Non-essential roles of cysteine residues in functional expression and redox regulatory pathways for canine glutamate/aspartate transporter based on mutagenic analysis

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A redox regulatory mechanism and a molecular link between oxidative and excitotoxic neurodegeneration have been postulated for high-affinity Na⁺-dependent glutamate transporters. In the present study, mutations were introduced at three cysteine residues in canine glutamate/aspartate transporter (GLAST) to investigate the functional significance of thiol groups in response to oxidation. Cys(–) GLAST, in which all cysteines were replaced by other amino acids, as well as other mutants with disruption of one of three cysteine residues, showed insoluble oligomer formation, which was considered to be due to spontaneous and excessive oxidation as observed in wild-type GLAST.

The mutant transporters also showed plasma-membrane localization and glutamate-transport kinetics that were very similar to those of wild-type GLAST. Glutamate-transport activities in COS-7 cells transfected with wild-type and Cys(–) GLAST were inhibited to the same degree when cells were exposed to Hg²⁺ and were recovered by the addition of thiol-specific reductant dithiothreitol. These findings suggest that cysteine residues are not critical in functional expression of GLAST and the redox-sensing pathway via glutamate transporters.

Key words: GLAST, glutamate transport, oligomer, oxidation.

INTRODUCTION

High-affinity Na⁺-dependent glutamate transporters participate in glutamate uptake into glial or neuronal cells to terminate excitatory neurotransmission at glutamatergic synapses [1]. In particular, glial transporters glutamate/aspartate transporter (GLAST) [2] and glutamate transporter-1 (GLT-1) [3] 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 disorders. The first substantial evidence *in vivo* was provided by the finding that selective depletion of GLAST, GLT-1 and excitatory amino acid carrier 1 (EAAC1) by chronic administration of antisense oligonucleotides in living rats led to the appearance of neurological symptoms, including seizures and progressive limb weakness [4].

Interestingly, recent studies have proposed that glutamate transporters exhibit redox-sensing properties; that is, glutamate uptake is regulated by the chemical redox state of reactive thiol groups of cysteine residues in the structure [5,6]. Different transporter subtypes appear to share this putative regulatory mechanism, since the transport activities of GLAST, GLT-1 and EAAC1 were equally and reversibly inhibited by oxidants [6]. Two cysteine residues, Cys¹⁸⁶ and Cys³⁷⁵ (GLAST numbering), are conserved among distinct glutamate-transporter subtypes, and thus appear to be involved in thiol–disulphide interconversion [5]. Further oxidation of thiol groups is likely to induce covalent bonds between the transporter molecules, leading to oligomer formation. Direct links between oxidative inhibition of glutamate transport via a redox regulatory mechanism and

neuronal death have been proposed in amyotrophic lateral sclerosis [5,7,8], Alzheimer's disease and traumatic and ischaemic injuries [5]. However, there is no direct evidence that demonstrates fundamental roles of cysteine thiol groups in the physiology and pathology of glutamate transporters.

The purpose of the present study was to define the functional significance of cysteine residues in GLAST protein. We have conducted a study on canine GLAST which shared high similarity, over 96%, with GLASTs from human and other animal species in the deduced amino acid sequence including three cysteine residues, Cys¹⁸⁶, Cys²⁵² and Cys³⁷⁵ [9]. Site-directed mutagenesis was employed to create mutant transporters in which each cysteine was replaced by other amino acids, and responses of the cysteine mutants expressed in COS-7 cells to oxidation were investigated.

EXPERIMENTAL

Preparation of crude membrane vesicles from canine cerebrum

Crude membrane vesicles from canine cerebrum were prepared according to Kanner [10]. Protein concentrations of the membrane vesicles were determined by the method of Bradford [11] with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using BSA as the standard.

Construction of cDNA clones encoding cysteine mutants of canine GLAST

Preparation of wild-type canine GLAST cDNA in the expression vector pCMVSPORT1 (Life Technologies, Rockville, MD, U.S.A.), which was designated pcmvGLAST/WT, was described in a previous study [9]. Several mutations at cysteine residues, Cys¹⁸⁶ → Gly (C186G), Cys²⁵² → Ala (C252A), Cys³⁷⁵ → Ala

Abbreviations used: DTT, dithiothreitol; EAAC1, excitatory amino acid carrier 1; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter-1.

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(C375A), and C252A and C375A (C252 A/C375A), were introduced into pcmvGLAST/WT by site-directed mutagenesis using a Transformer™ site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA, U.S.A.) according to the manufacturer's directions. Mutagenic primers used in the present study were: 5'-CTGGTGAAGCTGGCTTTAAACAG-3' (nt 544–567) for C186G, 5'-CTTCTCCATGGCCTTTGGACTTGTG-3' (nt 744–768) for C252A and 5'-CACATTCAAGGCCTTGAAGAGAAC-3' (nt 1113–1137) for C375A. Selection primers were as follows: 5'-CAAGCTTACGTGTGCATGCG-3' and 5'-GAGGATCCCTAGAGGGGCC-3' (nt 700–681 and 719–700 of pCMVSPORT1, respectively). pcmvGLAST/Cys(–), encoding the mutant GLAST that lacked all cysteine residues, was obtained by combining the C186G mutant and the C252A/C375A mutant using *SpeI* restriction sites (nt 735 of canine GLAST cDNA and nt 734 of pCMVSPORT1). Mutations were confirmed by sequencing the constructs using an ABI Prism BigDye terminator cycle sequencing ready reaction kit on an ABI Prism 377 DNA Sequencer (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.).

Expression of canine GLAST proteins in COS-7 cells

COS-7 cells were grown at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, U.S.A.), 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were transfected with cDNA clones using Trans IT LT-1 (PanVera Corp., Madison, WI, U.S.A.) following the manufacturer's instructions, and incubated for 48 h. pCMVSPORT1 vector alone was also transfected to produce mock-treated cells.

Analysis of canine GLAST proteins

The membrane proteins from canine cerebrum were separated by SDS/PAGE on 8% gels using Laemmli's system [12]. In some experiments, the proteins were solubilized in Tris-buffered saline (150 mM NaCl, 20 mM Tris/HCl and 2 mM EDTA, pH 7.5) containing 1% (v/v) Triton X-100 and 1.6 mM PMSF. Then they were kept on ice for 30–60 min. After centrifugation at 15000 *g* for 15 min at 4 °C, solubilized proteins in the supernatants were concentrated on Microcon YM-10 membrane filters (Millipore Corp., Bedford, MA, U.S.A.) and separated by SDS/PAGE. COS-7 cells transfected with the plasmids were solubilized in 1% (w/v) SDS containing 5% (v/v) 2-mercaptoethanol, or in 1% Triton X-100. When proteins from COS-7 cells solubilized in 1% SDS were analysed, the cell extracts were passed through QIAshredder resin (Qiagen, Hilden, Germany) to reduce the viscosity of the extracts, and concentrated in the same manner as described above.

The GLAST proteins were detected by immunoblotting with affinity-purified anti-GLAST antibodies [9] using an ECL chemiluminescence detection system (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Detection of canine GLAST proteins expressed at cell surface in COS-7 cells

Cell-surface expression of canine GLAST in COS-7 cells was estimated by immunoblotting of cell-surface proteins, which were labelled with NHS-S-S-biotin (Pierce Chemical Company, Rockford, IL, U.S.A.) and isolated on NutraAvidin beads (Pierce Chemical Company) according to the method described by Daniels and Amara [13]. Simultaneously, total expression of GLAST proteins was also analysed by immunoblotting

for whole-cell lysates prepared as described above. Expression of cell-surface GLAST proteins relative to the total amount of GLAST proteins within the cells was evaluated by densitometric scanning of the immunoblots using a GS-700 densitometer (Bio-Rad Laboratories).

Immunofluorescence microscopy

At 48 h after transfection, COS-7 cells grown on collagen-coated glass coverslips were washed with PBS. The cells were fixed with 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 for 15 min and blocked with 1% normal goat serum in PBS for 30 min. Subsequently the cells were incubated with anti-GLAST antibodies for 1 h at 4 °C, washed with PBS and then incubated with rhodamine-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After washing with PBS, the cells were mounted in ProLong antifade reagent (Molecular Probes, Eugene, OR, U.S.A.) and examined under a Nikon ECLIPSE microscope (Nikon, Tokyo, Japan).

Assay for Na⁺-dependent glutamate-transport activity

Transfected cells in 24-well plates were washed twice with Na⁺-free buffer consisting of 150 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose and 10 mM Hepes/Tris, pH 7.4. Then the cells were incubated at 37 °C in 200 µl of transport assay buffer [150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose and 10 mM Hepes/Tris, pH 7.4, containing 5–500 µM L-glutamate and 1–5 µCi/ml L-[3,4-³H]-glutamate (NEN Life Science Products, Rockville, MD, U.S.A.)]. NaCl was substituted for equimolar choline chloride to measure Na⁺-independent uptake. After incubation, the cells were washed three times with an excess volume of ice-chilled Na⁺-free buffer. The cells were lysed in 1% (v/v) Triton X-100 solution and deproteinized by the addition of an equal volume of 5% (w/v) trichloroacetic acid. After centrifugation at 15000 *g* for 15 min the resultant supernatants were counted for radioactivity using the solid scintillant ReadyCap (Beckman, Fullerton, CA,

Figure 1 Oligomer formation of GLAST proteins from canine brain membranes and from COS-7 cells transfected with wild-type GLAST

Membrane proteins from canine cerebrum (Brain) and proteins from COS-7 cells transfected with wild-type GLAST (COS) were subjected to SDS/PAGE and immunoblotting immediately after preparation (A), or after solubilization and incubation in 1% Triton X-100 on ice for 30 min in the presence (+) or absence (–) of 30 mM DTT (B). Migrating positions and apparent molecular masses of monomers (M), dimers (D) and oligomers with higher molecular masses (O) are indicated by arrowheads.

Figure 2 Expression of wild-type and cysteine mutants of canine GLAST in COS-7 cells

(A) COS-7 cells were transfected with wild-type canine GLAST (WT), its cysteine residue mutants [C186G, C252A, C375A and Cys(—)] and expression vector alone (Mock). After incubation for 48 h, cell-surface proteins were labelled with biotin. GLAST proteins in the whole cell lysate (Total) and biotinylated fraction (Surface) were detected by immunoblotting. Migrating positions of GLAST monomers (M) and dimers (D) are indicated. An asterisk indicates polypeptides derived from COS-7 cells and reacted with antibodies non-specifically. (B) Localization of the wild-type and the Cys(—) mutant GLAST in transfected cells was visualized by immunofluorescence microscopy with anti-GLAST antibodies. No signals were detected in the cells transfected with expression (Mock). Scale bars, 10 μ m. (C) Glutamate-transport activities were measured for the cells grown under the same conditions as described in (A). Data are expressed as means $\pm$ S.D. ($n = 4$) from Na⁺-dependent glutamate uptake at the concentration of 5 μ M.

U.S.A.). The Na⁺-dependent transport was calculated by subtraction of the values in the absence of Na⁺ from those in the presence of Na⁺. For kinetic analysis, endogenous Na⁺-dependent uptake of glutamate in COS-7 cells transfected with expression vector alone was subtracted from substrate uptakes in the cells transfected with GLAST cDNA clones. Preliminary time-course experiments in which incubations were carried out for 30 s to 30 min showed that glutamate uptake was almost linear for the initial 2.5 min (see Figure 3A, below). Therefore the initial uptake rates were determined by incubation of the cells for 30 s. Kinetic parameters, K_m for glutamate and V_{max} , were obtained from Woolf–Augustinsson–Hofstee plot analysis. In some experiments, cells were incubated in the transport assay buffer containing the indicated concentrations of HgCl₂ and dithiothreitol (DTT) prior to uptake assay (see the legend for Figure 3).

RESULTS

When membranes and cell extracts were subjected to immunoblotting immediately after preparation, only a single major band with an apparent molecular mass of 60 kDa was detected in the cerebrum, whereas two major bands, of 66 and 64 kDa polypeptides, were observed in the COS-7 cells transfected with wild-type GLAST cDNA (Figure 1A). The difference in electrophoretic mobility was determined to be due to different N-linked glycan structures since removal of *N*-glycan with several glycosidases resulted in reduction of apparent molecular masses of these monomer polypeptides to 50 kDa (results not shown). In contrast, when membranes from the brain and COS-7 cells were solubilized with non-ionic detergents such as Triton X-100 and allowed to stand for 30 min on ice, distinctly higher-molecular-mass signals corresponding to dimers (120 or 130 kDa) and higher-order oligomers (> 200 kDa) appeared on the immunoblots (Figure 1B), as reported for rat GLAST [14]. The rise in oligomer formation was not affected by the presence of excess amounts (up to 30 mM) of the thiol-specific reducing agent DTT upon and during solubilization of the membranes, although prevention of oligomer formation by DTT was reported by previous investigators [14]. These observations raised a question as to whether thiol residues in the GLAST polypeptide were important in the formation of oligomers. If not, a subsequent question was whether thiol residues were involved in redox-sensing properties of this transporter, since previous studies [5,6] have shown that the same cysteine residues could be involved in redox modulation and oligomer formation of glutamate transporters. To address these issues, several distinct cysteine mutants of the GLAST protein were prepared, and cell-surface expression, glutamate-transport activity, oligomer formation and responses to oxidants were examined in COS-7 cells.

Figure 2(A) shows typical profiles of immunoblots for GLAST polypeptides in total cell lysates and membrane-surface fractions prepared from COS-7 cells that were transfected with the wild type and several different mutants lacking one (C186G, C252A, C375A) or all [Cys(—)] of the cysteine residues. The immunoblot indicates that all the mutant GLASTs as well as the wild-type GLAST were synthesized and effectively transported to the plasma membrane in COS-7 cells. No signal for the GLAST polypeptide was observed in the cells transfected with expression vector alone. Densitometric scanning of the immunoblots, by which signal intensities of monomers, dimers and higher oligomers were integrated, revealed that approx. 60–90 % of the total amounts of GLAST polypeptides synthesized in these cells were found in cell-surface fractions. No statistically significant difference was observed among the constructs in the abundance of the cell-surface GLAST relative to the total amount in three

Figure 3 Characterization of Cys(–) GLAST in COS-7 cells

(A, B) Kinetic analysis of Na⁺-dependent glutamate uptake mediated by wild-type (WT) and Cys(–) GLAST. At 48 h after transfection with wild-type (left-hand panel) and Cys(–) (right-hand panel) GLAST, Na⁺-dependent glutamate uptake was measured at an extracellular glutamate concentration of 5 μM for the indicated time periods (A) and at various concentrations of glutamate for 30 s (B). Values are expressed as means ± S.D. (*n* = 4). (C) At 48 h after transfection, COS-7 cells expressing wild-type (white bars) and Cys(–) (black bars) GLAST were incubated for 10 min in transport assay buffer without additives, then for 1 min with the same buffer containing 10 μM HgCl₂, or incubated for the first 1 min with 10 μM HgCl₂ followed by a further incubation for 10 min in the presence of 3 mM DTT. Uptake in cells incubated for 10 min with 3 mM DTT alone was also measured. After washing out the buffer containing additives, glutamate uptake was assayed at an extracellular [glutamate] of 5 μM. Data represent a typical result from three independent experiments and show uptake as a percentage of that in mock-treated cells (means ± S.D., *n* = 4). Uptakes in mock-treated cells were 96.0 ± 13.4 pmol/well per 5 min (100 ± 14%) for wild type and 126.0 ± 7.6 pmol/well per 5 min (100 ± 6%) for Cys(–) GLAST. (D) Oligomer formation of Cys(–) GLAST in COS-7 cells. Cells were solubilized in 1% Triton X-100 followed by incubation on ice for 30 min in the presence (+) or absence (–) of 30 mM DTT followed by detection of the GLAST protein by immunoblotting. Migration positions of monomers (M), dimers (D) and higher-order oligomers (O) are indicated.

independent transfection experiments, although the C186G GLAST in the plasma-membrane fraction was more prominent than the wild type and other cysteine mutants. It should also be noted that the profiles for oligomer formation of the GLAST

protein were remarkably different between total and cell-surface fractions; that is, dimers and higher oligomers were the major constituents in the total lysate, whereas monomers were much more abundant in the cell-surface fraction (Figure 2A).

Cell-surface expression of wild-type GLAST and the Cys(–) mutant GLAST was also examined by indirect immunofluorescence. As shown in Figure 2(B), both wild-type GLAST and the Cys(–) mutant GLAST were predominantly localized to the plasma membrane and perinuclear region, whereas no signal for GLAST was detectable in mock-treated cells. Moreover, COS-7 cells transfected with wild-type GLAST or several distinct mutant GLASTs showed Na⁺-dependent glutamate-transport activities at a level very similar to each other when the uptake was measured at an extracellular glutamate concentration of 5 μM (Figure 2C). These results suggested that the mutant GLASTs lacking cysteine residues were equivalent to the wild-type GLAST with regard to localization to the plasma membrane, glutamate-transport activity and oligomer formation upon solubilization.

To further determine if cysteine residues are involved in the function and characteristics of the GLAST protein, we surveyed the kinetic properties and responses to oxidation of the Cys(–) mutant. We measured glutamate-uptake rates for the initial 30 s since glutamate uptake into COS-7 cells expressing the wild-type and the Cys(–) mutant GLAST showed a linear increase for the first 2.5 min (Figure 3A). As shown in Figure 3(B), Na⁺-dependent glutamate transport mediated by wild-type and Cys(–) GLAST showed kinetic characteristics similar to each other, which were dominated by a saturable component obeying Michaelis–Menten kinetics. The *K_m* and *V_{max}* values for glutamate were 46.8 μM and 189 pmol/well per 30 s in the cells transfected with wild-type GLAST and 35.6 μM and 199 pmol/well per 30 s in the cells transfected with the Cys(–) GLAST, respectively.

Glutamate uptake into COS-7 cells transfected with wild-type GLAST was significantly reduced when the cells were incubated with the thiol-specific modifier HgCl₂ at 10 μM (Figure 3C). The uptake recovered fully to the level in control cells after further incubation of the cells in the presence of DTT. Glutamate transport in the cells transfected with the Cys(–) mutant GLAST was also decreased compared with that observed in HgCl₂-treated cells expressing the wild-type transporter and recovered by the addition of DTT. DTT alone had no significant effect on glutamate uptake into these cells (Figure 3C).

Moreover, the Cys(–) mutant GLAST expressed in COS-7 cells also showed formation of dimers that were resistant to the SDS and mercaptoethanol included in the electrophoresis system. Oligomer formation of the Cys(–) mutant was not prevented by the addition of 30 mM DTT (Figure 3D) as described above for the GLAST proteins derived from the cerebrium and COS-7 cells transfected with the wild type (Figure 1).

DISCUSSION

Regulation of glutamate transporters via a redox-sensing mechanism [5,6] is an intriguing hypothesis, since oxidative inhibition or dysfunction of glutamate transporters could cause neurodegenerative disorders, including amyotrophic lateral sclerosis [5,7,8] and a decreased concentration of glutathione, which leads to hypersensitivity to some oxidants of red cells [9]. Trotti and colleagues [6] have hypothesized that thiol groups of cysteine residues in glutamate transporters are involved in redox modification of the transporter protein, based on their finding that thiol oxidants, including Hg²⁺ and 5,5'-dithiobis(2-nitrobenzoic) acid, potently and reversibly inhibited GLAST, GLT-1 and EAAC1, which were reconstituted in liposomes. Homomultimer formation

by non-covalent interactions is another characteristic shared by several distinct glutamate transporters, including GLAST, and has been suggested to be required for transport activity *in vivo* [14], whereas oxidation of thiol groups resulted in the appearance of oligomers on SDS/PAGE gels by the formation of covalent bonds between the transporter molecules [5,14]. Thus cysteine residues, particularly the residues conserved among different transporter subtypes, appeared to be involved in the redox regulatory mechanisms of the transporter protein.

The present study, however, demonstrated that the wild-type GLAST and some cysteine-mutant GLASTs, in which one or all cysteine residues within the structure of GLAST were substituted by other amino acids, had no significant difference in oligomer formation upon solubilization (Figures 1–3) and in glutamate-transport kinetics (Figures 2 and 3). This finding unequivocally indicated that the oligomerization process involved non-covalent and/or covalent interactions of GLAST polypeptides that occurred at amino acid residues other than cysteine. It is unlikely that oligomers are generated by interaction of N-linked oligosaccharide chains in the transporter molecules [15], since removal of N-glycan caused no reduction in oligomer contents (results not shown). It should be emphasized that, in the present study, the procedure used to isolate biotinylated proteins to some extent prevented formation of dimers and oligomers with higher molecular masses, while incubation of the solubilized proteins caused multimer formation in parallel experiments (Figure 2A). It is therefore suggested that association of biotin on the GLAST molecules and avidin masked some putative sites required for interaction of GLAST polypeptides and reduced oligomer formation.

The present study also demonstrated that the wild-type GLAST and the Cys(–) mutant GLAST were equally responsive to inhibition by Hg²⁺ (Figure 3C), indicating that this transport inhibition was not caused by modification of thiol groups in the GLAST protein. Direct inhibition of the transporter caused by some modification of Hg²⁺ of reactive groups other than thiol groups is not likely to be the case since inhibition of the transport via Cys(–) GLAST was totally reversed by the addition of the thiol-specific reductant DTT. Therefore, this inhibitory effect of Hg²⁺ is mainly attributed to an indirect effect on Na⁺-dependent transport. The most likely candidate was suppression of endogenous Na⁺/K⁺ pump activity by Hg²⁺ via modification of cysteine residues [16] leading to a reduced Na⁺ gradient across the plasma membrane. However, no remarkable reduction in glutamate uptake was observed even after incubation of the cells with 0.1 mM ouabain for 30 min (results not shown). Thus, the major target of Hg²⁺ remains unclear at present. We could not rule out the possibility that transport inhibition was caused by a combination of direct and indirect pathways and the effects of Hg²⁺ directed to thiols or other reactive groups in transporter protein were apparently hidden. If this is the case, however, the redox-sensing property of the GLAST protein would be less significant than that of the major target of Hg²⁺, at least in COS-7 cells.

Moreover, the present study also provided evidence that cysteine residues within the structure of GLAST are not critical

for glutamate-transport activity and trafficking of the newly synthesized transporter to the plasma membrane (Figures 2 and 3). In conclusion, the present study demonstrated that three cysteine residues in the GLAST protein are not critical for expression of the protein with transport activity and are not essential in the hypothetical redox regulatory mechanism of the transporter.

We thank Dr I. Koshino and Dr M. Matsumoto (University of Tokyo) for technical assistance. This study was supported by Grants in Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (grants 12460137 and 13556044 to M.I.).

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