Inherited Defects of Sodium-dependent Glutamate Transport Mediated by Glutamate/Aspartate Transporter in Canine Red Cells Due to a Decreased Level of Transporter Protein Expression*

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Kota Sato‡, Mutsumi Inaba‡‡, Yuki Suwa‡, Aya Matsuue‡, Yoshiaki Hikasa‡, Ken-ichiro Ono§, and Katsumoto Kagota‡

From the ‡Department of Veterinary Internal Medicine, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan and the §Laboratory of Veterinary Clinical Pathobiology, Department of Veterinary Medical Sciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

Canine red cells have a high affinity Na⁺/K⁺-dependent glutamate transporter. We herein demonstrate that this transport is mediated by the canine homologue of glutamate/aspartate transporter (GLAST), one of the glutamate transporter subtypes abundant in the central nervous system. We also demonstrate that GLAST is the most ubiquitous glutamate transporter among the transporter subtypes that have been cloned to date. The GLAST protein content was extremely reduced in variant red cells, low glutamate transport (LGlut) red cells characterized by an inherited remarkable decrease in glutamate transport activity. All LGlutDogs carried a missense mutation of Gly492 to Ser (G492S) in either the heterozygous or homozygous state. The GLAST protein with G492S mutation was fully functional in glutamate transport in Xenopus oocytes. However, G492S GLAST exhibited a marked decrease in activity after the addition of cycloheximide, while the wild type showed no significant change, indicating that G492S GLAST was unstable compared with the wild-type transporter. Moreover, LGlut dogs, but not normal dogs, heterozygous for the G492S mutation showed a selective decrease in the accumulation of GLAST mRNA from the normal allele. Based on these findings, we conclude that a complicated heterologous combination of G492S mutation and some transcriptional defect contributes to the pathogenesis of the LGlut red cell phenotype.

High affinity Na⁺-dependent glutamate transporters play important physiological roles in various mammalian tissues and cells. Several distinct glutamate transporters, GLAST,¹

GLT-1, EAAC1, EAAT4, and EAAT5 have been cloned, and their electrophysiological and pharmacological properties have been characterized (1–5). In the central nervous system, these transporters are highly differentially localized and participate in glutamate uptake into glial or neuronal cells to terminate excitatory neurotransmission (6). In particular, glial transporters GLAST and GLT-1 play critical roles to maintain the extracellular glutamate concentration at the submicromolar level, thereby preventing accumulation of glutamate in the synaptic cleft, which causes overstimulation of the receptors and neurodegeneration. Dysfunction of glutamate transporters has been considered to be involved in the pathogenesis of neurodegenerative diseases such as amyotrophic lateral sclerosis (7, 8), Alzheimer’s disease, trauma, and ischemia (8). In peripheral tissues, glutamate transporters are believed to have pivotal functions in epithelial transport and absorption of acidic amino acids (9, 10) and in modulation of glutathione synthesis (11). EAAC1 is presumed to be a transporter in the epithelia of the intestine and kidney, because its transcripts were identified in those peripheral tissues as well as in neurons (3). However, physiological and pathological functions of other transporter isoforms in peripheral tissues have not been well characterized.

We have been interested in the structure, function, and regulation of expression of the red cell glutamate transporter in dogs. Canine red cells possess a high affinity Na⁺- and K⁺-dependent l-glutamate and l-aspartate transport system (12, 13), despite the fact that most mammalian red cells are impermeable to these acidic amino acids (14). Dogs usually have red cells with low K⁺ and high Na⁺ concentrations (LK red cells) because they lose red cell Na,K-ATPase during reticulocyte maturation (15, 16). However, some Japanese Shiba and mongrel dogs have HK red cells with high Na,K-ATPase activity, resulting in high K⁺ and low Na⁺ concentrations, and this HK phenotype is inherited in an autosomal recessive manner (17). HK red cells show accelerated Na⁺/K⁺-dependent glutamate/aspartate uptake due to an increased concentration gradient of Na⁺ and K⁺ across the plasma membrane, leading to marked accumulations of intracellular glutamate, aspartate, and glutamine (12, 17). The increased concentration of glutamate further results in an elevated level of reduced glutathione and affects the redox state and protection against oxidative stress of the red cell (18). Interestingly, these breeds also include dogs

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¹ The abbreviations used are: GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter-1; EAAC1, excitatory amino acid carrier 1; EAAT4 and -5, excitatory amino acid transporter 4 and 5, respectively; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonic acid; LGlut, low glutamate transport; NGlut, normal glutamate transport; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; nt, nucleotides.

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Due to a Decreased Level of Transporter Protein Expression*
characterized by red (19) and nontoxic red cell glutamate transporter, generating variant HK cells without accumulation of glutathione. Their red cells were readily accessible to oxidants such as acetylphenyl hydrazine and generated many more Heinz bodies than normal HK cells or even LK red cells did. Such a hereditary defect of the glutamate transporter in mammals has never been described so far as we know, although several pathological studies on mice lacking glutamate transporters due to gene disruption has been reported (20–22). The observations in dogs suggested that the functions of the glutamate transporters contributed to protect cellular contents from oxidative damage in peripheral tissues. Defining the molecular basis that underlies the transport deficiency in canine red cells may facilitate our understanding of the regulatory mechanisms for expression of, and the physiological and pathological roles for, the glutamate transporter in various tissues, including the brain.

We have postulated that, based on observations of its kinetic and pharmacologic properties, the canine red cell glutamate transporter is EAAC1 (13). The purpose of the present study is to precisely define the glutamate transporter in canine red cells, thereby clarifying the underlying cause for the hereditary deficiency of the transport.

**EXPERIMENTAL PROCEDURES**

**Dogs**—The dogs used in this study were from a family of Japanese mongrel dogs that were a mixed breed of Japanese Shiba. Some pure Shiba and Beagle dogs were also used. These dogs were clinically healthy, and hematologic parameters of their red cells were within reference ranges except that HK red cells had a mean corpuscular volume slightly larger than that of LK red cells as demonstrated before (17).

**Isolation of RNA and DNA, Reverse Transcription, and PCR**—Methods for isolation of total RNA, poly(A)+ RNA, and genomic DNA, reverse transcription, PCR, and cloning of PCR products were described previously (23). RNAs were treated with DNase I. DNA sequencing was carried out using a Thermosequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) on an automated DNA sequencer ALF express (Amersham Pharmacia Biotech, Uppsala, Sweden) or using a TaqII as a cloning method (Life Technologies, Inc., Life Technologies, Inc., Rockville, MD) to create 5′- and 3′-stretched canine brain GLAST cDNA, pcGLAST. A bone marrow GLAST cDNA clone (pcGLASTbm) was also prepared in the same manner.

**Expression of Canine Glutamate Transporter in Xenopus Oocytes**—Oocytes (stage V and VI) were isolated from Xenopus laevis under ice-cold anesthesia and defolliculated by treatment with 0.2% collagenase in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES/Tris, pH 7.5). The oocytes were microinjected with 1–25 ng of capped synthetic GLAST RNAs (50 nl). GLAST RNAs were transcribed from pcGLAST or pcGLASTbm linearized with BglII immediately downstream from the termination codon or with MluI within the vector sequence using MaxiScript or MegaScript kit with T7 RNA polymerase and a cap analogue (Ambion, Austin, TX). The oocytes were incubated at 19 °C in ND96 containing 1.8 mM CaCl₂, 100 units/ml penicillin, and 100 μg/ml streptomycin for 36–72 h. In some experiments, cycloheximide (Wako Pure Chemical Industries, Osaka, Japan) was added to the medium at a concentration of 10 μg/ml, and incubation was continued for 12 h.

**Preparation of Antibodies to Canine GLAST**—Multiple antigen peptides were generated by coupling to bovine ovalbumin (N-(9-fluorenyl)methoxycarbonyl) solid phase method for the amino acid sequence of the carboxy-terminal domain of canine GLAST (NH₂-Asn-Ser-Val-Ile-Glu-Glu-Asn-(9-fluorenyl)methoxycarbonyl) to E. coli and purified with affinity chromatography on a column coupled with 5′- and 3′-stretched canine brain GLAST cDNA. Antibodies bound to the column were eluted with 0.5 M NaCl, 0.1 M glycine/Tris, pH 2.7, and neutralized immediately with Tris.

**Analyses of Membrane Proteins**—Red cell ghosts were prepared as described (16). Crude synaptic membranes from brain were prepared by homogenization and centrifugation (Kaner (25)).

Membrane proteins were separated by SDS-PAGE (Laemmli’s system) on 8% gels (23). The GLAST polypeptides were detected by immunoblotting using affinity-purified anti-GLAST antibodies and an ECL chemiluminescence detection system (Amersham Pharmacia Biotech). In some experiments, membrane proteins were solubilized in 2% (w/v) CHAPS (Dojin Laboratories, Kumamoto, Japan) and kept on ice for 30 min to induce oligomerization of the GLAST proteins, which leads to efficient detection of the polypeptides with the antibody solubilized in 1% sodium dodecyl sulfate with 2% 2-mercaptoethanol. Concentrations were then concentrated with ultrafiltration units (Ultrafree MC, 30,000 nominal molecular weight limit; Millipore Corp., Bedford, MA) and subjected to SDS-PAGE followed by immunoblotting.

In **Vitro Translocation of Canine GLAST**—pcGLAST was transcribed and translated using a TNT T7 coupled reticulocyte lysate system (Promega Corp., Madison, WI) with or without canine pancreatic microsomes (Promega) in the presence of [35S]methionine (EXPER-SSS;
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1,175 C/μmol; NEN Life Science Products). Translated products were analyzed by SDS-PAGE followed by exposure to Kodak BioMax MR films.

Deglycosylation Studies—Red cell membranes and crude synaptic membranes were deglycosylated using peptide:N-glycosidase F (NEN Life Science Products). Briefly, the membrane proteins (100–150 μg) were solubilized in 0.5% SDS, 1% β-mercaptoethanol at room temperature for 30 min. After the addition of 1/5 volume of 10% Nonidet P-40 and 0.5 m sodium phosphate, pH 7.5, samples were incubated with 2,000 units of peptide:N-glycosidase F at 37 °C for 1 h. Reactions were stopped by the addition of Laemmli’s sample buffer and subjected to SDS-PAGE and immunoblotting.

Glutamate Transport Assay in Red Cells and Xenopus Oocytes—Uptake of l-glutamate in canine red cells was measured as described previously (12, 13).

Oocytes injected with synthetic RNA were incubated at 19 °C in ND96 medium containing 1.3,4,5'-H glutamate (49 C/μmol; NEN Life Science Products) and 1.8 mM CaCl2 (100 μl/oocyte). After incubation, oocytes were washed three times with an excessive amount of ice-chilled ND96 medium. Each oocyte was transferred into a 1.5-ml tube chilled with 1,175 Ci/mmol; NEN Life Science Products). Translated products were analyzed by SDS-PAGE followed by exposure to Kodak BioMax MR films.

Restriction Enzyme Assay—A restriction enzyme assay was carried out to determine genotypes for G492S mutation. PCR fragments corresponding to nt 1,427–1,657 of canine GLAST cDNA were amplified using genomic DNA from dogs as the templates. The resulting PCR products were digested with NcoI and separated on 4% agarose gel.

Quantitation of GLAST mRNA—GLAST mRNA was quantitated by RT-PCR combined with a 5' -nuclease assay or SYBR green detection using the GeneAmp 5700 sequence detection system (Perkin-Elmer Applied Biosystems). A 5'-nuclease assay of GLAST mRNA was carried out with PCR primers 5'-TAT CGG CCA GTC-3' (nt 646–666) and 5'-TTG ACC CCA TTC ACA GAC CCT-3' in the presence of a TaqMan probe of 5'-AAT GTG TCG GAA GCC ATG GAG-3'.

RESULTS

Identification of Canine Red Cell Glutamate Transporter

Isolation of GLAST cDNA from the Brain and Bone Marrow Cells—In our preliminary experiments, PCR amplification of canine brain cDNA generated cDNA fragments corresponding to those of four different glutamate transporter subtypes, GLAST, GLT-1, EAAC1, and EAAT4. Nucleotide sequences of these cDNA fragments from dogs showed high similarities to those from other sources as described under "Experimental Procedures." However, when the same procedure was applied to cDNA from bone marrow cells, only cDNA fragments corresponding to GLAST were obtained, suggesting that the GLAST protein functions in canine erythroid cells.

Primers specific to the "GLAST-like" sequences obtained were prepared, and 5' - and 3' -RACE reactions were carried out using canine brain and bone marrow cDNAs as templates. The 5' - and 3' -RACE products were subcloned and combined. The cDNA clones from the brain (pcGLAST) and bone marrow cells (pcGLASTbm) were both about 3.8 kilobase pairs in length with a 1,629-bp open reading frame encoding a protein of 542 amino acid residues with the theoretical molecular mass of 59,757 Da. The size of the GLAST mRNA was confirmed by Northern blotting, although the signal intensity was very weak for bone marrow mRNA even when more than 10 μg of poly(A) + RNA was applied (data not shown). The deduced amino acid sequence showed high similarity, over 96%, to human, bovine, and rat GLASTs (Fig. 1) and significant but much lesser similarity to the other glutamate transporters, GLT-1, EAAC1, EAAT4, and EAAT5 (50–66%). Thus, recent models for mem-

brane topology of human GLAST (26, 27) with 10 or 11 membrane-spanning regions and cytoplasmic localization of NH2 and COOH termini can be adopted to the canine homologue. This prediction supposes two potential N-glycosylation sites, at Asn92 and Asn131, with N-glycosylation consensus sequences (Asn-X, where X represents any residue except Pro-Ser/Thr) within the putative second extracellular loop.

Analysis of GLAST mRNA Expression by RT-PCR—RT-PCR analysis indicated amplification of GLAST cDNA with strong signals in the cerebral cortex, cerebellum, and hippocampus (Fig. 2). Amplification was also observed in other tissues and cells except that a very faint band and no bands were obtained in reticulocytes and liver, respectively. It should be emphasized that signals for GLAST cDNA were detected clearly in reticulocytes and liver when the PCR cycles were increased (Fig. 2, right panels). Signals were also detected in other tissues examined, including colon, spleen, pancreas, thyroid gland, adrenal gland, and testis, with intensities similar to those of reticulocytes and liver (data not shown). Under the PCR conditions employed, no noticeable amplification of the transporter cDNA other than that of GLAST cDNA was observed in bone marrow cells and reticulocytes, although EAAC1 showed a very faint band of PCR products in bone marrow after extended PCR cycles. These results demonstrated a ubiquitous expression of GLAST transcripts in a variety of cells and tissues in dogs and indicated that canine erythroid cells contained the GLAST
mRNA but not the transcripts of other glutamate transporter genes.

Characterization of Glutamate Transport by Canine GLAST—Oocytes injected with synthetic RNA of canine GLAST showed high affinity Na⁺-dependent glutamate uptake that was completely abolished when the extracellular Na⁺ was replaced by choline. The Na⁺-dependent uptake was dominated by a saturable component obeying Michaelis-Menten kinetics (Fig. 3A). The $K_m$ value for l-glutamate obtained from a Lineweaver-Burk plot (Fig. 3B) was 36.3 μM. This value was slightly higher than that estimated for the uptake in canine red cells at 37 °C (7–14 μM; Refs. 12 and 13), whereas lower affinity was reported for other mammalian GLAST homologues in oocytes (70–80 μM) (1, 28–30).

Several structural analogues of L-glutamate were tested for their inhibitory effects on glutamate transport to compare the pharmacological properties of canine GLAST and canine red cells (Table I). Potent inhibition of the glutamate uptake was observed in response to three-3-hydroxyaspartate, L-glutamate, and L- and D-aspartate but not by D-glutamate. These were the properties common to all the brain glutamate transporters (31). Dihydroidkatinate and L-cysteine, which are selective inhibitors for human GLT-1 (32) and human EAAC1 (33), respectively, poorly inhibited the glutamate transport by canine GLAST. Thus, the responses of canine GLAST expressed in the oocytes were consistent with those of the glutamate transport system in canine red cells (Table I).

Identification of the GLAST Protein in Canine Red Cells—Affinity-purified antibodies to the synthetic peptide of GLAST reacted with 60-kDa proteins in membranes from the cerebral cortex, while the antibodies immunospecifically recognized polypeptides with an apparent molecular mass of 66 kDa in red cell membranes, as documented in Fig. 4A (Membranes). When the brain membranes were solubilized with CHAPS, the distinct higher molecular mass signals at 120 kDa appeared on the immunoblot (Fig. 4A, CHAPS extracts) as reported for rat GLAST (34). Likewise, 130-kDa bands appeared instead of the 66-kDa polypeptides in red cell membranes solubilized with CHAPS. The 130- and 120-kDa bands were dimers presumably induced by oxidation of the thiol groups not reducible by sulfhydryl reducing agents as reported by previous investigators for rat GLAST (34) and human GLUT-1 (35). Additional bands at 98–100, 72, and 45 kDa were visualized in red cell membranes when a large amount of protein was loaded onto the gels. These bands were probably derived from nonspecific reactions of the antibodies with band 3, protein 4.2, and actin, respectively, because corresponding bands were also observed in human red cells that lacked Na⁺-dependent glutamate transport activity (Fig. 4A).

It was interesting that the GLAST proteins synthesized in a reticulocyte lysate system had an apparent molecular mass of only 50 kDa in our SDS-PAGE system (Fig. 4B), much less than

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**Fig. 2.** RT-PCR analysis for mRNA expression of glutamate transporters in various canine tissues. mRNA of glutamate transporter isoforms was evaluated by RT-PCR using first-strand cDNAs reverse-transcribed from total RNAs of various tissues from an NGluT dog. PCR amplification was carried out for 30 cycles. The results after 40 cycles are shown in the right panel for bone marrow cells, reticulocytes, and liver (asterisks). Canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified as the internal control for 25 cycles.

**Fig. 3.** L-Glutamate transport in *Xenopus* oocytes expressing canine GLAST. A, *Xenopus* oocytes were injected with 25 ng of synthetic cDNA. After incubation for 48–60 h, glutamate uptake was measured in ND96 medium containing L-[3H]glutamate (5 μCi/ml) and various concentrations of L-glutamate at 19 °C for 5 min. Na⁺-dependent uptake was calculated by subtracting the mean values in the absence of Na⁺ from values obtained in the presence of Na⁺. Water-injected control oocytes exhibited an endogenous Na⁺-dependent glutamate uptake at a negligible level that was less than 0.3% that in the oocytes injected with synthetic RNA in each of the concentrations of glutamate. Data represent means ± S.D. (n = 6). B, Lineweaver-Burk plot of glutamate uptake shown in A.

**Table I**

Cross-inhibition with structural analogues of glutamate transport by canine GLAST expressed in oocytes

| Inhibitors               | Uptake (percentage of control) |
|--------------------------|--------------------------------|
|                          | Oocytes | Red cells* |
| None                     | 100.0 ± 21.8 | 100.0 ± 5.0 |
| L-Glutamate              | 46.2 ± 24.0 | 22.3 ± 0.3 |
| D-Glutamate              | 87.8 ± 21.3 | 76.7 ± 11.8 |
| L-Aspartate              | 43.9 ± 18.9 | 20.3 ± 0.9 |
| D-Aspartate              | 61.5 ± 27.9 | 53.2 ± 2.2 |
| three-3-Hydroxyaspartate | 15.7 ± 5.6  | 16.6 ± 0.4 |
| L-Cysteinesulfinate      | 43.5 ± 6.0  | 20.6 ± 1.0 |
| Dihydroidkatinate (500 μM) | 73.1 ± 21.2 | 58.4 ± 11.7 (1 mM) |
| L-Cysteine (500 μM)      | 73.3 ± 10.7 | 88.4 ± 1.0 |

* Reference data for dog red cells at 37 °C determined in our previous study (13) except for L-cysteine.
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Fig. 4. Analysis of canine GLAST proteins. A, membrane proteins from canine brain (cerebrum, 1 μg) and red cells (100 μg) and from human red cells (100 μg) were separated by SDS-PAGE on 8% gels and transferred onto polyvinylidene difluoride membranes (Membranes). GLAST proteins were detected using the affinity-purified anti-COOH-terminal peptide of canine GLAST. Membrane proteins solubilized with 2% CHAPS (CHAPS extracts) were also processed for immunoblotting. Monomers (M, 66 or 60 kDa) and dimers (D, 130 or 120 kDa) of GLAST polypeptides are indicated. Monomers (62 kDa) and dimers (120 kDa) were also detected in membranes from cerebellum (data not shown). Nonspecific bands corresponding to band 3 (100 kDa), protein 4.2 (72 kDa), and actin (45 kDa) are indicated with gray arrowheads. B, in vitro synthesis of canine GLAST protein. Canine GLAST cDNA was transcribed and translated in a TNT reticulocyte lysate system with [35S]methionine in the presence (●) or absence (○) of GLAST polypeptides are indicated with and dimers (D) were also processed for immunoblotting. CHAPS extracts (2% CHAPS) were also detected in membranes from cerebellum (data not shown). Membranes (M) and red cell ghosts (75 μg) were separated by SDS-PAGE on 8% gels and stained with Coomassie Brilliant Blue. The GLAST protein content was determined by densitometric scanning of the immunoblot. C, removal of N-glycan from the GLAST proteins in brain and red cells. The crude membranes from cerebrum (5 μg) and red cell ghosts (75 μg) were solubilized in 0.5% SDS, 1% β-mercaptoethanol at room temperature for 30 min. After the addition of 1/10 volume of 10% Nonidet P-40 and 0.5 M sodium phosphate, pH 7.5, they were incubated at 37 °C for 1 h in the presence (PNGase F) or absence (Mock) of peptide-N-glycosidase F, followed by SDS-PAGE and immunoblotting. Migrating positions of monomers (M) and dimers (D) of GLAST polypeptides are indicated with arrowheads.

Fig. 5. Pedigree of Japanese mongrel dogs, including LGluT dogs. A family consisting of three generations (I–III) of Japanese mongrel dogs and some other dogs were studied. The value shown below each symbol represents Na+-dependent glutamate transport activity in pmol/ml of cells/min. Phenotypes for glutamate transport activity (NGluT and LGluT) are indicated to the left of each symbol. A thick line in the symbol indicates individuals possessing HK/LK phenotype red cells. Genotypes for G492S mutation of GLAST were determined by PCR-RFLP (see “Experimental Procedures”) and are indicated to the right of the symbol. A typical profile of PCR-RFLP is shown in the inset.

mongrel dogs revealed the presence of a red cell phenotype with low glutamate transport (LGluT) as shown in Fig. 5. Dogs carrying red cells with the LK phenotype (dogs I-1, I-2, III-2, and III-3) and a dog with HK red cells (dog II-1) exhibited glutamate transport that was reduced to about 20–30% of that in the normal LK and HK cells, while red cells from other dogs in this family had transport activity within the reference ranges (normal glutamate transport (NGluT)). Moreover, two purebred Shiba dogs had LGluT red cells in which glutamate transport was hardly detectable (S-1 and S-2). Fig. 5 also shows that the LGluT phenotype was totally independent of HK/LK phenotypes.

Immunoblotting using antibodies to the GLAST peptide demonstrated that CHAPS extracts of red cells with normal transport contained the GLAST dimers (130 kDa) at fairly detectable levels, whereas red cells from LGluT dogs gave only faintly visible bands for the GLAST dimers (Fig. 6). Densitometric scanning of the immunoblot indicated that the content of immunoreactive GLAST polypeptides detected as dimers in LGluT cells was much less than 10% of that in the NGluT red cells or was nearly totally missing. There was no significant difference between NGluT and LGluT red cells in terms of their major membrane protein profiles on SDS gels stained with Coomassie Brilliant Blue. These findings indicated that impaired transport of glutamate in LGluT cells was in parallel with specific reduction of the GLAST protein content.

Genetic Analysis of the GLAST cDNA from Dogs with Reduced Red Cell Glutamate Transport—Sequencing analysis of the GLAST cDNA clones isolated from bone marrow cells of some LGluT dogs showed a missense mutation of G to A at nucleotide 1,594 that generated substitution of Gly992 to Ser (G492S). This mutation was confirmed by restriction enzyme assay of the PCR products from genomic DNA. It was not likely that this mutation was a simple polymorphism, since none were identified in genomic DNAs from 50 control dogs other than Shiba and Japanese mongrel dogs. However, pedigree analysis indicated that this G492S substitution did not simply cosegregate with the red cell phenotype of reduced glutamate transport by itself, since while red cells from individuals I-1, III-2, S-1, and S-2, homozygous for this mutation, exhibited the
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**Fig. 6. Immunoblot analysis of the GLAST protein in LGluT red cells.** Red cell membranes (100 μg) from dogs shown in Fig. 5 were solubilized in 2% CHAPS and concentrated by ultrafiltration. Proteins were separated on 8% SDS gels, and the GLAST proteins were detected using affinity-purified anti-GLAST antibodies. The black arrows indicate GLAST monomers and dimers, and shaded arrows indicate non-specific bands (see “Results” and legend to Fig. 4). NGluT and LGluT traits, genotypes for G492S mutation, and individual identifications are indicated. G/G, G/S, and S/S represent free (wild type), heterozygous, and homozygous for G492S mutation, respectively. The lower panel shows the band 3 (100-kDa) region of Coomassie Brilliant Blue-stained gel for comparison.

**Fig. 7. Quantitative analyses of GLAST mRNAs by RT-PCR-based assay.** A, GLAST mRNAs in bone marrow cells of NGluT and LGluT dogs were quantitated by RT-PCR combined with a 5’-nuclease assay and are shown in copy numbers normalized for 1,000 copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. B, GLAST cDNA fragments containing the G492S mutation loci were amplified from bone marrow cDNAs and were digested with NgoMIV as in PCR-RFLP analysis for G492S genotyping (see legend to Fig. 5). The digestion profile for the fragment amplified from genomic DNA of a dog heterozygous for the G492S mutation is shown on the left (gDNA). Abbreviations are as in Fig. 6.

LGlUt phenotype, the heterozygous state resulted in either LGlUt or NGluT red cells (Fig. 5). Therefore, we analyzed mRNA levels and characteristics of G492S GLAST to determine the significance of this mutation.

**Quantitation of GLAST mRNA in Bone Marrow Cells**—To quantitate GLAST mRNA levels in bone marrow cells, we employed a 5’-nuclease assay using TaqMan probes because the relative abundance of GLAST transcripts was insufficient for estimation by Northern blotting as described above. As shown in Fig. 7A, copy numbers of the GLAST mRNA, determined by amplification of the nt 646–725 sequence, in LGluT dogs heterozygous for G492S mutation were remarkably decreased compared with those in NGluT dogs. A similar decrease in total GLAST mRNA was also observed in an LGluT dog homozygous for G492S (S-2), while relative abundance of the GLAST mRNA in individual II-1 (homozygous for G492S, LGluT phenotype) was apparently increased.

PCR amplification of another target sequence including the G492S locus (nt 1, 427–1,657) gave a similar result (data not shown). Digestion of this sequence with NgoMIV (or NaeI) showed that GLAST transcripts from the G492S allele were obviously abundant in the amplification products of LGluT dogs, while PCR products of NGluT dogs carrying the G492S mutation had digestion profiles indistinguishable from those of genomic DNA (Fig. 7B). This indicated that a selective decrease in accumulation of GLAST mRNA with a normal coding frame occurred in LGluT dogs heterozygous for the G492S mutation, suggesting that some transcriptional defect of normal allele would contribute to generate a decreased level of GLAST proteins in a subset of the LGluT phenotype.

**Functions and Stability of G492S GLAST in Xenopus Oocytes**—Xenopus oocytes microinjected with synthetic RNA carrying the G492S mutation showed glutamate uptake with kinetic constants similar to that of wild-type GLAST (Fig. 8A). Co-injection of wild-type and G492S RNA caused no significant alteration in transport activity. Furthermore, functional expression of G492S GLAST was observed in COS-7 cells (data not shown).

We then examined whether the mutant protein was different from wild-type GLAST in terms of the stability or turnover rate. After incubation of oocytes for 12 h in the presence or absence of cycloheximide, glutamate transport activity of the wild type was not affected by the addition of this translation inhibitor. However, glutamate uptake in the oocytes injected with G492S GLAST and incubated with cycloheximide was only about 60% of that in control oocytes incubated without cycloheximide and was less than that observed before the addition of cycloheximide (Fig. 8B). It is not likely that the reduction in the activity of G492S GLAST was due to a decrease of the mutant RNA content within the oocytes, since the transport activity of G492S GLAST was increased when oocytes were not exposed to cycloheximide, as observed for the wild type (Fig. 8B). These findings demonstrated that G492S GLAST was fully functional for Na+-dependent glutamate transport but was unstable compared with the wild-type transporter.

**DISCUSSION**

GLAST is one of the most abundant glutamate transporters in the central nervous system (1) and has been believed to be rather specific to glial cells (37, 38). Our present study unequivocally demonstrated the presence of GLAST in a variety of peripheral tissues and cells, including red cells, as well as in the central nervous system. To our knowledge, this is the first demonstration of the ubiquitous expression of this glutamate transporter subtype. The molecular features of canine GLAST in erythroid cells and the brain were basically identical and were similar to those observed for previously identified GLAST homologues in terms of the affinity to glutamate, electrophoretic mobility on SDS gel, and characteristic dimeric association upon solubilization (Figs. 3 and 4).

Recent studies have shown that GLAST plays essential roles in the brain and retina for normal neurotransmission and protection of neuronal cells from the excitotoxicity of glutamate (8, 22, 39). The vulnerability to biological oxidants and possible involvement of an SH-based redox regulatory mechanism of glutamate transporters, including GLAST, have suggested a direct link between oxidation and neurodegeneration (8). Our previous studies demonstrated that glutamate transport in dog red cells regulates the synthesis and accumulation of reduced...
glutathione, affecting the susceptibility of the cells to oxidative stress (17, 18). Actually, LGlutT red cells with the HK phenotype had glutathione content reduced to 15–45% (1.3–3.5 mmol/liter of cells) of that in HK cells with normal glutamate transport. Typically, red cells from an LGluT/HK dog (S-2, Fig. 5) showed remarkably accelerated formation of Heinz bodies when incubated with acetylphenyl hydrazine,3 while NGluT/HK cells with a low glutathione concentration was resistant to this oxidative agent (18). These findings and the identification of GLAST in canine red cells indicate that one of the physiological roles of GLAST is to modulate the antioxidative defense of the cells. In turn, they also suggest that neuroprotection by glutamate transport in LGluT dogs was associated with a decrease of the transporter protein, which was the only causative mutation found in GLAST cDNA isolated from LGluT dogs, was unstable in the Xenopus oocyte expression system, while it was fully functional in terms of glutamate transport (Fig. 8). If G492S RNA serves as the major part of the GLAST gene transcript as observed in dogs II-1 and S-2 (Fig. 7), the instability of G492S GLAST would result in an aberrant deficiency of the transporter protein in erythroid cells, since they lose the translation machinery to synthesize proteins once they mature into red cells. According to the proposed membrane topology of the human GLAST protein, Gly492 resides within the well conserved intracellular COOH-terminal region (27) or in the 10th membrane-spanning β-sheet structure (28). Alteration of this amino acid residue might affect the stability of the protein. In this respect, recent reports on phosphorylation-regulated ubiquitination of two proteins, 1xBa (40) and β-catenin (41), are interesting. These studies demonstrated that phosphorylation of a specific Ser residue within the conserved target sequence led to ubiquitination and degradation of members of the 1xB and β-catenin families (41), and this ubiquitination target sequence consisted of a motif of Asp-Ser-Gly-H-X-Ser (where H represents a hydrophobic residue and X represents any residue), similar to the mutant sequence surrounding the G492S locus, Asp-Ser-Leu-Gly-Ala-Ser492.

Second, a quantitative decrease of mRNA derived from the normal allele, which was observed in LGluT dogs heterozygous for the G492S mutation, appeared to be an additional cause of the reduced accumulation of total GLAST mRNA (Fig. 7). The decreased level of mRNA in these animals (I-1 and I-2), in combination with the instability of GLAST with G492S mutation, would lead to a defect of the transporter protein. We obtained two types of GLAST cDNA from NGluT and LGluT dogs carrying the G492S mutation in the heterozygous state (Fig. 5), and these cDNAs were basically identical except for the G492S mutation. Neither alternative splicing products nor additional mutations that might result in instability of the transcripts were observed in PCR amplification of bone marrow cDNAs from these LGluT dogs using several distinct primer pairs encompassing 5′- and 3′-stretched ends (data not shown). Therefore, some transcriptional regulation may be responsible for the selective reduction of normal GLAST mRNA.

Thus, the G492S mutation and its heterologous combination with some putative transcriptional defect as causes for GLAST protein deficiency appear to fit the appearance of the LGlutT phenotype in the pedigree currently studied; i.e. dogs homozygous for the G492S mutation, and also those heterozygous for the G492S mutation and a selective decrease of normal GLAST mRNA, possess LGlutT red cells in which the protein content and transport activity of GLAST are reduced to much less than 50% of those in NGluT dogs (Figs. 5–7). The heterozygous existence of normal GLAST mRNA at a level even with that of the G492S mutation seems to be sufficient to compensate for the GLAST protein and transport activity, resulting in the NGluT phenotype (dogs I-3 and III-4; Figs. 5 and 7). Our observation that the GLAST transcripts in erythroid cells and the brain were identical in size and sequence suggests the use of the same promoter(s) in the brain and peripheral...
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cells. This is compatible with the finding that the promoter sequence of the 5′-flanking region of the murine GLAST gene has the characteristics of a housekeeping gene containing CCAAT box and GC-rich regions (42). These findings raise intriguing questions as to whether the GLAST protein level, CCAAT box and GC-rich regions (42). These findings raise 

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Inherited Defects of Sodium-dependent Glutamate Transport Mediated by Glutamate/Aspartate Transporter in Canine Red Cells Due to a Decreased Level of Transporter Protein Expression

Kota Sato, Mutsumi Inaba, Yuki Suwa, Aya Matsuu, Yoshiaki Hikasa, Ken-ichiro Ono and Katsumoto Kagota

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