Cellular distribution of glutamate transporters in the gastrointestinal tract of mice. An immunohistochemical and \textit{in situ} hybridization approach

Toshihiko Iwanaga\textsuperscript{1}, Marie Goto\textsuperscript{2} and Masahiko Watanabe\textsuperscript{1}

\textsuperscript{1}Department of Anatomy, Hokkaido University Graduate School of Medicine, and \textsuperscript{2}Removable Prosthetics, Department of Oral Functional Science, Hokkaido University Graduate School of Dentistry, Sapporo 060-8638, Japan

\textit{(Received 4 October 2005; and accepted 10 November 2005)}

\textbf{ABSTRACT}

\textit{L}-Glutamate transport by intestinal epithelial cells is an initial step of the entire glutamate metabolism pathway in the gut mucosa. The present study examined the cellular distribution of glutamate transporters in the digestive tract of adult mice using immunohistochemistry and \textit{in situ} hybridization technique. Expression of EAAC1 mRNA was more intense in the ileum, where the epithelium in crypts and the basolateral part of intestinal villi showed high levels of transcripts, suggesting an essential role of EAAC1 in differentiating or premature epithelial cells. Electron-microscopically, EAAC1 immunoreactivity was predominantly localized in the striated border of enterocytes. Immunoreactivity for GLT-1 was found in the lateral membrane of epithelial cells at the bottom of gastric glands and at the intestinal crypts, and also in the lateral membrane of secretory cells at the duodenal gland. GLAST immunoreactivity was restricted to the fundic and pyloric glands, and was especially intense in the neck portion of both glands. However, \textit{in situ} hybridization analysis failed to confirm the expression of GLT-1 and GLAST at the mRNA level, possibly due to limited sensitivity. The strong and specific luminal localization of EAAC1 in the intestinal epithelium suggests that EAAC1 is a predominant transporter of glutamate, at least in the lower part of the small intestine.

Glutamate is one of important excitatory neurotransmitters in the central nervous system, and also in the peripheral nervous system such as the enteric nervous system. Neurons and effector cells express a variety of glutamate receptor subtypes (more than 20) for this potent neurotransmitter. For glutamate to fulfill its diverse functions, the existence of a glutamate uptake system with high accumulative power is of critical importance, because the high-affinity glutamate transport prevents the extracellular glutamate concentration from reaching neurotoxic levels in the synaptic clefts. cDNAs encoding various mammalian glutamate transporter isoforms have been cloned and characterized. Grouping of protein products with similar functional characteristics has identified five subtypes of the excitatory amino acid transporter (EAAT) family: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5. The expression of GLT-1, EAAT4, and EAAT5 is mainly restricted to the brain and retina, whereas the expression of GLAST and EAAC1 transcripts has also been reported in non-neuronal tissues, including the kidney, heart, lung, liver, placenta, and small intestine (2, 3, 7, 13, 14, 17), thus suggesting the important roles of GLAST and EAAC1 in the uptake of nutrients and metabolites in non-neuronal organs.

Ingested glutamate is extensively metabolized to provide whole-animal energy and support N homeo-
stasis (18, 19, 29). This amino acid is also known to be an important energy source of intestinal epithelial cells themselves and to stimulate cellular proliferation. In order to utilize food-derived glutamate, the intestinal epithelium expresses glutamate transporters to take up glutamate abundantly present in the lumen of the digestive tract. Among different mammalian glutamate transporter isoforms, EAAC1 shows widespread distribution in a variety of tissues, including the intestine in rabbits (13), humans (17) and ruminants (11), and is responsible for the Na⁺-dependent uptake of glutamate via the striated border membrane. In addition to the presence of EAAC1, GLT-1 immunoreactivity is detected from the stomach to the colon in sheep and cattle (11). It has been reported that an elevated nutritional status up-regulates the content of EAAC1 protein (but not GLT-1 protein) in the ileal epithelium of sheep in parallel with increased tissue glutamate levels (12). Identification of the sites of expression is very important for a detailed understanding of functional roles of the glutamate transport system in the digestive tract. However, no information about the cellular or subcellular localization of glutamate transporters is available, except for an in situ hybridization analysis for EAAC1 in the rabbit intestine (13) and an immunohistochemical analysis for EAAC1 in the rat intestine during postnatal development (24). In order to understand how the gastrointestinal tract responds to food-derived glutamate, we investigated the cellular distribution of four types of glutamate transporters, i.e., GLAST, GLT-1, EAAC1 and EAAT4, throughout the gut of mice by the use of immunohistochemistry combined with in situ hybridization.

MATERIALS AND METHODS

Animals and tissue sampling. Adult male ddY mice (Japan SLC, Shizuoka, Japan), 10 weeks old, were used in the present study. For immunohistochemistry, the animals were deeply anesthetized with pentobarbital and perfused via the aorta with physiological saline, followed by 4% paraformaldehyde (0.1 M phosphate buffer, PB, pH 7.4). The tissues were removed from the glandular stomach, pyloric antrum, duodenum, jejunum (the middle region of the upper half of the small intestine), terminal ileum, cecum and proximal colon, and immersed in the same fixative for an additional 6 h at 4°C. As a positive control, the brain was used throughout the staining. For in situ hybridization, the mice were sacrificed by bloodletting from the heart under deep anesthesia with pentobarbital, and fresh tissues were removed from the regions of the gut listed above and frozen in liquid nitrogen. All experiments were performed under protocols following the Guidelines for Animal Experimentation, Graduate School of Medicine, Hokkaido University.

Immunohistochemistry. The paraformaldehyde-fixed tissues were then immersed in 30% sucrose dissolved in 0.1 M PB (pH 7.4) overnight, and frozen in liquid nitrogen. Sections, 10 μm in thickness, were cut in a cryostat and mounted on poly-L-lysine-coated glass slides. The sections were treated with 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 1 h, and then with 0.3% H₂O₂ in methanol for the inhibition of endogenous peroxidase activities. After preincubation with 10% normal goat serum for 30 min, the sections were incubated overnight with one of the antibodies raised in rabbits. Rabbit polyclonal antibodies against GLAST (25), GLT-1 (27), EAAC1 (αEAAC1 1-A, Alpha Diagnostic International, San Antonio, TX, USA), and EAAT4 (26) were used at the following dilutions: 0.25 μg/mL, 0.27 μg/mL, 0.25 μg/mL, and 0.25 μg/mL, respectively. Sections were then incubated with biotinylated goat anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan), and then with streptavidin-peroxidase (Nichirei), each for 1 h. The antigen-antibody reaction was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3’-diaminobenzidine and 0.002% H₂O₂ and examined with a light microscope.

In situ hybridization

Two non-overlapping 45-mer antisense oligonucleotide probes specific for mouse EAAC1 were synthesized. They were complementary to nucleotide residues 519-563 (TCAGGACATGTTCCTCAGAATCTTGTCAAGCCGTCTTCTCGC) and 1020-1264 (TCCGCTTTGCGTTGATGCGCTTCACGACGCTTCA) of mouse EAAC1 mRNA (accession no. D43797). These oligonucleotides were labeled with 35S-dATP, using terminal deoxynucleotidyl transferase (Promega, Madison, USA) at a specific activity of 0.5 × 10⁸ dpm/μg DNA. In situ hybridization analysis was performed as previously described (16). In brief, fresh frozen sections, 10 μm in thickness, were prepared and mounted on glass slides precoated with 3-amino-propyltriethoxysilane. They were fixed with 4% paraformaldehyde in 0.1 M PB for 15 min, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridi-
tion was performed at 42°C for 10 h by adding 35S-labeled oligonucleotide probes. Slides were rinsed once at room temperature for 30 min in 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate) containing 0.1% Sarkosyl, and twice at 55°C for 40 min in 0.1 × SSC containing 0.1% Sarkosyl, dehydrated through a graded series of ethanol, and air-dried. The sections were dipped into autoradiographic emulsion (NTB-2; Kodak, Rochester, NY), exposed at 4°C for 6 weeks and counterstained with hematoxylin after development.

In situ hybridization using two non-overlapping anti-sense probes exhibited consistent labeling in all the tissues examined. The specificity of hybridization was also confirmed by the disappearance of the signals when an excess of unlabeled antisense probe was added to the hybridization fluid.

Silver-intensified immunogold method for electron microscopy. Frozen sections, 15 µm in thickness, were prepared from the paraformaldehyde-fixed tissues and processed for the silver-intensified immunogold method. The sections on glass slides were incubated with the rabbit EAAC1 antibody at 2 mg/ml and subsequently reacted with goat anti-rabbit IgG covalently linked to 1-nm gold particles (1:200; BBIInternational, Golden Gate, UK). Following silver enhancement using HQ silver (Nanoprobes, Stony Brook, NY, USA), the sections were osmicated, dehydrated, and directly embedded in Epon (Nishshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with an aqueous solution of uranyl acetate and lead citrate for observation under an electron microscope (H-7100, Hitachi, Tokyo, Japan).

RESULTS

Preliminary in situ hybridization analysis of the mRNA expression of four glutamate transporters revealed intense signals for EAAC1 mRNA in the intestine. We detected intense signals for the transcripts of all four of these glutamate transporters in the brain (used as a positive control), but failed to find significant signals of GLT-1, GLAST, and EAAT4 mRNAs in the digestive tract. However, immunoreactivities for these four transporters were detectable in the stomach and intestine with specific antibodies used under the same conditions as used for the immunostaining of brain tissues (Fig. 1d).

EAAC1
Hybridization signals of EAAC1 mRNA were found mainly in the mucosal epithelium of the small intestine, and were especially intense in the ileum. Under the light microscope, the silver grains showing the expression sites gathered from the upper region of intestinal crypts to villus tips; the signals were more intense at the lower half of villi and tended to decrease in intensity toward the villus tips (Fig. 1a). The stomach and large intestine lacked any significant expression of EAAC1 mRNA.

Immunohistochemistry with anti-EAAC1 antibody detected intense immunoreactivity in the striated border (brush border) of enterocytes in the ileum (Fig. 1b, c). The labeled epithelial cells were distributed from the upper region of crypts to the villus tips, in agreement with the data from in situ hybridization. The base of the villi showed the most intense immunoreactivity, while the striated border in some regions of villous epithelium did not show the immunoreactivity. Goblet cells and Paneth cells lacked the immunoreactivity. In the duodenum and jejunum, intense immunoreactivity was restricted to the luminal surface at the upper region of crypts. There was no significant immunoreactivity in any region of the stomach or large intestine. Moderately intense immunoreactivity was shared by some neuronal cell bodies of the submucosal and myenteric nerve plexuses and a small number of endocrine cells dispersed within the epithelium.

The silver-intensified immunogold method using sections from the ileum revealed the subcellular localization of EAAC1 immunoreactivity. Under the electron microscope, the gold particles were concentrated in the microvilli of intestinal epithelial cells (Fig. 1e).

GLT-1
Immunostaining using anti-GLT-1 antibody revealed the presence of immunoreactivity at the bottom region of the fundic and pyloric glands, with a characteristic labeling pattern along the lateral cell membrane of epithelial cells. Similar stainability was seen to a variable extent in intestinal crypts from the duodenum to the colon (Fig. 2c). The most intense immunoreactivity on the lateral cell membrane was detectable in the duodenal gland of Brunner (Fig. 2a, b). Neuronal elements (possibly nerve fibers) in the myenteric nerve plexus throughout the gastrointestinal tract and a small number of enteroendocrine cells in the intestine were immunolabeled with anti-GLT-1 antibody.

GLAST
Immunoreactivity for GLAST in the mucosal layer...
Fig. 1 Expression of EAAC1 in the ileum and cerebellum. Light microscopic observation of a section from the ileum hybridized for EAAC1 mRNA (a). Dense aggregation of silver grains in the epithelium is seen from the upper region of the crypts to the basal half of the villi. The intense immunoreactivity for EAAC1 in the ileum is localized in the striated border in the upper region of crypts and intestinal villi (b, c). Note that the striated border in some regions of the villi lacks the immunoreactivity. Fig. 1d shows the immunoreactivity for EAAC1 in the cerebellum, which was used as a positive control; dendrites of Purkinje cells are immunolabeled, being dotted in appearance. Electron microscopically, gold particles showing the existence of EAAC1 are concentrated in tufts of microvilli in the epithelium of the ileum (e).
Glutamate transporter in the gastrointestinal tract

was restricted to the gastric and pyloric glands, especially in the neck portion of glands. The acid-secreting regions of the stomach showed a characteristic labeling pattern along the luminal sur-
face of mucous neck cells and parietal cells (Fig. 2d, e). The pyloric gland exhibited positive immunoreactivity in the lateral membrane of epithelial cells at the neck but not at the bottom of glands; the staining pattern was complementary in distribution to that seen with the GLT-1 antibody. Additional immunoreactivity for GLAST was found mainly in the myenteric nerve plexuses; the immunoreactivity was more intense in the stomach than in the intestine. Observation of whole mount preparations immunostained for GLAST showed limited expression of GLAST in the glial elements of the nerve plexus.

**EAAT4**

No significant immunoreactivity for EAAT4 was detected in the mucosal epithelium throughout the digestive tract, while a small number of nerve cell bodies in the submucous and myenteric nerve plexuses were positive in reaction (Fig. 2f). A number of nerve fibers distributed in the lamina propria, muscle layers and nerve plexuses displayed distinct immunoreactivity, especially in the stomach.

**DISCUSSION**

Dietary protein undergoes a series of degradative reactions catalyzed by proteolytic enzymes secreted from the stomach, pancreas and small intestine, resulting in the production of free amino acids and small peptides. Cellular transport of degraded protein products and free amino acids in the lumen is a key final step in their assimilation by the intestine and is mediated by carrier-transporters with various specificities. An *in vivo* study using fed piglets showed that luminal glutamate, rather than glutamine-derived glutamate, was the preferential source of glutamate for glutathione synthesis in the intestinal mucosa (23). However, little is known regarding the sites responsible for glutamate absorption and transport from the digesta, due in part to a lack of information about the cellular expression of the transporters. Although it is generally believed that the bulk of peptides and amino acids are transported in the proximal regions of the small intestine (1), for some types of amino acids and peptides the primary site of absorption may be the distal intestine (5, 24).

The expression of EAAC1 in the gastrointestinal tract has been examined in detail in several mammals. There is a distinct species-difference in the expression sites of gut EAAC1 among mammals. Northern blot analyses for EAAC1 mRNA from three different regions of the rat small intestine indicated a pronounced gradient of mRNA expression along the proximal-distal intestinal axis with the highest levels observed distally (5, 24). A high protein diet increased the mRNA level of EAAC1 in the middle intestine by 2-3 fold, suggesting that the small intestine has the ability to up-regulate its absorptive capacity for glutamate (5). Northern blot analysis of EAAC1 mRNA from rabbit tissues detected strong bands of EAAC1 transcripts in samples from the duodenum to the ileum, but not in samples from the colon (13). In contrast, another Northern blot study of human materials detected the transcripts in the colon as well as the small intestine (17). Extremely broad distribution of EAAC1 immunoreactivity has been reported in the alimentary canal of ruminants, including the forestomach, which is responsible for the net influx of glutamate produced by microorganisms in the rumen (11). The present immunohistochemical study combined with *in situ* hybridization analysis is the first attempt to identify in detail the expression sites of EAAC1. The expression of EAAC1 was highest in the ileum at both the mRNA and protein levels, suggesting that the distal region of the small intestine is an important area for the uptake of glutamate, in agreement with the findings of Northern blot analyses of the rat small intestine (5, 24). It is worth noting that intense expression of EAAC1 was localized at the upper region of crypts and the basal half of villi. This implies the active production of EAAC1 at an early stage of differentiation or at a premature stage of enterocytes, in contrast to the general idea that nutrient absorption is carried out by fully differentiated enterocytes, as indicated by the intestinal distribution of some peptide/glucose transporters in rodents (9, 20, 28) and by the expression of EAAC1 along the crypt-villus axis in the piglet small intestine (8). Our findings are in good agreement with the data from an *in vitro* study using Caco-2 cells, namely, the Na⁺-dependent glutamate transport system is established when cells cease to proliferate and undergo differentiation (15). They are also in accord with previous immunohistochemical findings for EAAC1 in the rat distal intestine during postnatal development (24).

Plasma membrane transport capacity is thought to be limiting for glutamate metabolism. Pharmacological studies using brush-border membrane vesicles prepared from human and rat intestinal mucosa suggest the localization of a sodium-dependent transport system for L-glutamate in the apical part of cells (4, 21, 22). However, the membrane-specific site of transporter expression was not morphologi-
cally determined in those studies. The present immunoelectron microscopic analysis clearly showed condensed localization of EAAC1 in the microvillous region of enterocytes.

As for the expression of GLT-1 in the gut, only the immunoblot analysis reported by Howell et al. (11) showed continuous expression from the forestomach to the colon in ruminants. This broad distribution of GLT-1 as well as EAAC1 may reflect the important role of glutamate as a primary source of amino acid-derived hydrocarbons in ruminants (6, 10). In contrast to the uniqueness of glutamate uptake and metabolism in ruminants, no significant signals of GLT-1 mRNA were detectable in the intestines of humans (17) or neonatal pigs (8). The present immunohistological study showed selective labeling of epithelial cells at the bottom of gastric glands and intestinal crypts, and in the duodenal gland. The specificity and significance of this positive immunoreactivity remain unknown, since in situ hybridization analysis of mRNA expression failed to detect significant signals of GLT-1 mRNA, possibly due to the limited sensitivity of our method. Furthermore, the localization of GLT-1 along the lateral cell membrane appears to be favorable for taking glutamate up from the blood circulation. Further experiments using highly sensitive in situ hybridization are needed to determine in detail the localization of GLT-1.

The expression of GLAST and EAAT4 has not been detected in the gastrointestinal tract of ruminants (11) or other mammals (8, 17). In contrast, RT-PCR assays (15) using the Caco-2 cell line of human colonic origin detected significant expression of GLAST mRNA in association with increased transport of L-glutamate, though the level of mRNA for EAAC1, the most predominant type of glutamate transporter expressed in the small intestine, was barely detectable. In the present study, positive immunoreactivity for GALST was restricted to the neck portion of glands in the gastric corpus and pyloric antrum, being complimentary to the GLT-1 immunoreactivity that was localized at the bottom region of the glands. Here again, questions remains about the specificity and significance of the immunolocalization of GLAST, as about those of GLT-1. EAAT4 in the brain is expressed in neurons such as cerebellar Purkinje cells (26). In the digestive tract, EAAT4 immunoreactivity was found only in the neuronal cell bodies and nerve fibers. The great abundance of EAAT4-immunoreactive nerve elements in the stomach suggests the involvement of EAAT4 in the glutamatergic neuro-regulation in this region.

Acknowledgements

This study was supported by a grant from International Glutamate Technical Committee.

REFERENCES


