Glutamate transporters (excitatory amino acid transporters, EAAT) play an important role in maintaining extracellular glutamate homeostasis and regulating glutamate neurotransmission. However, very few studies have investigated the regulation of EAAT expression. A binding sequence for the regulatory factor X1 (RFX1) exists in the promoter region of the gene encoding for EAAT3, a neuronal EAAT, but not in the promoter regions of the genes encoding for EAAT1 and EAAT2, two glial EAATs. RFX proteins are transcription factors binding to X-boxes of DNA sequences. Although RFX proteins are necessary for the normal function of sensory neurons in Caenorhabditis elegans, their roles in the mammalian brain are not known.

We showed that RFX1 increased EAAT3 expression and activity in C6 glioma cells. RFX1 binding complexes were found in the nuclear extracts of C6 cells. The activity of EAAT3 promoter as measured by luciferase reporter activity was increased by RFX1 in C6 cells and the neuron-like SH-SY5Y cells. However, RFX1 did not change the expression of EAAT2 proteins in the NRK52E cells. RFX1 proteins were expressed in the neurons of rat brain. A high expression level of RFX1 proteins was found in the neurons of cerebral cortex and Purkinje cells.

These results suggest that RFX1 enhances the activity of EAAT3 promoter to increase the expression of EAAT3 proteins. This study provides initial evidence for the regulation of gene expression in the nervous cells by RFX1.

Glutamate transporters (excitatory amino acid transporters, EAAT) are expressed in many organs and tissues including brain and kidney. They use the transmembrane gradients of Na\(^+\), K\(^+\), and H\(^+\) as a driving force to uptake glutamate from extracellular space into cells. Since glutamate is a major excitatory neurotransmitter, the role of EAAT in the function of the central nervous system has attracted significant attention. Evidence has suggested that EAATs play an important role in maintaining extracellular glutamate homeostasis and regulating glutamate neurotransmission.

Five EAATs have been characterized so far. EAAT1, EAAT2, and EAAT3 have been found in many regions and structures of the brain. EAAT4 is mainly expressed in the cerebellum. EAAT5 is expressed in the retina. In the brain, EAAT1 and EAAT2 are expressed in the glial cells. EAAT3 and EAAT4 are mainly found in the neurons.

Regulatory factor X (RFX) proteins are transcription factors that bind X-boxes of DNA sequences with a conserved 76-residue DNA binding domain in their molecules. There are five RFXs (RFX1–5) in the human. RFX1 is a prototypic mammalian RFX that contains 979 amino acids and was initially thought to interact with the X-box in the class II major histocompatibility complex gene. However, its role in the regulation of major histocompatibility complex expression has not been proven yet. Since the knockout of the RFX homologue in Caenorhabditis elegans results in severe sensory defects, it has been proposed that mammalian RFXs may play an important role in the nervous system. However, up until now, there is no report documenting the expression regulation of a neuronally expressed gene by RFXs in the mammalian nervous cells.

It has been shown that multiple agents, such as volatile anesthetics, cause acute changes of EAAT activity. In EAAT3, this acute change of activity is mediated by the redistribution of EAAT3 from intracellular compartments to the plasma membrane, the functional site of EAAT3. In contrast, very few studies have investigated the regulation of EAAT expression. There is no study on regulating the promoter activity of EAAT3 yet. By analyzing the sequences of the promoter regions for human EAAT1, EAAT2, and EAAT3, we found that only the EAAT3 promoter region contains a consensus binding sequence for RFX1. Thus, we hypothesize that RFX1 can specifically regulate the expression of EAAT3.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—Rat C6 glioma cells from American Type Culture Collection (Manassas, VA) were maintained in F-10 nutrient mixture (Ham's) (Invitrogen) supplemented with 15% horse serum and 2.5% fetal bovine serum at 37 °C. Rat normal kidney NRK-52E cells (American Type Culture Collection number CRL-1571) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum at 37 °C. Human neuroblastoma SH-SY5Y cells (American Type Culture Collection number CRL-2266) were cultured with a 1:1 mixture of Eagle’s minimum essential medium with non-essential acids and Ham’s F12 medium (Invitrogen), supplemented with 10% fetal bovine serum. Cells were transfected with RFX1 or EAAT3-Luc plasmids using the Transfection Reagent (Invitrogen).
FuGENE 6 transfection reagents (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

Cerebral cortical neurons in culture were prepared from fetuses of 16–18-day gestation Sprague-Dawley rats as we described before (11). They were maintained in Dulbecco’s modified Eagle’s medium containing 25 mM HEPES, 10% Ham’s F12 medium, 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (Invitrogen). The cultures were treated with 10 μg/ml cytosine-β-d-arabinofuranoside for 24 h to stop non-neuronal cell proliferation on days 4 and 5. Subsequent feeding was with the above described medium but without Ham’s F-12 to eliminate glutamate from feeding.

Treatment of Neuronal Cultures with Oligonucleotides—After the cells were in culture for 7–9 days, rat cerebral cortical neurons were treated with 5 μM RFX1 antisense or sense oligonucleotides for 96 h. The sequences of RFX1 antisense and sense oligonucleotides were from a previous publication (12): 5’-ggtcagctggaggg-3’ (antisense) and 5’-ccctccagtgacc-3’ (sense). The oligonucleotides were phosphorothioate derivatives to prolong their half-lives.

Plasmid Construction—RFX1 plasmid (pRFX1) containing the coding region for human RFX1 protein in pCMV vector was provided by Dr. Patrick Hearing (State University of New York at Stony Brook, Stony Brook, NY). EAAT3-Luc plasmid was constructed by inserting the human EAAT3 promoter region (from –1 to –482) including the putative RFX1 binding sequence, 5’-gggtggcggcggcaacggc-3’, into the pGL3-Luc vector (Promega Corp., Madison, WI) using the enzymes KpnI-BglII. The construct was then confirmed by DNA sequencing.

Luciferase Activity Assay—After being plated on 12-well plates (Corning Inc., New York, NY) for 20–24 h, C6 cells or SH-SY5Y cells at 50–70% confluence were transiently transfected with the EAAT3-Luc plasmid (0.5 μg/well) in the presence or absence of various amounts of pRFX1. The Renilla-luciferase expression vector (pRL/cytomegalovirus, 0.1 μg/well) also was co-transfected as an internal control. The amount of control cytomegalovirus plasmid DNA in the transfection mixture was adjusted to maintain that the total amount of DNA used was at 1 μg/well. At 24 h after the transfection, luciferase and Renilla-luciferase activities in the cells were measured using the Dual-Luciferase reporter assay system (Amersham Biosciences, Buckinghamshire, UK). Protein bands were detected using the enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Western Blotting—Equal amounts of whole cell extracts were separated on sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). The blots were probed with the primary antibodies and then with the horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry—As we described before (14), after being perfused with 100 ml of normal saline, adult Sprague–Dawley male rats were perfused transcardially with 100 ml of phosphate-buffered paraformaldehyde. Brains were removed and stored in the fixative for 2 days at 4 °C. Five-micrometer-thick coronal paraffin sections were cut for immunohistochem-
istry with the anti-RFX1 antibody. Immunoreactivity was visualized with an avidin-biotinylated horseradish peroxidase reaction with an ABC staining system from Vector Laboratories, Inc. (Burlingame, CA). Control incubations leaving out the primary or secondary antibodies were also performed.

Statistical Analysis—The intensity of RFX1, EAAT2, and EAAT3 protein bands was normalized to that of β-actin to control for errors in protein sample loading and transferring during the Western analysis when total cell lysates were used for analysis. The results of Western blotting are presented as means ± S.D. of the -fold change over the controls, with control being set as 1. The results of luciferase activity assays are means ± S.D. of the ratios of luciferase activity/Renilla-luciferase activity in the same sample. The data of glutamate uptake assay are means ± S.D. of the measured values in each sample. Statistical analysis was performed by unpaired t test or one-way analysis of variance followed by the Student-Newman-Keuls test for post hoc comparison as appropriate. A p < 0.05 was considered significant.

RESULTS

C6 glioma cells have been shown to express only EAAT3 and have been frequently used in the studies of the regulation of EAAT3 activity and expression (9, 15, 16). In this study, we have detected EAAT3 mRNA and protein (Figs. 1 and 2) but not the mRNA (Fig. 1) and protein (data not shown) of EAAT1 and EAAT2. The transfection of C6 cells with pRFX1 induced a time-dependent increase of the RFX1 proteins and EAAT3 proteins (Fig. 2). This increase peaked at 24 h after the transfection for RFX1 proteins (2.2 ± 0.4-fold the control, n = 3, p < 0.05 as compared with control) and for EAAT3 proteins (1.6 ± 0.1-fold the control, n = 3, p < 0.05 as compared with control). Consistent with the increased expression of EAAT3 proteins, glutamate uptake by C6 cells also time-dependently increased (Fig. 3). Since these cells only express EAAT3, this increased glutamate uptake should be EAAT3-mediated.

To determine whether the increased EAAT3 expression and activity by RFX1 was due to the modification of EAAT3 promoter activity, we first investigated whether there was a RFX1 DNA binding complex in C6 cells. In the nuclear extracts from C6 cells, the intensity of a supershifted band was increased with the transfection of pRFX1. This supershifted band detected by the RFX1 probe was abolished by a 33-fold excess of cold probe but was not affected by a mutated cold probe with a 5-base change in the sequence. The band was further supershifted by the incubation of the nuclear extracts with an anti-RFX1 antibody (Fig. 4). These results suggest that there are complexes that can bind to RFX1 in the nuclear extracts of C6 cells. We then studied whether RFX1 proteins modified the EAAT3 pro-
moter activity. When C6 cells were transfected with pRFX1, the expression of the reporter gene luciferase was increased in a dose-dependent manner (Fig. 5). These results suggest that the activity of EAAT3 promoter is increased by RFX1 proteins. Since a previous study showed that RFX1 and RFX3 decreased the promoter activity of microtubule-associated protein 1A gene in non-neuronal cells but not in neuron-like cells (17), we repeated our experiments in SH-SY5Y cells, a well-established neuron-like cell line. The transfection of these cells with pRFX1 also induced a dose-dependent increase of luciferase expression (Fig. 5).

We then determined whether the effects of RFX1 on EAAT3 expression were specific. NRK-52E cells express endogenous EAAT2 mRNA (Fig. 1) and proteins (Fig. 6). When these cells were transfected with pRFX1, the expression of RFX1 proteins was time-dependently increased (2.2 ± 0.2-fold the control at 24 h after the transfection, n = 3, p < 0.05 as compared with control). However, there was no change in EAAT2 protein expression (Fig. 6).

EAAT3 is a neuronal EAAT in the mammalian brains. However, the expression of RFX1 in the mammalian brains has not been determined yet. We showed by Western blotting and immunohistochemistry that rat cerebral cortex, hippocampus, and cerebellum expressed RFX1 proteins (Fig. 7). The cerebral cortical neurons and Purkinje cells expressed abundant RFX1 proteins that were distributed in both cytosol and nuclei (Fig. 7B). We then determined whether RFX1 affected the expression of endogenous EAAT3 in neurons. As shown in Fig. 8, the expression of EAAT3 in the rat cortical neurons in culture was significantly decreased when RFX1 expression was down-regulated by the RFX1 antisense oligonucleotides. The RFX1 sense oligonucleotides did not significantly change the expression of RFX1 or EAAT3. These results suggest that RFX1 plays a role in maintaining the basal expression of endogenous EAAT3 in the neurons.

**DISCUSSION**

RFXs have been found to regulate the expression of many genes including interleukin-5 receptor α chain and proliferating cell nuclear antigen (18, 19). Mutations in the DNA binding domain of RFX5 cause bare lymphocyte syndrome or major histocompatibility complex antigen class II deficiency (8). RFX1, the prototypic mammalian RFX, has been shown to act as a potent transactivator of enhancer I for the hepatitis B virus major surface antigen gene in cells that are of liver origin (20). However, the physiological significance of RFX1–3 in the mammalian cells is not yet clear. It has been shown that X-box sequences for RFX1, RFX2, and RFX3 are very similar to that for DAF-19 and are different from those for RFX4 and RFX5 (8, 21). DAF-19 is the RFX homologue in...
C. elegans, and knockout of the daf-19 gene results in severe sensory defects (8). Thus, RFX1–3 may play a role in the mammalian central nervous system. However, up until now, there is no report documenting the effects of these RFXs on gene expression in cells of central nervous system origin. Based on the results generated from cell lines of different origins, it was concluded that RFX1 and RFX3 inhibited the expression of microtubule-associated protein 1A in cells of non-central nervous system origin, such as HeLa cells, but not in cells of central nervous system origin, such as TGW and Neuro2A cells (17). We showed that RFX1 enhanced the activity of EAAT3 promoter in C6 and SH-SY5Y cells and the expression of EAAT3 proteins in C6 cells. C6 and SH-SY5Y cells are of central nervous system origin, and SH-SY5Y cells are neuron-like cells. Moreover, neurons in rat brain expressed RFX1 proteins, and knockdown of RFX1 proteins decreased EAAT3 expression in the rat cortical neurons in culture. Thus, our results provide initial evidence that RFX1 regulates gene expression in the nervous cells.

The determination of the expression of a gene in a cell is multifactorial. The interaction between transcription factors and the promoter of the gene contributes to the process. In analyzing the promoter regions of human EAAT1, EAAT2, and EAAT3 genes, we found that the promoter region of EAAT3 but not the promoter regions of EAAT1 or EAAT2 contained a binding sequence for RFX1. Consistent with this finding, our results showed that RFX1 enhanced the expression of EAAT3 but not EAAT2. These results suggest that the effects of RFX1 on EAAT3 are specific. EAAT3 is mainly neuronally expressed in vivo (1). We showed that neurons of rat brain expressed RFX1 proteins. In addition, knockdown of RFX1 by the RFX1 antisense oligonucleotides also reduced the expression of EAAT3 in rat neurons in culture. These results suggest an important role of RFX1 in regulating the expression of EAAT3 in neurons. These results may also help us understand why EAAT subtypes are expressed in a cell-type specific manner.

RFX1 enhanced EAAT3 expression. This effect may be mediated by the interaction between RFX1 proteins and the EAAT3 promoter. RFX1 is a transcription factor. There were RFX1 binding complexes in the nuclear extracts of C6 cells. The promoter. RFX1 is a transcription factor. There were RFX1 binding complexes in the nuclear extracts of C6 cells. The X-box in the EAAT3 gene is a positively acting regulatory element for the expression of this gene. Positively acting regulatory elements of the X-box after binding to RFXs have been shown in the promoters for genes, such as proliferating cell nuclear antigen and microtubule-associated protein 1A (17, 19). Thus, the functional change of a promoter by RFXs is context-dependent. The mechanisms for this phenomenon are not clear. Various interactions among different functional domains in the RFX proteins and other regulatory factors recruited during the process may contribute to the development of this phenomenon.
Our findings may have significant physiological implications. EAATs, through their functions of uptaking glutamate under physiological conditions, maintain extracellular glutamate homeostasis (1). Inhibition of EAAT activity in brain slices or cell cultures increased the peak glutamate concentration in the synaptic cleft and prolonged the glutamate-induced current, leading to a slowed excitatory postsynaptic current decay at some synapses (3–5). In a recent study, inhibition of neuronal EAATs, i.e. EAAT3, in hippocampal slices decreased the inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA)-mediated inhibitory postsynaptic current and miniature inhibitory postsynaptic current due to a reduction of GABA synthesis because glutamate uptaken by neuronal EAATs is a substrate for GABA synthesis (22). These results are consistent with the data from an early study showing that antisense knockdown of EAAT3 induced epilepsy in rats and that the hippocampal slices from these rats had a decrease in GABA synthesis, total GABA levels, and miniature inhibitory postsynaptic current (23). Thus, EAAT3 plays a role in maintaining the balance of glutamate/GABA neurotransmission. Consequently, factors, such as RFX1, which can regulate EAAT3 expression and activity, may have important physiological functions.

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