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The rat hepatoma cell line H4-II-E-C3 expresses high activities of the high-affinity glutamate transporter GLT-1A

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Abstract The rate of uptake of aspartate into the rat hepatoma cell line H4-II-E-C3 is very much higher than that exhibited by normal rat hepatocytes. Using an RT-PCR-based strategy, a glutamate transporter resembling mouse liver GLT-1A has been cloned from H4-II-E-C3 cells. Northern blotting confirmed that relatively high levels of mRNA for GLT-1A are expressed in hepatoma cells compared with negligible levels in rat hepatocytes. To our knowledge, this is the first report of the cloning of a high-affinity glutamate transporter from a transformed cell line and also the first demonstration of functional expression of GLT-1 outside the central nervous system. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Glutamate transporter; Rat hepatoma; GLT-1; High-affinity

1. Introduction

Glutamate transport across the plasma membrane into cells is mediated by a family of high-affinity sodium-dependent transporter proteins termed system X_{AG}^-. So far, five structurally distinct members of this family have been cloned and characterised. These all have similar kinetic properties and have been termed GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5. The pattern of individual glutamate transporter expression in the central nervous system (CNS) has been well characterised, and the metabolic function of these transporters in brain is well understood (for example see [1–3] for reviews).

In non-neuronal tissues, glutamate transporters are responsible for the uptake of acidic amino acids across the brush border membranes of epithelial cells, particularly in kidney, intestine and in cell lines derived from epithelial tissues. Where glutamate transporters have been cloned from such cells and tissues, these have been universally found to be the EAAC1. Expression of EAAC1 is highly regulated in some cell types. For example in the bovine renal epithelial cell line NBL-1, expression of EAAC1 activity is increased by both hyperosmotic stress and amino acid starvation [4]. There are few reports of expression of other glutamate transporters in non-neuronal tissues. Evidence for expression of GLAST and GLT-1 in human embryonic fibroblasts has been reported using flow cytometry [5]. Furthermore a variant of GLT-1 has been found to be expressed at very low levels in mouse liver by Northern blotting [6].

Little is known about glutamate transporter expression in tumour cells. It has been shown previously [7] that the rat hepatoma cell line H4-II-E-C3 expresses high rates of Na^+-dependent glutamate transport although normal rat hepatocytes express negligible transport activity. In order to obtain further information about changes in gene expression which occur during liver cell transformation, we have established the molecular identity of the glutamate transporter responsible for this activity in this hepatoma cell line.

2. Materials and methods

H4-II-E-C3 cells were maintained subconfluent in Hams nutrient F-12 medium supplemented with 10% (v/v) foetal bovine serum (Life Technologies, Paisley, UK), 2 mM glutamine and antibiotics as described previously [8]. Cells were used for transport experiments when they were approximately 80% confluent on 35 mm culture dishes. Transport experiments were performed as described previously [7].

Total RNA was prepared from cell cultures using Tri-reagent (Sigma, Poole, UK) as per manufacturer's instructions. First strand cDNA was synthesised using oligo$(dT)_{15}$ (Promega, Southampton, UK) and used as a template for PCR amplification using appropriate primers and Hi-Fidelity Taq polymerase according to the manufacturer's instructions (Roche, East Sussex, UK). In some experiments, a H4-II-E-C3 lambda-Zap II bacteriophage library from Stratagene (Cambridge, UK) was used as a template for PCR. PCR products were separated by agarose gel electrophoresis, gel-extracted (Qiaquick spin kit, Qiagen, West Sussex, UK), ligated into pGEM T-Easy vector (Promega) and used to transform $CaCl_2$-competent *E. coli*. Plasmid DNA was extracted (Genie II plasmid extraction kit, Helena Biosciences, Sunderland, UK) and subjected to automated sequencing (Applied Biosystems, Warrington, UK) using vector-specific M13 primers.

The primers used were as follows: F1 5′-GG(A/G)GA(A/C)AC-(C/T)CT(G/C)ATG(A/C)GGATGCTGAAG-3′; F2 5′-GCTGGA-ATTTTCCAAGCCTGGATCAC-3′; R1 5′-GC(A/T)ATGAA(C/G)-A(C/T)(A/G)GCTGCCACGGCTTC-3′; R2 5′-GTATATTA(C/T)-(A/T)T(G/C)TCCAC(G/C)ACC(A/G)TCATTGC-3′; FullF 5′-CCC-TCCTGTAGATAAGAGAATGGTCAG-3′; FullR 5′-GCTGCAG-GGGGTCTTTATTTTTCACG-3′; T3 5′-AAGCTCGAAATTAA-CCCTCACTAAAGGG-3′; T7 5′-AGTGAATTGTAATACGACT-CACTATAGGGC-3′.

For Northern analysis, total RNA prepared using Tri-reagent (Sigma) was run on a 2.2 M formaldehyde agarose gel [9] and blotted onto a nylon membrane (25 μg/lane). A 300 bp probe prepared by reverse transcription-PCR using primers F1 and R2 was labelled using the Redi-prime radiolabelling kit (Amersham, Buckinghamshire, UK) and [α-^{32}P]dCTP. Prehybridisation and hybridisation were carried out in a solution containing 45% formamide, 4×SSPE (1×SSPE contains 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 5% (w/v) dextran sulphate and 50 μg/ml sonicated salmon sperm DNA. Northern blots were probed overnight at 42°C. Blots were subsequently washed three times in

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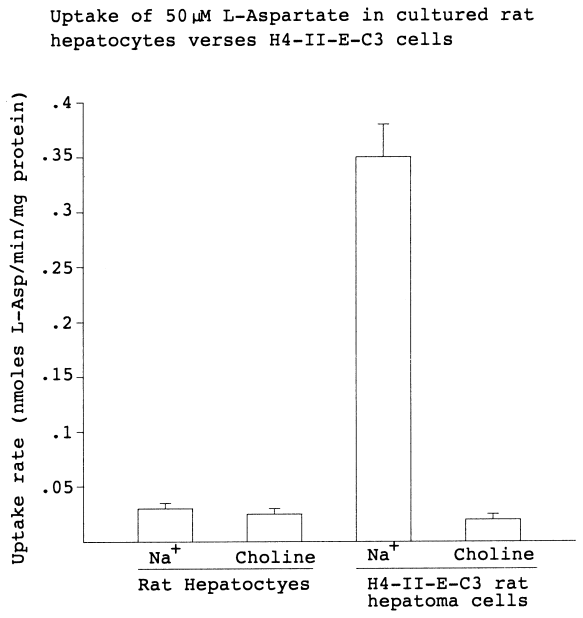

Fig. 1. Maximum rates of uptake of L-aspartate into H4-II-E-C3 hepatoma cells and rat hepatocytes. The hepatoma cells were incubated with 50 mM [L-^{3}H]aspartate in either Na^+- or choline-containing medium in the presence of 0.5 mM amino-oxyacetate to inhibit transamination reactions. The reaction was stopped after 3 min at room temperature; the uptake was linear with time over this period. The results are the mean ± S.E.M. of values from five separate culture dishes. The hepatocyte data are taken from [7]. Hepatocytes were prepared by a standard collagenase perfusion technique [13].

0.5×SSPE, 0.1% SDS at room temperature and a further three times in 0.5×SSPE, 0.1% SDS at 50°C. Blots were then exposed to film.

3. Results

Fig. 1 shows a comparison between the initial rates of uptake of L-aspartate in the hepatoma cell line H4-II-E-C3 and in normal rat hepatocytes in short-term culture. The hepatoma cells exhibited high rates of Na^+-dependent aspartate transport while no significant Na^+-dependent transport activity was present in the hepatocytes. Kinetic analysis of the transport activity in hepatoma cells showed that aspartate transport was competitively inhibited by L-glutamate and D-aspartate but not significantly by D-glutamate. The K_m for L-aspartate was in the range 5–10 μM (not shown). These results are consistent with transport via a member of the family of cloned high-affinity glutamate transporters. However, unlike the glutamate transporter EAAC1 expressed in the bovine renal epithelial cell line NBL-1 [4], the glutamate transporter expressed in H4-II-E-C3 cells was insensitive to regulation by hyperosmolarity or amino acid starvation (results not shown).

Initially it was assumed that this transporter was EAAC1 since no other glutamate transporter had been shown to be functionally expressed outside the CNS. However, extensive attempts to clone EAAC1 by screening a commercial H4-II-E-C3 cDNA library were unsuccessful, and no PCR products corresponding to known EAAC1 sequences were obtained using EAAC1-specific primers and H4-II-E-C3 cDNA as template. Members of the glutamate transporter family share significant regions of amino acid homology especially in transmembrane domains. Degenerate synthetic oligonucleotide primers (F1 and R1, Fig. 2) were designed to homologous sequences of rat brain EAAC1 and GLT-1. When these primers were used in PCR with H4-II-E-C3 cDNA as a template, a product of about 980 bp was obtained which on sequencing proved to be highly homologous to GLT-1, indicating that the hepatoma cell line expressed a glutamate transporter similar to GLT-1 rather than EAAC1

Fig. 2 illustrates the PCR cloning strategy used to obtain the full-length coding region of H4-II-E-C3 GLT-1 and it shows the positions of the primers used in relation to the rat brain GLT-1 cDNA sequence [10]. The 990 bp product (generated using primers F1 and R1) corresponds to bases 343–1333. A commercial H4-II-E-C3 lambda-Zap II bacteriophage cDNA library was used as a template for PCR. In this library, insert sequences are flanked by T3 and T7 promoter sequences. Primers corresponding to either T3 or T7 were used as anchor primers together with gene-specific primers (F2 or R2) designed to the 990 bp cDNA sequence already determined from H4-II-E-C3 cells in order to clone the 3′ and 5′ extremities of the molecule. The complete sequence was confirmed by PCR through the entire coding region using primers FullF and FullR. Three separate 1751 bp products were sequenced in both directions.

The full-length cDNA sequence together with the derived amino acid sequence is shown in the supplementary file (annex 1) which accompanies this article on the FEBSLetters website. The amino acid sequence is 94% similar to rat brain GLT-1 [10] and is greater than 99% identical to mouse liver GLT-1A [6]. Comparing the rat hepatoma amino acid sequence with the mouse liver GLT-1A sequence, there are five amino acid changes at positions 23, 65, 210, 507 and 518 from glutamate, alanine, leucine, histidine and valine in the H4-II-E-C3 protein to aspartate, serine, methionine, glutamine and isoleucine, respectively. Additionally there is an extra threonine residue at position 522 in the H4-II-E-C3 amino acid sequence which is absent in mouse liver GLT-1A. These sequence changes probably represent species differences because identical changes are observed between rat brain GLT-1 [10] and mouse brain GLT-1 ([6], accession no. AB007810). Utsunomyia-Tate et al. [6] have expressed mouse liver GLT-1A in *Xenopus oocytes* and shown that it encodes a functional sodium-dependent high-affinity glutamate transporter. Since the only amino acid differences between mouse liver GLT-1A and

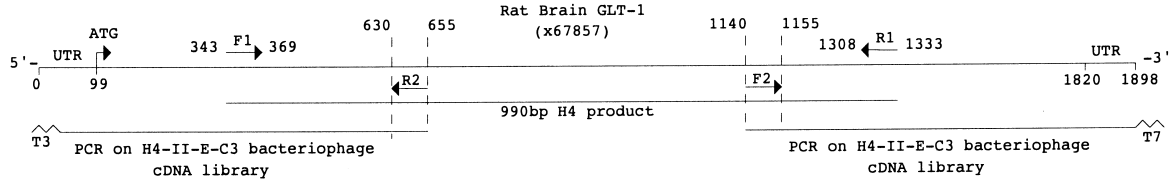

Fig. 2. PCR cloning strategy for the hepatoma glutamate transporter. See explanation in the text.

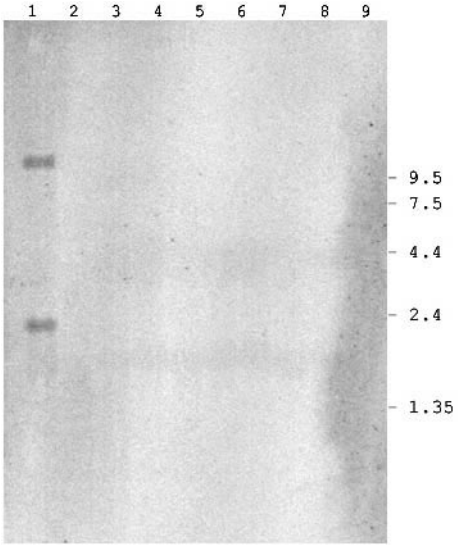

Fig. 3. Northern blot of total RNA prepared form several sources probed with 300 bp probe to GLT-1A. Loading of sample RNA is as follows: 1: H4-II-E-C3 cells; 2: whole rat liver; 3: isolated rat hepatocytes; 4: HepG2 cells; 5: JAR cells; 6: NBL-1 cells; 7: NRK cells; 8: HEK 293 cells; 9: rat kidney.

the rat hepatoma sequence are species-specific, we can deduce that the rat hepatoma cDNA sequence encodes a functional glutamate transporter.

To determine the relative abundance of mRNA encoding GLT-1A in hepatocytes and hepatoma cells, Northern blot analysis using a 300 bp GLT-1-specific probe was performed on total RNA (Fig. 3). In rat hepatocytes and whole rat liver, GLT-1A mRNA expression was undetectable under the conditions used. However, under identical conditions, two distinct bands of 11 kb and 2.4 kb representing GLT-1A mRNA were observed in H4-II-E-C3 cells. Additionally no GLT-1 mRNA was detectable in the bovine renal epithelial cell line NBL-1, the normal rat kidney epithelial cell line (NRK) or the human placenta choriocarcinoma cell line JAR, all of which are known to express high activities of glutamate transport.

4. Discussion

The results presented in this paper show that the high rates of Na^+-dependent aspartate transport observed in the rat hepatoma cell line H4-II-E-C3 are due to the expression of the rat analogue of mGLT-1A first cloned from mouse liver [6]. This transporter is not expressed at a functional level in isolated rat hepatocytes. To our knowledge, this is the first report of the cloning of a high-affinity glutamate transporter from a transformed cell line; it is also the first demonstration of the functional expression of GLT-1 from cells outside the CNS. Expression of GLT-1A is not observed in a number of other cell lines which express high rates of glutamate uptake and may be specific to cells derived from liver.

The physiological function of high-affinity Na^+-dependent glutamate transporter activity in tumour cells is not immediately obvious since these cells have a high internal concentration of glutamate derived from glutamine hydrolysis. Glutamate efflux in exchange for cystine by system xc- is essential for maintenance of intracellular glutathione levels [11]. Reuptake of glutamate by a high-affinity glutamate transporter has been proposed to maintain intracellular glutamate levels and also to influence system xc- activity [12]. In support of this, it has been shown that CHO-K1 cell mutants lacking high-affinity glutamate transport activity exhibit abnormally low system xc- activity, possess lower levels of intracellular glutathione and are more susceptible to damage by free radicals and anti-cancer drugs [12].

Hepatoma cells exhibit increased growth rates, energy requirements and altered metabolic profiles compared with normal hepatocytes. In principle, hepatoma cells can satisfy these needs in one or more of the following ways: increased expression of normal liver genes; anomalous expression of genes normally expressed in other tissues; or expression of novel isoforms of proteins possessing altered properties. In the case of high-affinity glutamate transport, it appears that the hepatoma cell line is upregulating expression of a gene which is expressed at very low levels in normal liver. It remains to be determined if upregulation of glutamate transport is an essential factor in cell transformation.

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References

[1] Seal, R.P. and Amara, S.G. (1999) Ann. Rev. Pharmacol. Toxicol. 39, 431–456.
[2] Danbolt, N.C., Chaudhry, F.A., Dehnes, Y., Lehre, K.P., Levy, L.M. and Ullensvang, K. (1998) Prog. Brain Res. 116, 23–43.
[3] Palacin, M., Estevez, R., Bertran, J. and Zorzano, A. (1998) Physiol. Rev. 78, 969–1054.
[4] Nicholson, B.N. and McGivan, J.D. (1996) J. Biol. Chem. 271, 12159–12164.
[5] Cooper, B., Chebib, M., Shen, J., King, N.J.C., Darvey, I.G., Kuchel, P.W., Rothstein, J.D. and Balcar, V.J. (1998) Arch. Biochem. Biophys. 353, 356–364.
[6] Utsunomiya-Tate, N., Endou, H. and Kanai, Y. (1997) FEBS Lett. 416, 312–316.
[7] McGivan, J.D. (1998) Biochem. J. 320, 255–260.
[8] Doyle, F.A. and McGivan, J.D. (1992) Biochim. Biophys. Acta 1104, 55–62.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press.
[10] Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[11] Sato, H., Tamba, M., Ishii, T. and Bannai, S. (1999) J. Biol. Chem. 274, 11455–11458.
[12] Igo, R.P. and Ash, J.F. (1998) Somat. Cell Mol. Genet. 24, 341–352.
[13] Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–520.