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Biochimica et Biophysica Acta 1191 (1994) 393–396

BBA Biochimica et Biophysica Acta

Short Sequence-Paper

Molecular cloning of human brain glutamate/aspartate transporter II

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(Received 25 October 1993; revised manuscript received 24 January 1994)

Abstract

Glutamate transporters are membrane-bound proteins which are localized in glial cells and/or pre-synaptic glutamatergic nerve endings and are essential for the removal and termination of action of synaptic glutamate. Several cDNAs encoding glutamate transporters have been isolated from mammalian tissues, including human cerebellum. Here, we screened cDNA libraries derived from human brain stem and cerebellum, and isolated a novel cDNA that encodes for a glutamate transporter. This cDNA predicts a protein which contains 565 amino acids and is homologous to a rat brain Na^+-dependent glutamate/aspartate transporter. The new cDNA is expressed in brain and is structurally distinct from the previously reported human glutamate transporter cDNA.

Key words: Glutamate transporter; cDNA; Human brain stem; Cloning; Sequence

Glutamate is known to serve as an excitatory neurotransmitter in mammalian CNS and has been implicated in neurodegenerative processes. Glutamate transport proteins are essential for the removal of synaptic glutamate via a high-affinity uptake process [1,2]. This process is shown to be altered in ischemia and some neuronal degenerations linked to excitotoxicity [3,4].

Because glutamate uptake depends on the co-transport of Na^+ and counter-transport of K^+, these proteins are called Na^+/K^+-dependent transporters [2]. Recent molecular biological studies led to the isolation of three distinct glutamate transporter cDNAs derived from rat brain [5,6] and rabbit small intestine [7]. We have also isolated a cDNA derived from human cerebellum which encodes for a glutamate transporter [8] and which is homologous to the rat transporter described by Storck et al. [5]. Since the human glutamate transporters are expected to exhibit multiplicity at the molecular level, we searched for additional carriers by screening a cDNA library constructed from human brain stem mRNA. A novel human cDNA, structurally distinct from the previously reported human cerebellar transporter, was isolated and characterized. The new human cDNA is expressed in brain and is homologous to another rat brain glutamate/aspartate transporter described by Pines et al. [6]. The novel human cDNA may prove useful in understanding brain glutamatergic mechanisms and in the study of neurodegenerative disorders linked to glutamatergic dysfunction.

A λZap cDNA library derived from human brain stem (Stratagene, CA) was screened using a RT-PCR-generated DNA probe corresponding to the coding region of a rat brain glutamate/aspartate transporter [6]. To synthesize this probe, total RNA was isolated from rat brain by the CsCl method [9] and was reverse transcribed (RT) using a Gibco-BRL Superscript preamplification kit. Synthesized DNA was then amplified by the polymerase chain reaction (PCR) using *taq* DNA polymerase (Perkin Elmer) and oligonucleotide primers (5′TCGCCACTGTCTCCAGACGTG3′; 5′GAATTGGCTGAGAATCGGTC3′) corresponding to the rat glutamate transporter [6]. Amplification was carried out as follows: denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for 2 min, 30 cycles. Library screening was carried out using standard protocols [9]. Hybridizations were performed at 42°C in 50% formamide/6 × SSC and the final wash was done at 65°C in 0.1 × SSC. Sequencing

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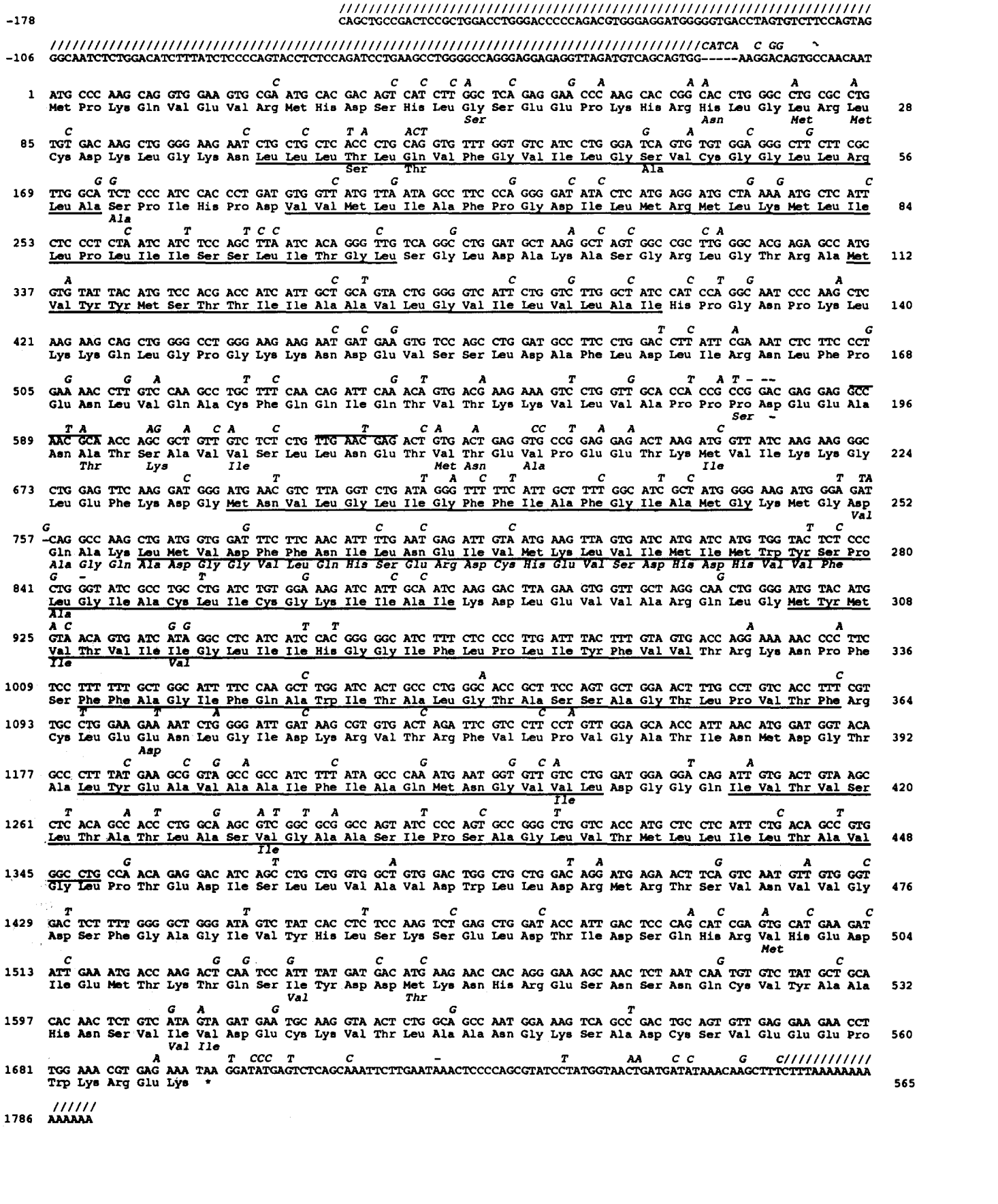
The sequence data reported in this paper have been submitted to the GenBank/EMBL/DDBG under the accession number U01824.

SSDI 0005-2736(94)00039-R



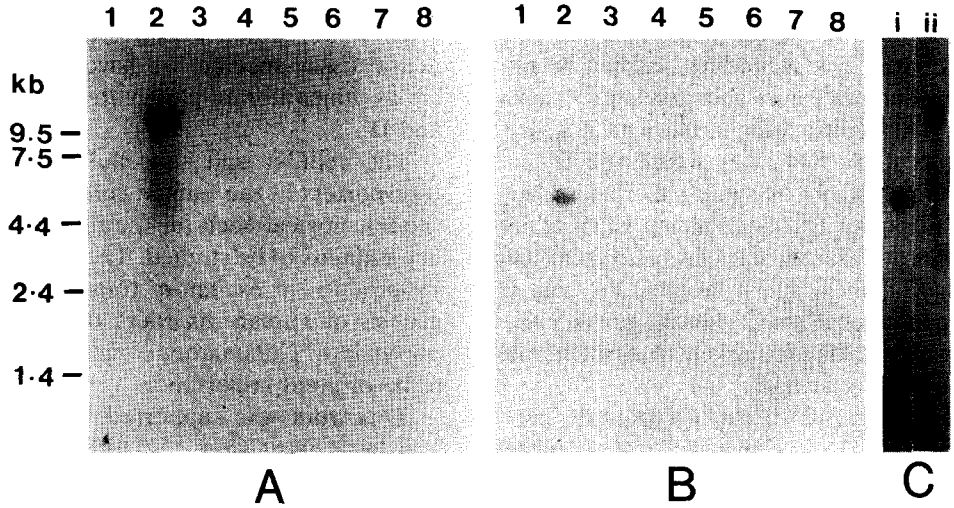


Fig. 2. Northern blot analysis of human tissue RNA. (A) Expression of human brain glutamate transporter II in human heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (5), skeletal muscle (6), kidney (lane 7), and pancreas (lane 8). Poly(A)$^+$ RNA (2 μg), isolated from the above tissues, was hybridized with the cloned cDNA as a probe and exposed to the film for 1 day. No detection of positive signal was found for tissues other than the brain even after the blot had been exposed to the film for up to 10 days. (B) Expression of human brain glutamate transporter I in human tissues. Poly A+ RNA from the tissues described in B was probed with the previously described human glutamate transporter I [8]. For this, the blot from A was regenerated and hybridized with the transporter I cDNA. This blot was exposed to the film for 7 days. (C) Expression of human brain glutamate transporters in retinoblastoma cell lines. Total RNA (20 μg) from human retinoblastoma cells was run in duplicates in an agarose gel, blotted and hybridized either with the cloned cDNA (human brain glutamate transporter II) (ii) or with the previously described human brain glutamate transporter I (i) [8]. These blots were exposed to the film for 5 days at −80°C. All hybridizations were done at 42°C (50% formamide, 5 × SSPE, 5 × Denhardt's solution, 2% SDS and 200 μg/ml denatured salmon sperm DNA) over night. The stringency of the final wash conditions was 65°C in 0.1 × SSC containing 0.1% SDS.

of the DNA was carried out on an Automated DNA Sequencer (Applied Biosystems, Model 373A) as well as manually by the dideoxy chain termination method [10] using the Sequenase kit (Version 2.0, US Biochemicals). For sequence confirmation, both strands of the DNA were sequenced. Analysis of the sequence data was done using Genetics Computer Group software.

The expression of the new human cDNA was studied by Northern blot analysis of various human tissues. Total RNA or poly(A)$^+$ RNA (Clontech) was isolated from different tissues and run on an agarose gel (1.2%) containing formaldehyde according to standard techniques [9]. Hybridizations were done at 42°C (50% formamide, 5 × SSPE, 5 × Denhardt's solution, 2% SDS and 200 μg/ml denatured salmon sperm DNA) overnight using the cloned cDNA (labeled with ^{32}P) as a probe. The stringency of the final wash conditions was 65°C in 0.1 × SSC containing 0.1% SDS. For comparison, total RNA and poly(A)$^+$ RNA blots, obtained as above, were also hybridized with the previously described human brain glutamate transporter cDNA [8] using identical conditions.

RT-PCR generated a 829 bp probe corresponding to the coding region of the rat glutamate transporter described by Pines et al. [6]. Screening of the human brain stem library led to the isolation of several positive clones. Three were sequenced and specified the same cDNA (Fig. 1) showing an 88% nucleotide sequence identity with the rat aspartate/glutamate transporter described by Pines et al. [6]. In addition, using the cloned cDNA as a probe, we screened another cDNA library derived human cerebellum [8]. This led to the isolation of several positive clones two of which were sequenced and were also found to specify the same cDNA. Due to a 5-nucleotide deletion at the 5'UTR, as compared to the rat brain cDNA [6], which puts the predicted N-terminal methionine of the rat transporter out of frame, the first in-frame methionine (Met-1) of the human transporter corresponds to Met-

Fig. 1. Nucleotide and deduced amino acid sequence of the human glutamate transporter cDNA. Nucleotides corresponding to the rat glutamate transporter [6] are shown in italics directly above the human nucleotide sequence only where they are different. Also, predicted amino acids corresponding to the rat cDNA [6] are shown in italics directly below the human amino acid sequence only where they are different. Insertions are marked by relevant bases and deletions by dashes. The nucleotide number is indicated on the left and the amino acid number at the right of the sequences. Positive nucleotide numbering begins at the putative translation initiation codon ATG. The stop codon is indicated with an asterisk. Underlined are putative transmembrane regions; the potential N-glycosylation sites are overlined. Non-homologous regions are indicated by vertical lines.

10 of the rat protein (Fig. 1). Also, compared to the rat cDNA, a single base deletion after position 756 of the human transporter (the corresponding position in rat cDNA is 779) introduces a frame shift for amino acids 253–281 (Fig. 1). This shift is eliminated with a single base addition at position 844 after which the frame reading of the human cDNA is similar to that of the rat glutamate transporter [6]. Sequencing of 5 additional cDNA clones of different sizes, isolated from the brain stem library (3 clones) and from a cDNA library derived from human cerebellum (2 clones), gave identical sequencing results making it unlikely that this frame shift was due to a cloning artifact.

The amino acid sequence of the polypeptide predicted by the human cDNA shows an 89% identity with that derived from the rat brain cDNA [6]. Omitting the stretch of 28 discordant amino acids which results from the above frame shift raises the amino acid sequence identity to greater than 95%. In contrast, the homology of the new human brain stem cDNA to the previously reported human glutamate transporter derived from cerebellum [8] was low (66%), thus indicating the diverse genetic origin of the two human transporters. This was also true for the glutamate transporters described by Storck et al. [5] and Kanai and Hediger [7].

The protein predicted by the new human cDNA shows nine putative transmembrane regions and two N-glycosylation consensus motifs (Fig. 1). The region of these motifs is conserved in both human glutamate transporters and is probably used for N-glycosylation.

Northern blot analysis of human tissues revealed that the cloned cDNA is expressed in brain and in retinoblastoma cell lines (Fig. 2). No message was detected in heart, liver, lung, kidney, pancreas, placenta or skeletal muscle (Fig. 2). Also, human ovary and testis gave a non-detectable message (data not shown). The size of the new glutamate transporter mRNA (11 kb) was considerably larger than that of the previously described human glutamate transporter [8] (Fig. 2). Since the two human glutamate transporters isolated thus far are expressed in brain and may be neural tissue-specific, we have designated these carriers as 'human brain glutamate/aspartate transporter I and II'.

The cellular and subcellular localization of these transporters in the human central nervous system, and the teleological need for the multiplicity of these carriers, remains to be studied. The role of these high-affinity proteins in excitatory transmission and the pathogenesis of human disorders that have been linked to presynaptic glutamatergic malfunction [3,4] may prove to be of great importance.

This work was supported by NIH grant NS-16871 and by a grant from the International Federation of Parkinson's Disease Societies. We thank Drs. D.D. Clarke and N. Robakis for their help in these studies and in reviewing the manuscript. We also thank Betsy Chalfin for her editorial assistance.

References

[1] Balcar, V.J., Borg, J. and Mandel, P. (1977) J. Neurochem. 28, 87–93.
[2] Amara, S.G. and Arriza, J.L. (1993) Curr. Opinion Neurobiol. 3, 337–344.
[3] Plaitakis, A. (1990) Ann. Neurol. 28, 3–8.
[4] Rothstein, J.D., Martin, L.J. and Kuncl, R.W. (1992) N. Engl. J. Med. 326, 1464–1468.
[5] Storck, T., Shulte, S., Hofmann, K. and Stoffel, W. (1992) Proc. Natl. Acad. Sci. USA 89, 10955–10959.
[6] Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathiesen, J., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[7] Kanai, Y. and Hediger, M.A. (1992) Nature 360, 567–471.
[8] Shashidharan, P. and Plaitakis, A. (1993) Biochim. Biophys. Acta 1216, 161–164.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.