Tohoku J. Exp. Med., 1999, **187**, 173-182

Identification of Truncated Human Glutamate Transporter

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MATSUMOTO, Y., ENOMOTO, T. and MASUKO, T. *Identification of Truncated Human Glutamate Transporter*. Tohoku J. Exp. Med., 1999, **187** (2), 173-182 —— Excitatory amino-acid carrier 1 (EAAC1) is a high affinity Na^+-dependent L-glutamate/D, L-Aspartate transporter protein. A truncated form of EAAC1 (tEAAC1) was identified by reverse transcription and polymerase chain reaction in the human cell line, ACHN, in which EAAC1 mRNA was highly expressed. The deduced amino acid sequence of tEAAC1 lacks 31-77 amino acids including the first extracellular domain. The mRNA encoding tEAAC1 was detected in various cells of human origin but not in cells of rat or mouse origin. The expression of tEAAC1 mRNA was proportional to that of full-length EAAC1 (fEAAC1) mRNA, suggesting common transcriptional regulation between tEAAC1 and fEAAC1. In addition, the expression of EAAC1 mRNA was relatively low or non-existent in non-adherent cells. ———— RT-PCR; EAAC1; EAAT3; glutamate transporter
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The EAAC1 is a 524 amino acid transporter protein possessing 10 transmembrane domains (Kanai and Hediger 1992). EAAC1 is expressed in brain, heart, liver, kidney and intestine (Kanai et al. 1994), whereas glutamate transporter (GLT-1) (Pines et al. 1992) and the L-glutamate/L-aspartate transporter (GLAST) (Storck et al. 1992), which are a high affinity glutamate transporters, are restricted to brain. These high affinity glutamate transporters terminate the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft in neuron (Fonnum 1984; Nicholls and Attwell 1990).

We have been studying CD98 which was originally identified as an early T cell activation antigen. The CD98 is strongly expressed in the cell surface of almost all tumor cells and activated lymphocytes. Although microinjection of CD98 complementary RNA to oocytes leads to an elevated activity of y+ like

Received October 30, 1998; revision accepted for publication February 3, 1999.
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amino acid transporter (Bertran et al. 1992; Wells and Hediger 1992; Wells et al. 1992; Chen et al. 1996; Miyamoto et al. 1996), CD98 protein possesses only one transmembrane domain. Thus it remains unclear whether CD98 is itself an amino acid transporter. Recently, we isolated the partial amino acid sequence of 40 kDa protein associated with rat CD98 with about 70% homology to human and rabbit EAAC1 (unpublished data). During analysis of whether CD98 is structurally or functionally associated with EAAC1, we found a truncated form of EAAC1 mRNA in human cells. In this study, we analyzed the physiological significance of the expression of the truncated form of EAAC1.

Materials and Methods

Cell lines

Cell lines used were as follows: ACHN renal, T24 bladder, HeLa cervix, ZR-75-1 breast carcinomas, Molt-4F lymphoma of human origin, FBC-1 bladder carcinoma, AH13 hepatoma, C6 glioma of rat origin, FM3A mammmary carcinoma, Hepa-I hepatoma, B16 melanoma, and P3X63-Ag 8.653 myeloma of mouse origin. These cells were cultured in Dulbecco's Modified Eagle Medium (D'MEM) supplemented with 5% heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH, USA) in a humidified CO_2 incubator.

Animals

Male Balb/c mice and F344 rats of age 6-8 weeks were purchased from Kumagai Animal Farm (Hamamatsu) and housed in a controlled environmemt at 22°C.

RNA preparation

Total RNA were extracted from 6×10^6-1×10^8 cells by the acid guanidinium phenol chloroform (AGPC) method (Chomczynski and Sacchi 1987). Cells were lysed with a denaturing solution containing 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol and 0.5% sarcosyl (GTC solution). Next, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water-saturated phenol and 0.2 volume of chloroform-isoamyl alchohol mixture (50 : 1) was sequentially added to the lysate with thorough mixing after the addition of each reagent. The final mixture was cooled on ice for 10 minutes and centrifuged at $10000\times g$ for 20 minutes at 4°C. The aqueous phase was mixed with an equal volume of 2-propanol and RNA was precipitated at −80°C for 5 minutes. The RNA was sedimented by centrifugation ($10000\times g$, 20 minutes, 4°C), dissolved in GTC solution, and precipitated again with 2-propanol. The RNA was washed with 75% ethanol, dried in air (5-10 minutes) and dissolved in diethylpyrocarbonate (DEPC)-treated water.

Reverse transcription

Reverse transcription (RT) were performed using a First-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden). Total RNA (3 μg) was dissolved in 4 μl of DEPC water, heat-denatured at 65°C for 10 minutes, diluted with the reaction buffer, and incubated at 37°C for 60 minutes. This solution was mixed with 2.5 μl of Bulk first-strand reaction mix, 0.5 μl of 200 mM dithiothreitol and 0.5 μl of 20 μg/μl random hexaoligonucleotides.

Polymerase chain reaction

The Polymerase chain reaction (PCR) reaction mixture consists of 2 μl of 2.5 mM dNTPs, 0.5 units of Taq DNA polymerase (Takara, Kyoto), 0.7 μl of 25 μM each primer, and 0.5 μl of first strand cDNA preparation from each sample, in a total volume of 20 μl in PCR buffer (Takara). Oligonucleotide primers (Table 1) were constructed based on the published sequences for human EAAC1 (GenBank accession number U06469) and ß-actin (Li et al. 1993). The PCR cycle consists of 25–40 rounds of denaturation at 94°C for 1 minutes, annealing at 55°C for 1 minutes and elongation at 72°C for 2 minutes.

Subcloning

The RT-PCR products were separated on a 1% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, ME, USA), and bands of note were cut out and purified by QIAEX II (Qiagen, Hilden, Germany). The recovered cDNA fragments were subcloned with T4 DNA ligase into pCR2.1 using the Original TA cloning kit (Invitrogen, San Diego, CA, USA).

DNA sequence

The cDNA fragments were sequenced by the dideoxy chain-termination method using an automated DNA Sequencer (Aplied Biosystems model 373A) according to the manufacturer's instructions. The entire sequences of full-length and truncated EAAC1 clones were determined on both strands using a combination of universal M13 primers and synthetic oligonucleotide primers made to specific EAAC1 sequences. DNA sequences were aligned using the DNASIS program (Takara).

Semiquantitative analysis of RT-PCR products

RT-PCR products were separated by gel electrophoresis in 2% agarose gels, and stained with ethidium bromide at a concentration of 0.5 μg/ml. Products were visualized under UV light at 312 nm and photographed. Amounts of PCR products were determined by computerized scanning densitometry (Image software, NIH, Maryland, MD, USA).

Results

During analysis of expression of EAAC1 mRNA by RT-PCR in ACHN human renal cancer cells, we detected three species of EAAC1 mRNA (Fig. 1). The sizes of these PCR products were 1575 base pair (bp), 1434 bp and 790 bp. Each PCR product was subcloned into T vectors, and sequenced. The nucleotide sequence of the 1575 bp PCR product was identical to the reported human EAAC1 sequence (Kanai et al. 1994) (fEAAC1 in this paper), while the 1434 bp and 790 bp PCR products were truncated EAAC1 with a deletion of 91–231 bp (tEAAC1 in this paper) and 219–1004 bp, respectively. Comparison of the amino acid sequence alignments of fEAAC1 and tEAAC1 are shown in Fig. 2.

To investigate the physiological meaning of the expression of tEAAC1 mRNA, expression of fEAAC1 and tEAAC1 mRNA was analyzed by RT-PCR of total RNA from various human tumor cell lines and lymphocytes with the primers hEAAC1S and hEA279A (Table 1). To measure the expression of fEAAC1 and tEAAC1 mRNA semiquantitatively, 25 to 40 cycles RT-PCR was carried out. A faint band of fEAAC1 (837 bp) was detected after 25 cycles in ACHN and HeLa cells, and both tEAAC1 (696 bp) and fEAAC1 were detected after 40 cycles in all human cells examined, except for Molt-4F lymphoma (Fig. 3A). The amounts obtained by 35 cycles were normalized to the amount of ß-actin obtained by 25 cycles (Fig. 3B). These data indicate that the expression of tEAAC1 mRNA is proportionate to that of fEAAC1 mRNA.

To examine the expression of fEAAC1 and tEAAC1 mRNA in rodent animals, RT-PCR was carried out on total RNA from various normal tissues and tumor cells of mouse and rat origin (Fig. 4). The nested PCR was carried out

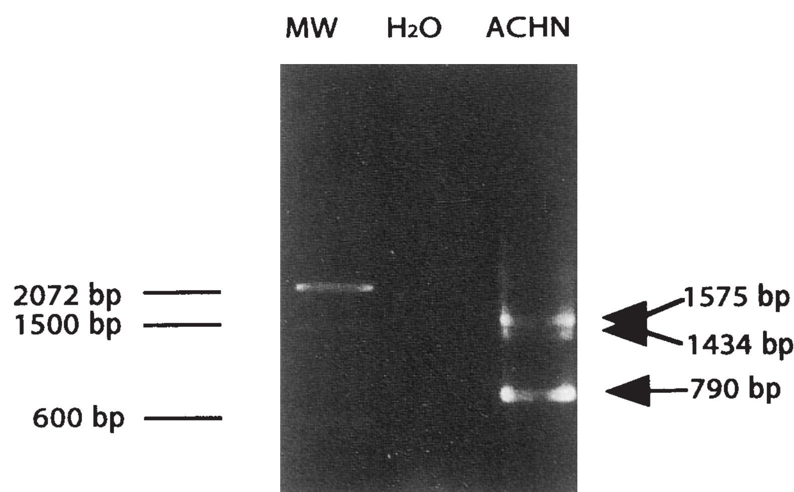

Fig. 1. RT-PCR detection of mRNAs encoding full-length (1575 bp) EAAC1 and truncated (1434 bp, 790 bp) EAAC1. Representative ethidium bromide-stained 1% agarose gel shows PCR products obtained by amplification of cDNA from ACHN human renal carcinoma RNA with primers of hEAAC1S and hEAAC1A (Table 1).

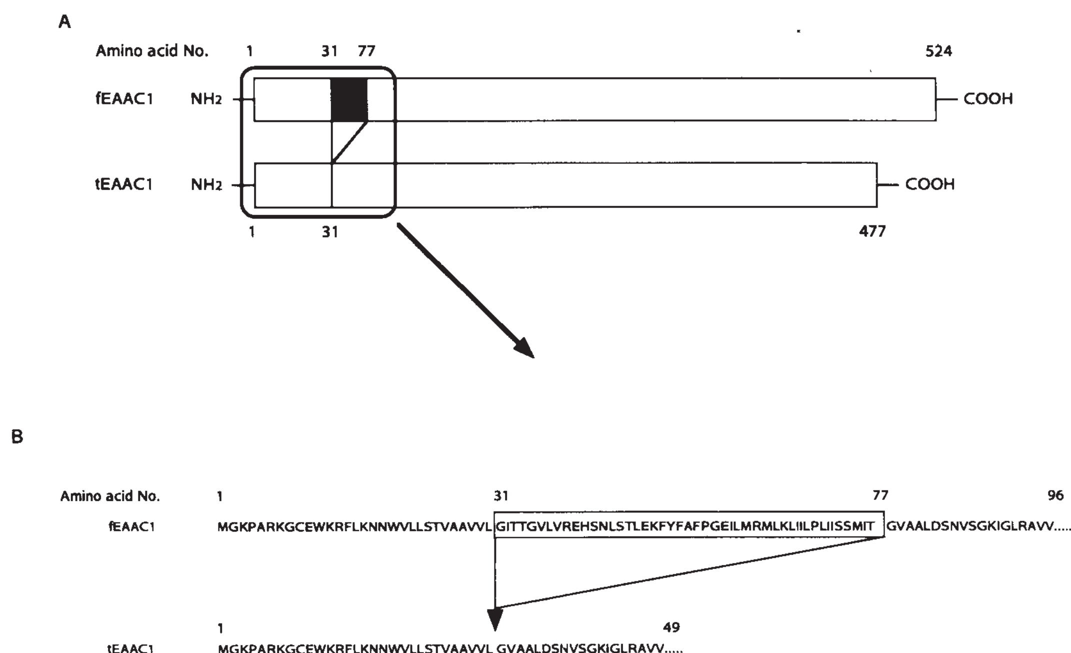

Fig. 2. Comparison of the protein structure of fEAAC1 with that of tEAAC1.
(A) Possible structural model for fEAAC1 and tEAAC1. ■, Truncated domain.
(B) The circle-box domain of (A) is expanded, and the residues of truncated domain are boxed. Omitted sequences are identical between fEAAC1 and tEAAC1.

TABLE 1. *Oligonucleotide primers used in RT-PCR*

Primer	Sequence (5′-3′)
EAAC1 primer	
hEAAC1S	CCG AAT TCA TGG GGA AAC CGG CGA GGA AAG GAT GCG A (Eco RI)
mrEAAC1S	ATG GGG AAG CCC ACG AGC TC
hEA163A	TTG TAC TGC TGA AAA CAG GC
hEA279A	AAT ATT TCC CAG TCT TCA ACT TCT ATG AT
hEAAC1A	CCC TGC AGC TAG AAC TGT GAG GTC TGG GTG AAT GAG A (Pst I)
β-Actin primer	
β-Actin sense	CCT AAG GCC AAC CGT GAA AAG
β-Actin anti	TCT TCA TGG TGC TAG GAG CCA

because standard PCR resulted in poor results. In the nested PCR, the first PCR was performed using the primers of mrEAAC1S and hEA279A, and the second PCR was done using the primers of mrEAAC1 and hEA163A (Table 1). The sequence of mrEAAC1S primer is conserved between mouse and rat EAAC1. On the other hand, the sequences of hEA163A and hEA279A primers are conserved among human, mouse and rat EAAC1. Each PCR was done with 30 cycles. Full-length EAAC1 was detected in both mouse and rat cells, however, truncated

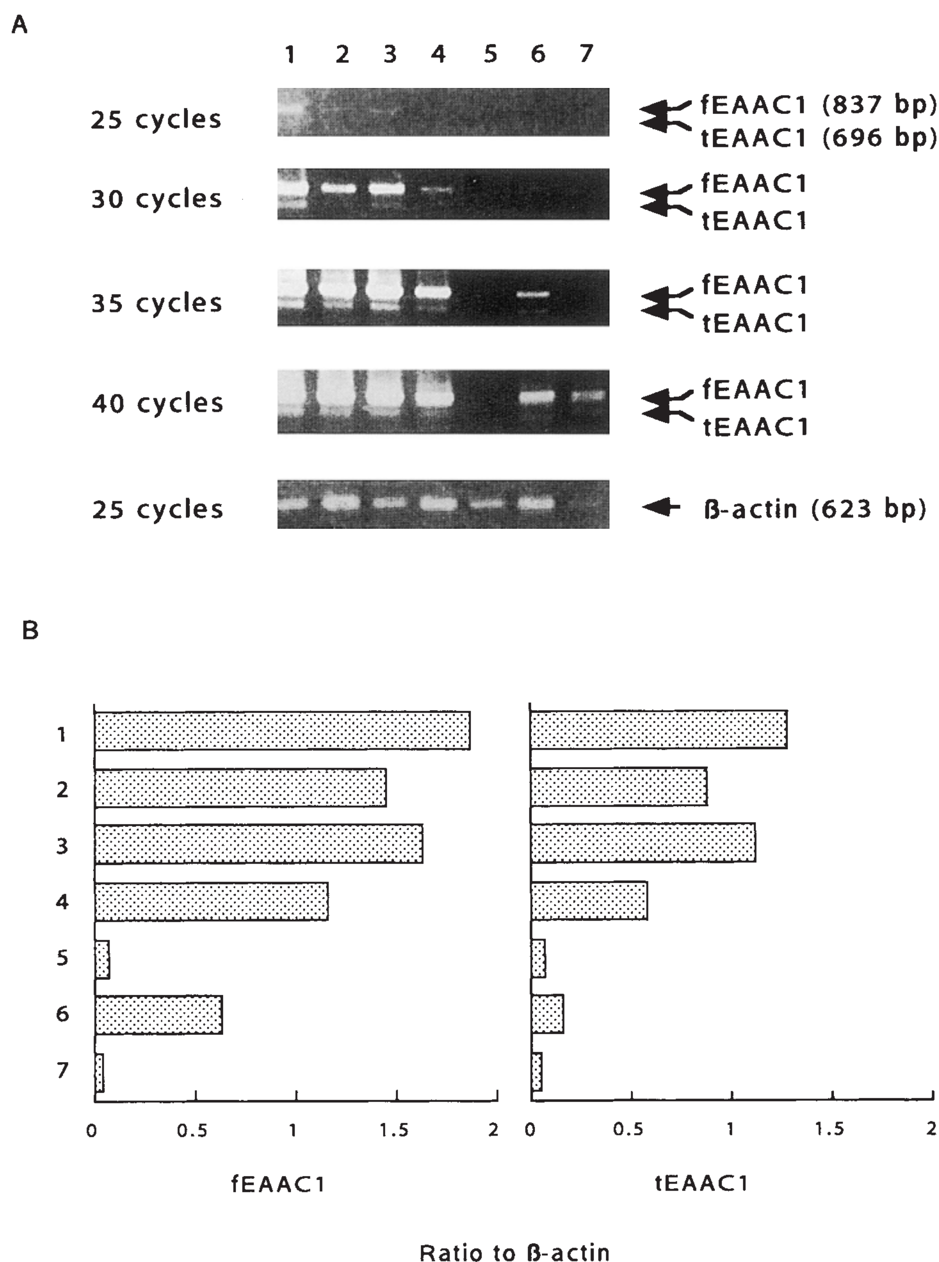

Fig. 3. Expression of fEAAC1 and tEAAC1 mRNA in various human tumor cells. In A, typical agarose gel (2%) electrophoresis of RT-PCR products at 25–40 cycles is shown. In densitometric analyses of relative expression of fEAAC1 (B) and tEAAC1 (C), relative amounts of PCR products were normalized to those of β-actin, and results were expressed as the value of fEAAC1 (35 cycles)/β-actin (25 cycles) or tEAAC1/β-actin. 1: ACHN, 2: T24, 3: HeLa, 4: ZR-75-1, 5: Molt-4F, 6: Activated lymphocytes, 7: Fresh lymphocytes.

EAAC1, corresponding to human tEAAC1, was not detected. As to plastic-nonadherent cells, such as FM3A, X63 and AH13, neither full-length nor tEAAC1-PCR fragments were detected (Fig. 4).

Discussion

We identified three types of human EAAC1 mRNA in ACHN human renal

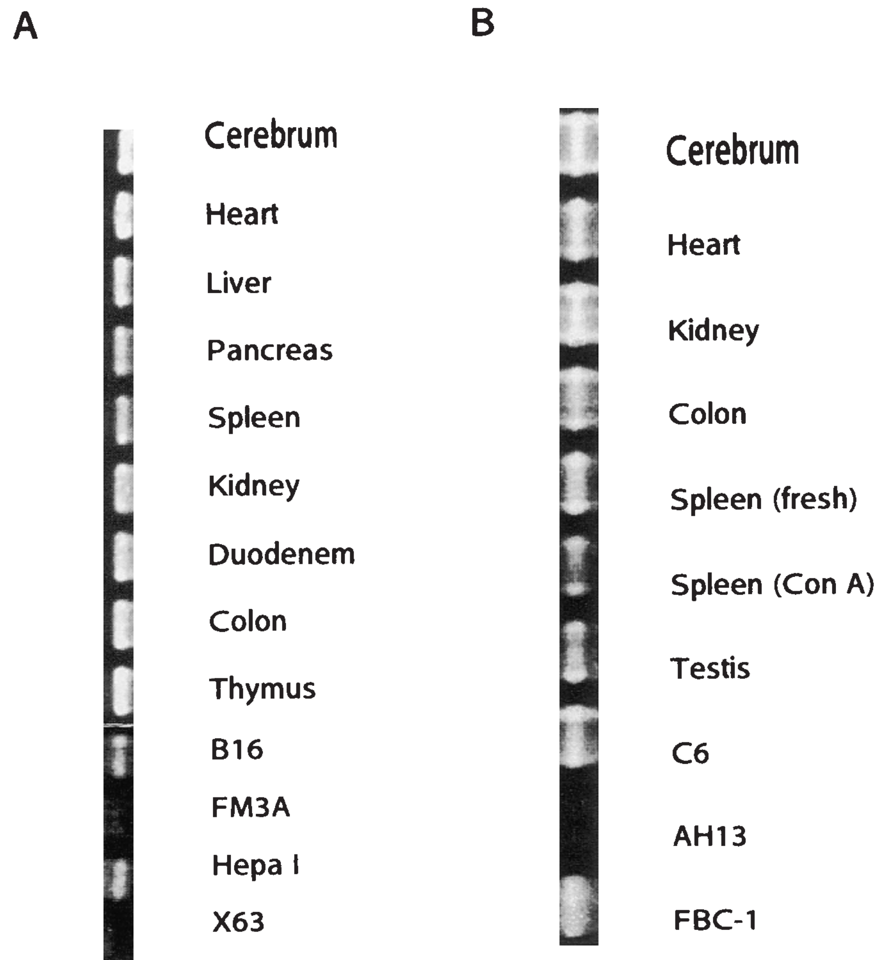

Fig. 4. Expression of EAAC1 mRNA in normal tissues and tumor cells of mouse (A) and rat (B). Typical agarose gel electrophoresis of RT-PCR products. The primers used in PCR amplification for EAAC1: mrEAAC1S and hEA163A. An alignmment of primers were shown in table 1.

cancer cells by RT-PCR. One was a mRNA encoding fEAAC1 (Kanai et al. 1994), and the other two were truncated EAAC1. When the two truncated EAAC1 mRNAs were translated to amino acid sequence, one (tEAAC1) coded EAAC1 lacking amino acids 31-77, and the other was non-productive because of a frame-shift. Since the truncation in tEAAC1, which corresponds to amino acids 31-77 of fEAAC1, exists between the first (18-38 amino acid) and the second (62-82 amino acid) transmembrane domain, a dramatical structual change of tEAAC is expected. One possible structure of tEAAC1 is shown in Fig. 5A, in which the position of extracellular and intracellular domains of tEAAC1 are reversed as compared with fEAAC1. This structure is less likely and probably non-functional, because this form of tEAAC1 has N-glycosylation sites in the intracellular domain and protein kinase C phosphorylation sites in the extracellular domain. Another possibility is shown in Fig. 5B, in which the region corresponding to the first and second transmembrane domains of fEAAC1 are buried in the cell membrane.

Since amino acids in the C terminal region are relatively conserved among

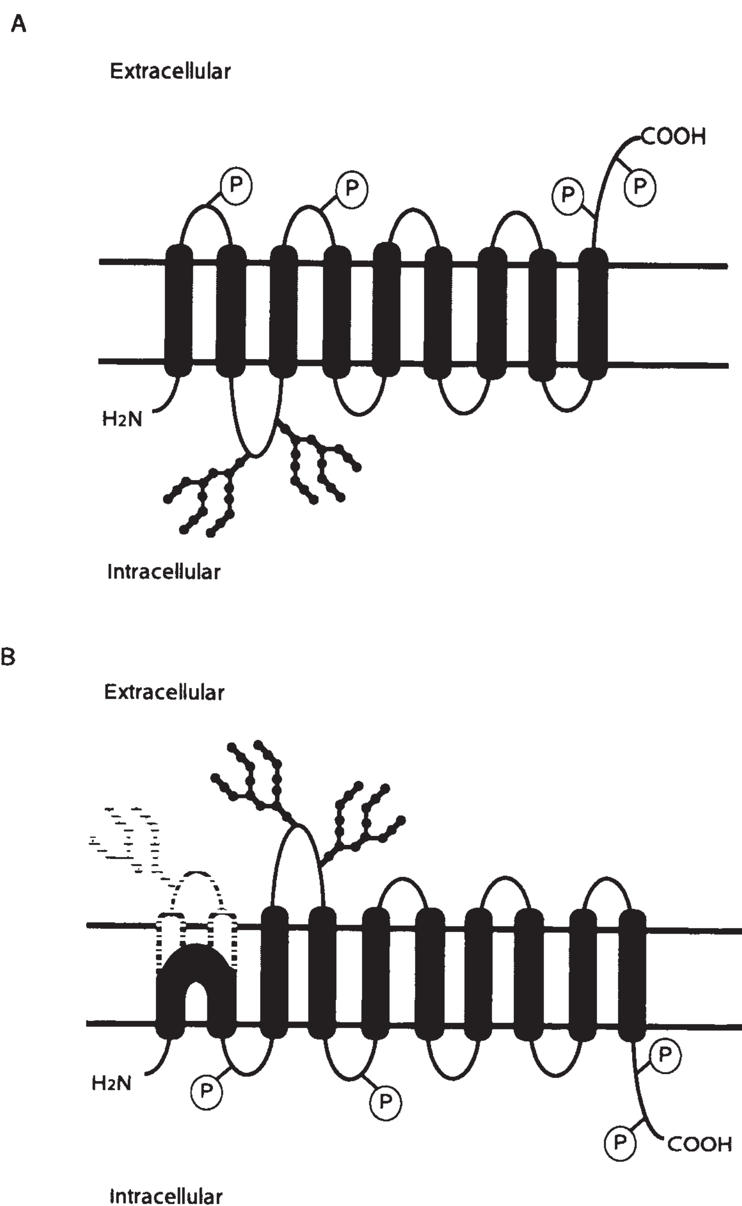

Fig. 5. The supposed structure of tEAAC1. Putative membrane spanning regions are dipicted as spindle boxes. Putative N-glycosylation sites and protein kinase C phosphorylation sites are marked as oligosaccharide "trees" and Ⓟ, respectively. A broken line of B shows a truncated region of fEAAC1.

EAAC1 and glutamate transporter family proteins (Arriza et al. 1997), the C terminal region seems to possess transporter activity, and the N terminal region may be a regulator. In this context, the truncation of the N terminal region in EAAC1 may affect ligand binding by changing affinity to glutamate or may inactivate EAAC1 in the role sensor of the extracellular environment including extracellular glutamate concentration or hypertonic stress.

Recently, two truncated mRNAs of GLT-1, coding one of glutamate transporter family proteins, were cloned (Lin et al. 1998; Nagai et al. 1998). These mRNA were an alternative splicing form of GLT-1 mRNA without exon 9 and a truncated transcriptive form containing an intronic sequence of the 3′ end of exon

7. The latter form is truncated at the C terminal region and has a dominant negative function. This observation is in accordance with our hypothesis that the C terminal region of EAAC1 possesses transporter activity. Although truncated regions are different between GLT-1 and EAAC1, the presence of truncated glutamate transporter family proteins may suggest a common role of these species as the regulator of glutamate transport.

RT-PCR analysis has revealed that fEAAC1 and tEAAC1 mRNA are expressed in a various human tumor cell lines and lymphocytes and the expression of tEAAC1 is proportionate to that of fEAAC1. In contrast, in the case of mouse and rat cells, full-length EAAC1 was detected but the truncated EAAC1 was not. Although we could not detect the truncated from of mouse and rat EAAC1 mRNA, a possibility still remains that other truncated forms exist in these cells.

In summary, our data cleary shows the existence of tEAAC1 mRNA in various human cell lines and lymphocytes. The fact that expression of tEAAC1 mRNA is proportionate to that of fEAAC1 mRNA in various cells suggests common transcriptional regulation of two types of EAAC1. Since tEAAC1 mRNA is expressed in a wide variety of human cells including normal lymphocytes, we assume that the expression of tEAAC1 is not necessarily related to pathogenic events but rather plays physiological roles in normal cells. Further analysis of the protein products of tEAAC1 mRNA are required to understand the precise physiological role of tEAAC1.

Acknowledgment

This work was supported by Grants for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan.

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