Expression of apical membrane l-glutamate transporters in neonatal porcine epithelial cells along the small intestinal crypt-villus axis

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Fan, Ming Z., James C. Matthews, Nadege M. P. Etienne, Barbara Stoll, Dale Lackeyram, and Douglas G. Burrin. Expression of apical membrane l-glutamate transporters in neonatal porcine epithelial cells along the small intestinal crypt-villus axis. Am J Physiol Gastrointest Liver Physiol 287: G385–G398, 2004. First published March 25, 2004; 10.1152/ajpgi.00232.2003.—Enteral L-glutamate transport across the apical membrane. Kinetic data suggested that l-glutamate transport by the enterocyte apical membrane vesicles prepared by Mg2+ precipitation and differential centrifugation, with membrane potential clamped by SCN−. Initial l-glutamate uptake results suggested the presence of B0 and XAG transport systems, but the XAG system was predominant for uptake across the apical membrane. Kinetic data suggested that l-glutamate uptake through the XAG system was associated with higher maximal transport activity but lower transporter affinity in crypt than in villus cells. Molecular identity of the XAG glutamate transporter, based on immunoblot and RT-PCR analysis, was primarily the defined excitatory amino acid carrier (EAAC)-1. EAAC-1 expression was increased with cell differentiation and regulated at transcription and translation levels from crypt to upper villus cells. In conclusion, efficiency and capacity of luminal l-glutamate uptake across the apical membrane are regulated by changing expression of the XAG system transporter gene EAAC-1 at transcription and translation levels as well as maximal uptake activity and transporter affinity along the intestinal crypt-villus axis in the neonate.

excitatory amino acids; gut mucosa; transporter affinity; gene expression; neonates

REGULATION OF GUT MUCOSAL growth in the neonate is a fundamental issue of mucosal biology (21). The small intestinal mucosa is a layer of epithelium compartmentalized into the villi and the crypt region (9, 58). The villi bear mature cells that arise from multipotent stem cells, which undergo proliferation and differentiation as they migrate from the crypt compartment (9). Enterocytes constitute up to 90% of epithelial cells in the crypt and >95% of villus cells (9, 58). Thus small intestinal mucosal growth is dominated by the proliferation and differentiation of enterocytes (17).

Amino acids are key nutrients that are essential for gut mucosal growth, because they serve as metabolic fuels (6, 61) and precursors for the synthesis of protein (2, 55), nucleosides, and polyamines (6). L-Glutamate is one of the most intensively investigated amino acids in gut mucosal growth and metabolism (50, 60). Earlier studies showed that enteral glutamate was extensively metabolized by the gut mucosa in dogs and rats (42, 61). With the use of stable isotope tracer techniques in vivo, it was further shown that enteral glutamate was preferentially metabolized to CO2 and, specifically, used as a precursor for the biosynthesis of glutathione, arginine, and proline by the gut mucosa in neonatal pigs fed milk-based diets (51).

L-Glutamate transport by the enterocyte apical membrane is the initial step of the entire glutamate metabolism pathway in the gut mucosa. Physiological characterization of intestinal amino acid transport systems responsible for glutamate uptake in the mammalian species has been largely conducted with brush-border membrane vesicles prepared from total mucosal scrapings of mature animals (3, 49). Yet neonatal intestinal epithelial cells effectively transport enteral glutamate across the apical membrane remains unclear.

L-Glutamate transport across the enterocyte apical membrane was shown to be through the Na+-dependent high-affinity XAG system and/or the low-affinity B0 system (3, 29, 49). Although both transport activities couple the absorption of glutamate to Na+, major differences exist between these transport activities. Whereas the B0 system functions primarily as a neutral amino acid transporter, recognizing l-glutamate only at pH less than about 5.5 (24), l-glutamate and l- and d-aspartate are the principal substrates of the XAG system, which functions as an obligate countertransporter of K+ (3, 10, 30, 49). In contrast to the B0 system, the molecular identities of four proteins [glutamate/aspartate transporter 1 (GLAST-1), excitatory amino acid carrier (EAAC)-1, and excitatory amino acid transporters (EAAT)-4 and -5] capable of XAG system activity have been described in brain and various nonbrain tissues (12, 23, 47, 56). Recently, EAAC-1 expression has been examined in the rat (11, 53) small intestinal mucosa, EAAC-1 and GLT-1 proteins have been identified throughout the intestinal epithelium of sheep...
and cattle (18, 19), and GLAST-1 is known to be expressed by the cryptlike cell line IEC-17 (38, 39) and the differentiating human colorectal carcinoma Caco-2 cell line (37). With regard to the site of glutamate uptake activity along intestinal villi, early studies conducted with quantitative autoradiographic techniques suggested that the expression of various amino acid transport systems was limited to the upper third of the villi (8). Despite these advances in our understanding of intestinal glutamate uptake, overall, little is known about L-glutamate transport activity or the proteins in the apical membrane of epithelial cells, especially as these cells differentiate during their migration up the crypt–villus axis in the neonate.

Therefore, the objectives of this study were to establish the major apical membrane transport activities for L-glutamate absorption and identify specific glutamate transport proteins of epithelial cells at different stages of differentiation along the small intestinal crypt–villus axis in neonatal pigs fed a milk protein-based formula. Given the extensive metabolism of enteral glutamate in the neonate, we hypothesized that glutamate transporter activity and expression would be higher in differentiated upper villus cells than in proliferative crypt cells. Because upper villus cells are differentiated into an absorptive phenotype, we expected the activity and transporter content of glutamate transporters to be higher than in proliferative crypt cells.

MATERIALS AND METHODS

Materials. L-[14C]-Glutamic acid (specific activity 1.70–1.88 TBq/mmol) was obtained from Amersham (Arlington Heights, IL); Eco-lume scintillant from ICN Pharmaceuticals (Costa Mesa, CA); dye reagent from Bio-Rad Laboratories (Sandy, NJ); and bovine serum albumin (fraction V), L-glutamic acid, d-mannitol, Trizma-HCl, HEPES, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and other chemicals and reagents from Sigma (St. Louis, MO).

Animals and tissue preparation. A total of 30 piglets, taken from different sows at the age of 7 days, were individually housed in stainless steel metabolic cages equipped with feeders in a room maintained at 28°C. The piglets were fed three times a day a liquid, milk protein-based formula containing 25% lactose, 25% protein, and 10% fat (Litter Life, Merricks, Middleton, WI). At 16 days of age, the piglets were euthanized for isolation of small intestinal epithelial cells.

Sequential isolation of epithelial cells along the crypt–villus axis. We recently developed a procedure for sequentially isolating enterocytes along the crypt–villus axis (14) by adapting the distended intestinal sac technique previously developed in rats (58, 59). By aid of a 50-ml syringe fitted with a feeding needle, the divided segments were then quickly filled with a preincubation buffer (27 mM sodium citrate, 0.2 mM PMSF, and 0.5 mM DTT, pH 7.4), oxygenated with 19:1 (vol/vol) O2–CO2, and warmed in a water bath (37°C). The filled intestinal segments were sealed with two pairs of hemostatic forceps, immersed in saline (154 mM NaCl) in 2-liter glass beakers, and incubated for 15 min. After preincubation, the intestinal segments were filled with an isolation buffer (1.5 mM Na2EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 2 mM t-glucose, oxygenated with the O2–CO2 mixture, and warmed at 37°C) for the sequential isolation of 12 cell fractions (F1–F12) from the villus tip to the bottom crypt.

For in vitro glutamate transport measurements on individual cell fractions, a total of 12 cell fractions were isolated from 24 piglets, and the isolated cell fractions were stored separately for each pig. The F1–F12 cell fractions were collected through six consecutive 10-min incubations and buffer removal. The F7–F12 cell fractions were collected through six consecutive 15-min incubations and buffer removal. Each cell fraction was washed twice with 150 ml of oxygenated cell suspension buffer (155 mM KCl, pH 7.4), and the cells were retained through centrifugation at 400 g for 4 min at 4°C. The washed cells were immediately frozen at −80°C for future isolation of brush-border membrane vesicles for transport measurements. Isolated cell fractions were also pooled between the proximal and the distal intestinal segments for each piglet.

For molecular identification of the putative glutamate transporters, three major cell fractions, consisting of the upper (F1–F4), the middle (F5–F8), and the crypt (F9–F12) cell fractions, were then sequentially isolated through three consecutive incubations at 40, 50, and 60 min, respectively, from six additional piglets with the use of the distended intestinal sac technique described above. Isolated cell fractions from each piglet were immediately frozen in liquid nitrogen and stored at −80°C.

Preparation of apical membrane vesicles. Apical membrane vesicles were isolated by Mg2+ precipitation and differential centrifugation according to a previously established procedure (13, 28). Specifically, for each batch of apical membrane vesicle preparation, ~5 g of frozen cell fractions collected from two piglets were thawed in ice-cold homogenate buffer (50 mM d-mannitol, pH 7.4) at 20 ml of the homogenate buffer per gram of cells and homogenized by a Polytron homogenizer. The resulting homogenate was pooled and centrifuged at 2,000 g for 15 min. After the top foam layer was removed and the pellets were discarded, the supernatant was mixed with 1 M MgCl2 to contain 10 mM MgCl2, stirred for 15 min, and then centrifuged at 2,400 g for 15 min. After the top foam layer was discarded, the supernatant was centrifuged at 19,000 g for 30 min to generate crude apical membrane pellets. The crude apical membrane pellets were then suspended in a suitable amount of a membrane suspension buffer (150 mM KSCN and 180 mM D-mannitol, pH 7.0) and centrifuged at 39,000 g for 30 min to generate the final apical membrane vesicle pellets. The final pellets were resuspended with a 25-gauge needle in a suitable volume of the same membrane suspension buffer. The final membrane vesicle suspension was assayed for protein content and diluted with the same buffer to contain 4 mg of protein/ml for subsequent transport measurements. Several aliquots of the final membrane vesicle suspension were taken for the assays of marker enzyme activities.

Protein and marker enzyme assays. Protein was determined by using the Bio-Rad protein dye reagent and bovine serum albumin (fraction V) as a standard. All the following enzyme assays were carried out under the conditions that enzyme reactions were linear with time. Aminopeptidase N activity was assayed according to an established procedure (32). The incubations were conducted in a final volume of 0.200 ml containing membrane suspension or cell homogenate (2.5–20 μg protein), 50 mM Na2HPO4, and 28.0 mM L-alanine–p-nitroanilide hydrochloride at 37°C and pH 7.0 (adjusted with 0.1 M H3PO4 and 0.1 M NaOH) for 20 min. The K+-stimulated p-nitrophenylphosphatase activity was measured according to a previously established procedure (41). The incubations were carried out in a final volume of 1.000 ml containing membrane suspension or cell homogenate (1–10 μg protein), 90 mM KCl or NaCl, 10 mM MgCl2, 5.0 mM...
Na$_2$EDTA, 2.0 mM p-nitrophenyl phosphate Tris salt, and 10 mM Tris-HCl at 37°C and pH 7.0 (adjusted with 0.5 M Tris-base and 0.5 M Tris-HCl) for 10 min. The succinate dehydrogenase activity was measured according to King (26). The incubations were carried out in a final volume of 1.500 ml containing membrane suspension or cell homogenate (2.5 μg protein), 75 mM disodium phosphate, 0.67 mM K$_2$Fe(CN)$_6$, and 80.0 mM disodium succinate at 37°C and pH 7.0 (adjusted with 0.5 M Tris-base and 0.5 M Tris-HCl) for 10 min. The d-glucose-6-phosphatase activity was measured according to Hübischer and West (20). The incubations were carried out in a final volume of 0.400 ml containing membrane suspension or cell homogenate (2.5 μg protein), 29 mM d-glucose-6-phosphate barium salt, 4.0 mM Na$_2$EDTA, 2.0 mM KF, and 200 mM sodium maleate at 37°C and pH 6.0 (adjusted with 0.5 M NaOH) for 10 min. The acid phosphatase activity was measured according to Hübischer and West. The incubations were carried out in a final volume of 0.400 ml containing membrane suspension or cell homogenate (2.5 μg protein), 15 mM β-glycerol phosphate, 4.0 mM Na$_2$EDTA, and 52.0 mM sodium acetate at 37°C and pH 5.4 (adjusted with 0.5 M acetic acid and 0.5 M NaOH) for 10 min.

In vitro transport measurements. In vitro transport experiments were carried out with the rapid filtration procedure (13), which was conducted on the day of membrane vesicle preparation. After protein assay, 1 ml of membrane vesicle suspension was equilibrated for an additional 30 min before transport measurements. Membrane vesicles were preloaded with a buffer containing 180 mM ω-mannitol, 150 mM KSCN, and 10 mM Trizma-HCl, pH 7.0. The uptake buffer for measuring L-glutamate transport via the high-affinity X$_{AG}$ system contained 2.4 mM L-[3H]glutamate, 120 mM ω-mannitol, 60 mM L-phenylalanine, 150 mM NaSCN, and 10 mM Trizma-HCl, pH 6.0. The uptake buffer for measuring L-glutamate transport via the B$_0$ system contained 2.4 mM L-[3H]glutamate, 120 mM ω-mannitol, 60 mM ω-aspartate, 150 mM NaSCN, and 10 mM Trizma-HCl, pH 7.0. Fifty microliters of uptake buffer were first pipetted into the bottom of a polystyrene tube (100 × 15 mm), and then 10 μl of apical membrane vesicle suspension was spotted onto the side of the tube in two separate drops directly above the uptake buffer with a Microman pipette. After the tube was warmed for 20 s at room temperature (24°C), uptake incubation was initiated by a foot-switch-activated Bibromixer, and the process was terminated by the addition of 1.125 ml of ice-cold wash solution (180 mM ω-mannitol, 150 mM NaSCN, 10 mM Trizma-HCl, and 0.1 mM MgCl$_2$ at pH 7.0 or 6.0 for measuring L-glutamate transport via the X$_{AG}$ system). One millilitre of the uptake mixture was then rapidly pipetted onto 0.45-μm cellulose acetate filters (poresized with 20 mM L-glutamate, pH 7.4, to minimize nonspecific binding of these 2 amino acids to the filters) mounted in a Manifold filtration unit, which was connected to a vacuum source. On the basis of our preliminary measurements, a 6-s incubation was used to measure initial L-[3H]glutamate tracer transport rate at 2 μM. Timing was performed with an electronic timer-intervalometer (model 545, GraLab). The filters were immediately washed three times with 5 ml of ice-cold wash solution. The remaining solution in the incubation tubes was collected and at the end of the experiment was pooled and counted for the average initial radioactivity in the uptake medium. After a 30-min extraction in 5 ml of Ecolume scintillator, filters were counted with a liquid scintillation analyzer with automatic quench correction.

The components of L-glutamate transport via the X$_{AG}$ and the B$_0$ systems were partitioned according to a previously established protocol (27). L-glutamate transport by the X$_{AG}$ system was measured in the presence of 50 mM L-phenylalanine, a typical B$_0$ system substrate, in the uptake medium to block possible L-glutamate transport by the B$_0$ transporter (see legends to Figs. 1 and 2). L-glutamate transport by the B$_0$ system was measured in the presence of L-ω-aspartate, a typical X$_{AG}$ system substrate, in the uptake medium to block possible L-glutamate transport by the X$_{AG}$ transporter (see legends to Figs. 1 and 2).

The nonspecific binding of L-glutamate to the cellulose acetate filters was corrected by subtraction of radioactivity measured with the same uptake buffers in the absence of membrane vesicles.

Each uptake experiment was conducted in triplicate. Three separate uptake experiments were conducted using three different batches of membrane vesicle suspension prepared from cell fractions pooled from two piglets. Inasmuch as isolated cell fractions were also pooled between the proximal and distal intestinal segments for each piglet, all amino acid transport results represented the whole small intestine of the neonatal piglet fed the liquid formula.

Immunoblot analysis for expression of EAAC-1 and GLT-1. Cell fractions were homogenized in a buffer consisting of 50 mM ω-mannitol, 10 mM HEPES, 1 mM Na$_2$EDTA, 0.2 mM PMSF, and 2 μg/ml each of N-a-p-tosyl-l-lysine ketone, N-tosyl-l-phenylalanine chloromethyl-ketone, leupeptin hemisulfate, aprotinin, and pepstatin A and stored at −80°C until immunoblot analyses or further processed to isolate apical membrane and then stored at −80°C until immunoblot analysis. Analysis of transporter protein expression by enterocyte populations was conducted by immunoblot analysis using 50 μg of homogenate or membrane protein and antibodies and procedures of Matthews et al. (34). The amount of transporter immunoreactivity measured among cell populations was normalized to crypt values within an animal to control for differences in hybridization intensity among blots.

Extraction of RNA and determination of EAAC-1, GLT-1, and 18S ribosomal mRNA expression by RT-PCR and Southern blot analyses. For RNA analyses, 600–2,000 mg of each cell fraction were homogenized in 5 ml of TRizol according to instructions of the manufacturer (GIBCO-BRL, Grand Island, NY), and recovered total RNA was suspended in RNase-free, distilled and deionized water and stored at −80°C. Five micrograms of total RNA were reverse-transcribed using Superscript II reverse transcriptase and random and oligo(dT) primers according to instructions of the manufacturer (GIBCO-BRL). The PCR primers were as follows: 5'-GGGACAGATCTGGTG-GATT-3' and 5'-GGATCCCTCTTGGCCACG-3' (EAAC-1) and 5'-GAAAACCCCCATTCTCCCTTTT-3' and 5'-CCGACTGGGAG-
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Fig. 2. Kinetics of l-glutamate transport via the X AG system into apical membrane vesicles prepared from upper villus epithelial cells isolated along the small intestinal crypt-villus axis in 16-day-old neonatal pigs. A: data analyzed according to the classical kinetic model (28, 30). B: data analyzed according to the tracer inhibitory kinetic model (28, 30).

For ethidium bromide detection and Southern blot analyses, RT-PCR products were separated by electrophoresis through a 1.2% agarose gel in Tris-acetic acid-EDTA buffer and stained with ethidium bromide. The sizes of the RT-PCR products were estimated by regression of migration distance compared with a 100-bp DNA standard ladder (GIBCO-BRL). Subsequently, for Southern analysis, the same gels were denatured (0.4 M NaOH-1.5 M NaCl) for 30 min, rinsed twice with distilled water, neutralized (0.5 M Tris-HCl-1.5 M NaCl, pH 7.5) for 30 min, and transferred by downward capillary action onto a 0.45-μm positively charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Arlington Heights, IL). After they were blotted, the membranes were air dried and cross-linked by ultraviolet light.

Oligonucleotides with sequences internal to our partial-length porcine EAAC-1 sequence and previously reported 18S ribosomal RNA protein were designed and used for Southern analysis. The sequences of these “nested” probes were 5‘-ATCCCTTGTCCACCTGGTTCTCTCTTTCA-3‘ and 5‘-ATCCCTGTGTTGGCCCTCCGTAATCCTTTTAA-3‘, respectively. After synthesis (Invitrogen, San Diego, CA), the oligonucleotides were end labeled with [γ-32P]ATP (3000 Ci/mmol; Amersham) using a forward 5‘-terminal cDNA end-labeling reaction kit (GIBCO-BRL). Briefly, 5 pmol of oligonucleotide were incubated with 25 μCi of [γ-32P]ATP and 10 U of T4 polynucleotide kinase for 10 min at 37°C. The reaction was terminated by addition of Na2EDTA to a final concentration of 5 mM. Radiolabeled oligonucleotides were separated from unincorporated [γ-32P]ATP molecules using a G-50 Sephadex spin column (Amersham Pharmacia, Piscataway, NJ), after phenol-chloroform separation of the aqueous phase using standard molecular biology techniques. In serial hybridization experiments with the blots, recovered 32P-labeled oligonucleotides were boiled for 5 min, mixed with 5 ml of 1.25 M NaCl-0.075 M sodium citrate, pH 7.0. Subsequent washes were also carried out for 2 min but varied in SSC concentration. Typically, blots were serially washed once in 2.5× SSC and then with 1.25× SSC. After an appropriate exposure period of the membrane to autoradiographic film (Amersham Pharmacia), digital images of the autoradiographic film (Amersham Pharmacia), digital images of the autoradiographic film were recorded, and relative intensities of bands were determined as previously described (57). The ratios of EAAC-1 to 18S ribosomal RNA protein cDNA intensities were computed to account for potential variation of RT-PCR product generation, loading, and transfer and, thus, allowed for the relative comparison of EAAC-1 expression among the epithelial cell fractions.

Generation of partial-length porcine EAAC-1 cDNA. The EAAC-1 RT-PCR product was cloned into the EcoRI multiple cloning site of pCR II (Invitrogen) plasmid vector according to instructions of the manufacturer. The identity of the cDNA was determined by DNA sequencing by the University of Florida DNA Sequencing Laboratory (Gainesville, FL). The nucleotide sequence reported here resides in the GenBank/EMBL data bank (accession no. AY195622). Sequence alignments and comparisons to the previously reported porcine 18S ribosomal protein (GenBank accession no. AF102857).

For EAAC-1 and 18S ribosomal RNA, PCR conditions were 25 cycles at 94°C for 30 s, 51°C for 30 s, and 68°C for 1 min with a final extension at 68°C. For GLT-1, 35 cycles at 94°C for 1 min, 50°C for 45 s, and 72°C for 1 min with a final extension at 72°C for 7 min were performed. The PCR products were first denatured (94°C) of cDNA in the presence of MgCl2 for EAAC-1 and GLT-1, respectively. For all reactions, thermal cycling was performed using Taq DNA polymerase (GIBCO-BRL) in 50 μl and 2 mM MgCl2, and cycle time was optimized for product formation. Within a cell fraction of an individual animal, the same RT product was used for EAAC-1, GLT-1, and 18S ribosomal RNA PCR, but as separate PCR.

GACGAATC-3‘ (GLT-1). The expected size of the EAAC-1 RT-PCR product was 369 bp (corresponding to bp 118–141 and 499–516, respectively, of the previously reported porcine 18S ribosomal protein (GenBank accession no. AF102857).

expected size of the EAAC-1 RT-PCR product was 159 bp (corresponding to bp 118–141 and 499–516, respectively, of the previously reported porcine 18S ribosomal protein (GenBank accession no. AF102857).

The expected size of the GLT-1 RT-PCR product was 159 bp (corresponding to bp 118–141 and 499–516, respectively, of the previously reported porcine 18S ribosomal protein (GenBank accession no. AF102857).

The PCR primers used for amplification of a potential 398-bp 18S ribosomal protein cDNA were 5‘-CGGGCCGGGTGAGGTTTCGC-3‘ and 5‘-CGGGCCGGGTGAGGTTTCGC-3‘. These primers corresponded to bp 118–141 and 499–516, respectively, of the previously reported porcine 18S ribosomal protein (GenBank accession no. AF102857).
other GenBank entries were performed using BLAST 2.0 software (blast@ncbi.nlm.nih.gov).

Calculations and kinetic and statistical analyses of transport data. The initial rates of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer and/or total \(L\)-glutamate uptake under various experimental conditions were calculated according to our previously established procedure (13)\): \[ J = \left(\frac{(R_E \cdot R_p) \times S}{W \times t}\right) (1) \]

where \(J\) is initial rate of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer and/or total \(L\)-glutamate uptake into membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})\}, R_E\) is radioactivity in disintegration per minute (dpm) of filters (dpm/filter), \(R_p\) is radioactivity for nonspecific binding to cellulose membrane filters (dpm/filter), \(S\) is extravesicular \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer and/or total \(L\)-glutamate concentration (pM or nM)}, R_i\) is radioactivity in the uptake medium (dpm/µl), \(W\) is the amount of membrane protein provided for the incubations (mg protein), and \(t\) is the time of incubation for initial uptake (s).

Kinetic parameter estimates of the \([\text{L-}\text{glutamate transporter affinity, the maximal transport activity, and the transmembrane diffusion were determined according to a previously established tracer inhibitory kinetic model (28, 31)\): \[ J = \left(\frac{J_{\text{max}} \times S_{\text{tracer}}}{(K_m + S_{\text{tracer}})} + J_{\text{diff}}\right) (2) \]

where \(J\) is initial rate of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer uptake into membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})}\], \(J_{\text{max}}\) is the maximal rate of \([\text{L-}\text{glutamate transport into membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})}\], \(S_{\text{tracer}}\) is extravesicular concentration of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer (2 µM)}\), \(K_m\) is the transporter affinity (mM), \(S_{\text{tracer}}\) is extravesicular concentration of unlabeled \(L\)-glutamate (mM), and \(J_{\text{diff}}\) is transmembrane diffusion rate of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer in the membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})}\).

Kinetic parameter estimates of the \([\text{I-}\text{glutamate transporter affinity, the maximal transport activity, and the transmembrane permeability constant were determined according to a previously established classical kinetic model (62)\): \[ J = \left(\frac{J_{\text{max}} \times S}{(K_m + S)} + S \times K_D\right) (3) \]

where \(J\) is initial rate of \([\text{I-}\text{glutamate uptake into membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})}\], \(J_{\text{max}}\) is the maximal rate of \([\text{I-}\text{glutamate transport into membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1})}\], \(K_m\) is the transport activity (mM), \(S\) is the total extravesicular concentrations of labeled and unlabeled \(I\)-glutamate (mM), and \(K_D\) is the transmembrane permeability constant of \([\text{I-}\text{glutamate in the membrane vesicles (pmol \text{mg}^{-1} \text{ protein}^{-1} \text{s}^{-1} \text{mM}^{-1})}\) calculated by linear analysis of \([\text{I-}\text{glutamate uptake rates against the total extravesicular \(L\)-glutamate concentrations}\].

All kinetic parameter estimates of \([\text{I-}\text{glutamate transport activities were analyzed according to Eqs. 2 and 3 using the Fig.P curve-fitting program (1993, Biosoft, Cambridge, UK). Comparison of kinetic parameter estimates was conducted by using the pooled two-tailed Student’s \(t\)-test (7)\].

The effect of cell population on relative expression of \(X_{\text{AG}}\) system transporter protein and mRNA was evaluated using the general linear model procedure of SAS (SAS Institute, Cary, NC). When appropriate, protected Fisher’s least significant difference procedures of the SAS were used to compare cell population means.

RESULTS

Characterization of neonatal porcine enterocytes isolated along the crypt-villus axis. The small intestinal epithelial cells enriched in enterocytes were isolated along the small intestinal crypt-villus axis with the distended intestinal sac method (14). The 12 cell fractions (F1–F12) were characterized by \(\sim\)8-fold decreases in a villus cell marker enzyme, alkaline phosphatase-specific activity, and 8- to 18-fold increases in the crypt cell marker in vivo \(\text{[H]}\)thymidine incorporation from F1 (tip villus cells) to F12 (bottom crypt cells). On the basis of the distribution patterns of the villus and the crypt cell markers, the 12 cell fractions were grouped as the upper villus (F1–F4), middle villus (F5–F8), and crypt (F9–F12) cells, representing differentiated, differentiating, and proliferating enterocytes, respectively. We monitored the cell viability especially for the batches of cells used in the molecular work and observed no differences among the three major cell fractions in cell viability, which was 90–95% by trypan blue exclusion.

The 16-day-old neonatal piglets in this study were associated with a mean \pm SE (\(n = 30\)) fresh weight of 209 \pm 9.8 g and length of 7.02 \pm 0.2 m for the small intestine at 4.7 \pm 0.4 kg body wt. We also observed that \(\sim\)50% of the total fresh weight of the small intestine was epithelial cells enriched in enterocytes. Furthermore, the upper villus, middle villus, and crypt region accounted for \(\sim 28.4 \pm 0.6, 45.5 \pm 0.7, \) and 26.5 \pm 0.8% (mean \pm SE, \(n = 30\)) of the total epithelial cells, respectively, on a wet weight basis.

Characterization of the apical membrane prepared from neonatal porcine enterocytes isolated along the crypt-villus axis. Our previous work showed that more aminopeptidase N than other apical membrane hydrolases, such as lactase, sucrase, and alkaline phosphatase, was expressed in the crypt epithelial cells (14). We therefore chose to use this enzyme-specific activity as a marker for the enterocyte apical membrane. The apical membrane vesicles prepared from all 12 cell fractions (F1–F12) were enriched 6- to 10-fold in the aminopeptidase N specific activity compared with their corresponding cell homogenates.

Enrichment of the enzyme marker for the enterocyte basolateral membrane, \(\text{K}^+\)-stimulated phosphatase-specific activity was less than onefold in the apical membrane prepared from all cell fractions compared with their corresponding cell homogenates, indicating little contamination of the apical membrane preparation with the enterocyte basolateral membrane.

Enrichments of other enzyme markers, including the mitochondria membrane marker succinate dehydrogenase specific activity, the endoplasmic reticulum membrane marker d-glucose-6-phosphatase specific activity, and the lysosome membrane marker acid phosphatase specific activity, were \(< 1.7\) fold in the apical membrane prepared from all cell fractions compared with their corresponding cell homogenates, suggesting little contamination of the apical membrane preparation with the other intracellular membrane fractions.

Initial rates of \([\text{L-}\text{G}-\text{H}]/\text{glutamate tracer transport in the apical membrane of neonatal porcine enterocytes along the crypt-villus axis. It has been well documented that high-affinity \(X_{\text{AG}}\) and low-affinity \(B^0\) systems are responsible for transporting \(L\)-glutamate across the mammalian enterocyte apical membrane (3, 49). It is thus logical to first compare the relative importance of the high-affinity \(X_{\text{AG}}\) and the low-affinity \(B^0\) systems in transporting glutamate across the apical membrane of proliferating, differentiating, and differentiated neonatal porcine epithelial cells. Inasmuch as Na\(^+\)-dependent \(L\)-glutamate transport via the \(B^0\) system across the enterocyte apical membrane is an electrogenic process, membrane potential across the cell membrane is an important driving force in transporting amino acids under normal physiological conditions (3, 10). Our previous work revealed a sharply decreasing gradient in ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase and ouabain-
insensitive Na\textsuperscript{+}-ATPase activities from the tip villus to the bottom crypt cells (14), suggesting a decreasing membrane potential gradient associated with the neonatal porcine epithelial cells during cell proliferation and differentiation. To eliminate possible contributing effects from differences in membrane potential and the apical membrane permeability among the cell fractions, the membrane-permeable anion SCN\textsuperscript{−} was used in the membrane suspension buffer and also in amino acid uptake buffers to clamp the membrane potential (15).

Initial rates of l-[G-\textsuperscript{3}H]glutamate tracer transport across the apical membrane via the high-affinity X\textsubscript{AG} system showed a cubic pattern of progressive increases (P < 0.05) from the tip villus (F1) to the bottom crypt epithelial cells (F12) at the tracer substrate concentration of 2.0 μM (Fig. 1). At the same substrate concentration, initial rates of l-[G-\textsuperscript{3}H]glutamate tracer transport across the apical membrane via the low-affinity B\textsuperscript{0} system showed no differences (P > 0.05) from the tip villus (F1) to the bottom crypt (F12) epithelial cells (Fig. 1). Furthermore, initial rates of l-[G-\textsuperscript{3}H]glutamate tracer transport via the high-affinity X\textsubscript{AG} system were much higher (P < 0.05) than those via the low-affinity B\textsuperscript{0} system in all cell fractions, indicating that glutamate transport via the high-affinity X\textsubscript{AG} system is the major pathway for absorbing the enteral glutamate.

Kinetics of l-glutamate transport via the X\textsubscript{AG} transporter in the apical membrane prepared from neonatal porcine enterocytes along the crypt-villus axis. Inasmuch as initial rates of l-[G-\textsuperscript{3}H]glutamate tracer transport across the apical membrane via the B\textsuperscript{0} system were very low in the presence of increasing levels of unlabeled l-glutamate, we were unable to determine the kinetics of glutamate transport via the B\textsuperscript{0} system in the apical membrane vesicles. Kinetics of glutamate transport via the X\textsubscript{AG} system were determined with apical membrane vesicles prepared from the upper villus (F1–F4), middle villus (F5–F8), and crypt (F9–F12) cells under the condition that the membrane potential in the apical membrane vesicles was clamped with SCN\textsuperscript{−}.

We compared two well-defined kinetic models to quantify glutamate transport kinetics, inasmuch as both models allowed simultaneous determination of the component of transmembrane diffusion (28, 31, 62), and very few previous literature reports simultaneously compared both models in the same studies. The tracer inhibitory kinetic model uses initial tracer l-[G-\textsuperscript{3}H]glutamate transport rate as the independent variable, the concentrations of unlabeled l-glutamate as the dependent variable, and the l-[G-\textsuperscript{3}H]glutamate tracer concentration (2 μM) as an input parameter. Thus the kinetic curve associated with this model (Eq. 2) visually resembles competitive inhibition kinetics, namely, the inhibition of tracer l-[G-\textsuperscript{3}H]glutamate uptake by unlabeled l-glutamate (Figs. 2A, 3A, and 4A). Kinetics of l-glutamate transport were also analyzed with the classical model (Eq. 3) after partition of the transmembrane diffusion component, and the saturable component was found to be within 0–0.22 mM l-glutamate (Figs. 2B, 3B, and 4B).

The kinetics of glutamate transport were determined with the apical membrane vesicles prepared from the pooled upper villus (Fig. 2), the pooled middle villus (Fig. 3), and the pooled crypt (Fig. 4) cells. The kinetics of l-glutamate transport in the apical membrane vesicles of the upper, middle, and crypt enterocyte fractions were best fitted by a single transport system (Figs. 2–4). The relation between initial tracer l-[G-\textsuperscript{3}H]glutamate transport rates and the logarithmically transformed, unlabeled l-glutamine concentrations is presented in Fig. 5.

Kinetic parameter estimates of glutamate transport activities analyzed with the tracer inhibitory kinetic model, including J\textsubscript{max}, K\textsubscript{m}, and J\textsubscript{diffus}, were determined and are compared in Table 1. There were differences (P < 0.05) in J\textsubscript{max} between the upper villus, middle villus, and crypt groups: J\textsubscript{max} was highest in the crypt, intermediate in the middle villus, and lowest in the upper villus epithelial cells (7.31 ± 2.25, 6.56 ± 1.53, and 5.09 ± 1.75 pmol·mg protein\textsuperscript{−1}·s\textsuperscript{−1}, respectively). There were differences (P < 0.05) in K\textsubscript{m} between the upper villus, middle villus, and crypt epithelial cells: K\textsubscript{m} was highest in the upper villus, intermediate in the middle villus, and lowest in the crypt enterocyte fraction (42.6 ± 16.5, 63.5 ± 16.3, and 56.0 ± 19.1 μM, respectively). There were also differences (P < 0.05) in the apparent transmembrane tracer l-glutamate diffusion rate among the upper villus, middle villus, and crypt...
epithelial cells: 0.0308 ± 0.0091, 0.0246 ± 0.0063, and 0.0391 ± 0.0102 pmol·mg protein\(^{-1}\)·s\(^{-1}\), respectively.

Kinetic parameter estimates of glutamate transport activities analyzed with the classical kinetic model, including \(J_{\text{max}}\), \(K_m\), and \(K_D\), were determined and are compared in Table 1. The two kinetic models gave similar \((P > 0.05)\) \(J_{\text{max}}\) and \(K_m\) estimates; however, they calculated different types of transmembrane diffusion parameters (Table 1). There were differences \((P < 0.05)\) in the \(J_{\text{max}}\) values between the upper villus, middle villus, and crypt enterocyte groups: \(J_{\text{max}}\) was highest in the upper crypt, intermediate in middle villus, and lowest in upper villus epithelial cells \((7.49 ± 1.61, 6.12 ± 1.04, \text{and} 5.05 ± 0.90 \text{ pmol·mg protein}^{-1}·\text{s}^{-1}\), respectively). There were differences \((P < 0.05)\) in \(K_m\) among the upper villus, middle villus, and crypt epithelial cells: 14.9 ± 1.1, 5.7 ± 1.6, and 17.7 ± 1.6 pmol·mg protein\(^{-1}·\text{M}^{-1}\)·s\(^{-1}\), respectively.

Five mammalian glutamate transporters featuring the high-affinity \(X_{\text{AG}}\) transport system have been cloned and characterized (22, 37). Four of the five cloned glutamate transporters, GLAST, GLT-1, EAAC-1, and EAAT-4, are defined in animals, whereas the respective glutamate transporters are named EAAT-1, EAAT-2, EAAT-3, and EAAT-4 in humans (22).

To test the hypothesis that \(X_{\text{AG}}\) transporter systems were responsible for the observed \(\alpha\)-aspartate-sensitive glutamate uptake by neonatal porcine epithelial cell populations, the putative presence of EAAT-4, GLAST-1, GLT-1, and/or EAAC-1 in homogenates of upper, middle, or crypt enterocytes was evaluated by immunoblot analysis. The presence of

### Table 1. Kinetic parameter estimates of \(Na^+\)-dependent \(L\)-glutamate transport through high-affinity \(X_{\text{AG}}\) system into apical membrane vesicles from proliferating, differentiating, and differentiated epithelial cells isolated along the crypt-villus axis in neonatal pig

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>(J_{\text{max}}) (pmol·mg protein(^{-1}·\text{s}^{-1}))</th>
<th>(K_m) (\text{M})</th>
<th>(J_{\text{attu}}) (pmol·mg protein(^{-1}·\text{M}^{-1})·s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper villus</td>
<td>5.09 ± 1.75*</td>
<td>42.6 ± 16.5*</td>
<td>0.0308 ± 0.0091*</td>
</tr>
<tr>
<td>Tracer inhibitory model</td>
<td>5.05 ± 0.90*</td>
<td>41.6 ± 16.0*</td>
<td>14.9 ± 1.1*</td>
</tr>
<tr>
<td>Classical model</td>
<td>6.62 ± 1.04†</td>
<td>68.1 ± 24.8†</td>
<td>9.7 ± 1.6†</td>
</tr>
<tr>
<td>Middle villus</td>
<td>6.56 ± 1.53†</td>
<td>63.5 ± 16.3†</td>
<td>0.0246 ± 0.0063†</td>
</tr>
<tr>
<td>Tracer inhibitory model</td>
<td>6.12 ± 1.04†</td>
<td>68.1 ± 24.8†</td>
<td>9.7 ± 1.6†</td>
</tr>
<tr>
<td>Classical model</td>
<td>7.31 ± 2.25†</td>
<td>56.0 ± 19.1†</td>
<td>0.0391 ± 0.0102†</td>
</tr>
<tr>
<td>Crypt</td>
<td>7.49 ± 1.61†</td>
<td>58.7 ± 16.7†</td>
<td>17.7 ± 1.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE. \(J_{\text{max}}\), maximal transport rate (pmol·mg protein\(^{-1}·\text{s}^{-1}\)); \(K_m\), transporter affinity (\text{M}); \(J_{\text{attu}}\), apparent transmembrane diffusion rate (pmol·mg protein\(^{-1}·\text{M}^{-1}\)·s\(^{-1}\)). Estimates, were derived using a tracer inhibitory and classical model as described in MATERIALS AND METHODS. Parameter estimates for tracer inhibitory model were derived with \(P < 0.05\) \((n = 57)\). Parameter estimates for classical model were derived with \(P < 0.05\) \((n = 24\) for \(J_{\text{max}}\) and \(K_m\), \(n = 57\) for \(J_{\text{attu}}\)). Means in the same column with different symbols (*, †, ‡) differ, \(P < 0.05\).
EAAT-4 and GLAST-1 was not detected in any cell populations (data not shown). Immunoreactive bands of ~167 and 181 kDa were detected in the homogenate and the apical membrane preparations from all cell populations isolated from two of six pigs (Fig. 6). To further evaluate the potential expression of GLT-1 protein by porcine small intestinal epithelia, RT-PCR analysis for GLT-1 was performed on the animal with the highest expression of GLT-1 protein (pig 2). Expression of GLT-1 mRNA was not detected by RT-PCR followed by ethidium bromide visualization (Fig. 7A). In contrast, GLT-1 was expressed by a control tissue (sheep liver) (17). Repetition of this analysis for pig 2 and an initial analysis for the other five pigs also failed to detect expression of GLT-1 mRNA (Fig. 7B).

In contrast to the sporadic expression of GLT-1, EAAC-1 protein was detected in homogenates of all cell populations of all six pigs (Fig. 8). Typically, predominant immunoreactive bands of 72.5 and 56.5 kDa were detected, consistent with glycosylated and nonglycosylated forms of EAAC-1. Between cell populations, the relative amount of EAAC-1 expressed between the upper and middle villus did not differ ($P < 0.05$), but both cell populations contained more EAAC-1 ($P < 0.05$) than did crypt cells (Fig. 9). Specifically, the upper and middle villus enterocyte populations contained 63 and 78% more ($P < 0.05$) EAAC-1 than did crypt cells (Fig. 9).

Fig. 6. Immunoblot analysis of glutamate transporter (GLT)-1 protein expression by proliferating, differentiating, and differentiated populations of epithelial cells isolated along the small intestinal crypt-villus axis in 16-day-old neonatal pigs. Detection of GLT-1 protein in 50 µg of homogenate protein of the upper villus (U), middle villus (M), and crypt (C) populations of enterocytes was performed by immunoblotting using a rat anti-GLT-1 antibody. Six animals were evaluated for GLT-1 expression. Data are from 1 of the 2 pigs that tested positive for GLT-1 expression. Representative blots for the 4 pigs that failed to express GLT-1 are not shown.

Fig. 7. RT-PCR analysis of GLT-1 mRNA expression by proliferating and differentiated populations of epithelial cells isolated along the small intestinal crypt-villus axis in 16-day-old neonatal pigs. Total RNA (5 µg) extracted from upper villus, middle villus, and crypt populations of epithelial cells isolated from 16-day-old pigs ($n = 6$) was analyzed for GLT-1 mRNA using primers corresponding to human GLT-1 mRNA and RT-PCR conditions described in MATERIALS AND METHODS. A preliminary RT-PCR experiment (A) includes positive (GLT-1 product of ~150 bp) and negative control reactions, which are representative of control reactions run for each of the individual RT-PCR analyses for GLT-1 mRNA expression by each animal (B).
To determine whether the pattern of EAAC-1 mRNA expression paralleled that of protein, the presence and relative amount of mRNA expressed were evaluated by RT-PCR and Southern blot analysis (Fig. 10). To account for potential differences in RT-PCR product generation resulting from unequal amounts of RT cDNA and/or efficiency of EAAC-1 PCR among cell populations, the amount of EAAC-1 RT-PCR product quantified was normalized to the amount of 18S cDNA generated in the same fraction. Within cell fractions, the same RT product was used for EAAC-1 and 18S PCR. RT-PCR detection was used instead of Northern blot analysis because of the limited amount of RNA available from cell populations. The 18S ribosomal RNA (398 bp) was detected in all cell populations from all pigs by ethidium bromide staining because of the limited amount of RNA available from cell populations.

In contrast to the uniform expression of 18S ribosomal protein RNA by cell populations, EAAC-1 RT-PCR product (369 bp) was not visible by ethidium bromide detection in the middle villus and crypt fractions of three of six animals (Fig. 10C) but was detected in all fractions of all cells by Southern blot analysis (Fig. 10D). Densitometric analysis of the Southern blots demonstrated that the amount of 18S ribosomal cDNA product did not differ \(P = 0.92\) among enterocyte fractions (Fig. 11A). In contrast, the amount of EAAC-1 cDNA product recovered from the cell populations differed \(P < 0.05\). Specifically, the upper villus cell population expressed 81\% \(P = 0.04\) and 76\% \(P = 0.03\) more EAAC-1 mRNA, respectively, than did the middle villus or crypt cell fractions (Fig. 11B). When the amount of EAAC-1 cDNA product formed was normalized to the amount of 18S cDNA produced from the same RT product, the same response was observed. Specifically, the amount of EAAC-1 cDNA product recovered from the upper villus cell population was 83 and 76\% \(P < 0.05\) greater than that recovered from the middle villus or crypt cell fraction (Fig. 11C).

To confirm the identity of the “EAAC-1” RT-PCR cDNA and to determine its exact sequence, the EAAC-1 cDNA generated by RT-PCR from the upper villus cell fraction was TA cloned and sequenced. Consistent with the use of primers designed from the human sequence, the cDNA contained 369 bp and shared 94\% nucleotide identity and 97\% amino acid similarity with bp 735–1,104 of the corresponding region of human EAAC-1 (GenBank accession no. U06469; Fig. 12).

**DISCUSSION**

On the basis of the initial glutamate tracer uptake data, we have identified \(X_{AG}\) as the major system responsible for transporting the enteral \(L\)-glutamate across the apical membrane of proliferating and differentiated enterocytes in the neonate, whereas activity of the \(B^0\) system was limited. Inasmuch as membrane potential of the apical membrane vesicles was clamped with SCN\(^-\), possible effects from differences in membrane potential and transmembrane \(L\)-glutamate diffusion were excluded. The glutamate concentration used in the initial transport experiments was 2 \(\mu\)M for the \(X_{AG}\) and \(B^0\) transport systems. Transport rates measured at this substrate concentration, as reported in Fig. 1, were not the maximal transport rates. Thus the initial transport rates measured at 2 \(\mu\)M and reported...
in Fig. 1 could not reflect the maximal glutamate transport rate via the B° transport system. The tracer glutamate transport activity via the B° system in the presence of unlabeled L-glutamate was very low, and we failed to measure meaningful initial tracer glutamate transport rates at higher substrate concentrations for measuring kinetics. Thus we were experimentally unable to obtain glutamate transport kinetic estimates for the B° system. Therefore, we cannot comment on potential changes in the maximal glutamate transport rate via the B° system among the cell fractions.

Several previous studies used different techniques to examine the expression of amino acid transport activity during the enterocyte proliferation and differentiation (8, 37, 44–46). Very early work using a quantitative autoradiographic technique in studies with adult animals demonstrated that amino acid and peptide transporter activities were limited to the differentiated upper villus enterocytes (8, 25). Results of those studies contradict recent observations from differentiating Caco-2 cells (37, 44–46) which show that the uptake of dipolar-neutral and cationic amino acids was higher in proliferating than in differentiated cells. On the other hand, the studies of amino acid transport activity in association with cell differentiation in cell line models are usually limited by the fact that it is difficult to differentiate the measured amino acid transport activities between the apical and basolateral membranes. Furthermore, Caco-2 cells are of colorectal origin and may exhibit certain differences from enterocytes with regard to in vivo conditions, including the different expression pattern of amino acid transport systems.

We have shown that the apical membrane maximal L-glutamate transport activity via the XAG/H system was high in proliferating and differentiating midvillus epithelial cells and low in differentiated upper villus enterocytes (8, 25). Results of those studies contradict recent observations from differentiating Caco-2 cells (37, 44–46) which show that the uptake of dipolar-neutral and cationic amino acids was higher in proliferating than in differentiated cells. On the other hand, the studies of amino acid transport activity in association with cell differentiation in cell line models are usually limited by the fact that it is difficult to differentiate the measured amino acid transport activities between the apical and basolateral membranes. Furthermore, Caco-2 cells are of colorectal origin and may exhibit certain differences from enterocytes with regard to in vivo conditions, including the different expression pattern of amino acid transport systems.

We have shown that the apical membrane maximal L-glutamate transport activity via the XAG/H system was high in proliferating and differentiating midvillus epithelial cells and low in differentiated upper villus enterocytes, a pattern distinctly different from that reported in differentiating Caco-2 cells (37). Moreover, it is possible that the pattern of expressing L-glutamate activity in proliferating, differentiating, and differentiated enterocytes we observed in the neonatal pig changes with stage of development, an issue not addressed in this study. Two major factors affecting the maximal amino acid transport activity ($J_{\text{max}}$) are the number of transporters expressed and

![Fig. 10. RT-PCR analysis of 18S ribosomal protein RNA (18S; A) and EAAC-1 (C) mRNA expression by proliferating, differentiating, and differentiated populations of epithelial cells isolated along the small intestinal crypt-villus axis in neonatal pigs. Total RNA (5 μg) extracted from upper villus, middle villus, and crypt (C) populations of enterocytes isolated from 16-day-old pigs (n = 6) was analyzed for 18S and EAAC-1 mRNA using primers corresponding to human 18S ribosomal protein and EAAC-1 mRNA and RT-PCR conditions described in MATERIALS AND METHODS. Expected product sizes were 398 and 369 bp, respectively. Negative control reaction products are shown in the last 3 lanes of each gel. RT-PCR products contained in gels in A and C were transferred to membranes, and Southern blot analysis was performed for 18S (B) and EAAC-1 (D) using nested primers and conditions described in MATERIALS AND METHODS.](http://ajpgi.physiology.org/doi/abs/10.1152/ajpgi.00473.2003)
transporter affinity. Expression of a large number of transporter proteins and a high glutamate transporter affinity could potentially result in a large $J_{\text{max}}$. Our data showed that $X_{\text{AG}}$ glutamate transporter affinity was significantly lower in crypt and middle villus than in upper villus cells. It is well established that transporters with a low villus affinity (a large $K_m$ value) are usually associated with a large $J_{\text{max}}$ value. In this situation, changes in $J_{\text{max}}$ alone could not truly reflect differences in glutamate transporter efficiency among the cell populations at different stages of proliferation and differentiation. Therefore, significant changes in the transporter affinity are believed to be an additional mechanism regulating transport capacity and efficiency. We suggest that the efficiency of $\ell$-glutamate transport across the apical membrane is likely affected by changes in transporter maximal activity and affinity along the neonatal small intestinal crypt-villus axis.

The $X_{\text{AG}}$ transporter affinity for $\ell$-glutamate ($K_m$) determined from this study (42–68 $\mu$M) for the neonatal pig was generally within the range of the values (4–150 $\mu$M) reported in the literature (10, 27, 49). $K_m$ among upper villus, middle villus, and crypt enterocytes determined from this study was more variable than the values (107–118 $\mu$M) reported in differentiating Caco-2 cells (37, 40). Differences in species, developmental stages, and experimental conditions were likely responsible for differences in the $K_m$ values between studies (13). In this study, the differences in $K_m$ may be attributed to the following factors. First, apical membrane-bound amino acid transporters are glycoproteins, and studies indicate that significant villus-crypt changes occur in the glycosylation of the apical membrane-bound proteins (35). Changes in the glycosylation might have been, in part, responsible for the different $K_m$ values among the different enterocyte fractions. Yet changes in glycosylation were not responsible for the apical membrane hydrolase lactase affinity (5). However, all of the apical membrane proteins are regulated independently; thus, we could not rule out this possibility for this high-affinity $X_{\text{AG}}$ transporter protein at this time. Second, changes in the microvillus lipid composition and membrane fluidity, although not determined in this study, as was demonstrated in several previous studies (1, 4), were also possibly responsible for the different $K_m$ in the different cell fractions. On the other hand, a significant component of transmembrane diffusion observed with both kinetic models of analyses was consistent with previous studies with the membrane vesicle approach (13, 31, 62), suggesting the leakiness of membrane vesicles under the in vitro preparation condition as well as the intrinsic leaky nature of cell membranes (Table 1). In addition, the data obtained with both kinetic models pointed to the fact that the apical membrane of the upper villus and the crypt cells was more permeable than that of the middle villus cells, probably because of their differential combined effects of cell proliferation, differentiation, and luminal factors.

Transmembrane uptake of the enteral $\ell$-glutamate is the initial step of its entire first-pass metabolism pathway in the gut mucosa. Enteral $\ell$-glutamate metabolism in the gut mucosa has been extensively investigated with in vivo animal studies (50, 52, 54, 60). However, the metabolic fate of glutamate at the cellular level remains unclear, especially within enterocytes. Inasmuch as proliferating crypt epithelial cells are likely to be metabolically different from differentiating upper villus enterocytes, the metabolic fate of glutamate in proliferating and differentiating crypt cells warrants further investigation.

To test the hypothesis that the $X_{\text{AG}}$ transporter system was responsible for the observed $\ell$-aspartate-sensitive glutamate uptake by cell membrane preparations, the putative presence of

![Fig. 11. Quantitative analysis of Southern blot data for 18S ribosomal protein RNA (18S) and EAAC-1 mRNA expression by upper villus, middle villus, and crypt populations of epithelial cells isolated from 16-day-old neonatal pigs. Densitometric analysis of 18S and EAAC-1 protein mRNA detected in Fig. 10D was performed on all bands. Control experiments were performed to ensure that absorbance values were in the linear range of the densitometer and film. Values are means ± SE ($n = 6$), expressed as arbitrary densitometric units, for 18S (A) and EAAC-1 (B) RT-PCR products. C: data presented and evaluated as normalized to crypt values. Bars that share a common superscript (a and b) do not differ ($P > 0.05$).](http://ajpgi.physiology.org/Downloaded_from/)
EAAT-4, GLAST-1, GLT-1, and/or EAAC-1 in homogenates of the upper, middle, or crypt cells was evaluated by immunoblot analysis. That EAAT-4 and GLAST-1 proteins were not detected in small intestinal epithelia of neonatal pigs, but EAAC-1 was, is consistent with that observed for developing rats (53), mature ruminant animal species (18), and growing lambs (19). In contrast, the sporadic expression (2 of 6 animals evaluated) of GLT-1 by neonatal pigs differed from that by the ruminant animal species (18, 19), inasmuch as GLT-1 was detected in small intestinal epithelium by all tested animals. Whether the relatively sporadic expression of GLT-1 by neonatal pigs reflects species-specific or ontogenic regulation of GLT-1 remains to be determined.

Immunoblot analysis of cell homogenates also demonstrated that more EAAC-1 were expressed in differentiated upper and middle villus cells than in proliferating crypt cells (Fig. 9). When the kinetic parameter estimates for $X_{AG}$ glutamate transport system activities among cell populations, as summarized in Table 1, are considered in terms of EAAC-1 content, it is clear that a greater EAAC-1 protein content is associated with a lower maximal transport activity and changing transporter affinity. Collectively, these data suggest that another, $\alpha$-aspartate-insensitive, glutamate transport activity (other than the $X_{AG}$ system) may be expressed in less-differentiated cells or the functional properties of EAAC-1 change with differentiation. With regard to the first possibility, the apical membrane of rat placenta possesses such an $Na^{+}$-dependent glutamate uptake activity (34). Conversely, a cytosolic protein [GTRAP-3–18] that binds and decreases EAAC-1 affinity for glutamate has been identified (Genbank accession no. AF240182). When GTRAP-3–18 and EAAC-1 were overexpressed in 293C18 cells, a loss of glutamate affinity was achieved without a change in uptake velocity. In the present study, however, decreases in glutamate uptake affinity as cell populations differentiated were accompanied by increases in the maximal velocity. Our observations that EAAC-1 is the main identifiable $X_{AG}$ transporter system expressed in all enterocyte populations coupled with kinetic analysis suggest that there is a single transporter system in these cell fractions. It is most likely that the $X_{AG}$ system transporter EAAC-1 is the only $Na^{+}$-dependent glutamate transporter expressed by these epithelial cells. However, the existence of additional transporters cannot be excluded.

With regard to how the differential expression of EAAC-1 is regulated, the data of Figs. 9 and 11 indicate that the increase in EAAC-1 protein content of differentiated cells likely resulted from a combination of increased gene transcription and posttranscriptional events. That is, whereas the relative content of EAAC-1 protein and mRNA was proportional in the upper villus and crypt cell populations, the amount of EAAC-1 mRNA in the middle villus cells was low compared with protein content. This discordance between relative abundance of steady-state EAAC-1 mRNA and protein in neonatal pig enterocyte populations is consistent with that observed for EAAC-1 regulation in other tissues and developmental models (34, 48).

In conclusion, this study demonstrated that the high-affinity $X_{AG}$ system was the major pathway for transporting luminal $L$-glutamate across the apical membrane of proliferating, differentiating, and differentiated neonatal porcine epithelial cells. There were significant differences in the apical membrane glutamate maximal uptake activity and transporter affinity and the in the neonatal porcine enterocyte along the crypt-villus axis. EAAC-1 was the major $X_{AG}$ glutamate transporter identified, and its expression was increased with cell differentiation and regulated at the transcription and translation levels in the neonatal porcine enterocyte along the crypt-villus axis. The finding of a reduced maximal glutamate transport activity in association with a significantly increased transporter affinity associated with the $X_{AG}$ system in the upper villus cells supports the concept that efficiency of luminal $L$-glutamate uptake across the apical membrane is different along the intestinal crypt-villus axis in the neonate. However, the changes in EAAC-1 expression in isolated cells are consistent with the idea that glutamate transport is upregulated in differentiated villus enterocytes. Further study is warranted to establish whether the metabolic fate of glutamate is indeed different in undifferentiated crypt cells compared with differentiated enterocytes.

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