Amino Acids (2014) 46:1697–1705
DOI 10.1007/s00726-014-1731-1

ORIGINAL ARTICLE

Cysteine mutagenesis reveals alternate proximity between transmembrane domain 2 and hairpin loop 1 of the glutamate transporter EAAT1

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Received: 2 November 2013 / Accepted: 18 March 2014 / Published online: 2 April 2014

Abstract Excitatory amino acid transporter 1 (EAAT1) plays an important role in restricting the neurotoxicity of glutamate. Previous structure–function studies have provided evidence that reentrant helical hairpin loop (HP) 1 has predominant function during the transport cycle. The proposed internal gate HP1 is packed against transmembrane domain (TM) 2 and TM5 in its closed state, and two residues located in TM2 and HP2 of EAAT1 are in close proximity. However, the spatial relationship between TM2 and HP1 during the transport cycle remains unknown. In this study, we used chemical cross-linking of introduced cysteine pair (V96C and S366C) in a cysteine-less version of EAAT1 to assess the proximity of TM2 and HP1. Here, we show that inhibition of transport by copper(II)(1,10-phenanthroline)$_3$ (CuPh) and cadmium ion (Cd^{2+}) were observed in the V96C/S366C mutant. Glutamate or potassium significantly protected against the inhibition of transport activity of V96C/S366C by CuPh, while TBOA potentiated the inhibition of transport activity of V96C/S366C by CuPh. We also checked the kinetic parameters of V96C/S366C treated with or without CuPh in the presence of NaCl, NaCl + L-glutamate, NaCl + TBOA, and KCl, respectively. The sensitivity of V96C and S366C to membrane-impermeable sulfhydryl reagent MTSET [(2-trimethylammonium) methanethiosulfonate] was attenuated by glutamate or potassium. TBOA had no effect on the sensitivity of V96C and S366C to MTSET. These data suggest that the spatial relationship between Val-96 of TM2 and Ser-366 of HP1 is altered in the transport cycle.

Keywords Alternate proximity · TM2 · HP1 · EAAT1

Abbreviations

EAAT1	Excitatory amino acid transporter 1
HP	Hairpin loop
TM	Transmembrane domain
CuPh	Copper(II)(1,10-phenanthroline)$_3$
TBOA	D,L-*Threo*-β-benzyloxyaspartate
MTSET	(2-Trimethylammonium)-methanethiosulfonate

Introduction

Excitatory amino acid transporters (EAATs) play important roles in terminating excitatory neurotransmission by taking up extracellular glutamate. Glutamate transporters are the members of solute carrier family 1 (SLC1), which also includes two Na^+-dependent neutral amino acid transporters (Arriza et al. 1993; Utsunomiya-Tate et al. 1996). At present, five plasma membrane glutamate transporter subtypes (EAAT1–EAAT5) have been identified in humans (Arriza et al. 1994, 1997; Fairman et al. 1995; Kanai and Hediger 1992; Pines et al. 1992; Storck et al. 1992).

The crystal structure of a prokaryotic Glt_{Ph} from *Pyrococcus horikoshii*, homologue to the eukaryotic glutamate transporters, reveals that Glt_{Ph} consists of three uniform subunits, with each protomer containing eight transmembrane domain (TM) 1–8 and two highly conserved

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reentrant helical hairpin loops (HP1 and HP2) (Yernool et al. 2004). It has been indicated that HP2 forms the external gate of the transporter, while HP1 has been assumed to form the internal gate of the transporter (Brocke et al. 2002; Yernool et al. 2004; Boudker et al. 2007; Reyes et al. 2009; Crisman et al. 2009). Glutamate transports co-transport three sodium ions and one proton, and counter-transport one potassium ion for each molecule of glutamate (Brew and Attwell 1987; Kanner and Bendahan 1982; Kavanaugh et al. 1997; Levy et al. 1998; Pines and Kanner 1990; Wadiche et al. 1995; Zerangue and Kavanaugh 1996).

Previous evidence has supported that HP1 and TM2 have different roles during the transport cycle. As the proposed internal gate of transporter, the inward movement of HP1 relative to TM7 and TM8 results in the opening of an aqueous pathway from the substrate-binding site to the cytoplasm (Yernool et al. 2004), and the residues in HP1 are relevant to the translocation pore during the transport process (Grunewald and Kanner 2000; Slotboom et al. 1999). HP1 and HP2 together with TM7 and TM8 form the binding pocket of Glt_{Ph} (Yernool et al. 2004). In addition, glutamate transporters and their homologue Glt_{Ph} also mediate uncoupled anion conductance (Ryan and Vandenberg 2002; Ryan and Mindell 2007), and TM2 plays a key role in forming the chloride channel (Ryan et al. 2004). A membrane-associated domain involving in transport cycle was reported in close proximity to the extracellular area of TM2 (Ryan et al. 2004), and TM2 is highly conserved between transporter subtypes. These studies provide information that besides mediating chloride conductance and forming the unchanged trimeric interface, TM2 has an "insightful" role in the transport process through proximity to other domain of glutamate transporter (Groeneveld and Slotboom 2007; Ryan et al. 2004). Residues Q93 of TM2 and V452 of HP2 in EAAT1 form a spontaneous disulfide bond and are close in space (Ryan et al. 2004). The equivalent residues of Q93 and V452 in EAAT3 (K64 of TM2 and V420 of HP2) are also in close proximity in the inward-facing conformation of EAAT3 (Crisman et al. 2009).

However, the spatial relationship between residues of TM2 and HP1 in EAAT1 during the transport cycle remains unknown. The aim of this study was to assess the proximity of residues in TM2 and HP1 in the cysteine-less version of EAAT1. In this study, we engineered pair of cysteine residues (V96C/S366C) into TM2 and HP1 (Fig. 1), and we used two types of functional assays to infer the proximity of the engineered cysteine pair. The

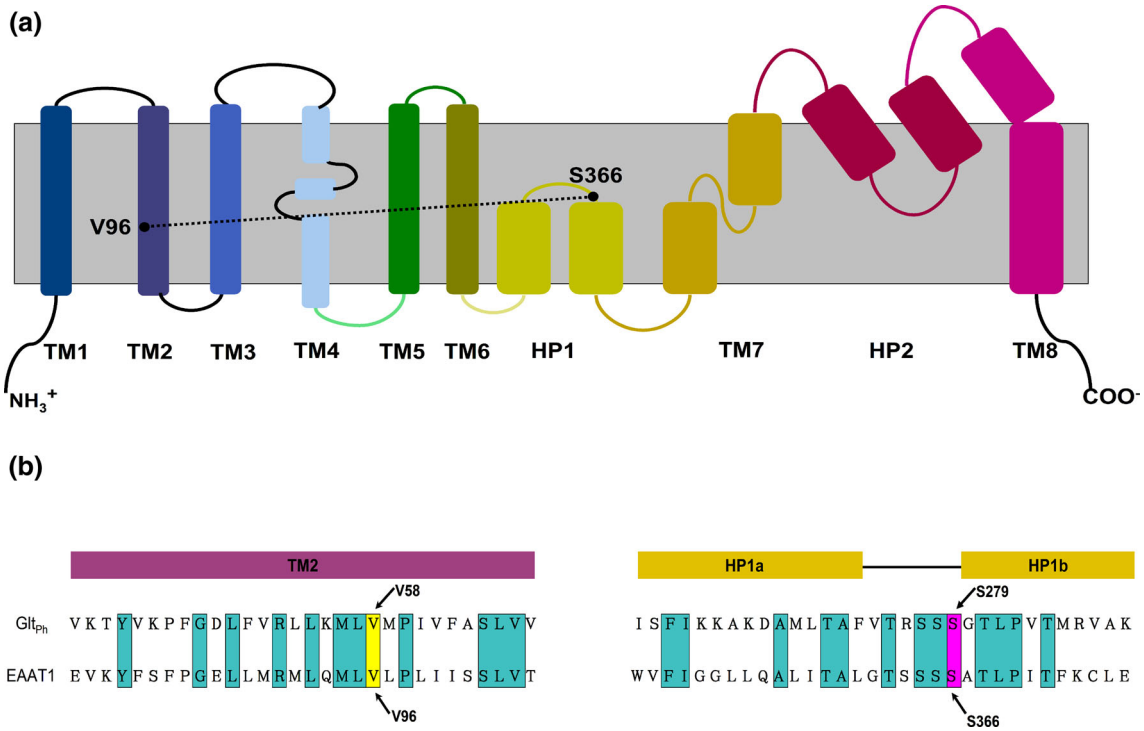

Fig. 1 Membrane topology model of EAAT1 (**a**), and sequence alignment of TM2 and HP1 of Glt_{Ph} and EAAT1 (**b**). The membrane topology of EAAT1 shown is based on the structure of Glt_{Ph}. The *black dots* show the approximate locations of the following cysteine substitutions: V96C and S366C (**a**). TM helices 1–8 and two reentrant hairpin loops (HP1 and HP2) are labeled (**a**). Regions of high homology of Glt_{Ph} and EAAT1 are highlighted in *aqua green* (**b**). V58 of Glt_{Ph} and V96 of EAAT1 are highlighted in *yellow* (**b**). S279 of Glt_{Ph} and S366 of EAAT1 are highlighted in *magenta* (**b**). The *black arrows* show the locations of V58/V96 and S279/S366 of TM2 and HP1 (**b**)

double mutant was subjected to conditions of oxidative cross-linking in the presence and absence of transporter ligands. We measured the reactivity of mutants V96C and S366C to the membrane-impermeable sulfhydryl reagent [(2-trimethylammonium) methanethiosulfonate] (MTSET) in the presence of substrates, inhibitors, sodium, and potassium, respectively. We provide evidence that the spatial relationship between TM2 and HP1 is altered during the transport cycle.

Materials and methods

Generation and subcloning of mutants

Site-directed mutagenesis was performed by using single-strand uracil-containing DNA derived from the cysteine-less EAAT1 (CL-EAAT1) in the vector pBluescript SK (-) (Stratagene) as described previously (Kunkel et al. 1987; Kleinberger-Doron and Kanner 1994). EAAT1/GLAST (glutamate/aspartate transporter) cDNA used in this work was from *Rattus norvegicus*. Following yielding the sense strand, another primer was designed to be antisense of the uracil-containing single-stranded DNA. Restriction enzymes *Eco*RI and *Bsr*GI or *Bsr*GI and *Sac*I were used to subclone the mutations into the CL-EAAT1 construct. Subcloned DNAs were sequenced in both directions between these unique sites.

Cell growth and expression

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 200 U/ml penicillin, 200 µg/ml streptomycin, and 2 mM glutamine. HeLa cells plated on 24-well plates were infected with the recombinant vaccinia/T7 virus vTF as described previously (Fuerst et al. 1986), 30 min later, then transfected with cDNA encoding CL-EAAT1 or mutant transporter, using the transfection reagent DOTAP as described previously (Keynan et al. 1992). Cells were incubated at 37 °C until transport assay.

Transport

HeLa cells were grown in 24-well plates for glutamate uptake assays. After 16–20 h transfection, the culture medium was aspirated and washed twice with the choline solution (150 mM choline chloride, 5 mM KP_i, pH 7.4, 0.5 mM $MgSO_4$, and 0.3 mM $CaCl_2$). Each well was then incubated with 200 µl sodium solution (150 mM NaCl, 5 mM KP_i, pH 7.4, 0.5 mM $MgSO_4$, and 0.3 mM $CaCl_2$) supplemented with 0.4 µCi (0.15 µM) of D-[^{3}H]-aspartate, and incubated for 10 min at room temperature, and the reaction was stopped by adding the ice-cold sodium solution twice. To dissolve the cells, 1 % SDS was added to the culture holes and the radioactivity was assessed by liquid scintillation counting.

Inhibition studies with sulfhydryl reagent and oxidizing agent

For inhibition studies, HeLa cells were washed once with choline solution. Each well was then incubated for 5 min at room temperature with 200 µl of the preincubation medium supplemented with MTSET or copper(II)(1,10-phenanthroline)$_3$ (CuPh) (the different compositions of preincubation medium and the concentrations of MTSET and CuPh are indicated in the figure legends). Cells were washed twice with choline solution. Then the transporter-mediated uptake was assayed as described above. Our preliminary experiments revealed the optimized concentrations of MTSET and CuPh according to the mutants in different experiments. Each experiment was performed at least three times.

Kinetics

HeLa cells were transfected with the CL-EAAT1 and V96C/S366C, and then they were preincubated for 5 min with or without of 250 µM CuPh in the presence of NaCl, NaCl + L-glutamate, NaCl + TBOA, and KCl, respectively. Subsequently, the cells were washed. Transport was measured with 100 nM D-[^{3}H]-aspartate in final unlabeled D-aspartate concentrations of 1, 5, 10, 50, 100, 250, 500, and 1,000 µM for 10 min at room temperature. K_m and V_{max} were calculated by nonlinear fitting to the generalized Hill equation using built-in functions of Origin 6.1 (Microcal). K_m is expressed in µM, and V_{max} is expressed as a percent of that of CL-EAAT1 untreated with CuPh in the presence of NaCl.

Restoration of activity by dithiothreitol (DTT)

To confirm the formation of disulfide bond in application of CuPh, we observed the restoration transport activity by using DTT. CuPh-catalyzed cross-linking was performed as described above, then the medium was aspirated, and the cells were washed twice with 1 ml of the choline solution. Cells were incubated with 20 mM DTT for 5 min. Subsequently, they were assayed for D-[^{3}H]-aspartate transport as described above.

Inhibition of transport by cadmium chloride (Cd^{2+})

HeLa cells were washed once with choline solution. Each well was incubated with the indicated concentration of Cd^{2+} in transport solution (150 mM NaCl, 5 mM KP_i, pH

7.4, 0.5 mM $MgSO_4$, and 0.3 mM $CaCl_2$) with the tritium-labeled substrate for 10 min at room temperature, and then, they were assayed for D-[^{3}H]-aspartate transport as described above.

Results

Inhibition of activity in V96C/S366C by thiol cross-linking and cadmium coordination

In order to eliminate the influence between induced and endogenous cysteines, we substituted the endogenous cysteines of EAAT1 with other amino acids. The background of the cysteine-less version of EAAT1 (CL-EAAT1), which contains the C186S, C252A and C375G substitutions, has ~70 % of the activity of wild type-EAAT1. Consequently, we used CL-EAAT1 to assess the proximity of residues in TM2 and HP1. The double mutant V96C/S366C exhibited 38.7 ± 4.2 % of the transport activity of CL-EAAT1 ($n = 3$), and the single cysteine mutants V96C and S366C showed 91.6 ± 3.4 and 45.2 ± 4.8 %, respectively. To determine whether the cysteine pair, V96C/S366C, was capable of forming a disulfide bond, we expressed the mutant in HeLa cells and then measured the accumulation of radiolabeled D-aspartate in the presence of the cross-linking reagent CuPh. It exhibited a dramatic decrease in the transport activity following exposure to CuPh (Fig. 2a, b). We observed that increasing concentrations of the cross-linking agent (200–800 μM) led to a greater reduction in aspartate transport. In 200 μM CuPh, the activity of V96C/S366C transporter was 58.4 ± 4.3 % (Fig. 2a), and in 800 μM, the activity was 27.8 ± 2.6 % of that of control (Fig. 2a). The transport activity of V96C/S366C exhibited ~27 % of that of control ($n = 3$) after preincubation with 800 μM CuPh (Fig. 2b), while the single cysteine mutants V96C and S366C showed ~91 and ~93 %, respectively (Fig. 2b). This inhibition was not seen in cells expressing either CL-EAAT1 or the single cysteine mutants V96C and S366C (Fig. 2b), indicating that the inhibition of transport by oxidative cross-linking required a cysteine at both positions. The lack of inhibition by CuPh was also observed in cells cotransfected with V96C and S366C (Fig. 2b), which demonstrated that the inhibition by CuPh was only observed when the cysteines at positions V96C and S366C were present on the same polypeptide, but not when the two cysteines resided on different polypeptides. This suggests that the cysteines at positions V96C and S366C come into close proximity within single subunits. Following preincubation with 800 μM CuPh, the double mutants were incubated with the reducing agent DTT to reduce any disulfide bonds that may have formed between

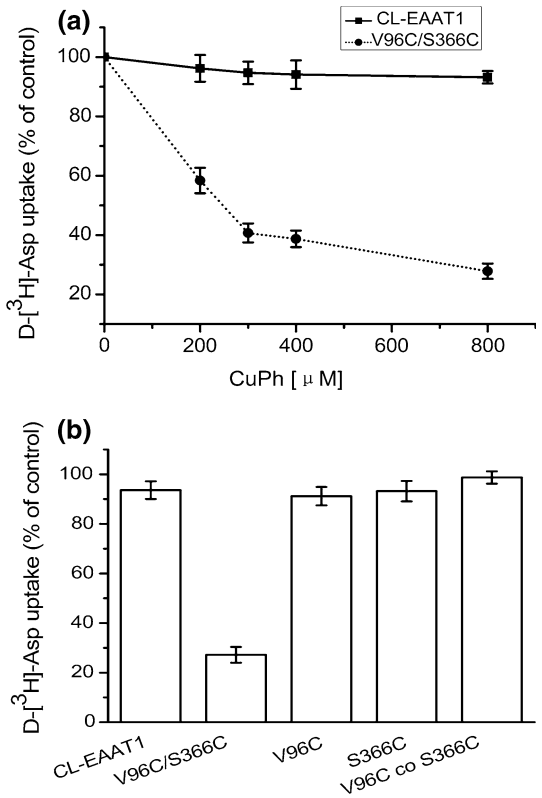

Fig. 2 Effect of CuPh on the activity of cysteine mutants. HeLa cells were incubated with CuPh for 5 min, respectively. Cells were washed twice with choline solution. Then they were assayed for D-[^{3}H]-aspartate transport. V96C/S366C mutants and CL-EAAT1 were pretreated with 200, 300, 400, 800 μM CuPh, respectively (**a**). In the case of V96C/S366C double cysteine mutant, single cysteine mutants V96C and S366C, and cells cotransfected with V96C and S366C, they were preincubated with 800 μM CuPh, respectively. Co-expression of two single cysteine mutants in HeLa cells is marked by "co" (**b**). Data are expressed as a percent of the uptake measured in the absence of CuPh treatment and are the mean ± SE of three experiments done in triplicate

the introduced cysteines. As expected, the transport activity of V96C/S366C inhibited by CuPh could be restored by a subsequent incubation with 20 mM DTT. Following treatment with 20 mM DTT, the V96C/S366C transporter activity was reversed to 85.4 ± 4.6 % ($n = 3$), where the CL-EAAT1 activity was 94.3 ± 3.7 %. Thus, the restoration of activity by application of DTT confirms the formation of a disulfide bond.

A complementary method used to determine proximity of V96C and S366C is the formation of a high affinity cadmium binding site in which a cadmium ion (Cd^{2+}) interacts with the cysteinyl side chains and the affinity of the interaction is dramatically increased when the Cd^{2+} could be coordinated by two cysteines. Subsequent application of 500 μM Cd^{2+} had very little effect on the aspartate uptake of single mutants V96C and S366C (Fig. 3), while an inhibition of ~74 % of activity was

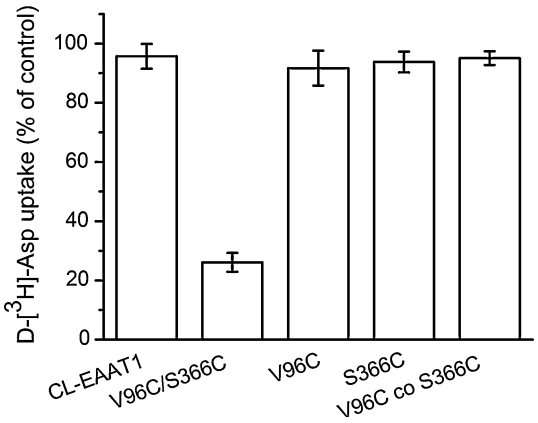

Fig. 3 Effect of Cd^{2+} on the activity of cysteine mutants. HeLa cells expressing V96C/S366C double cysteine mutant, single cysteine mutants V96C and S366C, and cells cotransfected with V96C and S366C, which were all in the background of CL-EAAT1. Cells were washed once with choline solution, then incubated with 500 μM cadmium chloride in transport solution with the tritium-labeled substrate for 10 min at room temperature, and were assayed for D-[^{3}H]-aspartate transport. Co-expression of two single cysteine mutants in HeLa cells is marked by "co". Data are expressed as a percent of the uptake measured in the absence of cadmium chloride treatment and are the mean ± SE of three experiments done in triplicate

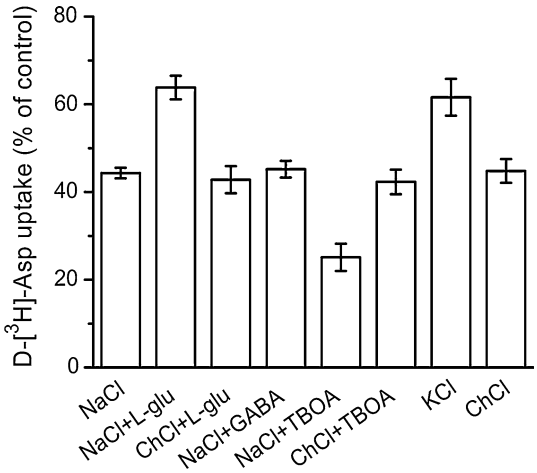

Fig. 4 Transport activity of V96C/S366C cysteine mutants in the effect of external medium by CuPh. HeLa cells expressing V96C/S366C mutant were preincubated for 5 min in the presence and absence of 250 μM CuPh. The indicated medium as follows, NaCl, NaCl + 1 mM L-glutamate, ChCl + 1 mM L-glutamate, NaCl + 1 mM GABA, NaCl + 20 μM TBOA, ChCl + 20 μM TBOA, KCl, choline chloride. Then they were assayed for D-[^{3}H]-aspartate transport. Data are expressed as a percent of the uptake measured in the absence of CuPh treatment and are the mean ± SE of three experiments done in triplicate

observed on the V96C/S366C mutant (Fig. 3). Consistent with the inhibition by CuPh (Fig. 2b), the inhibition by Cd^{2+} was also only observed when the cysteine pairs were introduced in the same polypeptide. This result further suggests that the cysteines introduced at positions Val-96 and Ser-366 come in close proximity within the transporter monomer. From these results, we predicted that Val-96 of TM2 was indeed in close proximity to Ser-366 of HP1.

Effect of glutamate and TBOA on cross-linking in double cysteine transporters

In the following experiments, we wanted to observe the effect of external medium on the activity of the transporter. There was little change in the transport activity when sodium was replaced by choline in cells expressing V96C/S366C (Fig. 4). When the external sodium was supplemented with glutamate or replaced by potassium, which increases the proportion of transporters in the inward-facing conformation (Reyes et al. 2009; Shlaifer and Kanner 2007), a significant reduction in the degree of inhibition by CuPh appeared (Fig. 4). This result indicates that the V96C and S366C residues are far apart in the inward-facing state. The protection by L-glutamate was not observed either in the absence of sodium (choline replacement) (Fig. 4) or substitution with GABA (Fig. 4), which is not the substrate of EAAT1. TBOA is assumed to bind at the same site on the transporter as glutamate but not to undergo translocation, and it is expected to increase the proportion of outward-facing transporters (Boudker et al. 2007). In our work, TBOA potentiated the inhibition of CuPh (Fig. 4). When sodium was replaced by choline, TBOA had no significant effect on this inhibition (Fig. 4).

To examine the effect of CuPh on the kinetic parameters of the transporter for the substrate, the K_m and V_{max} values were determined (Table 1). In the presence or absence of CuPh under the various conditions, there was no significant difference between the K_m values for D-aspartate of V96C/S366C and that of CL-EAAT1 (Table 1), but V96C/S366C had significantly lower V_{max} than that of CL-EAAT1 (Table 1). For V96C/S366C mutant, as compared to the treatment with CuPh in the presence of NaCl, the V_{max} values got higher when it was treated with CuPh in the presence of NaCl + L-glutamate or in the presence of KCl, while the V_{max} value got lower when it was treated with CuPh in the presence of NaCl + TBOA (Table 1).

Aqueous accessibility of the cysteines introduced at positions 96 and 366

Changes in accessibility of the engineered cysteine residues, rather than in their distance, could result in the modulation of CuPh inhibition effect. The introduced cysteines (V96C or S366C) could react with MTSET via their sulfhydryl groups in an aqueous environment, thus locks the conformation of transporter, and finally decreases the activity of transporter. In this work, application of MTSET inhibited the transport activity of V96C and

Table 1 Kinetic parameters of CL-EAAT1 and V96C/S366C treated with or without CuPh

	V_{max}^{a}	K_{m}^{a}	CuPh	
			V_{max}^{a}	K_{m}^{a}
CL-EAAT1				
NaCl	100	54.1 ± 5.9	96.2 ± 7.6	56.3 ± 7.2
V96C/S366C				
NaCl	35.1 ± 6.8	58.7 ± 8.5	16.5 ± 4.3	60.9 ± 9.1
NaCl + L-glu	41.9 ± 5.0	55.9 ± 7.2	25.6 ± 5.1	54.1 ± 4.7
NaCl + TBOA	40.4 ± 7.1	60.3 ± 4.5	10.7 ± 3.4	58.6 ± 6.4
KCl	44.7 ± 3.2	56.6 ± 6.4	27.2 ± 3.8	59.7 ± 8.3

[a] K_m and V_{max} values were calculated for CL-EAAT1 and V96C/S366C treated with or without CuPh under the various conditions. K_m is expressed in μM, and V_{max} is expressed as a percent of that of CL-EAAT1 untreated with CuPh in the presence of NaCl. Values represent the mean ± SE of three independent experiments done in triplicate

S366C mutants (Fig. 5a, b). Preincubation of V96C with 1.0 mM MTSET resulted in 20 % reduction in transporter uptake (Fig. 5a), while preincubation of S366C with 0.1 mM MTSET resulted in nearly 60 % reduction in transporter uptake (Fig. 5b). In the mutants V96C and S366C, glutamate or potassium protected against the inhibition by MTSET (Fig. 5a, b). However, TBOA showed no effect on the V96C and S366C mutants in application of MTSET (Fig. 5a, b).

Discussion

Glutamate transporters play important roles in terminating excitatory neurotransmission. Recently, researchers have indicated that, in addition to alleviating glutamate excitotoxicity, glutamate transporters also protect the central nervous system through antioxidant defenses (Had-Aissouni 2012), and they also participate in regulating learning, memory, and behavior (Pita-Almenar et al. 2012). So malfunction of glutamate transporters may contribute to the neurodegenerative diseases from different pathway, and the study of glutamate transporters has extremely significance in the medical field. Upregulation of glutamate transporters has potent therapeutic effects in treating neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Amyotrophic lateral sclerosis (Leung et al. 2012; Morel et al. 2013).

In this work, we provide insight into the spatial relationship between V96 of TM2 and S366 of HP1 in EAAT1 during different transport phases. Here, we show that CuPh and Cd^{2+} could inhibit the transport activity of the V96C/S366C cysteine mutants (Figs. 2, 3). We conclude that the residues Val-96 and Ser-366 become so close and that in the presence of CuPh, a disulfide bond is formed between V96C/S366C. Once the disulfide bond is formed, the

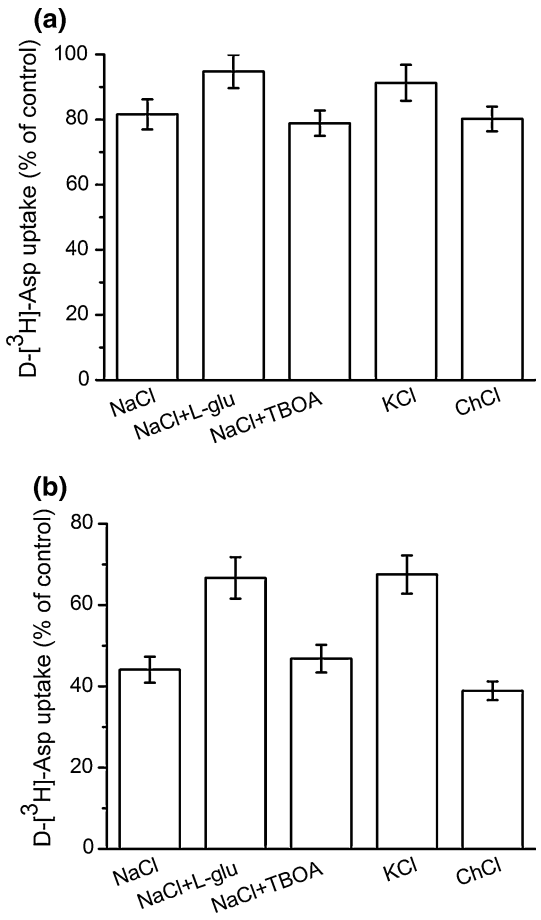

Fig. 5 Transport activity of single cysteine V96C and S366C mutants in the effect of the external medium by MTSET. HeLa cells expressing the single cysteine mutants V96C (**a**) or S366C (**b**) were preincubated for 5 min in the presence or absence of 1.0 (**a**) or 0.1 (**b**) mM MTSET. The indicated medium as follows, NaCl, NaCl + 1 mM L-glutamate, NaCl + 20 μM TBOA, KCl, choline chloride. Then they were assayed for D-[^{3}H]-aspartate transport. Data are expressed as a percent of the uptake measured in the absence of MTSET treatment and are the mean ± SE of three experiments done in triplicate

structure of the transporter is locked in that state. Moreover, we found only 85.4 % of transport activity of V96C/S366C variant was restored after reduction using DTT, due to that CuPh could also result in copper-thiol bridges in addition to disulfide bonds. However, the reduction in transporter activity observed on cross-linking may be resulted from directly inhibiting glutamate and sodium binding or by obstructing the conformational changes during the transport cycle. In the following experiments, we observed the aqueous accessibility of the cysteines introduced at position 96 or 366 via impermeant sulfhydryl reagent MTSET.

Previous study reported that application of 10 mM membrane-impermeable sulfhydryl reagent MTSES (2-sulfonatoethyl-methanethiosulfonate) for 5 min significantly changed the L-glutamate-activated conductance of V96C mutant and verified that residue Val-96 was accessible to the aqueous environment in EAAT1 (Ryan et al. 2004). In our experiment, we used 1 mM MTSET for 5 min and resulted in 20 % reduction in uptake (Fig. 5a). We speculate the difference possibly results from that TM2 forms part of the chloride permeation pathway, and thus, this region appears to be selective for anions over cations (Ryan et al. 2004). Amara's group found S366C at the C-terminal of domain 7 (later this domain was suggested forming HP1) in EAAT1 was highly reactive to MTSET (Seal et al. 2000). In addition, several studies also indicated that consecutive and conserved serine residues in HP1 were in an aqueous environment, and these residues were accessible from both sides of the membrane (Slotboom et al. 1999; Seal et al. 2000). Preincubation of A364C in GLT-1 with 1 mM MTSET for 5 min inhibited more than 80 % of D-[^{3}H]-aspartate transport (residue 364 of the glutamate transporter GLT-1 is equivalent to residue 366 of EAAT1) (Grunewald and Kanner 2000). Furthermore, Ser-366 locates on the top of HP1, with its apex facing the extracellular. Our results also indicated that preincubation of S366C with 0.1 mM MTSET resulted in inhibition of nearly 60 % uptake (Fig. 5b), which is consistent with the previous results.

For the V96C/S366C cysteine mutants, when the external sodium was supplemented with glutamate or replaced by potassium, it showed protective effect on the inhibition by CuPh (Fig. 4). In addition, glutamate or potassium had a protective effect on the inhibition of transport of single cysteine mutants V96C and S366C by MTSET (Fig. 5a, b). From these results, we can conclude that glutamate or potassium can cause a relative movement between TM2 and HP1. Because the trimeric interface involving TMs 2, 4, and 5 is known to be unchanged during transport (Groeneveld and Slotboom 2007), we assume that this conformational change would most likely involve HP1. Furthermore, in the inward-facing conformation of the glutamate transporters, after binding to the substrate, the protein core consisting of HP1, TM7, HP2, and TM8 moves inward relative to the rest of the transporter to form a cytoplasm facing conformation (Crisman et al. 2009). So in the inward-facing conformation, HP1 is far apart from TM2 in the mammalian transporter.

TBOA increased the inhibition by CuPh (Fig. 4), whereas it had no effect on the inhibition of transport of V96C and S366C by MTSET (Fig. 5a, b). During the transport cycle, the closure of HP1 induces the empty

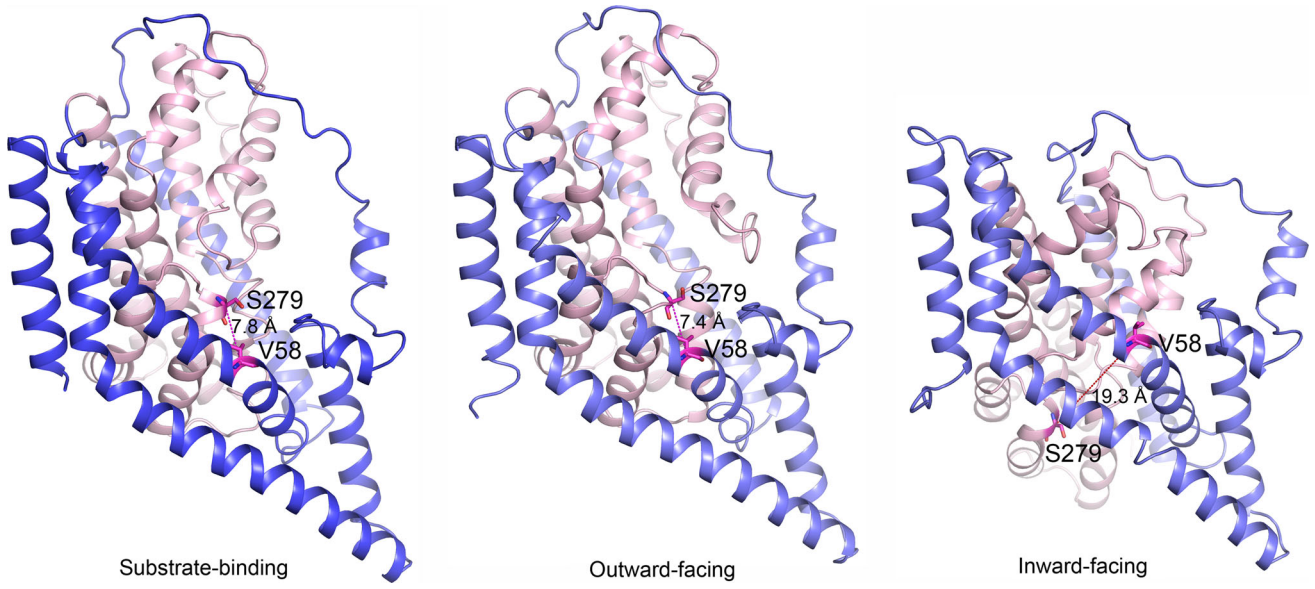

Fig. 6 The distances between V58 and S279 of Glt_{Ph} in different transport phase. The distances between V58 and S279 of Glt_{Ph} in the substrate-binding, outward-facing and inward-facing structures are shown here. These residues correspond to V96 and S366 of EAAT1, respectively. These structures are viewed from the side with the *dash lines* indicating the distances between the Cα atoms of V58 and S279

occluded state, followed by the opening of HP2. Moreover, TBOA binding blocks transport by keeping HP2 in an open conformation (Boudker et al. 2007). Thus, we speculate that in the out-facing conformation of transporter, which is induced by TBOA, the opening of HP2 causes HP1 move downwards to TM2. In addition, during the transport cycle, Val-96 and Ser-366 lie in the deep of transporter, and thus, TBOA has no obvious effect on the inhibition by MTSET.

After exposure to CuPh, the K_m values of V96C/S366C had no change but the V_{max} values reduced (Table 1). The V_{max} values of V96C/S366C increased when it was treated with CuPh in the presence of NaCl + L-glutamate or KCl, while the V_{max} value decreased when it was treated with CuPh in the presence of NaCl + TBOA, comparing with the treatment of CuPh in the presence of NaCl (Table 1). These data suggest that the reaction of V96C/S366C and CuPh modifies transporters partly. The extent of modification by CuPh got lower when replaced NaCl with NaCl + L-glutamate or KCl, while it got higher when replaced with NaCl + TBOA.

As the glutamate transporter homologue, Glt_{Ph} shares about 37 % amino acid sequence identity with the EAATs, and it seems to be a good model to study the structure and function of EAATs. V96 and S366 correspond to V58 and S279 of Glt_{Ph}, respectively (Fig. 1b). We measure the distances of V58 and S279 of Glt_{Ph} in different transport states of Glt_{Ph} and compare with the data in EAAT1 from our work (Fig. 6). Where at this pair positions, the distance is 7.8 Å apart in the substrate-bound state of Glt_{Ph} (PDB ID 1XFH) (Yernool et al. 2004) (Fig. 6), suggesting that V58 (V96 of EAAT1) and S279 (S366 of EAAT1) are in close proximity in Glt_{Ph}. A marked reduction in the inhibition by CuPh was observed when the external sodium was supplemented with glutamate or replaced by potassium in our results (Fig. 4), suggesting V96 and S366 are far apart in the inward-facing state, which is consistent with situation in the inward-facing state of Glt_{Ph} (PDB ID 3KBC), as the distance is 19.3 Å apart (Reyes et al. 2009) (Fig. 6). In our work, cross-linking between V96 and S366 potentiated in the presence of TBOA possibly results from the transporter is locked in an open-to-out state where TM2 and HP1 are closer to each other, while the distance between V58 and S279 is 7.4 Å in the outward-facing state of Glt_{Ph} (PDB ID 2NWW) (Boudker et al. 2007) (Fig. 6).

Hence, our results indicate that relevant movement occurs between V96 of TM2 and S366 of HP1 during different transport phases in the glutamate transporter EAAT1, and yet to better understand the proximity of HP1 and TM2 during the transport cycle, further structural and functional studies are essential.

Acknowledgments We thank Peng Zhang for making Fig. 6. This work was supported by the National Natural Science Foundation of China Grant 31170734, the International Centre for Genetic Engineering and Biotechnology Grant CRP/CHN11-01, the Specialized Research Fund for the Doctoral Program of Higher Education Grant 20114433120002, the Science and Technology Planning Project of Guangdong Province 2012B050200003, the Science and Technology Planning Project of Guangzhou 2013J4500018, and the Specialized Research Fund for talent introduction of colleges and universities of Guangdong Province Grant 2011430-1.

Conflict of interest The authors declare that they have no competing financial interests.

References

Arriza JL, Kavanaugh MP, Fairman WA, Wu YN, Murdoch GH, North RA, Amara SG (1993) Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. J Biol Chem 268(21):15329–15332

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from motor cortex. J Neurosci 14(9):5559–5569

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci USA 94(8):4155–4160

Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E (2007) Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. Nature 445(7126):387–393

Brew H, Attwell D (1987) Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature 327(6124):707–709

Brocke L, Bendahan A, Grunewald M, Kanner BI (2002) Proximity of two oppositely oriented re-entrant loops in the glutamate transporter GLT-1 identified by paired cysteine mutagenesis. J Biol Chem 277(6):3985–3992

Crisman T, Qu S, Kanner BI, Forrest L (2009) Inward-facing conformation of glutamate transporters as revealed by their inverted-topology structural repeats. Proc Natl Acad Sci USA 106(49):20752–20757

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375(6532):599–603

Fuerst TR, Niles EG, Studier FW, Moss B (1986) Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc Natl Acad Sci USA 83(21):8122–8126

Groeneveld M, Slotboom DJ (2007) Rigidity of the subunit interfaces of the trimeric glutamate transporter GltT during translocation. J Mol Biol 372(3):565–570

Grunewald M, Kanner BI (2000) The accessibility of a novel reentrant loop of the glutamate transporter GLT-1 is restricted by its substrate. J Biol Chem 275(13):9684–9689

Had-Aissouni L (2012) Toward a new role for plasma membrane sodium-dependent glutamate transporters of astrocytes: maintenance of antioxidant defenses beyond extracellular glutamate clearance. Amino Acids 42(1):181–197

Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360(6403):467–471

Kanner BI, Bendahan A (1982) Binding order of substrates to the sodium and potassium ion coupled L-glutamic acid transporter from rat brain. Biochemistry 21(24):6327–6330

Kavanaugh MP, Bendahan A, Zerangue N, Zhang Y, Kanner BI (1997) Mutation of an amino acid residue influencing potassium coupling in the glutamate transporter GLT-1 induces obligate exchange. J Biol Chem 272(3):1703–1708

Keynan S, Suh YJ, Kanner BI, Rudnick G (1992) Expression of a cloned gamma-aminobutyric acid transporter in mammalian cells. Biochemistry 31(7):1974–1979

Kleinberger-Doron N, Kanner BI (1994) Identification of tryptophan residues critical for the function and targeting of the gamma-aminobutyric acid transporter (subtype A). J Biol Chem 269(4):3063–3067

Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 154:367–382

Leung TC, Lui CN, Chen LW, Yung WH, Chan YS, Yung KK (2012) Ceftriaxone ameliorates motor deficits and protects dopaminergic neurons in 6-hydroxydopamine-lesioned rats. ACS Chem Neurosci 3(1):22–30

Levy LM, Warr O, Attwell D (1998) Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na^+-dependent glutamate uptake. J Neurosci 18(23):9620–9628

Morel L, Regan M, Higashimori H, Ng SK, Esau C, Vidensky S, Rothstein J, Yang Y (2013) Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT-1. J Biol Chem 288(10):7105–7116

Pines G, Kanner BI (1990) Counterflow of L-glutamate in plasma membrane vesicles and reconstituted preparations from rat brain. Biochemistry 29(51):11209–11214

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature 360(6403):464–467

Pita-Almenar JD, Zou SW, Colbert CM, Eskin A (2012) Relationship between increase in astrocytic GLT-1 glutamate transport and late-LTP. Learn Mem 19(12):615–626

Reyes N, Ginter C, Boudker O (2009) Transport mechanism of a bacterial homologue of glutamate transporters. Nature 462(7275):880–885

Ryan RM, Mindell JA (2007) The uncoupled chloride conductance of a bacterial glutamate transporter homolog. Nat Struct Mol Biol 14(5):365–371

Ryan RM, Vandenberg RJ (2002) Distinct conformational states mediate the transport and anion channel properties of the glutamate transporter EAAT-1. J Biol Chem 277(16):13494–13500

Ryan RM, Mitrovic AD, Vandenberg RJ (2004) The chloride permeation pathway of a glutamate transporter and its proximity to the glutamate translocation pathway. J Biol Chem 279(20):20742–20751

Seal RP, Leighton BH, Amara SG (2000) A model for the topology of excitatory amino acid transporters determined by the extracellular accessibility of substituted cysteines. Neuron 25(3):695–706

Shlaifer I, Kanner BI (2007) Conformationally sensitive reactivity to permeant sulfhydryl reagents of cysteine residues engineered into helical hairpin 1 of the glutamate transporter GLT-1. Mol Pharmacol 71(5):1341–1348

Slotboom DJ, Sobczak I, Konings WN, Lolkema JS (1999) A conserved serine rich stretch in the glutamate transporter family forms a substrate-sensitive reentrant loop. Proc Natl Acad Sci USA 96(25):14282–14287

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89(22):10955–10959

Utsunomiya-Tate N, Endou H, Kanai Y (1996) Cloning and functional characterization of a system ASC-like Na^+-dependent neutral amino acid transporter. J Biol Chem 271(25):14883–14890

Wadiche JI, Amara SG, Kavanaugh MP (1995) Ion fluxes associated with excitatory amino acid transport. Neuron 15(3):721–728

Yernool D, Boudker O, Jin Y, Gouaux E (2004) Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. Nature 431(7010):811–818

Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. Nature 383(6601):634–637