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Preliminary Communication

Cloning and Characterization of a *Drosophila melanogaster* cDNA Encoding a Glutamate Transporter

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A *Drosophila* cDNA encoding a glutamate transporter was cloned and examined. The predicted protein (479 amino acid residues) shows significant sequence identity with mammalian counterparts. The protein expressed in *Xenopus* oocytes had a glutamate transport activity. Northern blot analysis showed that the transcript increased in amount developmentally. This expression pattern is different from those of *Drosophila* glutamate receptors.

Key words: cDNA cloning; *Drosophila*; expression; glutamate transporter; insect

The essential amino acid glutamate plays an important excitatory role as a neurotransmitter. In mammals, glutamate transporters are carrier proteins that suppress excitation in the nervous system by removing glutamate from the extracellular spaces.[1)] Recently, because glutamate is important in memory, learning, and the death of nerve cells, mammalian glutamate transporters have been extensively characterized using molecular biological techniques.[2,3)] Glutamate transporter proteins consist of about 550 amino acid residues with the typical membrane protein profile and are structurally markedly similar to each other. As for invertebrates, only a few glutamate transporters are known so far, while glutamate and its analogs affect invertebrate nervous systems.[4–7)]

Insects are highly progressed organisms among invertebrates. The fruit fly *Drosophila melanogaster* is in particular a powerful tool for studying invertebrates as well as insects due to accumulation of the genetic background and applicability of molecular biological techniques. In *Drosophila*, glutamate is suggested to be related to neural transmission including memory and learning.[8,9)] This led us to an attempt to characterize a *Drosophila* glutamate transporter to elicit the function of glutamate in invertebrates. We here report molecular cloning of a *Drosophila* glutamate transporter cDNA and its transport activity. We also show the developmental expression of the transcript.

In order to obtain a partial cDNA for screening a cDNA library, RT-PCR was done based on the amino acid sequences ATINMDG and AAIFIAQ (Oligonucleotide primers 5′-GCTACNATHAAYATGGAYGG-3′ and 5′-TGNGCDATGAARATAGCNGC-3′ (N:G/A/T/C; H:A/T/C; Y:T/C; R:G/A)), which are conserved among glutamate transporters known so far. For PCR amplification, first strand cDNA was synthesized from 1 μg of adult *Drosophila* poly(A)$^+$ RNA purchased from Clontech Laboratory using oligo(dT)$_{12-18}$ (Boehringer Mannheim) as a primer, and used for PCR. The resulting PCR product was cloned into a pCRII vector (Invitrogen), and sequenced by the dideoxy chain termination method on an Applied Biosystem model 373A DNA sequencer (Applied Biosystem). A probe 5′-ATGGACGGAACGGCTCTCTATGAGGCTGT-3′ was synthesized based on the above sequence and labeled with digoxigenin (DIG)-ddUTP using a DIG Oligonucleotide 3′ Labeling Kit (Boehringer Mannheim), and then used for screening an adult whole body *Drosophila* cDNA library in λgt 10 (Clontech Laboratory). Hybridized plaques were detected by chemical luminescence using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The cDNA inserts of the isolated positive plaques were amplified using the λgt 10 primers (Takara), and directly sequenced using the λgt 10 primers and synthetic primers. The nucleotide sequences thus obtained were analyzed using a GENETYX-MAC software (Software Development). The cDNA sequence was 2,689 base long and contained a longest open reading frame from nucleotide 387–1826 encoding a protein 479 residue long (D86739). In the 3′ non-coding region, seven mRNA instability motifs ATTTA[10)] were found, while A typical polyadenylation signal AATAAA or ATTAAA is not found.

The predicted protein showed significant sequence similarity with mammalian glutamate transporters (40–45%), a *Caenorhabditis elegans* counterpart Ceglut-1 (45%) and the caterpillar glutamate transporter TrnEAAT1 (60%).[3,4,7,11)] A Kyte-Doolittle hydropathy

The nucleotide sequence reported in this paper has been submitted to DDBJ/EMBL/GenBank Data Banks and appear in the DNA databases under the accession number D86739.

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Abbreviations: cDNA, complemental DNA; DIG, digoxigenin; mRNA, messenger RNA; PCR, polymerase chain reaction; RT, reverse transcriptase

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                                                                                  *    **    **** ** **  **       **   *     **      ***
Dglt-1       1                  MTRPKQDGGKFKAFMQENVLTMATVIGVFVGGLIGFIIKNSTGEWSKREIMYISFPGEIFLRMLKCLIVPLLVSSITSAIGGLDLSMSSKIATRAITYYFVTTI  104
TrnEAAT1     1                   MPLQIRRNRCTSFLRENLLIIFTIIGVIAGSLLGCGLRFSGHEWSRRDVMYFQYPGELFLRMLKSLIVPLAVSSIVSAIGSLDLSLSGKVGLRAIIYYMTTTI  103
hEAAT2       1  MPKQVEVRMHDSHLGSEEPKHRHLGLRLCDKLGKNLLLTLQVFGVILGSVCGGLLRLASPIHPDVVMLIAFPGDILMRMLKMLILPLIISSLITGLSGLDAKASGRLGTRAMVYYMSTTI  120

                 *  **  **    **                   *                          *                                              * *****  *
Dglt-1     105  SAVILGICLVTTLRPGQGAKIVETQTESIDKASKVLTPDTLMDLVRNMFTDNIIQSTMFQHRTE-IYE--------------------NTSISPAQPMENWEFKSAQREGSNVLGLVMFS  203
TrnEAAT1   104  CAVMLGIALVTTIKPGKETTYTQPNATKV-ISKDTLTSDTLLDLIRNVFPENLAQATIASYRTKLIYDK-------------------NDTKSRLGELETYQIQGEYQSGSNVLGLVCFS  203
hEAAT2     121  IAAVLGVILVLAIHPGNPKLKKQLGPGKKNDEVSSLDAFLDLIRNLFPENLVQACFQQIQTVTKKVLVAPPPDEEANATSAVVSLLNETVTEVPEETKMVIKKGLEFKDGMNVLGLIGFF  240

                   *   * *        ***  * *  *       * ****   *   ** *          ** *  **  **  **           *   *    *   *   ** ** **  ***
Dglt-1     204  VILGTTIGRMREKGQLLQDFFTTLSEAMMTITSWVIWISPLGVAFLIAAKIIEMESIAATIQSLGWYFITVMIGLFLHGFGTIAVIFFLGTRRLPYRYIAKLSQVLATAFGTGSSSATMP  323
TrnEAAT1   204  IVLGITLGKMGEKARPLQDFFHSLSEAMMIITGWVIWLSPLGVFFLVTAKIMEIDDFGDLVGRLGKYFFTVLLGLFLHGFGTLRILFILATKKLPCKYIAKMGQVMATAFGTASSSATMP  323
hEAAT2     241  IAFGIAMGKMGDQAKLMVDFFNILNEIVMKLVIMIMWYSPLGIACLICGKIIAIKDLEVVARQLGMYMVTVIIGLIIHGGIFLPLIYFVVTRKNPFSFFAGIFQAWITALGTASSAGTLP  360

                 *  *     * * * **** * ***************** ****        * * *** *** ** *** ** *******  *  * *   * *** ***********  **  *  *
Dglt-1     324  LTIKC-LDNMGIDPRVTRFVIPVGATINMDGTALYEAVAALFIAQYREMSYSFGTIVAVSITATAASIGAAGIPQAGLVTMVMVLDTVGLEPKDVSLIIAVDWLLDRFRTTINVMCDALG  442
TrnEAAT1   324  ITIGC-CDDMGLDPRITRFVIPIGATINMDGTALYEAVAAIFIAQLRKVEMSFGKIIAVSVTATAASIGAAGIPQAGLVTMVMVLDTVNLPAEDVSLILAVDWLLDRFRTTINVVCDALG  442
hEAAT2     361  VTFRCLEENLGIDKRVTRFVLPVGATINMDGTALYEAVAAIFIAQMNGVVLDGGQIVTVSLTATLASVGAASIPSAGLVTMLLILTAVGLPTEDISLLVAVDWLLDRMRTSVNVVGDSFG  480

                   *  **
Dglt-1     443  TILVNHLSKNDLASVDRLNAEPHELLELGPNGHEMKE                                                                                    479
TrnEAAT1   443  AIIVTSLSQGDIDKSRALNEREAAPSHELTELEKGDH                                                                                    479
hEAAT2     481  AGIVYHLSKSELDTIDSQHRVHEDIEMTKTQSIYDDMKNHRESNSNQCVYAAHNSVIVDECKVTLAANGKSADCSVEEEPWKREK                                   565
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Fig. 1. Amino Acid Comparison of the Deduced Dglt-1 Protein with TrnEAAT1 and hEAAT2.

The deduced amino acid sequence of Dglt-1 was aligned with those of a caterpillar (TrnEAAT1) and a human (hEAAT2) glutamate transporters. All residues common between the three proteins are shown by asterisks. Bold sequences indicate the positions of primers for the RT-PCR. Underlined residues correspond to the oligonucleotide probe for screening the cDNA library.

analysis[12] on the predicted protein named Dglt-1 (*Drosophila* glutamate transporter-1) showed that the protein contained six prominent hydrophobic domains up to residues 290. In addition, Dglt-1 contained two small hydrophobic peaks at residues $\sim$310 and $\sim$350, and a long hydrophobic stretch at residues $\sim$380–$\sim$430. This hydropathy was quite similar to that of TrnEAAT1 (data not shown). Figure 1 shows an alignment of amino acid sequences of the Dglt-1, TrnEAAT1, and one of the human glutamate transporters, hEAAT2. The *N*- and *C*-terminal regions of Dglt-1 that are situated in intracellular domains are shorter than those of hEAAT2, and the truncated residues of Dglt-1 result in a shorter extracellular loop. The *C*-terminal half is more nearly identical to that of hEAAT2, suggesting that the *C*-terminal region should be responsible for the function. Dglt-1 had two possible *N*-glycosylaion sites at residues 40 and 172 that are situated between hydrophobic domains 1 and 2, and 3 and 4, respectively. Five protein kinase C (PKC)-dependent phosphorylation sites are predicted. One of them at Ser^{88} is situated between predicted membrane-spanning domains 2 and 3. This phosphorylation site is conserved among glutamate transporters, and the corresponding serine residue in the rat glutamate transporter GLT-1 was actually the major phosphorylated site induced by a phorbol ester, suggesting that Dglt-1 may be regulated by PKC-dependent phosphorylation in the same manner.[13]

To clarify the function of the Dglt-1 protein, glutamate transporter activity of the protein was measured. The *Dglt-1* cDNA was subcloned into the *Eco*RI site of a pBluescript II SK$^-$ vector (Stratagene) and linearized with *Not*I. Capped cRNA was obtained using an *in vitro* transcription kit (mMESSAGE mMACHINE: Ambion). Then, the glutamate transport activity was measured as reported previously.[5] As shown in Fig. 2, *Xenopus* oocytes injected with the *Dglt-1* cRNA showed L-[^{14}C]glutamate uptake to 4 fold above that of water-injected control oocytes. This result indicated that Dglt-1 was defined as a glutamate transporter. The transport activity of Dglt-1 was the same as that of the *C. elegans* glutamate transporter Ceglut-1 (data not shown).

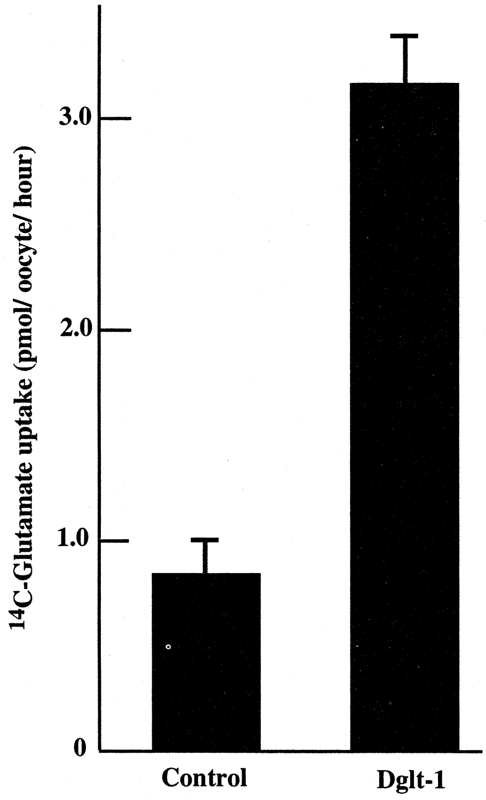

Fig. 2. L-Glutamate Transport via Dglt-1.

L-[^{14}C]glutamate uptakes into the *Xenopus laevis* oocytes injected with in vitro synthesized *Dglt-1* cRNA or DEPC-treated water were measured. Data points represent averages from at least 5 oocytes. *Ordinate* is uptake of labeled glutamate.

Finally, in order to analyze the *Dglt-1* gene expression, Northern blotting was done on the transcript in several stages. *Drosophila* mRNAs prepared from embryos (stages 12 to 17), larva (second- and third-instar), and adults (until 16 hours after eclosion) that were pur-

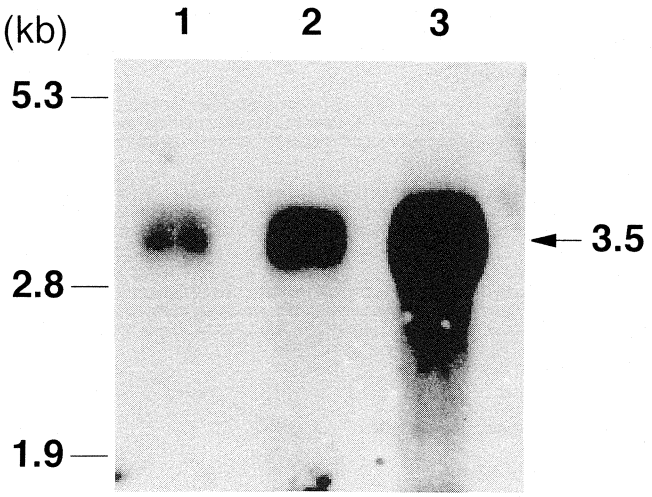

Fig. 3. Developmental Northern Blot Analysis on *Drosophila* mRNAs.

Approximately 1.0 μg of poly(A)$^+$ RNA prepared from embryos (lane 1), larva (lane 2), and adults (lane 3) were electrophoresed. Digoxigenin-labeled *Dglt-1* cDNA was used for a probe. Hybridized bands were detected by a chemical luminescence method.

chased from Clontech Laboratory were electrophoresed on a 1.0% agarose gel in the presence of 2.2 M formaldehyde, and blotted onto a nylon membrane (GeneScreen Plus: NEN). The amplified cDNA insert was purified, labeled with DIG-dUTP (Boehringer Mannheim), and used as a probe. Hybridization was done by the protocol provided by the manufacture. After washing the membrane in 0.1 × SSC, 0.1% SDS at 68°C, hybridized fragments were detected as described above. As shown in Fig. 3, a single hybridizing band at 3.5 kb was detected in embryonic, larval, and adult stages. The abundance of the transcript increased developmentally. In our preliminary experiment, whole-mount *in situ* hybridization on stage 16 embryos showed that the gene expression was restricted to the central nervous system especially in the embryonic brain and the ventral nerve cord (data not shown). As for *Drosophila* ionotropic glutamate receptor genes *DGluR-I* and *DNMDAR-I*, the genes are highly expressed in embryonic and adult stages.[14,15] By contrast, the abundances of the transcripts in the first-instar larva are quite low and continually decrease to undetectable levels during larval development. As for a *Drosophila* metabotropic glutamate receptor gene *DmGluRA*, the gene expression is only detected in the embryonic stage by *in situ* hybridization and in the adult stage by the RT-PCR method.[16] Thus, the expression of the *Dglt-1* gene in larval stage seems quite characteristic.

In this study, we cloned and characterized a *Drosophila melanogaster* cDNA encoding a glutamate transporter named Dglt-1. Interestingly, the *Dglt-1* gene is highly expressed in the larval stage, while the glutamate receptor genes are hardly transcribed in the same stage. The glutamate transporter may solely function without the receptors during larval development. Our further study will show detailed spacial distribution of the *Dglt-1* transcript and regulation of the gene expression in the near future.

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References

1) Kanai, Y., Smith, P. C., and Hediger, A. M. A., New family of neurotransmitter: The high-affinity glutamate transporters. *FASEB J.*, **7**, 1450–1459 (1993).
2) Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Hori, S., Takimoto, M., and Wada, K., Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science*, **276**, 1699–1702 (1997).
3) Castagna, M., Shayakul, C., Trotti, D., Sacchi, V. F., Harvey, W. R., and Heediger, M. A., Molecular characteristics of mammalian and insect amino acid transporters: Implication for amino acid homeostasis. *J. Exp. Biol.*, **200**, 269–286 (1997).
4) Kawano, T., Takuwa, K., and Nakajima, T., Molecular cloning of a cDNA for the glutamate transporter of the nematode *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.*, **228**, 415–420 (1996).
5) Kawano, T., Takuwa, K., and Nakajima, T., Structure and activity of a new form of the glutamate transporter of the nematode *Caenorhabditis elegans*. *Biosci. Biotechnol. Biochem.*, **61**, 927–929 (1997).
6) Radice, A. D. and Lustigman, S., Cloning and characterization of cDNAs encoding putative glutamate transporters from *Caenorhabditis elegans* and *Onchocerca volvulus*. *Molec. Biochem. Parasitol.*, **80**, 41–53 (1996).
7) Donly, B. C., Richman, A., Hawkins, E., Mclean, H., and Caveney, S., Molecular cloning and functional expression of an insect high-affinity Na^+-dependent glutamate transporter. *Eur. J. Biochem.*, **248**, 535–542 (1997).
8) Fagnou, D. D. and Tuchek, J. M., The biochemistry of learning and memory. *Mol. Cell. Biochem.*, **149**, 279–286 (1995).
9) Saitoe, M., Tanaka, S., Takata, K., and Kidokoro, Y., Neural activity affects distribution of glutamate receptors during neuromuscular junction formation in *Drosophila* embryos. *Dev. Biol.*, **184**, 48–60 (1997).
10) Shaw, G. and Kamen, R., A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, **46**, 659–667 (1986).
11) Arriza, J. L., Eliasof, S., Kavanaugh, M. P., and Amara, S. G., Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 4155–4160 (1997).
12) Kyte, J. and Doolittle, R. F., A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132 (1982).
13) Casado, M., Bendahan, A., Zafra, F., Danbolt, N. C., Aragon, C., Gimenez, C., and Kanner, B. I., Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J. Biol. Chem.*, **268**, 27313–27317 (1993).
14) Ultsch, A., Schuster, C. M., Laube, B., Schloss, P., Schmitt, B., and Betz, H., Glutamate receptors of *Drosophila melanogaster*: Cloning of a kainate-selective subunit expressed in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10484–10488 (1993).
15) Ultsch, A., Schuster, C. M., Laube, B., Betz, H., and Schmitt, B., Glutamate receptors of *Drosophila melanogaster*. Primary structure of a putative NMDA receptor protein expressed in the head of the adult fly. *FEBS Lett.*, **324**, 171–177 (1993).
16) Parmentier, M. L., Pin, J. P., Bockaert, J., and Grau, Y., Cloning and functional expression of a *Drosophila* metabotropic glutamate receptor expressed in the embryonic CNS. *J. Neurosci.*, **16**, 6687–6694 (1996).