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Neuronal Glutamate Transporter EAAT4 Is Expressed in Astrocytes

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ABSTRACT High-affinity excitatory amino acid transporters (EAATs) are essential to terminate glutamatergic neurotransmission and to prevent excitotoxicity. To date, five distinct EAATs have been cloned from animal and human tissues: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5. EAAT1 and EAAT2 are commonly known as glial glutamate transporters, whereas EAAT3, EAAT4, and EAAT5 are neuronal. EAAT4 is largely expressed in cerebellar Purkinje cells. In this study, using immunohistochemistry and Western blotting, we found that EAAT4-like immunoreactivity (ir) is enriched in the spinal cord and forebrain. Double-labeled fluorescent immunostaining and confocal image analysis indicated that EAAT4-like ir colocalizes with an astrocytic marker, glial fibrillary acidic protein (GFAP). The astrocytic localization of EAAT4 was further confirmed in astrocyte cultures by double-labeled fluorescent immunocytochemistry and Western blotting. Reverse transcriptase-polymerase chain reaction analysis demonstrated mRNA expression of EAAT4 in astrocyte cultures. Sequencing confirmed the specificity of the amplified fragment. These results demonstrate that EAAT4 is expressed in astrocytes. This astrocytic localization of neuronal EAAT4 may reveal a new function of EAAT4 in the central nervous system.

INTRODUCTION

Glutamate is the predominant excitatory amino acids (EAAs) in the mammalian central nervous system (CNS) and is involved in many important physiological processes and pathological conditions (Kanai, 1997; Robinson and Dowd, 1997). High-affinity excitatory amino acid transporters (EAATs) are essential to terminate glutamatergic neurotransmission and to prevent excitotoxicity (Danbolt, 2001; O'Shea, 2002). To date, five distinct EAATs have been cloned from animal and human tissues: EAAT1 (GLAST) (Arriza et al., 1994; Kawakami et al., 1994), EAAT2 (GLT-1) (Arriza et al., 1994; Shashidharan et al., 1994), EAAT3 (EAAC1) (Kanai and Hediger, 1992; Arriza et al., 1994), EAAT4 (Fairman et al., 1995), and EAAT5 (Arriza et al., 1997). EAAT family members display ~ 50–55% amino acid sequence identity. They each contain 10 hydrophobic domains, with both the N- and C-termini in the cytoplasm (Wahle and Stoffel, 1996).

The EAAT subtypes differ in regional, cellular, and developmental distribution (Robinson and Dowd, 1997; Schluter et al., 2002). EAAT1 immunoreactivity (ir) is highly abundant in cerebellar Bergmann glia, and EAAT2 ir is concentrated in astrocytes in the hippocampus and cerebral cortex (Rothstein et al., 1994; Lehre et al., 1995). EAAT3 is highly expressed in the cortex, hippocampus, caudate–putamen, and peripheral tissues (Rothstein et al., 1994). EAAT4 is confined to cerebellar Purkinje cells (Yamada et al., 1996; Dehnes et al., 1998), with little expression in other brain regions (Massie et al., 2001). EAAT5 is expressed predominantly in the retina (Arriza et al., 1997). EAAT1

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and EAAT2 are commonly known as glial glutamate transporters, whereas EAAT3, EAAT4, and EAAT5 are thought to be primarily neuronal (Rothstein et al., 1994; Anderson and Swanson, 2000; Maragakis and Rothstein, 2001).

The physiological and pathological roles of EAATs in the brain have been well characterized (Meldrum et al., 1999; Lievens et al., 2000; Maragakis and Rothstein, 2001; Trotti et al., 2001; Proper et al., 2002). However, little is known about the characterization of EAATs in the spinal cord. EAAT2 ir is restricted to and enriched in the spinal gray matter of rats, mice, and humans (Milton et al., 1997; Fray et al., 1998; Sasaki et al., 2001), whereas EAAT1 ir is primarily localized in superficial laminae of the dorsal horn (Rothstein et al., 1995; Vera-Portocarrero et al., 2002). EAAT3 ir is mainly localized in ventral horn motoneurons and dorsal horn superficial laminae. After spinal cord injury, rapid upregulation of EAAT1, EAAT2, and EAAT3 has been reported (Vera-Portocarrero et al., 2002). Whether EAAT4 is expressed in spinal cord remains elusive, although Western blot studies have indicated that EAAT4 is undetectable (Furuta et al., 1997) or weakly expressed (Nagao et al., 1997) in spinal cord homogenates.

In the course of investigating the role of EAATs in the spinal cord after injury, we observed that EAAT4 ir is enriched throughout the spinal cord of rats and mice. Further studies using double-labeled immunofluorescent staining, confocal image analysis, and reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrated that EAAT4 is expressed in astrocytes. This astrocytic localization of EAAT4 may reveal a new role of EAAT4 in the central nervous system.

MATERIALS AND METHODS

Cortical Astrocyte Cultures

Cortical astrocyte cultures were prepared using standard methods. Briefly, the cerebral cortices were dissected from the brain of P7 mouse pups. After removal of the meninges, the tissues were dissociated by trypsinization and trituration. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum and penicillin/streptomycin and plated on uncoated 75 cm^2 flasks at an approximate density of 50,000 cells/cm^2. The cultures were maintained in humidified 5% CO_2 incubator at 37°C. The medium was changed 24 h after plating and then every 3–4 days. The cells were grown and became confluent after 10–14 days. At this point, more than 90% of the adherent cells exhibited the flattened, polygonal appearance typical of type I astrocytes. Non-astrocytes were detached from the flasks by shaking and removed by changing the medium. The remaining cells were dissociated by trypsinization (0.1% trypsin-0.04% EGTA) and plated on uncoated six-well plates at a density of 10,000 cells/cm^2. The cells became confluent again 10–14 days after the plating.

RNA Isolation, RT-PCR, T-A Cloning and Sequencing

Total RNA was isolated from the snap-frozen tissues or cultured astrocytes with TRIzol reagent (Gibco-BRL, Rockville, MD). Two micrograms of RNA, pretreated with DNase I for 2 h at 37°C, was used to synthesize cDNA using SuperScript II reverse transcriptase with random hexanucleotide as primers. The EAAT4-specific primers were designed according to the known sequence of mouse EAAT4 (genebank number D83262): upstream primer, 5′-CCGCCAGATCAAGTACTTC-TCCT-3′; downstream primer, 5′-GCAGAGCTGGAAG-AGGTACCC-3′ (corresponding to nucleotides 427–450 and 1338–1361, respectively). A total of 35 reaction cycles (94°C for 30 s, 55°C for 45 s, 72°C for 1.5 min) was preceded by a hot start at 94°C for 3 min and concluded with an extension step at 72°C for 10 min. As negative controls, RNA without RT was used as template or the cDNA in the reaction mixture was substituted with water. Amplified products were analyzed by horizontal agarose gel electrophoresis and visualized via ethidium bromide staining. A 1 kb DNA marker was used as molecular weight reference.

The PCR fragment of expected size was excised from the agarose gel, purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), and ligated into pCR II vector (Invitrogen, Carlsbad, CA). After transformation of competent *E. coli* DH5α cells, the plasmid was extracted with the Miniprep Wizzard kit (Promega, Madison, WI). The cloned fragment was sequenced with the T7 promotor primer by the Perkin-Elmer ABI 373 DNA Sequencer with XL upgrade and Big Dye chemistry.

Mammalian Expression Vector Cloning and Transfection

N-terminally Flag-epitope-tagged pCMV-Tag vector (Stratagene, La Jolla, CA) constructs encoding rat EAAT4 (pCMV-Flag-rEAAT4) were generated by RT-PCR from rat forebrain RNA. N-terminally Myc-epitope-tagged pCMV-Tag vector (Stratagene) constructs encoding mouse EAAT4 (pCMV-Myc-mEAAT4) were generated by PCR from SuperScript mouse brain cDNA library (Invitrogen). All vectors were confirmed by sequencing, immunocytochemistry, and Western blot analysis.

The human embryonic kidney (HEK) 293T cell line or monkey kidney COS-7 cell line were maintained in high-glucose DMEM containing 10% fetal bovine serum and penicillin/streptomycin. For immunofluorescent microscopy, cells were seeded on eight-well plastic chamber slides. For Western blot and immunoprecipitation experiments, cells were seeded on 10 cm dishes. Cells were transfected the following day by the standard calcium phosphate precipitation.

Western Blot and Immunoprecipitation

Normal healthy adult Sprague-Dawley rats or C57BL/6 mice were anaesthetized in a chamber with halothane and decapitated. The spinal cords and brains were removed, frozen instantly in liquid nitrogen, and stored at −80°C until further processing. Dissection of gray and white matter of the spinal cord was performed as previously described (Hu et al., 1999).

Snap-frozen tissues, cultured astrocytes, or transfected HEK293T cells were solubilized in Triton X-100-based lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and phosphatase inhibitor cocktail I (Sigma, St. Louis, MO). Lysates were incubated through end-over-end mixing at 4°C for 30 min. The nuclear and cellular debris were cleared by centrifugation at 20,000 *g* for 20 min at 4°C. The protein concentrations of the supernatant were determined with *Dc* Protein Assay kit from BioRad (Hercules, CA). Proteins were denatured via boiling for 5 min in sodium dodecyl sulphate (SDS) sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) in tris-glycine buffer, and transferred to nitrocellulose membrane (BioRad). The SeeBlue prestained standard (Invitrogen) was used as molecular weight reference. Blots were blocked in 5% nonfat dry milk/tris-buffered saline (pH 7.6) plus 0.1% Tween-20 (TBS-T) for 1 h and then incubated overnight at 4°C with affinity-purified rabbit anti-rEAAT4 polyclonal antibody against a 21 aa synthetic peptide near the rEAAT4 C-terminus (1:1,000; Alpha Diagnostic, San Antonio, TX) in 2% milk/TBS-T. After incubation for 1 h with horseradish peroxidase-conjugated antirabbit antiserum (1/2,000; Amersham Biosciences, Piscataway, NJ) in 2% milk/TBS-T, immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences). All washing steps were performed with TBS-T.

For immunoprecipitation, the lysates from HEK293T cells overexpressing Flag-rEAAT4 were incubated overnight at 4°C with 0.5 μg anti-Flag (Sigma) monoclonal antibody or mouse control IgG. The immune complexes were precipitated with GammaBind G Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden), washed four times with 0.6 M NaCl lysis buffer, and then eluted by boiling for 5 min in SDS sample buffer. The eluted proteins were fractionated on SDS-PAGE and detected by Western blotting with anti-Flag monoclonal antibody (1:2,000) or anti-rEAAT4 polyclonal antibody (1:1,000).

Routine Immunohistochemistry

For immunohistochemical studies, free-floating sections of spinal cord and brain were stained as described previously (Hu et al., 1999; Hausmann et al., 2002). Briefly, animals were transcardially perfused with 4% paraformaldehyde. The spinal cords and brains were dissected and postfixed overnight in the same fixative. All tissue samples were cryopreserved with buffered 25% sucrose. Serial transverse or longitudinal sections were cut frozen on the sliding microtome at a thickness of 40 μm. The sections were permeated with 0.5% Triton X-100 and blocked with 10% normal goat serum for 30 min and then incubated with primary anti-rEAAT4 polyclonal antibody (1:1,000) overnight at 4°C. The following day, sections were washed and incubated with secondary goat antirabbit biotinated antibody (1:200) for 1 h. The sections were then incubated in ABC compound (Vector Laboratories, Burlingame, CA) followed by diaminobenzidine (DAB) staining. Staining specificity was determined through omitting the primary antibody or preabsorbing it with synthetic rEAAT4 peptide (Alpha Diagnostic) for additional sections in parallel experiment.

Immunofluorescent Staining and Confocal Image Analysis

For sequential double-labeled immunofluorescent staining, the cultured cells, after fixation with 4% paraformaldehyde for 30 min, or the tissue sections were pretreated with blocking solution of 10% normal goat serum and 0.5% Triton X-100 overnight at 4°C. The sections were incubated with the primary anti-rEAAT4 antibody at 1:400 in blocking solution overnight at 4°C. After washing, the Alexa Fluor 488 (green)-linked secondary goat antirabbit antibody (Molecular Probes, Eugene, OR) 1:400 was added for 1 h. Then different monoclonal antibodies against Flag epitope or various cell markers were used. Neurons were identified with an anti-MAP2 (Sigma) antibody at 1:200. Astrocytes were stained with an antiglial fibrillary acid protein (GFAP) antibody (Chemicon International, Temecula, CA) 1:400. Oligodendrocytes were stained with an anti-RIP antibody (DSHB, Iowa, IA) 1:40. The Alexa Fluor 594 (red) secondary goat antimouse antibody (Molecular Probes) 1:200 in blocking solution was used. Fluorescences were analyzed under the confocal Axiovert 100 M microscope with the LSM 510 scanning module (Carl Zeiss, Thornwood, NY).

Quantitative Image Analysis

The images from Western blot and RT-PCR were scanned and analyzed with the Quantity One software from BioRad. The data are presented as adjusted volume (intensity) times total area.

RESULTS

Characterization of Anti-rEAAT4 Antibody

To characterize the commercially available affinity-purified polyclonal antibody against the C-terminus of rEAAT4, we cloned full-length rEAAT4 into a Flag-

tagged mammalian expression vector, transfected it into HEK293T cells, and purified this fusion protein via immunoprecipitation with an anti-Flag antibody. Western blot analysis of the purified fusion protein with the anti-rEAAT4 polyclonal antibody and anti-Flag monoclonal antibody demonstrates that anti-rEAAT4 antibody specifically recognizes Flag-tagged rEAAT4 fusion protein (Fig. 1A). This antibody also recognizes endogenous EAAT4 in both rat spinal cord and brain, and the immunoreactive bands are completely eliminated when the antibody (0.5 μg/ml) was preabsorbed with 5 μg/ml synthetic rEAAT4 peptide (Fig. 1B). Western blotting of mouse brain and spinal cord tissues as well as Myc-tagged mouse EAAT4 fusion protein shows a similar pattern of immunoreactive bands (Fig. 1C). Both endogenous and exogenous expressions of EAAT4 are present largely as fuzzy multiple bands of higher molecular weight, even under reducing conditions (Fig. 1C). These multiple bands are observed predominantly in cerebellum as evidenced by Western blotting for different region of brain (Fig. 1D), which is in accord with other previous studies (Bristol and Rothstein, 1996; Yamada et al., 1996, 1997; Dehnes et al., 1998; Lin et al., 1998). However, both endogenous (Fig. 1B–D) and exogenous (Fig. 1A and C) EAAT4 appear as a lower-molecular-weight band roughly at ~ 60 kDa, which can also be blocked by a competitive peptide (Fig. 1B). A small difference in the mobility of the lower-molecular-weight band appears among tissues and transfected cells (Fig. 1C and D). In the cerebellum, the small band is not increased in proportion to the multiple bands when compared to other regions (Fig. 1D).

To confirm the specificity of anti-rEAAT4 antibody, double-labeled immunocytochemistry and confocal image analysis were performed in HEK293T and COS-7 cells overexpressing Flag-tagged rEAAT4 (Fig. 2) or Myc-tagged mEAAT4 (data not shown). As shown in Figure 2, the rEAAT4-like ir colocalizes with the Flag-like ir. The staining is predominantly on the plasma membrane, which is consistent with the predicted transmembrane topology of EAAT4 protein (Wahle and Stoffel, 1996) and the endoplasmic reticulum/Golgi apparatus (Fig. 2).

EAAT4 Protein Expression in Astrocytes of Spinal Cord and Brain

EAAT4 expression has been widely demonstrated in cerebellar Purkinje cells (Yamada et al., 1996; Dehnes et al., 1998; Massie et al., 2001). To test if neuronal EAAT4 is expressed in spinal cord, we performed routine immunohistochemical studies. The affinity-purified anti-rEAAT4 antibody stains extensively throughout the spinal cord of rat (Fig. 3) and mouse (data not shown). The staining pattern resembles astrocytes, not neurons. In the white matter, long and enriched network processes are immunoreactive for EAAT4 with highest levels appearing around the peripheral rim. In the gray matter, the positive processes approach blood vessels and neurons. Dilution of the primary antibody reduces the staining and preabsorption of anti-rEAAT4 antibody (0.5 μg/ml) with a synthetic rEAAT4 peptide (5 μg/ml) quenches the immunoreactivity (Fig. 3B).

To confirm the cellular distribution of EAAT4 ir, double-labeled immunofluorescent staining and confocal image analysis were performed. In the spinal cord, EAAT4-like ir is colocalized with the astrocytic marker GFAP (Fig. 4, top row) but not with a neuronal marker MAP2 (Fig. 4, second row from top) or an oligodendrocyte marker RIP (Fig. 4, third row from top). As a positive control for EAAT4 ir, the rat cerebellar cortex was stained (Fig. 4, bottom row). Consistent with previous reports (Yamada et al., 1996; Furuta et al., 1997; Itoh et al., 1997; Nagao et al., 1997; Dehnes et al., 1998), the Purkinje cells are intensively immunoreactive for EAAT4, whereas granule cells are not (Fig. 4, bottom row). This antibody also stains astrocytes extensively in forebrain and hindbrain (Fig. 5). Like GFAP staining, EAAT4 staining, with processes forming a dense network, is predominantly in the white matter, maximally in the subventricular zone and subpial layer.

EAAT4 Protein Expression in Cultured Astrocytes

To provide direct evidence for the presence of EAAT4 protein in astrocytes, mouse cortical astrocyte cultures were used for immunocytochemistry and Western blotting. EAAT4-like ir colocalizes with GFAP-positive astrocytes (Fig. 6A). Western blotting of cultured astrocytes shows the same size bands as that in spinal cord tissues (Fig. 6B). To confirm EAAT4 protein expression in astrocytes, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), which have been previously shown to upregulate EAAT expression (Zelenaia et al., 2000; Suzuki et al., 2001), were used to treat the cultured astrocytes. As shown in Figure 6B, treatment with bFGF (10 ng/ml) or EGF (50 ng/ml) for 24 h increases EAAT4 expression in cultured astrocytes.

EAAT4 mRNA Expression in Astrocytes

To confirm the presence of EAAT4 in astrocytes, RT-PCR analysis was performed in cultured astrocytes, demonstrating a strong band of the predicted size (Fig. 7). EAAT4 mRNA is also highly expressed in the mouse spinal cord and brain (Fig. 7). The amplified fragments from both astrocytes and spinal cord tissues were cloned into pCR II TA-cloning vector and sequenced with the T7 promotor primer. Analysis of the sequence through Genebank database demonstrates that the RT-PCR-amplified fragment exactly matches mouse EAAT4 and shares 94% and 89% homology to rat and human EAAT4, respectively.

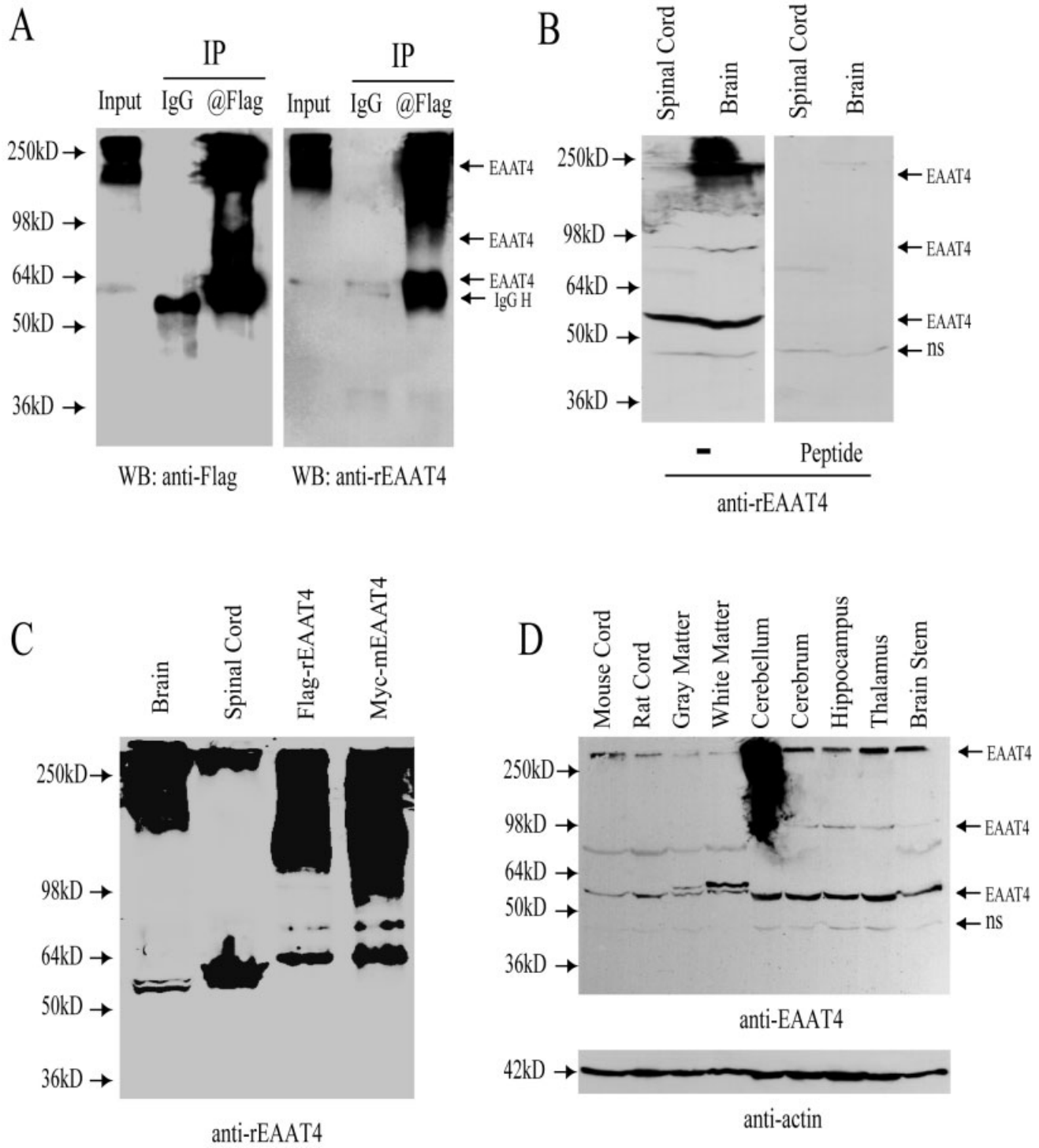

Fig. 1. Characterization of antirat EAAT4 antibody. **A:** Exact matching of rEAAT4 immunoreactivity with Flag-rEAAT4 fusion protein. HEK293T cells were transfected with pCMV-Flag-rEAAT4 vector by standard calcium phosphate precipitation. After 24 h, the overexpressed Flag-rEAAT4 fusion protein was immunoprecipitated (IP) with anti-Flag monoclonal antibody or control mouse IgG. Western blots (WB) were duplicated and analyzed separately with anti-Flag monoclonal antibody or anti-rEAAT4 polyclonal antibody. **B:** Peptide blockade of rEAAT4 immunoreactivity. The whole homogenates of rat spinal cord and brain at amount of 50 μg protein were resolved in 10% SDS-PAGE and Western blotting was performed with anti-rEAAT4 antibody (0.5 μg/ml). Preabsorption with rEAAT4 peptide (5 μg/ml) completely abolished the specific band of EAAT4 immunoreactivity. Ns: nonspecific band. **C:** Comparison of endogenous and exogenous expression of EAAT4. The lysates from mouse brain and spinal cord and HEK293T cells overexpressing Flag-rEAAT4 or Myc-mEAAT4 fusion protein were immunoblotted with anti-rEAAT4 antibody. **D:** Regional distribution of EAAT4 in spinal cord and brain. Proteins are loaded at 50 μg each lane followed by Western blotting with anti-rEAAT4 antibody. The same blot was reprobed with anti-actin monoclonal antibody after stripping.

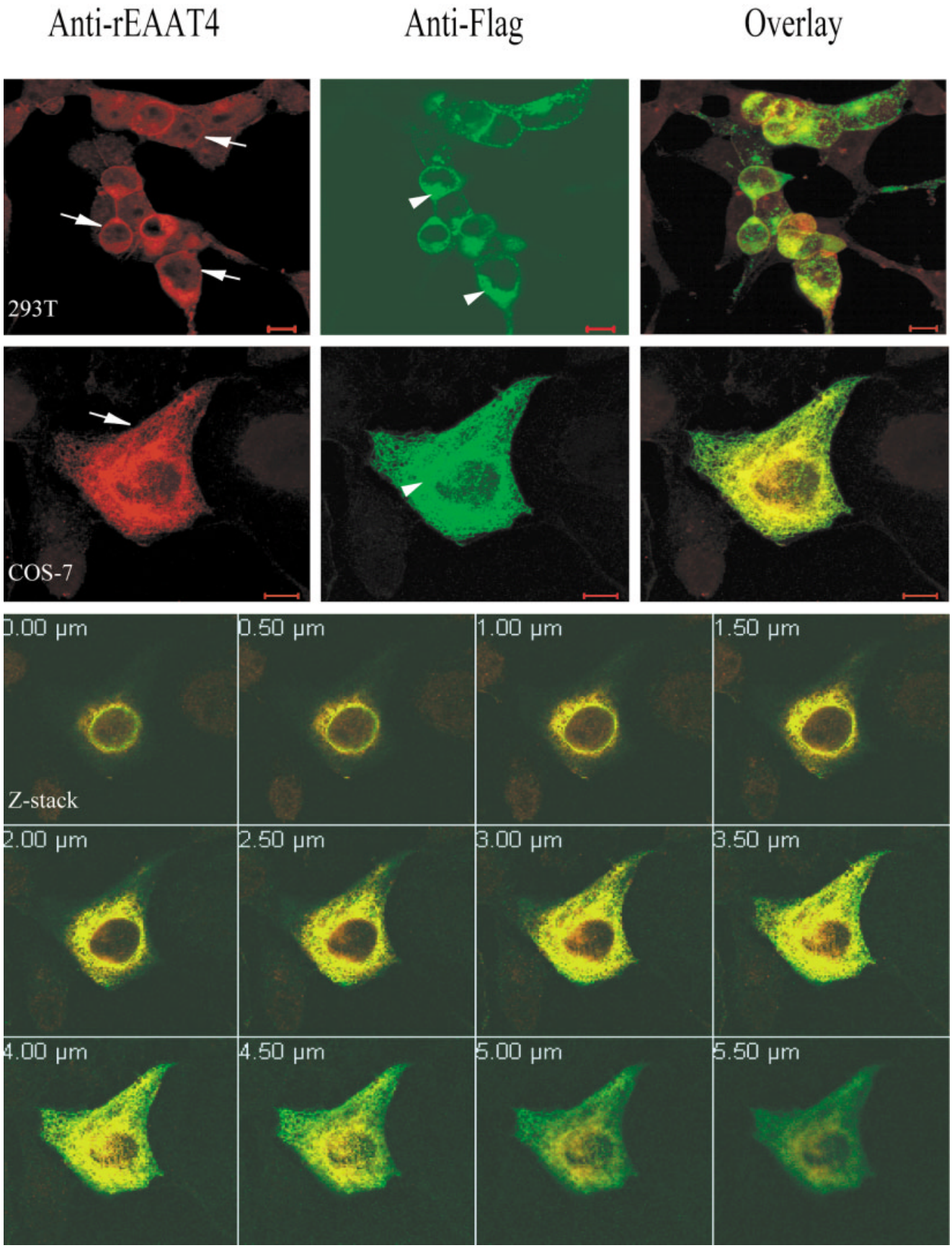

Fig. 2. Colocalization of rEAAT4-like immunoreactivity with Flag-rEAAT4 fusion protein. HEK293T or COS-7 cells were transfected with pCMV-Flag-rEAAT4 and 24 h later sequential double-labeled immunofluorescent staining and confocal image analysis were performed. Note the staining around the plasma membrane (arrow) and endoplasmic reticulum/Golgi apparatus (arrow head). Scale bar, 10 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

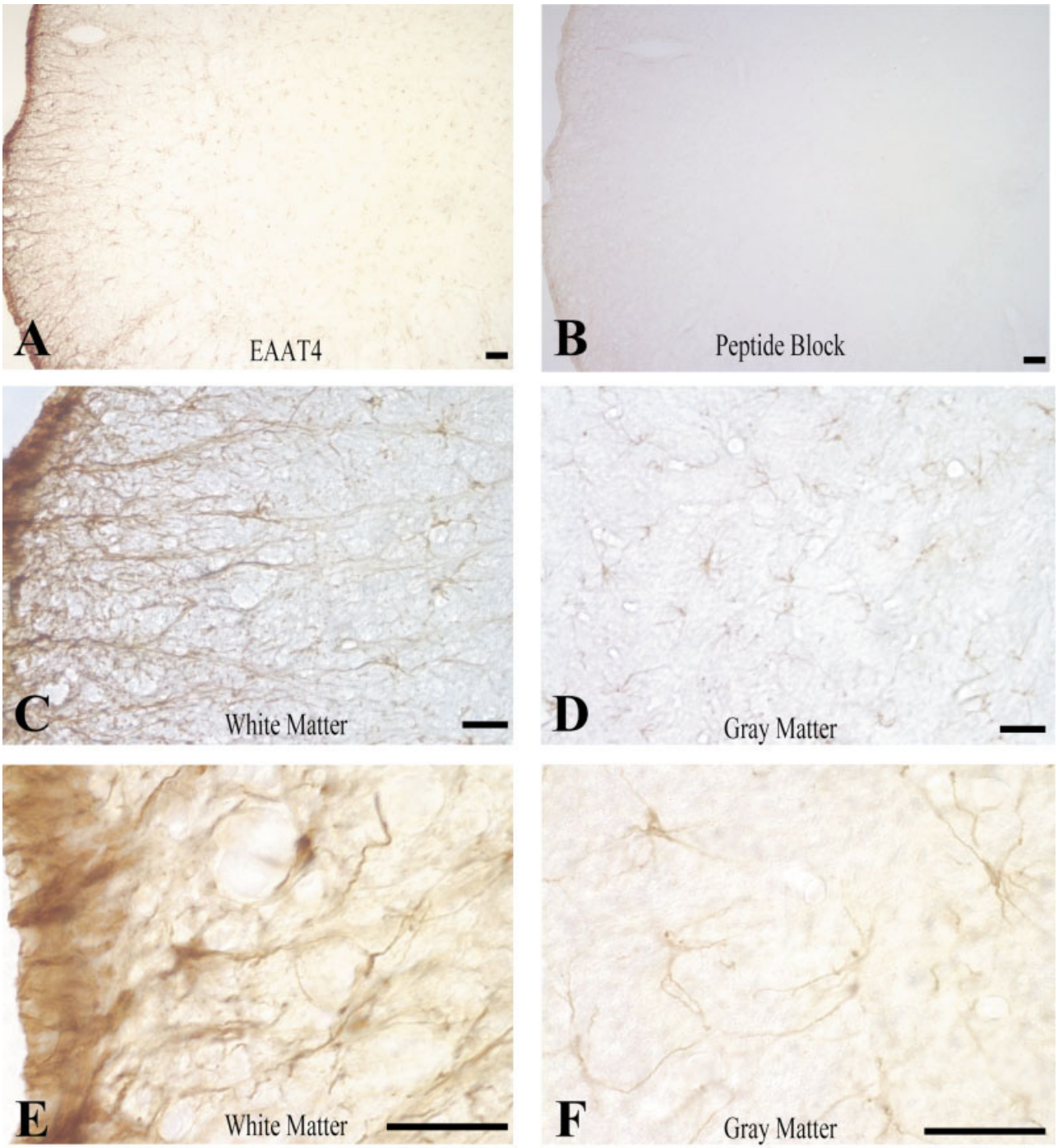

Fig. 3. EAAT4 immunohistochemical staining in rat spinal cord. Affinity-purified anti-rEAAT4 antibody stains extensively throughout the spinal cord. The staining pattern resembles astrocytes but not neurons. Preabsorption of anti-rEAAT4 antibody (0.5 μg/ml) with an rEAAT4 peptide (5 μg/ml) quenches the immunoreactivity. **C/E** and **D/F** are amplified from the white matter and gray matter in **A**, respectively. **B:** Peptide block. Scale bar, 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

DISCUSSION

The important finding in this study is that the previously described neuronal glutamate transporter EAAT4 is expressed in astrocytes at both protein and mRNA levels. We used a combination of immunostaining, Western blotting, RT-PCR, and sequence analysis to demonstrate EAAT4 expression in astrocytes.

The affinity-purified anti-rEAAT4 antibody used in this study is a commercially available polyclonal antibody raised against a synthesized peptide from the cytoplasmic C-terminus of rat EAAT4, which shares

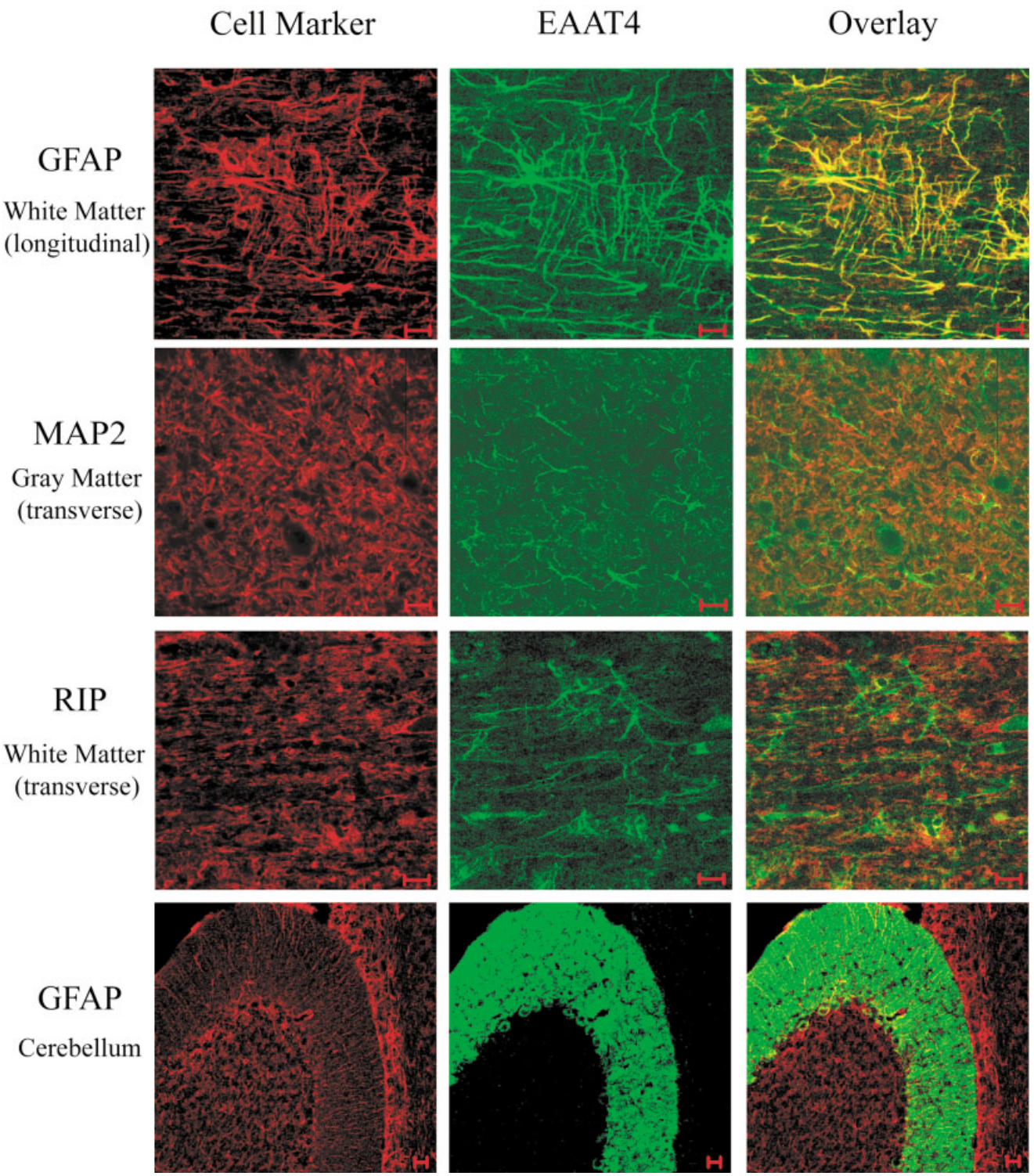

Fig. 4. Double-labeled immunofluorescent staining of rat spinal cord and confocal image analysis. EAAT4-like immunoreactivity is totally colocalized with GFAP-like immunoreactive astrocytes in rat spinal cord (top row) but not with neuronal marker MAP2 (second row from top) and oligodendrocyte marker RIP (third row from top). As a positive control in cerebellum, EAAT4 is expressed in Purkinje cells (bottom row). Scale bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

100% homology to mouse EAAT4 and 95% homology to human EAAT4 but no homology to the other subtypes of EAATs (Lin et al., 1998). This antibody recognizes the purified Flag-tagged rEAAT4 fusion protein as shown by immunoprecipitation and Western blotting with an anti-Flag antibody. Double-labeled confocal

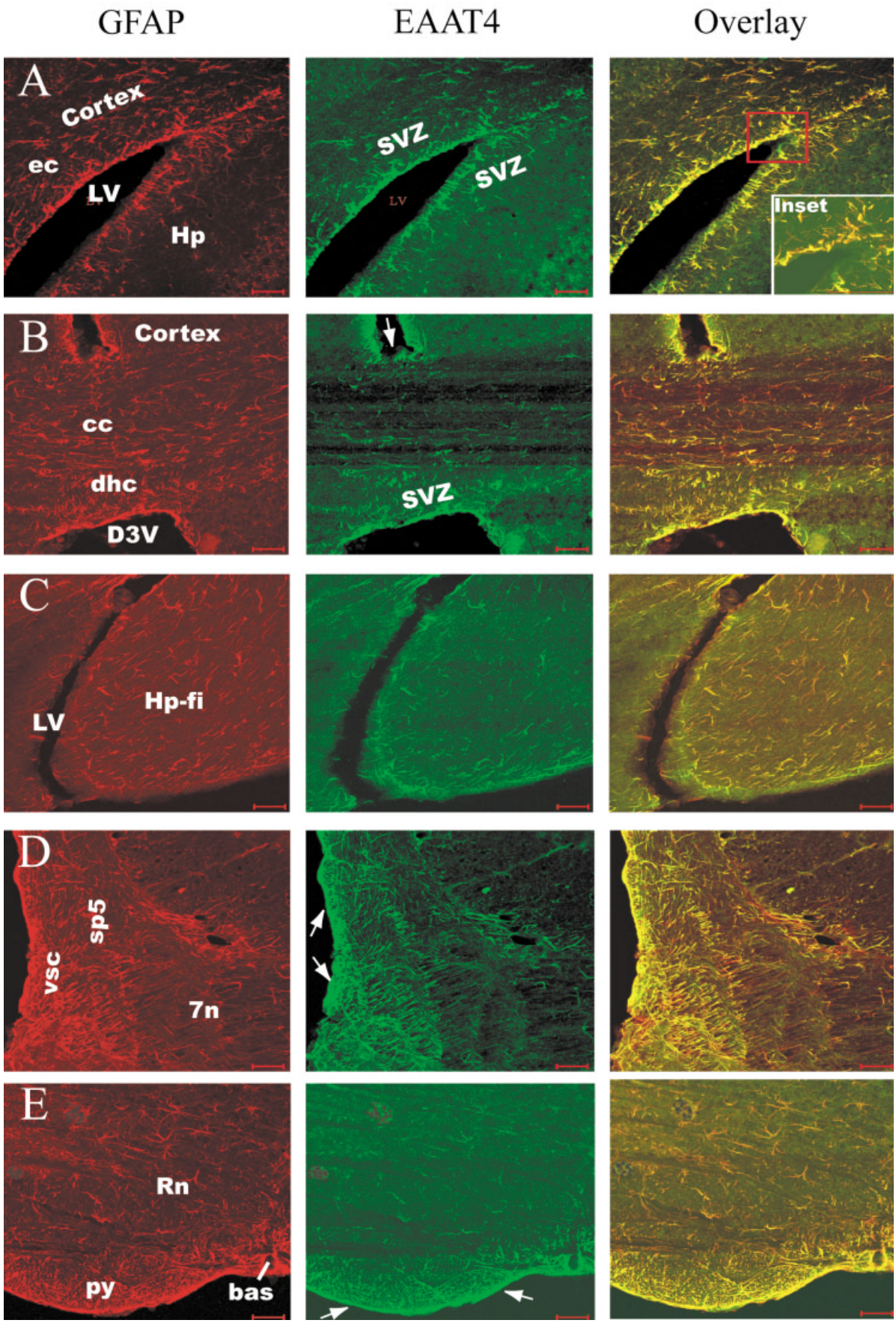

Fig. 5. Double-labeled immunofluorescent staining of mouse brain and confocal image analysis of selective regions of white matter in forebrain (**A**–**C**) and hindbrain (**D** and **E**). EAAT4-like immunoreactivity (green) is predominantly in subventricular zone (SVZ) and subpial region (arrow) and colocalizes with many GFAP-positive cells (red). bas, basilar artery; cc, corpus callosum; D3V, dorsal third ventricles; dhc, dorsal hippocampal commissure; ec, external capsule; 7n, facial nucleus; fi, fimbria of hippocampus; Hp, hippocampus; LV, lateral ventricles; py, pyramidal tract; Rn, raphe nucleus; sp5, spinal trigeminal tract; vsc, ventral spinocerebellar tract. Scale bar, 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

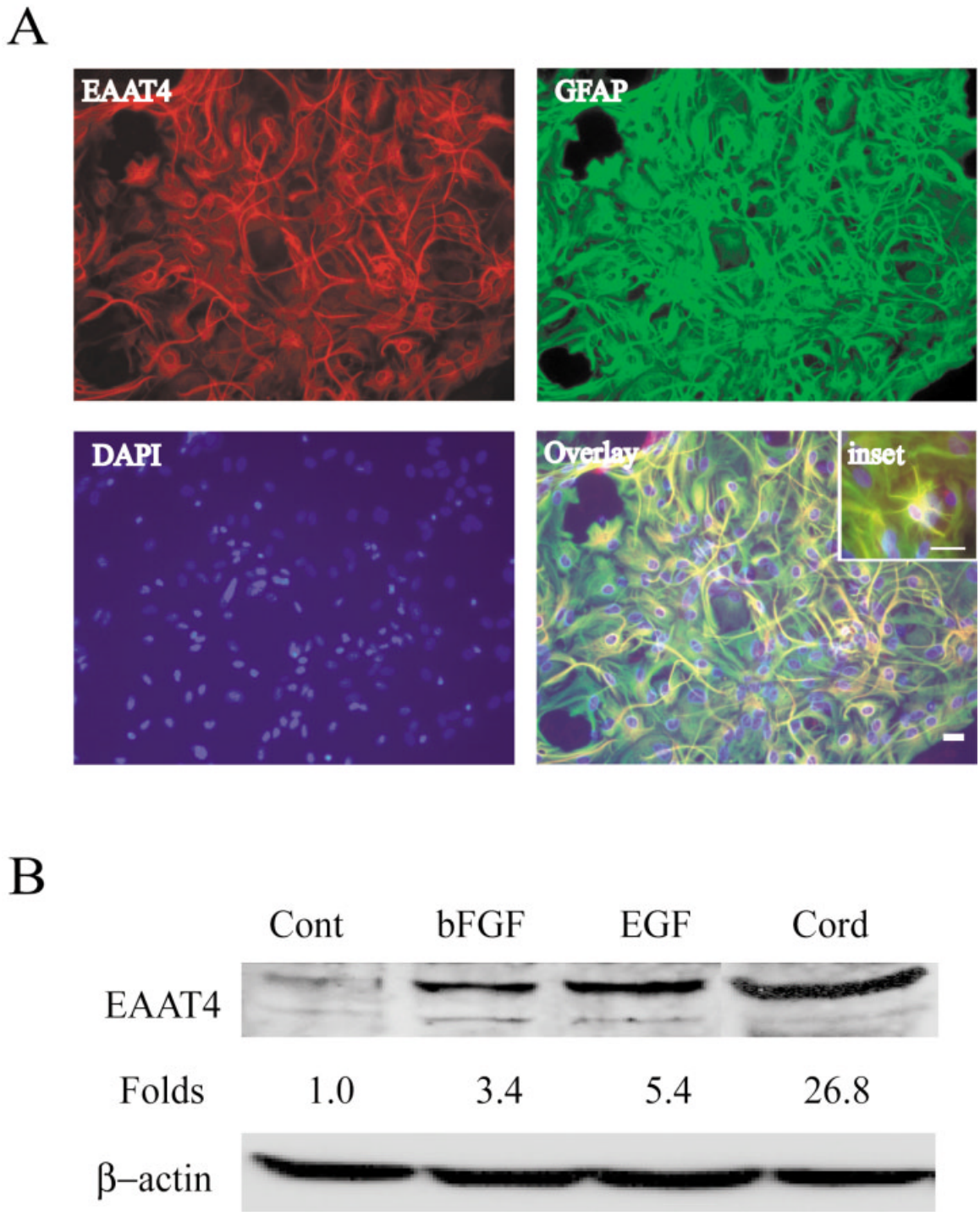

Fig. 6. EAAT4 protein is expressed in cultured cortical astrocytes. **A:** Double-labeled immunofluorescent staining and DAPI staining. Astrocytes were prepared from P7 mouse pups and maintained for 28 days. The insect shows another field at higher amplification. Scale bar, 50 μm. **B:** Western blot analysis. Cultured astrocytes were treated with or without bFGF 10 ng/ml or EGF 50 ng/ml for 24 h and the whole cell lysates were analyzed by Western blot with anti-rEAAT4 polyclonal antibody and the same blot was reprobed with anti-β-actin monoclonal antibody. The whole lysates from rat spinal cord was used as control. Number between the panels represents the relative level compared with the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

image analysis of Flag-rEAAT4 fusion protein confirms the colocalization of EAAT4-like and Flag-like immunoreactivities. The endogenous EAAT4 in spinal cord and brain tissues appears as the same bands (including small and multiple large bands) as the exogenous Flag-rEAAT4 fusion protein in Western blotting with this anti-rEAAT4 antibody (Fig. 1). These studies strongly suggest that the commercially available antibody rec-

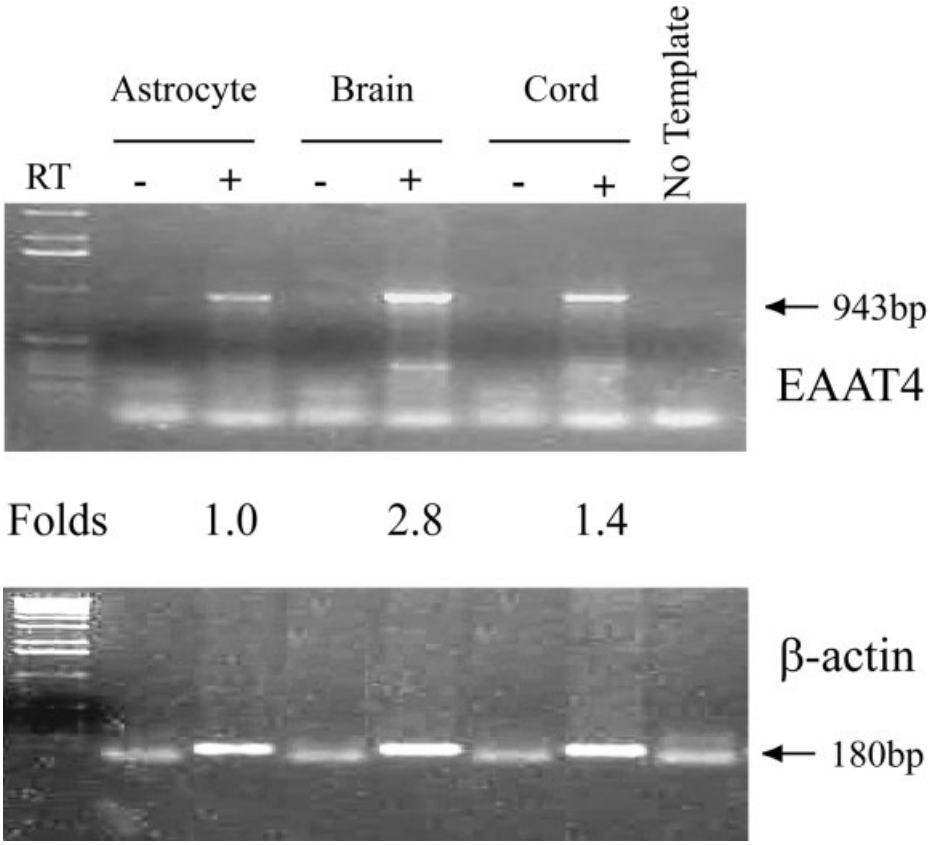

Fig. 7. EAAT4 mRNA expression in mouse astrocyte cultures and spinal cord tissues. The cDNA was reverse-transcribed (RT) from equal amount of total RNA pretreated with DNase I for 2 h and amplified by PCR with mouse EAAT4 primers. No template represents negative control. The amplified fragments were confirmed by sequencing. Number between the panels represents the relative level of mRNA expression.

ognizes EAAT4 expressed endogenously and exogenously.

In the cerebellum and transfected cells, multiple immunoreactive bands of higher molecular weight were observed for EAAT4, consistent with multiple bands for EAAT1 and EAAT2 (Haugeto et al., 1996; Dehnes et al., 1998; Danbolt, 2001). These higher bands may be attributed to oxidation of sulf-hydral groups and irreversible crosslinking to form large molecular mass aggregates (Haugeto et al., 1996; Dehnes et al., 1998). However, these higher bands from both the cerebellum and transfected cells could not be separated in our experiments using various reducing protocols as previously described (Dehnes et al., 1998; Massie et al., 2001). The chemical properties of the EAAT4 aggregates remain to be elucidated.

A smaller band in our Western blotting experiments was observed for both endogenous and exogenous expression of EAAT4 (Fig. 1). The molecular weight is approximately ~ 60 kDa, the predicted size based on the amino acid sequence (561 residues) of rEAAT4 (Maeno-Hikichi et al., 1997; Lin et al., 1998), which is in agreement with several previous reports (Nagao et al., 1997; Schlag et al., 1998). However, other reports have shown that EAAT4 has a molecular weight of ~ 65–70 kDa (Furuta et al., 1997; Dehnes et al., 1998). In the present study, a difference in the mobility of the small band was also observed among tissues and transfected cells (Fig. 1C and D). It is also reported that a single band in astrocyte-enriched cultures is 20 kDa smaller than that in cerebellar homogenates (Schlag et al., 1998). These differences, though elusive, might be due to the variable protein amounts loaded and different crosslinking status (Haugeto et al., 1996; Schlag et al., 1998). Why the band at the appropriate molecular weight in the cerebellum is not increased in proportion to the EAAT4 multimer band compared with other tissues may be due to the irreversible crosslinking. To confirm the specificity of these small bands, peptide sequencing would be required.

To date, there are no detailed studies on the expression of EAAT4 in the spinal cord (Furuta et al., 1997; Nagao et al., 1997). The present study provides the first evidence that EAAT4 is expressed in spinal cord astrocytes. The immunohistochemical studies detect EAAT4-like ir in the astrocytes of both white matter and gray matter in the spinal cord. The strongest astrocytic immunostaining was localized around the rim of white matter, indicating that the white matter may be more resistant to excitotoxic injury by removing

glutamate through EAAT4. This may provide a possible explanation for the peripheral rim of spared tissue and axons in the spinal cord after injury in animals and human (Beattie and Bresnahan 2000).

One previous report mentioned in the discussion that EAAT4-like ir was found at low level in forebrain astrocytes (Furuta et al., 1997). In the present study, we demonstrate that EAAT4 ir is extensively expressed in astrocytes of forebrain and hindbrain, especially in subventricular zone and subpial region. The astrocytic expression of EAAT4 protein is supported by previous observations in rat astrocyte-enriched cortical cultures (Schlag et al., 1998) and the present study using mouse cortical astrocytes. We also show that EAAT4 is expressed at the mRNA level in mouse astrocytes. Although previous studies have shown that EAAT4 mRNA is expressed in brain tissue (Bristol and Rothstein, 1996; Yamada et al., 1997; Lin et al., 1998; Massie et al., 2001), the present study is the first demonstration that EAAT4 mRNA is expressed in spinal cord tissue. Interestingly, the distribution of EAAT4 ir in adult forebrain reveals a noticeable similarity to the expression of Lewis X, a marker for neural stem cells (Capela and Temple, 2002). The maximal expression of EAAT4 ir in subventricular zone hints at its correlation to neural stem cells. The potential role of EAAT in neurogenesis deserves further investigation.

It is generally accepted that EAAT1 and EAAT2 are glial while EAAT3, EAAT4, and EAAT5 are neuronal (Rothstein et al., 1994; Anderson and Swanson, 2000; Maragakis and Rothstein, 2001). However, this interpretation has been challenged by recent studies. While EAAT2 is primarily an astrocytic transporter, a splice variant of EAAT2 is preferentially expressed in neurons and nonastrocytic glial cells in the same cellular and subcellular distribution as EAAT3 (Schmitt et al., 2002). EAAT2 is the predominant nerve terminal glutamate transporter (Suchak et al., 2003). Despite the abundance of EAAT2 in the CNS, examples of its expression in vitro as determined in primary astrocytes (Gegelashvili et al., 1997; Swanson et al., 1997), rat C6 glioma cell line, and human U373 astrocytoma cell line (Dowd et al., 1996; Palos et al., 1996; Dunlop et al., 1999) cultures are limited. Protein expression of EAAT2 has been described in cultures of primary hippocampal and cortical neurons (Mennerick et al., 1998; Wang et al., 1998) and the NT2 cell line (Dunlop et al., 1998). These cultures exhibit L-glutamate transport activity sensitive to dihydrokainate, a selective EAAT2 inhibitor (Dunlop et al., 1998; Mennerick et al., 1998; Wang et al., 1998). EAAT1 is also detectable in cultured hippocampal neurons (Perego et al., 2000; Plachez et al., 2000). In contrast, EAAT3 expression has been shown in some astrocytes (Conti et al., 1998), rat C6 glioma cell line (Dowd et al., 1996; Palos et al., 1996), and human U373 astrocytoma cell line (Dunlop et al., 1999). EAAT4 has been found in cultured cortical astrocytes (Schlag et al., 1998). EAAT5 is expressed in both neurons and glias (Arriza et al., 1997). In the present study, we demonstrate that EAAT4 is expressed in astrocytes of spinal cord and brain, whereas the neuronal expression of EAAT4 is predominantly in the cerebellum (Bristol and Rothstein, 1996; Yamada et al., 1996, 1997; Dehnes et al., 1998; Lin et al., 1998) and, to a less extent, in cortical neurons (Massie et al., 2001). Taken together, the segregation of neuronal and glial EAATs is no longer tenable.

It is currently interpreted that EAAT3 may act primarily as a source of metabolic glutamate for neurons and EAAT2 may mediate most of forebrain glutamate transport, whereas EAAT1 may be important in the cerebellum during development. Since glutamate has neurotrophic as well as neurotoxic activities, it is thus of primary importance to understand the role of the different glutamate transporter subtypes in controlling the extracellular glutamate concentration during development, neurotransmission, and neurotoxicity. Astrocytes play a key role in removing excess extracellular glutamate. The regional distribution of EAAT4 in the spinal cord is different from the previously reported astrocytic transporter EAAT1 and EAAT2, which are predominantly localized in the gray matter of spinal cord (Rothstein et al., 1995; Milton et al., 1997; Fray et al., 1998; Sasaki et al., 2001; Vera-Portocarrero et al., 2002). Therefore, EAAT4 may be an important regulator for the glutamate transmission and excitotoxicity in the spinal cord, especially in the white matter.

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