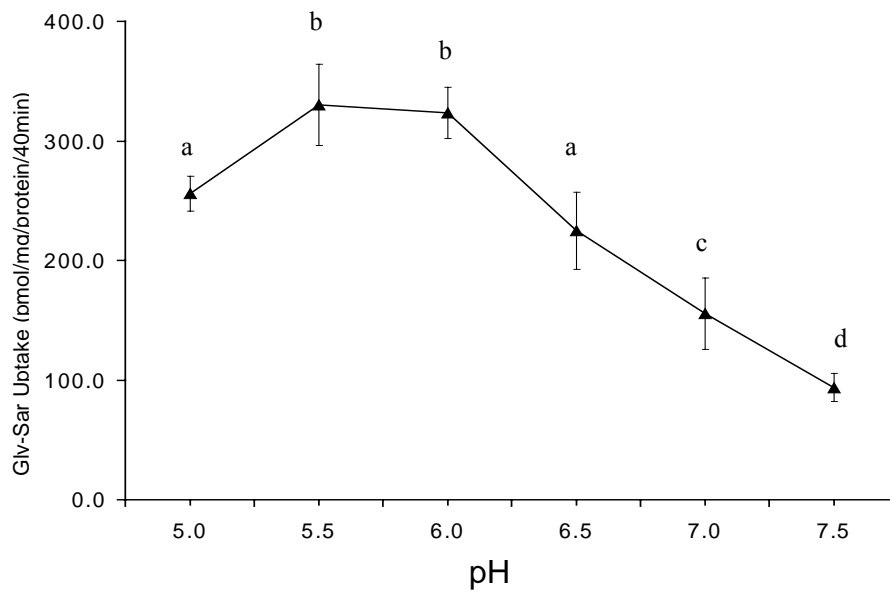


Figure 5.2. pH dependency in oPepT1-transfected CHO cells. CHO cells were transfected with pBK-oPepT1 expression vector. Transfected cells were then incubated with uptake buffer containing [^3H]-Gly-Sar at pH 5.0-7.5. Values are means \pm SEM, n = 6 total wells in 2 transfections. Means without a common letter differ, P < 0.05.

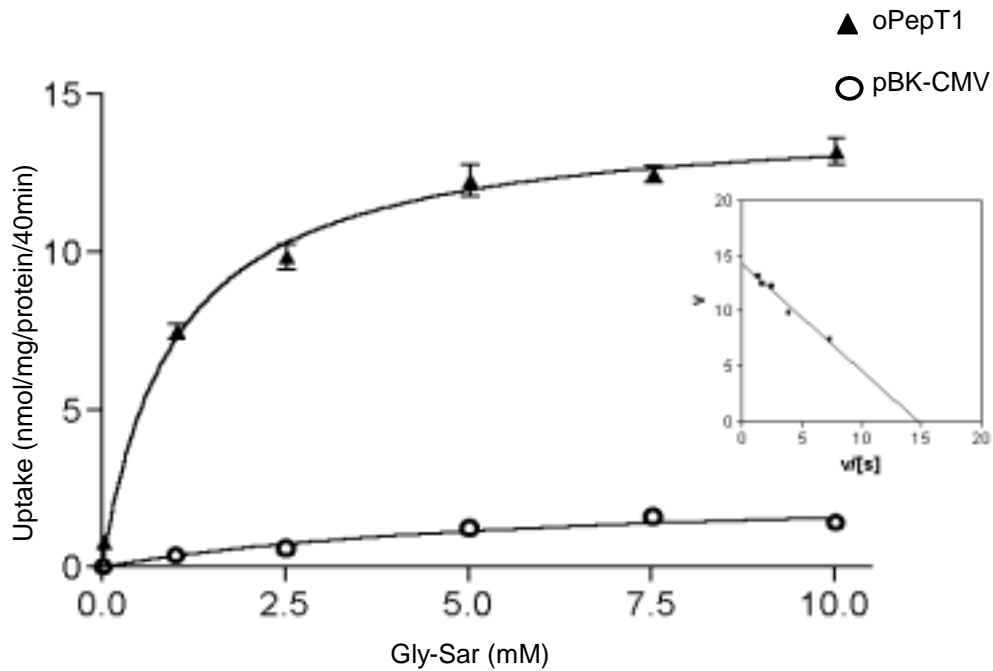


Figure 5.3. Kinetic parameters of Gly-Sar were measured in CHO cells transfected with pBK-oPepT1 expression vector. Transfected CHO cells were incubated with six Gly-Sar concentrations (0.02 to 10 mM). Vector pBK-CMV without insert was transfected into CHO cells as a control. Values are means \pm SEM, $n = 6$ total wells in 2 transfections. Inset: Eadie-Hofstee plot of the Gly-Sar uptake in pBK-oPepT1-transfected CHO cells.

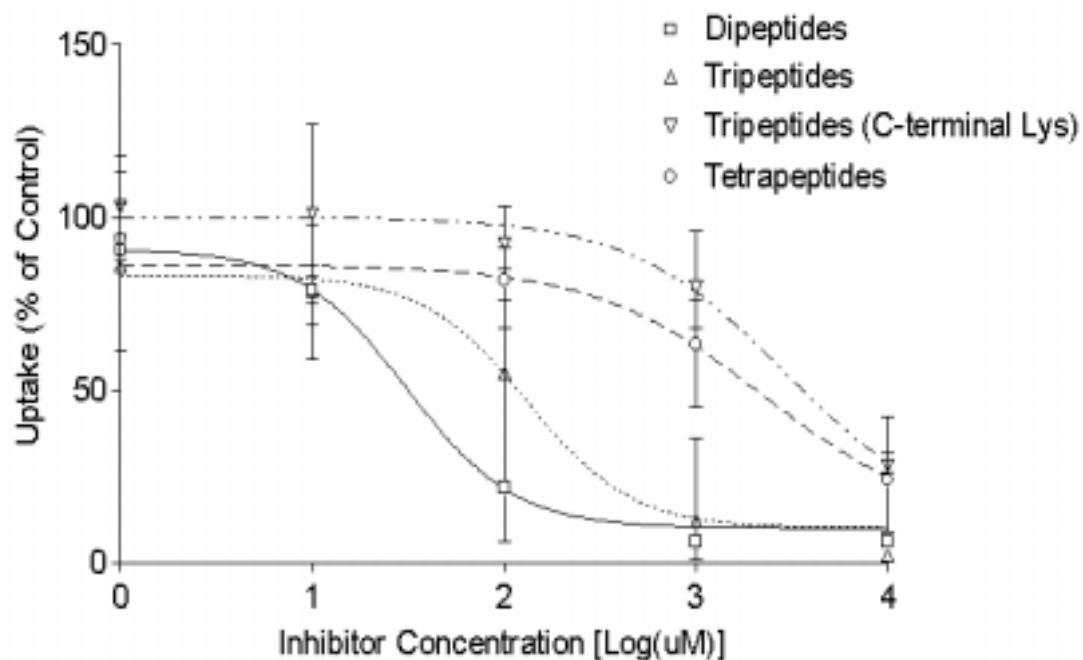


Figure 5.4. Inhibition of Gly-Sar uptake by different classes of peptides. Inhibition studies were performed by coincubating [^3H]-GlySar ($20\ \mu\text{M}$) with individual peptide substrates at concentrations of 1, 10, $100\ \mu\text{M}$ and 1 and $10\ \text{mM}$ at pH 6.0. Seven dipeptides, three tripeptides, three C-terminal Lys-containing tripeptides, and three tetrapeptides were evaluated. Values are means \pm SEM of indicated peptide groups.

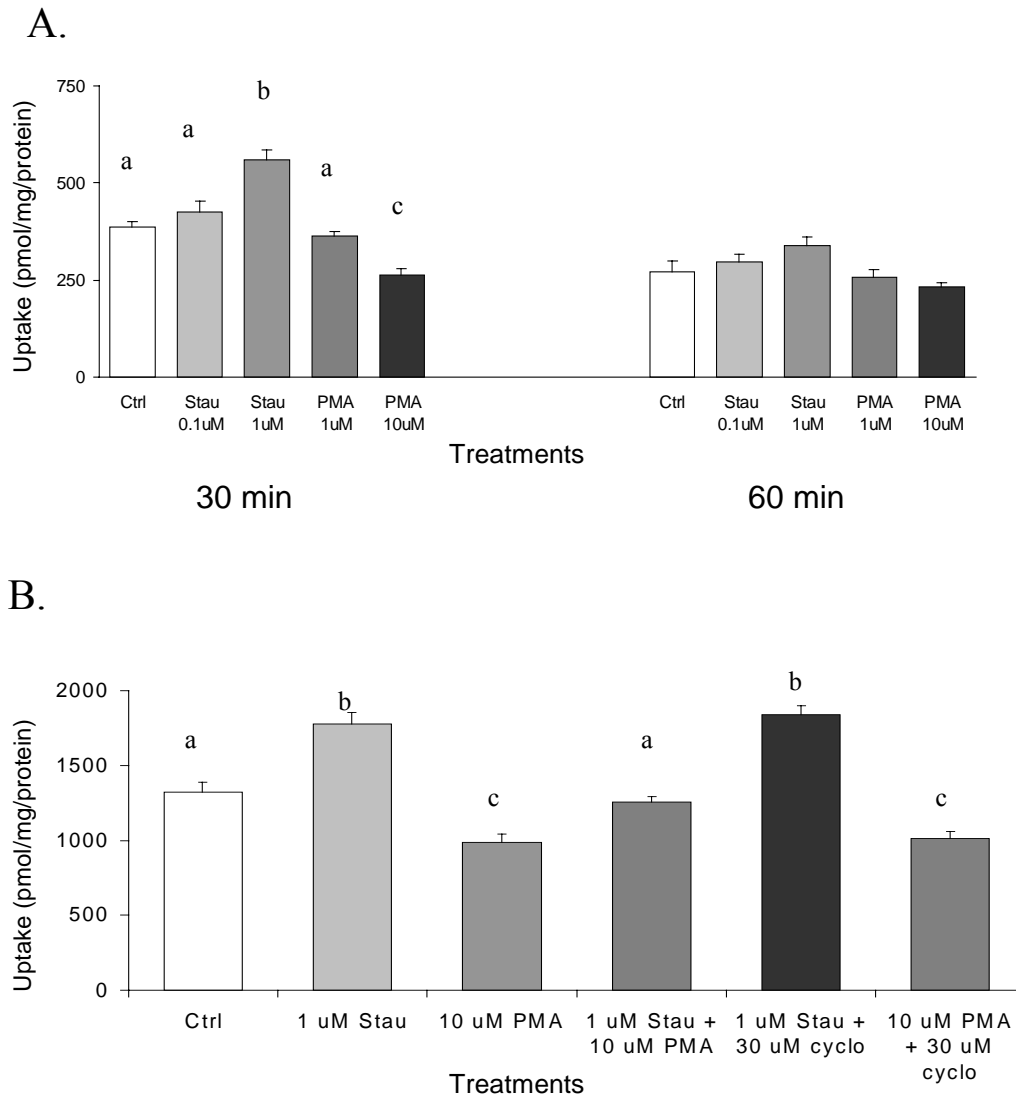


Figure 5.5. Influence of a protein kinase C activator or inhibitor on Gly-Sar uptake in pBK-oPepT1 transfected CHO cells. pBK-oPepT1-transfected CHO cells were preincubated for different time periods with the effectors, or buffer alone as a control before measurement of Gly-Sar uptake. Values are means \pm SEM, $n = 6$ total wells in 2 transfections. Stau: staurosporine; PMA: phorbol 12-myristate-13-acetate; Cyclo: cycloheximide. Means without a common letter differ, $P < 0.05$.

A. Effects of pre-treatment time and concentration on Gly-Sar uptake. Pretreatment for 30 min differed ($P < 0.05$) from pretreatment for 60 min before uptake measurement.

B. Effects of co-incubation of PKC activator and inhibitor and protein synthesis inhibitor on Gly-Sar uptake.

Chapter VI

Epilogue

The cloning of peptide transporters, PepT1 and PepT2, from mammalian species opens a new era in peptide transport protein nutrition research. Unique features of these peptide transporters, such as protonmotive force of the transport, broad yet specific substrate range, and highly conserved transporter structure have been revealed largely following the successful cloning of these two transporters. As reviewed in chapter II of this dissertation, recent studies have been focused on the mechanisms of peptide transport by PepT1 and PepT2, the possible applications of peptide transport, and more importantly, the regulation of peptide transport in animals.

To achieve the ultimate goal of better nutritional management, a clearer understanding of peptide transport mechanisms is needed. From current information, it is clear that all di- and tripeptides are not equivalent substrates of cPepT1 and oPepT1. Desirable configurations of substrates are yet to be elucidated. Instead of testing all possible substrates, which are over 8,000 di- and tripeptides, it might be a better approach to use the *Xenopus* oocyte expression system and CHO cells that over-express cPepT1 or oPepT1 as screening tools to test existing protein supplements derived from partial hydrolysis. The two systems are proven to be applicable to test for better uptake performance. If feeding animals with partial protein hydrolysis based on selections for better peptide uptake by PepT1 result in enhanced growth performance, this will be an indication of direct relationship between peptide transport and animal growth.

Identification and analysis of gene structures of cPepT1 and oPepT1 are needed for future development of targeted gene knockout mouse models. Targeted gene knockout mice will provide a great tool to facilitate research on importance of peptide transporters. With available knockout mice, functions of the normal peptide transport system are disrupted or greatly attenuated. The closely related physiological and nutritional events will be affected and damage resulting from the lack of peptide transporters will be revealed. Experiments can also be done with the knockout mice to study the possibly changed growth performance and physiological behaviors in response to developmental stimuli or dietary modifications. These changes may also reveal the relevance of peptide transporters to whole animal nutrition and physiology.

In order to further understand the regulatory mechanisms of peptide transport, promoter regions of cPepT1 and oPepT1 need to be identified and analyzed. Structural features of the promoter regions of the genes will provide insight into the possible regulatory elements located upstream of the transporter genes and help identify the regulatory pathways of peptide transport.

Once the promoter regions of the genes are found, investigation should focus on searching for the possible presence of both amino acid and peptide responsive *cis*-elements on the promoter regions of cPepT1 and oPepT1. This will facilitate research on identifying the mechanisms of gene upregulation by amino acids or peptides. It is reported that PepT1 expression is upregulated by peptide substrates (Thamotharan et al., 1998; Walker et al., 1998). Our data also indicate that the level of cPepT1 mRNA increases when feeding higher dietary protein. There is evidence showing that upregulation of some genes by

amino acid supplementation is mediated through the activator protein-1 (AP-1, Li et al., 1995; Varga et al., 1994). Activation of transcription by dietary protein remains a complicated area for investigation because of the variety of end products from protein digestion. Activation of PepT1 transcription by free amino acids and peptide substrates is through different pathways, as suggested by Shiraga et al. (1999). Besides the binding sites for common activators/regulators such as Sp1, AP-1, Jun-B, and c-Myc, homeodomain transcription factor *cdx-2* may be playing a role in the genes' response to developmental initiation and differentiation as shown in our studies on cPepT1. There is no doubt that the *cis*-regulatory elements identified will lead to future discovery of trans- factors that regulate the amino acids or peptides activating pathways and developmental initiation pathways.

Once the promoter regions of the genes are located, investigation should also focus on searching for the possible presence of the regulatory elements that are related to the induction of PepT1 during nutrient restriction, which will help to elucidate the mechanisms by which cells or animals survive stress. As discussed in this dissertation, there is accumulating evidence showing that nutrient restriction, especially dietary protein limitation, is one of the regulatory factors in upregulation of PepT1. The effects of amino acid starvation on gene expression of several proteins have been studied. For example, C/EBP homologous protein mRNA abundance was strongly induced by methionine deprivation whereas asparagine synthetase mRNA level was increased by limitation of histidine, asparagine, or cysteine (Jousse et al., 2000). There are several proposed pathways involved in regulation induced by amino acid starvation (e.g., amino acid

response pathway and endoplasmic reticulum stress response pathway). Studies have already identified several corresponding regulatory elements on the responsive genes. There is little information on whether the upregulations induced by amino acid starvation and amino acid availability are the same or of totally separate pathways. Whether the upregulatory mechanisms of PepT1 expression by nutrient restriction are the same as the regulatory pathways by amino acid is unknown at the moment. The knowledge gained on these novel regulatory pathways will help to elucidate the mechanisms cells and animals initiate to survive stress.

Studies should include the regulation of PepT1 expression at the translational level. There is evidence showing that amino acid starvation induces translation of an amino acid transporter (Cat-1) mRNA (Fernandez et al., 2001). The proposed mechanism is the existence of an internal ribosomal entry sequence (IRES) located within the 5'-untranslated region that interacts with other unknown responsive factors to induce 5' cap-independent translation of the Cat-1 protein in response to amino acid limitation. This is a novel pathway providing a cellular stress response to induce the synthesis of proteins essential for cell survival. The findings from studies on regulation by amino acid availability at the translational level open up possibilities for further investigation of translational control of PepT1 expression.

Post-translational control of PepT1 gene expression can be studied by further investigation of phosphorylation of cPepT1 and oPepT1. There are several potential phosphorylation sites identified from the proposed cPepT1 and oPepT1 amino acid sequences. To investigate the mechanisms of regulation of PepT1 expression by

phosphorylation, putative PKC sites on the cPepT1 and oPepT1 may be mutated by DNA sequence manipulation and the effect of PKC on peptide transport by PepT1 may then be investigated. Phosphorylation by PKC is a very important event associated with protein activity and especially membrane protein translocation. Further confirmation of response of cPepT1 and oPepT1 to phosphorylation in the *in vivo* situation will also help in determining the possible post-translational controls of peptide transport.

Using genomics and proteomics techniques, multiple genes that are closely associated with physiological and nutritional conditions under which PepT1 gene expression is regulated can be identified simultaneously. Proteins from the identified genes may be factors that respond to available amino acids or peptides. These proteins may also be the regulators for gene expression of PepT1. These will help in the study of regulatory pathways for regulation of PepT1 by dietary protein, by nutrient starvation, or by developmental stages.

In summary, future studies should follow up on the further characterization of substrate affinity by exploring available protein hydrolysis products as preferable substrates of cPepT1 and oPepT1. This will also provide insight into better diet formulation to enhance growth performance. Promoter analysis of cPepT1 and oPepT1 should be a preliminary task in order for future research on transcriptional control of PepT1 expression. The identification of *cis*-regulatory elements will lead to discovery of trans-regulatory factors and maybe the whole regulatory pathways through which regulations of both substrate availability and nutrient deprivation take place. With the availability of antibodies against cPepT1 and oPepT1 and protein analysis tools, the translational and

post-translational regulation of cPepT1 and oPepT1 can be evaluated. Ultimately, the nutritional importance and physiological relevance of peptide transport will be revealed.

Literature Cited

- Abe, H., Satoh, M., Miyauchi, S., Shuto, S., Matsuda, A. and Kamo, N. 1999.
Conjugation of dipeptide to fluorescent dyes enhances its affinity for a dipeptide transporter (PEPT1) in human intestinal Caco-2 cells. *Bioconjug. Chem.* 10: 24-31.
- Adibi, S. A. 1997. The oligopeptide transporter (Pept-1) in human intestine: biology and function. *Gastroenterology* 113:332-340.
- Amasheh, S., Wenzel, U., Boll, M., Dorn, D., Weber, W.-M., Clauss, W., and Daniel, H. 1997a. Transport of charged dipeptides by the intestinal H⁺/peptide symporter PepT1 expressed in *Xenopus laevis* oocytes. *J. Membrane Biol.* 155:247-256.
- Amasheh, S., Wenzel, U., Weber, W.-M., Clauss, W., and Daniel, H. 1997b.
Electrophysiological analysis of the function of the mammalian renal peptide transporter expressed in *Xenopus laevis* oocytes. *J. Physiol.* 504:169-174.
- Baldwin, S. A. 1993. Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta.* 1154: 17-49.
- Baron-Delage, S., Capeau, J., Barbu, V., Chastre, E., Levy, P., Gespach, C., and Cherqui, G. 1994. Reduced insulin receptor expression and function in human colonic Caco-2 cells by ras and polyoma middle T oncogenes. *J. Biol. Chem.* 269: 18686-18693.

- Berlitz, F., Julien, S., Tsocas, A., Chariot, J., Carbon, C., Farinotti, R., and Rozé, C. 1999. Neural modulation of cephalixin intestinal absorption through the di- and tripeptide brush border transporter of rat jejunum in vivo. *J. Pharmacol. Exp. Ther.* 288:1037-1044.
- Berlitz, F., Maoret, J.-J., Paris, H., Laburthe, M., Farinotti, R., and Rozé, C. 2000. α_2 -adrenergic receptors stimulate oligopeptide transport in a human intestinal cell line. *J. Pharmacol. Exp. Ther.* 294: 466-472.
- Bishop, W. P. and Wen, J. T. 1994. Regulation of Caco-2 cell proliferation by basolateral membrane epidermal growth factor receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.* 267: G892-G900.
- Biviano, A. B., Martinez del Rio, C., and Philips, D. L. 1993. Ontogenesis of intestinal morphology and intestinal disaccharidases in chicken fed contrasting purified diets. *J. Comp. Physiol. B.* 163: 508-518.
- Boll, M., Markovich, D., Weber, W.-M., Korte, H., Daniel, H., and Murer, H. 1994. Expression cloning of a cDNA from rabbit small intestine related to proton-coupled transport of peptides, β -lactam antibiotics and ACE-inhibitors. *Eur. J. Physiol.* 429:146-149.
- Bommakanti, R. K., Bokoch, G. M., Tolley, J. O., Schreiber, R. E., Siemsen, D. W., Klotz, K. N., and Jesaitis, A. J. 1992. Reconstitution of a physical complex between the n-formyl chemotactic peptide receptor and G protein. Inhibition by pertussis toxin-catalysed ADP ribosylation. *J. Biol. Chem.* 267: 7576-7581.

- Brandsch, M., Ganapathy, V., and Leibach, F. H. 1995. H⁺-peptide cotransport in Madin-Darby canine kidney cells: expression and calmodulin-dependent regulation. *Am. J. Physiol.* 368:F391-F398.
- Brandsch, M., Miyamoto, Y., Ganapathy, V., and Leibach, F. H. 1994. Expression and protein kinase C-dependent regulation of peptide/H⁺ co-transport system in the Caco-2 human colon carcinoma cell line. *Biochem. J.* 299:253-260.
- Buddington, R. K., and Diamond, J. M. 1989. Ontogenetic development of intestinal nutrient transporters. *Annu. Rev. Physiol.* 51:601-619.
- Buddington, R. K. and Diamond, J. M. 1990. Ontogenetic development of monosaccharide and amino acid transporters in rabbit intestine. *Am. J. Physiol.* 259: G544-G555.
- Chen, H., Pan, Y-X., Wong, E. A., Bloomquist, J. R., and Webb, Jr., K. E. 2000a. Molecular cloning and functional expression of a chicken intestinal peptide transporter (cPepT1) in *Xenopus* oocytes and CHO cells. *Poultry Sci.* 79(Suppl 1): 41.
- Chen, X-Z., Steel, A., and Hediger, M. A. 2000b. Functional roles of histidine and tyrosine residues in the H⁺-peptide transporter PepT1. *Biochem. Biophys. Res. Commun.* 272: 726-730.
- Chen, H., Wong, E. A., and Webb, Jr., K. E. 1999. Tissue distribution of a peptide transporter mRNA in sheep, dairy cows, pigs, and chickens. *J. Anim. Sci.* 77:1277-1283.

- Cross, H. S. and Quaroni, A. 1991. Inhibition of sucrase-isomaltase expression by EGF in the human colon adenocarcinoma cells Caco-2. *Am. J. Physiol. Cell Physiol.* 261: C1173-C1183.
- Daniel, H. 1996. Function and molecular structure of brush border membrane peptide/H⁺ symporters. *J. Membrane Biol.* 154:197-203.
- Daniel, H., and Adibi, S.A. 1994. Functional separation of dipeptide transport and hydrolysis in kidney brush border membrane vesicles. *FASEB. J.* 8:753-759.
- Daniel, H. and Herget, M. 1997. Cellular and molecular mechanisms of renal peptide transport. *Am. J. Physiol.* 273:F1-F8.
- Döring, F., Dorn, D., Bachfischer, U., Amasheh, S., Herget, M., and Daniel, H. 1997. Functional analysis of a chimeric mammalian peptide transporter derived from the intestinal and renal isoforms. *J. Physiol.* 497:773-779.
- Döring, F., Walter, J., Will, J., Föcking, M., Boll, M., Amasheh, S., Clauss, W., and Daniel, H. 1998a. Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. *J. Clin. Invest.* 101:2761-2767.
- Döring, F., Will, J., Amasheh, S., Clauss, W., Ahlbrecht, H., Daniel, H. 1998b. Minimal molecular determinants of substrates for recognition by the intestinal peptide transporter. *J. Biol. Chem.* 273: 23211-23218.
- Dudley, M. A., Schoknecht, P. A., Dudley, Jr., A. W., Jiang, L., Ferraris, R. P., Rosenberger, J. N., Henry, J. F., & Reeds, P. J. (2001). Lactase synthesis is

- pretranslationally regulated in protein-deficient pigs fed a protein-sufficient diet. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280: G621-G628.
- Erickson, R. H., Gum, Jr., J. R., Lindstrom, M. M., McKean, D., and Kim, Y. S. 1995. Regional expression and dietary regulation of rat small intestinal peptide and amino acid transporter mRNAs. *Biochem. Biophys. Res. Commun.* 216:249-257.
- Fafournoux, P., Bruhat, A., and Jousse, C. 2000. Amino acid regulation of gene expression. *Biochem. J.* 351: 1-12.
- Fei, Y.-J., Kanal, Y., Nussberger, S., Ganapathy, V., Leibach, F. H., Romero, M. F., Singh, S. K., Boron, W. F., and Hediger, M. A. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature.* 368:563-566.
- Fei, Y.-J., Liu, J.-C., Fujita, T., Liang, R., Ganapathy, V., Leibach, F. H. 1998. Identification of a Potential Substrate Binding Domain in the Mammalian Peptide Transporters PEPT1 and PEPT2 Using PEPT1-PEPT2 and PEPT2-PEPT1 Chimeras. *Biochem. Biophys. Res. Commun.* 246:39-44.
- Fei, Y.-J., Liu, W., Prasad, P. D., Kekuda, R., Oblak, T. G., Ganapathy, V., and Leibach, F. H. 1997. Identification of the histidyl residue obligatory for the catalytic activity of the human H⁺/peptide cotransporters PEPT1 and PEPT2. *Biochemistry.* 36:452-460.
- Fei, Y.-J., Sugawara, M., Liu, J.-C., Li, H. W., Ganapathy, V., Ganapathy, M. E., and Leibach, F. H. 2000. cDNA structure, genomic organization, and promoter analysis of the mouse intestinal peptide transporter PEPT1. *Biochim. Biophys. Acta.* 1492: 145-154.

- Fernandez, J., Yaman, I., Mishra, R., Merrick, W. C., Snider, M. D., Lamers, W. H., and Hatzoglou, M. 2001. Internal ribosome entry site-mediated translation of a mammalian mRNA is regulated by amino acid availability. *J. Biol. Chem.* 276: 12285-12291.
- Ferraris R. P., and Diamond, J. M. 1989. Specific regulation of intestinal nutrient transporters by their dietary substrates. *Annu. Rev. Physiol.* 51:125-141.
- Ferraris R. P., Diamond J., and Kwan, W. W. 1988. Dietary regulation of intestinal transport of the dipeptide carnosine. *Am. J. Physiol.* 255:G143-G150.
- Ferraris, R. P., Villenas, S. A., and Diamond, J. 1992. Regulation of brush-border enzymes activities and enterocyte migration rates in mouse small intestine. *Am. J. Physiol.* 262: G1047-G1059.
- Ferruzza, S., Ranaldi, C., Di Girolamo, M., and Sambuy, S. 1995. The transport of lysine across monolayers of human cultured intestinal cells (Caco-2) depends on Na (+)-dependent and Na (+)-independent mechanisms on different plasma membrane domains. *J. Nutr.* 125: 2577-2585.
- Fisher, D. A. and Lakshmanan, J. 1990. Metabolism and effects of epidermal growth factor and related growth factors in mammals. *Endocr. Rev.* 11: 418-442.
- Freeman, T. C., Bentsen, B. S., Thwaites, D. T., and Simmons, N. L. 1995a. H⁺/di-tripeptide transporter (PepT1) expression in the rabbit intestine. *Eur. J. Physiol.* 430:394-400.
- Freeman, T. C., Collins, A. J., Heavens, R. P., and Tivey, D. R. 1993. Genetic regulation of enterocyte function: a quantitative in situ hybridization study of lactase-phlorizin

- hydrolase and Na⁺-glucose cotransporter mRNAs in rabbit small intestine.
Pflugers Arch. 422:570-576.
- Freeman, T. C., Howard, A., Bentsen, B. S., Legon, S., and Walters, J. R. F. 1995b.
Cellular and regional expression of transcripts of the plasma membrane calcium
pump PMCA1 in rabbit intestine. *Am. J. Physiol.* 269:G126-G131.
- Ganapathy, V., Brandsch, M., and Leibach, F. H. 1994. Intestinal transport of amino acids
and peptides. *Physiology of the gastrointestinal tract*, 3rd Ed. Raven Press, New
York, pp 1773-1794.
- Ganapathy, V. and Leibach, F. H. 1999. *Textbook of Gastroenterology*. Ed. Yamada, T.
Lippincott Williams and Wilkins, Philadelphia, PA. 456-467.
- Ganapathy, M. E., Wei, H., Wang, H., Ganapathy, V., and Leibach, F. H. 1998.
Valacyclovir: A substrate for the intestinal and renal peptide transporters PEPT1
and PEPT2. *Biochem. Biophys. Res. Commun.* 246:470-475.
- Goldin, A. L. 1992. Maintenance of *Xenopus laevis* and oocyte injection. *Methods*
Enzymol. 207:266-279.
- Gonzalez, E., & Vinardell, M. P. (1996). Ontogenetic development of proline intestinal
transport in the domestic fowl. *Br. Poult. Sci.* 37:383-394
- Groneberg, D. A., Nickolaus, M., Springer, J., Döring, F., Daniel, H., and Fischer, A.
2001. Localization of the peptide transporter PEPT2 in the lung-implications for
pulmonary oligopeptide uptake. *Am. J. Pathol.* 158: 707-714.
- Guandalini, S. and Rubino A. 1982. Development of dipeptide transport in the intestinal
mucosa of rabbits. *Pediatr. Res.* 16: 99-103.

- Haley, J. D. 1990. Regulation of epidermal growth factor receptor expression and activation: a brief review. *Symp. Soc. Exp. Biol.* 44: 21-37.
- Han, H. K., Rhie, J. K., Oh, D. M., Saito, G., Hsu, C. P., Stewart, B. H., and Amidon, G. L. 1999. CHO/hPEPT1 cells overexpressing the human peptide transporter (hPEPT1) as an alternative in vitro model for peptidomimetic drugs. *J. Pharm. Sci.* 88:347-350.
- Hansen, T. v. O. 2001 Cholecystokinin gene transcription: promoter elements, transcription factors and signaling pathways. *Peptides.* 22: 1201-1211.
- Harcouët L., Lebrec, D., Rozé, C., Carbon, C., and Farinotti, R. 1997. Increased intestinal absorption of cefixime by nifedipine in the rat intestinal perfusion model: evidence for a neural regulation. *Pharmacol. Exp. Ther.* 281:738-745.
- Hauser, M., Donhardt, A. M., Barnes, D., Naider, F., and Becker, J. M. 2000. Enkephalins are transported by a novel eukaryotic peptide uptake system. *J. Biol. Chem.* 275: 3037-3041.
- Hediger, M. A., and Rhoads, D. B. 1994. Molecular physiology of sodium-glucose cotransporters. *Physiol. Rev.* 74:993-1026.
- Himukai, M., Konno, T., and Hoshi, T. 1980. Age-dependent change in intestinal absorption of dipeptides and their constituent amino acids in the guinea pig. *Pediatr. Res.* 14: 1272-1275.
- Hofmann, K., Bucher, P., Falquet, L., and Bairoch, A. 1999. The PROSITE database, its status in 1999. *Nucleic Acids Res.* 27: 215-219.

- Ihara, T., Tsujikawa, T., Fujiyama, Y., and Bamba, T. 2000. Regulation of PepT1 peptide transporter expression in the rat small intestine under malnourished conditions. *Digestion* 61: 59-67.
- Jean, C., Rome, S., Mathe, V., Huneau, J. F., Aattouri, N., Fromentin, G., Achagiotis, C. L., & Tome, D. (2001) Metabolic evidence for adaptation to a high protein diet in rats. *J. Nutr.* 131:91-98
- Jiang, L. and Ferraris, R. P. 2001. Developmental reprogramming of rat GLUT-5 requires de novo mRNA and protein synthesis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280: G113-G120.
- Jousse, C., Bruhat, A., Ferrara, M., and Fafournous, P. 2000. Evidence for multiple signaling pathways in the regulation of gene expression by amino acids in human cell lines. *J. Nutr.* 130: 1555-1560.
- Kay, B. K., & Peng, H. B. 1991. *Xenopus laevis*: Practical uses in cell and molecular biology. *Methods Cell Biol.* 36:679-81.
- Kekuda, R., Torres-Zamorano, V., Fei, Y. J., Prasad, P. D., Li, H. W., Mader, L. D., Leibach, F. H., and Ganapathy, V. 1997. Molecular and functional characterization of intestinal Na (+)-dependent neutral amino acid transporter B⁰. *Am. J. Physiol.* 272: G1463-G1472.
- King, D. E., Asem, E. K., and Adeola, O. 2000. Ontogenetic development of intestinal digestive functions in white pekin ducks. *J. Nutr.* 130: 57-62.

LITERATURE CITED

- Knütter, I., Theis, S., Hartrodt, B., Born, I., Brandsch, M., Daniel, H., and Neubert, K. 2001. A novel inhibitor of the mammalian peptide transporter PEPT1. *Biochem.* 40: 4454-4458.
- Koeln, L. L. and K. E. Webb, Jr. 1982. Peptide, erythrocyte and plasma amino acid transport across the gastrointestinal tract and liver of calves. *Fed. Proc.* 41:948-953.
- Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125-8148.
- Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.
- Lee, H.-M., Greeley, Jr., G. H., and Englander, E. 2001. Age-associated changes in gene expression patterns in the duodenum and colon of rats. *Mech. Ageing Dev.* 122: 355-371.
- Leibach, F. H. and Ganapathy, V. 1996. Peptide transporters in the intestine and the kidney. *Annu. Rev. Nutr.* 16: 99-119.
- Li, L., Gotta, S., Mauviel, A., and Varga, J. 1995. L-tryptophan induces expression of collagenase gene in human fibroblasts. *Cell. Mol. Biol. Res.* 41: 361-368.
- Lin, C-J, Akarawut, W., and Smith, D. E. 1999. Glycylsarcosine transport by enalapril in rabbit renal brush border membrane vesicles: interaction of ACE inhibitors with high-affinity H⁺/peptide symporter. *Pharm. Res.* 16:609-615.
- Liu, W., Liang, R., Ramamoorthy, S., Fei, Y.-J., Ganapathy, M. E., Hediger, M. A., Ganapathy, V., and Leibach, F. H. 1995. Molecular cloning of PEPT 2, a new

- member of the H⁺/peptide cotransporter family, from human kidney. *Biochim. Biophys. Acta.* 1235:461-466.
- Liang, R., Fei, Y.-J., Prasad, P. D., Ramamoorthy, S., Han, H., Yang-Feng, T. L., Hediger, M. A., Ganapathy, V., and Leibach, F. H. 1995. Human intestinal H⁺/peptide cotransporter cloning, functional expression, and chromosomal localization. *J. Biol. Chem.* 270:6456-6463.
- Lubkowitz, M. A., Barnes, D., Breslav, M., Burchfield, A., Naider, F., and Becker, J. M. 1998. *Schizosaccharomyces pombe* isp4 encodes a transporter representing a novel family of oligopeptide transporters. *Mol. Microbiol.* 28: 729-741.
- Lubkowitz, M. A., Breslav, M., Naider, F., and Becker, J. M. 1997. An oligopeptide transport gene from *Candida albicans*. *Microbiology* 143: 387-396.
- MacDonald, R. S., Thornton, Jr., W. H., and Bean, T. L. 1993. Insulin and IGF-1 receptors in a human intestinal adenocarcinoma cell line (Caco-2): regulation of Na⁺ glucose transport across the brush border. *J. Recept. Res.* 13:1093-1113.
- Mackenzie, B., Fei, Y. J., Ganapathy, V., and Leibach, F. H. 1996. The human intestinal H⁺/oligopeptide cotransporter hPEPT1 transports differently-charged dipeptides with identical electrogenic properties. *Biochim. Biophys. Acta* 1284:125-128.
- Malandro, M. S., and Kilberg, M. S. 1996. Molecular biology of mammalian amino acid transporters. *Annu. Rev. Biochem.* 65: 305-336.
- Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feltner, D. E., Nair, R., Becker, E. L., and Ward, P. A. 1984. Purification and identification of formyl-methionyl-

- leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J. Biol. Chem. 259: 5430-5439.
- Matthews, D. M. 1991. Protein Absorption: Development and Present State of the Subject. Wiley-liss, Inc. New York.
- Matthews, J. C., Wong, E. A., Bender, P. K., Bloomquist, J. R., and Webb, Jr, K. E. 1996. Demonstration and characterization of dipeptide transport system activity in sheep omasal epithelium by expression of mRNA in *Xenopus laevis* oocytes. J. Anim. Sci. 74:1720-1727.
- McCormick, B. A., Hofman, P. M., Kim, J., Carnes, D. K., Miller, S. I., and Madara, J. L. 1995. Surface attachment of *Salmonella typhimurium* to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. J. Cell Biol. 131:1599-1608.
- McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K., and Madara, J. L. 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by *Salmonella typhimurium*. J. Immunol. 160: 455-466.
- Meredith, D., Boyd, C. A. R., Bronk, J. R., Bailey, P. D., Morgan, K. M., Collier I. D., and Temple, C. S. 1998. 4-Aminomethylbenzoic acid is a non-translocated competitive inhibitor of the epithelial peptide transporter PepT1. J. Physiol. 512:629-634
- Merlin, D., Steel, A., Gewirtz, A. T., Si-Tahar, M., Hediger, M. A., and Madara, J. L. 1998. hPepT1-mediated epithelial transport of bacteria-derived chemotactic

- peptides enhances neutrophil-epithelial interactions. *J. Clin. Invest.* 102: 2011-2018.
- Miyamoto, K.-I., Shiraga, T., Morita, K., Yamamoto, H., Haga, H., Taketani, Y., Tamai, I., Sai, Y., Tsuji, A., and Takeda, E. 1996. Sequence, tissue distribution and developmental changes in rat intestinal oligopeptide transporter. *Biochim. Biophys. Acta.* 1305:34-38.
- Mordrelle, A. Jullian, E., Costa, C., Cormet-Boyaka, E., Benamouzig, R., Tomé, D., and Huneau, J.-F. 2000. EAAT1 is involved in transport of L-glutamate during differentiation of the Caco-2 cell. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279: G366-G373.
- Moundras, C., Rémésy, C., & Demigné, C. (1993) Dietary protein paradox: decrease of amino acid availability induced by high-protein diets. *Am. J. Physiol.* 264:G1057-G1065.
- Nicholson, B and McGivan, J. D. 1996. Induction of high affinity glutamate transport activity by amino acid deprivation in renal epithelial cells does not involve an increase in the amount of transporter protein. *J. Biol. Chem.* 271: 12159-12164.
- Nicklin, P. L., Irwin, W. J., Hassan, I. F., Mackay, M., and Dixon, H. B. 1995. The transport of acidic amino acids and their analogues across monolayers of human intestinal absorptive (Caco-2) cells in vitro. *Biochim. Biophys. Acta.* 1269: 176-186.

- Nielsen, C. U., Amstrup, J., Steffansen, B., Frokjaer, S., and Brodin, B. 2001. Epidermal growth factor inhibits glycylsarcosine transport and hPepT1 expression in human intestinal cell line. *Am. J. Physiol. Gastrointest. Liver Physiol.* 281: G191-G199.
- Noy, Y. & Sklan, D. (1995). Digestion and absorption in the young chick. *Poultry Sci.* 74: 366-373.
- Obst, B. S. & Diamond, J. M. (1992). Ontogenesis of intestinal nutrient transport in domestic chickens (*Gallus gallus*) and its relation to growth. *Auk* 109:451-464
- Palacin, M., Estevez, R., Bertran, J., and Zorzano, A. 1998. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* 78: 969-1054.
- Pan, Y., Bender, P. K., Akers, R. M., and Webb, Jr., K. E. 1996. Methionine-containing peptides can be used as methionine sources for protein accretion in cultured C₂C₁₂ and MAC-T cells. *J. Nutr.* 126:232-241.
- Pan, Y. and Webb, Jr., K. E. 1998. Peptide-bound methionine as methionine sources for protein accretion and cell proliferation in primary cultures of ovine skeletal muscle. *J. Nutr.* 128: 251-256.
- Pan, Y-X., Wong, E. A., Bloomquist, J. R., and Webb, Jr., K. E. 1997. Poly(A)⁺ RNA from sheep omasal epithelium induces expression of a peptide transport protein(s) in *Xenopus laevis* oocytes. *J. Anim. Sci.* 75:3323-3330.
- Pan, Y-X., Wong, E. A., Bloomquist, J. R., and Webb, Jr., K. E. 2001. Expression of a cloned ovine gastrointestinal peptide transporter (oPepT1) in *Xenopus* oocytes induces uptake of oligopeptides in vitro. *J. Nutr.* 131: 1264-1270.

- Paulsen, I. T., and Skurray, R. A. 1994. The POT family of transport proteins. *Trends Biochem. Sci.* 19: 404-404.
- Ramamoorthy, S., Liu, W., Ma, Y-Y., Yang-Feng, T. L., Ganapathy, V., and Leibach, F. H. 1995. Proton/peptide cotransporter (PEPT2) from human kidney: Functional characterization and chromosomal location. *Biochim. Biophys. Acta.* 1240:1-4.
- Rubio-Aliaga, I., Boll, M., and Daniel, H. 2000. Cloning and characterization of the gene encoding the mouse peptide transporter PEPT2. *Biochem. Biophys. Res. Comm.* 276: 734-741.
- Saito, H., Okuda, M., Terada, T., Sasaki, S., and Inui, K.-I. 1995. Cloning and characterization of a rat H⁺/peptide cotransporter mediating absorption of beta-lactam antibiotics in the intestine and kidney. *J. Pharmacol. Exp. Therap.* 275:1631-1637.
- Saito, H., Terada, T., Okuda, M., Sasaki, S., and Inui, K.-I. 1996. Molecular cloning and tissue distribution of rat peptide transporter PEPT2. *Biochim. Biophys. Acta.* 1280:173-177.
- SAS Institute Inc. (1989) SAS user's guide: statistics. SAS Institute, Cary, NC.
- Sawada, K., Terada, T., Saito, H., Hashimoto, Y., and Inui, K.-I. 1999a. Recognition of L-Amino Acid Ester Compounds by Rat Peptide Transporters PEPT1 and PEPT2. *J. Pharmacol. Exp. Ther.* 291:705-709.
- Sawada, K., Terada, T., Saito, H., Hashimoto, Y., and Inui, K.-I. 1999b. Effects of glibenclamide on glycylsarcosine transport by the rat peptide transporters PEPT1 and PEPT1. *Br. J. Pharmacol.* 128: 1159-1164.

- Seal, C. J. and Parker, D. S. 1991. Isolation and characterization of circulating low molecular weight peptides in steer, sheep and rat portal and peripheral blood. *Comp. Biochem. Physiol.* 99B: 679-685.
- Shen, H., Smith, D. E., and Brosius, III, F. C. 2001. Developmental expression of Pept1 and PepT2 in rat small intestine, colon, and kidney. *Pediatr. Res.* 49: 789-795.
- Shiraga, T., Miyamoto, K.-I., Tanaka, H., Yamamoto, H., Taketani, Y., Morita, K., Tamai, I., Tsuji, A., Takeda, E. (1999). Cellular and molecular mechanisms of dietary regulation on rat intestinal H⁺/peptide transporter PepT1. *Gastroenterology.* 116: 354-362.
- Soderlund, D. M., Bloomquist, J. R., Wong, F., Payne, L. L., and Knipple, D. C. 1989. Molecular neurobiology: Implications for insecticide action and resistance. *Pestic. Sci.* 26:359-374.
- Soriano, M. E. & Planas J. M. (1998). Developmental study of α -methyl-D-glucoside and L-proline uptake in the small intestine of chicken. *Poult. Sci.* 77:1347-1353.
- Steel, A., Nussberger, S., Romero, M. F., Boron, W. F., Boyd, C. A. R., and Hediger, M. A. 1997. Stoichiometry and pH dependence of the rabbit proton-dependent oligopeptide transporter PepT1. *J. Physiol.* 498:563-569.
- Taub, M. E., Moss, B. A., Steffansen, B., and Frokjaer, S. 1997. Influence of oligopeptide transporter binding affinity upon uptake and transport of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar in filter-grown Caco-2 monolayers. *Int. J. Pharm.* 156 (2): 219-228.

- Terada, T, Saito, H., Mukai, M., and Inui, K. 1996. Identification of the histidine residues involved in substrate recognition by a rat H⁺/peptide cotransporter, PEPT1. *FEBS Lett.* 394:196-200
- Terada, T, Saito, H., Mukai, M., and Inui, K. 1997. Recognition of β -lactam antibiotics by rat peptide transporters, PEPT1 and PEPT2, in LLC-PK₁ cells. *Am. J. Physiol.* 273:F706-F711.
- Terada, T., Saito, H., Sawada, K., Hashimoto, Y., and Inui, K.-I. 2000a. N-terminal halves of rat H⁺/peptide transporters are responsible for their substrate recognition. *Pharm. Res.* 17: 15-20.
- Terada, T., Sawada, K., Irie, M., Saito, H., Hashimoto, Y., and Inui, K.-I. 2000b. Structural requirements for determining the substrate affinity of peptide transporters PepT1 and PepT2. *Pflügers Arch.-Eur. J. Physiol.* 440: 679-684.
- Terada, T., Sawada, K., Ito, T., Saito, H., Hashimoto, Y., and Inui, K.-I. 2000c. Functional expression of novel peptide transporter in renal basolateral membranes. *Am. J. Physiol. Renal. Physiol.* 279: F851-F857.
- Terada T., K. Sawada, Saito, H., Hashimoto, Y., and Inui, K.-I. 1999. Functional characteristics of basolateral peptide transporter in the human intestinal cell line Caco-2. *Am.J. Physiol.* 276:G1435-G1441.
- Terada, T., Sawada, K., Saito, H., Hashimoto, Y., and Inui, K.-I. 2000d. Inhibitory effect of novel oral hypoglycemic agent nateglinide (AY4166) on peptide transporters PEPT1 and PEPT2. *Eur. J. Pharmacol.* 392: 11-17.

LITERATURE CITED

- Thamotharan, M., Bawani, S. Z., Zhou, X., and Adibi, S. A. 1998. Mechanism of dipeptide stimulation of its own transport in a human intestinal cell line. *Proc. Assoc. Am. Physicians.* 110: 361-368.
- Thamotharan, M., Bawani, S. Z., Zhou, X., and Adibi, S. A. 1999. Hormonal regulation of oligopeptide transporter Pept-1 in a human intestinal cell line. *Am. J. Physiol.* 276 (Cell Physiol. 45): C821-C826.
- Tolza, E. M. and Diamond, J. M. 1992. Ontogenetic development of nutrient transport in rat intestine. *Am. J. Physiol.* 263: G593-G604.
- Varga, J., Li, L., Mauviel, A., Jeffrey, J., and Jimenez, S. A. 1994. L-tryptophan in supraphysiologic concentrations stimulates collagenase gene expression in human skin fibroblasts. *Lab. Invest.* 70: 183-191.
- Vazquez, J. A., Daniel, H., and Adibi, S. A. 1993. Dipeptides in parenteral nutrition: from basic science to clinical applications. *Nutr. Clin. Pract.* 8: 9-105.
- Walker, D., Thwaites, D. T., Simmons, N. L., Gilbert, H. J., and Hirst, B. H. 1998. Substrate upregulation of human small intestinal peptide transporter, hPEPT1. *J. Physiol.* 507: 696-706.
- Wang, H., Fei, Y-J., Ganapathy, V., and Leibach, F. H. 1998. Electrophysiological characteristics of the proton-coupled peptide transporter PEPT2 cloned from rat brain. *Am. J. Physiol.* 275: C967-C975.
- Wenzel U., Diehl, D., Herget, M., Kuntz, S., and Daniel, H. 1999. Regulation of the high-affinity H⁺/peptide cotransporter in renal LLC-PK1 cells. *J. Cell Physiol.* (UNITED STATES). 178:341-348.

LITERATURE CITED

Wenzel, U., Meissner, B., Döring, F., and Daniel, H. 2001. PepT1-mediated uptake of dipeptides enhances the intestinal absorption of amino acids via transport system b⁰,⁺. *J. Cell. Physiol.* 186: 251-259.

Ziegler, T. R., Estivariz, C. F., Gu, L. H., Wallace, T. M., Diaz, E. E., Pascal, R. R., Galloway, J. R., Wilcox, and Leader, L. M. 1998. Oligopeptide transporter PEPT1 mRNA expression in human intestinal mucosa: gut gradient, cellular localization and colonic upregulation in short bowel syndrome (SBS). *Gastroenterology.* 114: A436.

Vita

Hong Chen, daughter of Qimin Chen and Yuwen Liu, was born in March 1968, in Lanzhou, Gansu, P. R. of China. She graduated from Lanzhou University, with a Bachelor of Science degree in Cell Biology in July 1990. She married YuanXiang Pan in August 1992. She started her study in molecular nutrition at Virginia Tech in August 1996. She received her Master of Science degree in 1998. She received financial support from the John Lee Pratt Animal Nutrition Fellowship for five years.

She is a member of the American Society of Animal Science, the Poultry Science Association, and the American Physiological Society.
