Existence of an Endogenous Glutamate and Aspartate Transporter in Chinese Hamster Ovary Cells

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Abstract

Chinese hamster ovary cells show endogenous high-affinity Na⁺-dependent glutamate transport activity. This transport activity is kinetically similar to a glutamate transporter family strategically expressed in the central nervous system and is pharmacologically unlike glutamate transporter-1 or excitatory amino acid carrier 1. The cDNA of a glutamate/aspartate transporter (GLAST)-like transporter was obtained and analyzed. The deduced amino acid sequence showed high similarity to human, mouse, and rat GLAST. We concluded that a GLAST-like glutamate transporter exists in Chinese hamster ovary cells that might confer the endogenous high-affinity Na⁺-dependent glutamate transport activity evident in these cells.

Keywords

GLAST; CHO cell; glutamate transporter

L-Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS) that contributes not only to fast synaptic neurotransmission, but also to complex physiological processes like memory, learning, neuronal plasticity, and neuronal cell death. To terminate the action of glutamate and maintain its extracellular concentration below excitotoxic levels, Na⁺-dependent high affinity glutamate transporters (termed excitatory amino acid transporters (EAATs)), located on the plasma membrane of neurons and glial cells, rapidly remove glutamate from the extracellular space. The family consists of five distinct transporters, glutamate/aspartate transporter (GLAST), glutamate transporter (GLT)-1, excitatory amino acid carrier (EAAC) 1, EAAT4, and EAAT5, cloned from mammals [1–7]. GLAST is widely expressed in the brain [8] and also shows a broad distribution in peripheral organs other than the CNS [9]. Several studies also showed that there are two alternative splicing variants of GLAST. GLAST-1a, lacking exon 3, is expressed in rat bone and brain [10]. EAAT1ex9skip, which lacks the entire exon 9, is expressed in human brain [11].

Chinese hamster ovary (CHO) cell line constitutes one of the most common cell lines selected for expression of external genes. To investigate the property of the Na⁺-dependent glutamate transporters, the expression of glutamate transporters in CHO cells has been carried out in some studies. Although the properties of the glutamate transporters have been made more explicit, these studies also implied that there existed an endogenous Na⁺-dependent transport system for glutamate/aspartate in CHO cells, which brought more difficulties to the research [12–15]. However, no published data are available about the nature of the endogenous Na⁺-dependent transport.

The present report shows the existence of a high-affinity Na⁺-dependent glutamate transporter in CHO cells. This transporter was pharmacologically similar to a glial GLAST. The cDNA sequence for the GLAST-like transporter was also obtained and analyzed.

Materials and Methods

Cell culture

The CHO cell line was obtained from the China Center for Type Culture Collection (Shanghai, China) then maintained in RPMI 1640 medium (Gibco, Carlsbad, USA)

DOI: 10.1111/j.1745-7270.2007.00354.x
containing 10% fetal bovine serum (Gibco).

**Transport studies**

Measurement of glutamate uptake was carried out on intact CHO cells plated in 48-well plates. Briefly, near-confluent cells (approximately 60,000 cells per well) were rinsed three times with HEPES-buffered saline (137 mM NaCl, 0.7 mM K_2HPO_4, 1 mM MgCl_2, 1 mM CaCl_2, 5.5 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4 with Tris) or with HEPES-buffered saline in which NaCl was replaced by choline chloride. The cells were then exposed to 100 µl HEPES-buffered saline containing 100 nM L-[3H]-glutamate (Amersham Pharmacia Biotech, Little Chalfont, UK) as necessary with cold substrate for 20 min at 37 ºC. The reaction was terminated by removing the transport medium and washing the cells three times with ice-cold sodium-free HEPES-buffered saline. Cells were then solubilized in 2 M KOH solution and aliquots were measured by a liquid scintillation analyzer (Tri-Carb 2900TR; Packard, Downers Grove, USA). To estimate the kinetic constants of glutamate uptake, different concentrations of L-glutamate were used, varying from 1 to 100 µM. To analyze the sensitivity of the transport activity to selected inhibitors dihydrokainate (DHK; Sigma-Aldrich, St. Louis, USA), L-cysteine (Shanghai Shiyi Chemicals Reagent Co., Shanghai, China), and DL-threo-β-hydroxyaspartic acid (THA; Sigma-Aldrich), the uptake for 1 µM glutamate in the presence of appropriate concentrations (1 µM to 1 mM) of various compounds was measured. All tests were replicated between three and five times.

**Reverse transcription-polymerase chain reaction (RT-PCR) and cDNA sequencing**

Total RNA from CHO cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer’s instructions. The extracts were then treated with DNase for 15 min at room temperature prior to first-strand cDNA synthesis with random 6 mers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). The first-strand cDNA was then subject to 30 cycles of PCR amplification (denature: 94 ºC, 1 min; anneal: 55 ºC, 1 min; extension: 72 ºC, 1 min) with 2 U Taq DNA polymerase (TaKaRa, Shiga, Japan) and primer pairs as follows: 5’ primer (P1), 5’-GCCGGTGATAATGTTGGAT-3’; 3’ primer (P2), 5’-CTCATCTTTGTTTCTGCT-3’; 5’ primer (P3), 5’-ATGCNAAAGGACACCGG-3’; and 3’ primer (P4), 5’-AAAGGTATGTTAGGTTG-3’. These primers were designed based on the conserved sequences of GLAST cDNA between human (GenBank accession No. NM_004172, from base pair 245 to 1873), rat (GenBank accession No. NM_019225, from base pair 204 to 1835), and mouse (GenBank accession No. NM_148938, from base pair 490 to 2121). As the *Cricetulus* GLAST sequence is not known, we used the following strategy in our study. First, we used P1 and P2 to obtain product 1 that corresponds to base pair 1336−2121 of NM_148938, or to base pair 1050−1835 of NM_019225 (or to base pair 1091−1873 of NM_004172). Then product 2 was obtained by using P3 and P4, which corresponds to base pair 490−1607 of NM_148938, or to base pair 204−1321 of NM_019225 (or to base pair 245−1362 of NM_004172). The above two PCR products were cloned into the pGEM-T vectors (Promega) and sequenced. The data were analyzed to obtain the whole CHO-GLAST sequence.

**Results**

**CHO cells showed a robust ability to uptake [3H]-glutamate**

As shown in Fig. 1, [3H]-glutamate uptake measured in CHO cells showed high affinity with a *K_m* of approximately 27.1±5.6 µM and a maximum translocation velocity (*V_m*)

![Fig. 1](http://abbs.oxfordjournals.org/)

**Representative kinetics of [3H]-glutamate [Glu] transport into Chinese hamster ovary cells**

(A) Cells were incubated with HEPES-buffered saline containing 10 µCi/ml [3H]-glutamate and different concentrations of L-glutamate for 20 min at 37 ºC. The uptake in the wells in which NaCl was substituted by choline chloride served as the negative control and was subtracted when presented in the plot. Data represent mean±SD (n=6). (B) Woolf-Hanes plot analysis was carried out using the data shown in (A).
of approximately 67.5±11.5 pmol/well/min. The $K_m$ value for glutamate uptake was more similar to the value for glutamate transporter subtypes isolated from the CNS [16, 17] than to the value for the neutral amino acids’ transporters [8,18].

**Uptake dependent on extracellular Na$^+$**

We further investigated whether the uptake was dependent on extracellular Na$^+$. As indicated in Fig. 2, when extracellular NaCl in the incubation medium was replaced by choline chloride, $[^3]$H-glutamate uptake by CHO cells was reduced to only approximately 6% of the control, in which cells were incubated in normal extracellular Na$^+$ concentration.

![Fig. 2](http://www.abbs.info; www.blackwellpublishing.com/abbs)

**Fig. 2** Na$^+$-dependence of glutamate/aspartate transporter-mediated L-glutamate transport into Chinese hamster ovary (CHO) cells

Transport of L-glutamate into CHO cells was measured in the presence or absence of Na$^+$. The incubation medium contained various concentrations of NaCl and choline chloride (pH 7.4) as indicated. Data represent the mean±SD percent of the normal Na$^+$ concentration control ($n$=6).

**Uptake inhibited by different glutamate uptake inhibitors**

Cells were incubated with several glutamate transporter inhibitors at a concentration range from 1 µM to 1 mM. Of the three different inhibitors tested, GLT-1 inhibitor DHK [16] and EAAC1 inhibitor L-cysteine [19] showed poorer potential (IC$_{50}$>1 mM) in inhibiting $[^3]$H-glutamate accumulation into CHO cells than the non-selective inhibitor THA (IC$_{50}$= 30.5±9.4 µM) (Fig. 3). Based on these findings, we surmised that there might be a GLAST-like transporter in CHO cells.

![Fig. 3](http://www.abbs.info; www.blackwellpublishing.com/abbs)

**Fig. 3** Inhibitory effects of different glutamate inhibitors against glutamate transport activity in Chinese hamster ovary (CHO) cells

The uptake of 1 µM glutamate was measured in CHO cells in the presence of three inhibitors, dihydrokainate (DHK); L-cysteine (L-cys), and DL-threo-β-hydroxyaspartic acid (THA). Values represent the mean±SD ($n$=6).

**Analysis of the sequence of the CHO-GLAST**

The cDNA obtained from CHO cells was a 1632 bp length open reading frame encoding a protein of 543 amino acid residues (GenBank accession No. EF155647). The deduced amino acid sequence showed high similarity to human (95%), mouse (98%), and rat (98%) GLASTs (Fig. 4), and significant but much less similarity to the other EAATs (50%–66%).

**Discussion**

To study the properties of the glutamate transporters, it would be more useful to have a mammalian cell line expressing these molecules. CHO cells, which constitute one of the most common cell lines selected for expression of external genes, displayed endogenous Na$^+$-dependent glutamate/aspartate uptake activity [15]. It is fortunate that there exist mutant CHO cell lines lacking Na$^+$-dependent glutamate transport [12]. Levy et al. successfully established an inducible GLT-1 expression system in the mutant CHO line Dd-B7 and investigated the stoichiometry of this transporter [13,14]. Nevertheless, the nature of the endogenous Na$^+$-dependent glutamate transport activity in CHO cells is unknown.

Our results showed that there exists a robust, Na$^+$-dependent, high-affinity glutamate transport activity in CHO cells with a $K_m$ value more similar to the value for glutamate transporter subtypes isolated from the CNS. This transport
Fig. 4 Alignment of deduced amino acid sequences of Chinese hamster ovary glutamate/aspartate transporter (CHO-GLAST) with other mammalian GLASTs and the two splicing variants

The deduced amino acid sequence of CHO-GLAST was aligned with those of human, mouse, and rat GLASTs, and the two splicing variants, GLAST1a (lacks exon 3, expressed in rat bone and brain) and excitatory amino acid transporter EAAT1ex9skip (lacks exon 9, expressed in human brain). Conserved amino acid residues are shaded.

The complete nucleotide sequence of CHO-GLAST cDNA has been submitted to the GenBank database with the accession number EF155647.
activity can be effectively inhibited by the non-selective glutamate transporter inhibitor THA, but not the selective GLT-1 inhibitor DHK or EAAC1 inhibitor L-cysteine, indicating that it is unlikely the transport activity involves a GLT-1-like or an EAAC1-like transporter. The total RNA of CHO cells was then obtained and PCR was carried out using GLAST-specific primer pairs. The acquired product was sequenced and the deduced amino acid sequence was analyzed. Results showed that the GLAST-like transporter obtained from CHO cells was highly similar to the human, mouse, and rat GLAST and that the CHO-GLAST was not a splicing one of the known GLASTs. It revealed that there are six amino acid substitutions (60, 105, 140, 200, 269, and 555) in this sequence that are conserved across human, rat, and mouse. The three N-terminal substitutions (60, 105, and 140) lie in the first three of the six hydrophobic regions, proposed to span the plasma membrane in an α-helical manner [20]. The substitution 200 lies in the putative extracellular loop between transmembrane domains III and IV, and the substitution 269 lies in the loop between transmembrane domains IV and V. The substitution 535 lies in the C-terminal tail. However, amino acid residues that are important for GLAST functions are still conserved in this sequence, including experimentally verified N-glycosylation sites of GLAST (N206 and N216) and the residues pivotal to the binding of glutamate (Y405 and R479) [20]. Thus, the transport function of this sequence might not be significantly different to those known GLASTs, and more detailed study concerning CHO-GLAST should be pursued.

We also carried out RT-PCR to detect whether or not GLT-1, EAAC1, EAAT4, and EAAT5 exist in CHO cells by using the same strategy as described in “Materials and Methods”. Unfortunately, no PCR product is available. However, this can not exclude the existence of other glutamate transporters. To clarify this problem, further work is needed that includes more extensive pharmacological approaches, knockdown or knockout CHO-GLAST, and immunological approaches.

GLAST/EAAT1 is strategically localized in retinal Muller cells and cerebellar Bergmann glia [7]. However, recent research showed that GLAST also localized at tissues other than synaptical regions, such as bone [21], cartilage [22], the reproductive system [23], adrenal and pituitary glands [24], and lactating mammary glands [25]. It is also indicated that GLAST showed a ubiquitous expression in a variety of peripheral tissues and organs, including ovary [9,26]. Therefore, it is reasonable that GLAST transporter exists in CHO cells, derived from Chinese hamster ovary.

Igo and Ash developed a mutant CHO cell line lacking Na+-dependent glutamate transport [12]. Some researchers concerned with glutamate transporters, and using the CHO expressing system, chose the mutant CHO line Dd-B7 to exclude the influence induced by endogenous glutamate transport activity [13,14]. Others chose to highly express the particular transporter to diminish the influence of the endogenous glutamate transport activity [15]. In this study, considering the high overexpression (20-fold) over the endogenous transport activity, the data concerning EAAT2 is reasonable. With regard to the data relating to EAAT1 (GLAST), for which the expression activity was only 4 folds over the endogenous transport activity, it should be taken into account that benzodiazepines might influence the two GLASTs differently. However, information regarding the kind of species from which the GLAST sequence in that study was sourced is not available, making it difficult to reevaluate the related results. Thus, the fact that there is an endogenous functional expression of GLAST in CHO cells should be carefully considered when interpreting functional data obtained from overexpression of transporters in this system.

In conclusion, this is the first attempt to uncover the molecular basis of the glutamate transport activity in CHO cells. The results suggested an existence of a GLAST transporter in CHO cells, and this transporter might contribute to the observed endogenous Na+-dependent, high-affinity glutamate transport in CHO cells.

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