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A New Monoclonal Antibody, A3B10, Specific For Astrocyte-Lineage Cells Recognizes Calmodulin-Regulated Spectrin-Associated Protein 1 (Camsap1)

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Recent studies of adult neurogenesis of the mammalian central nervous system have suggested unexpected plasticity and complexity of neural cell ontogenesis. Redefinition and reconstitution of cell classification and lineage relationships, especially between glial and neural precursors, are an urgent and crucial concern. In the present study, we describe a new monoclonal antibody, A3B10, which was produced by immunizing mice with the membrane fraction prepared from astrocyte-enriched primary neural cell cultures. Immunohistochemistry of brain sections, including brains from glial fibrillary acidic protein (GFAP)-deficient mice and primary mixed neural cell cultures, as well as immunoblot analysis and immunoelectron microscopy, have revealed that 1) A3B10 recognizes a majority of cells in ependyma in neonatal and adult rats, 2) A3B10 stains almost all $GFAP^+$ cells and some $S100\beta^+$ cells in the corpus callosum, 3) A3B10 specifically stains astrocytes in vitro in primary cultures of rat embryonic cerebral hemispheres, 4) A3B10 equally stains ependymal cells of wild-type and GFAP-deficient mice, and 5) A3B10 antigen might construct intermediate filament bundles with GFAP and/or vimentin. These data suggested that the antibody labels a wide array of astorcytic-lineage cells including astrocytes, astrocyte precursors, and neural stem cells. Screening a cDNA library derived from rat embryonic brain has revealed that the antibody recognizes calmodulin-regulated spectrin-associated protein 1 (Camsap1). Thus this antibody may provide not only a new marker to identify astrocyte-lineage cells but also a new target molecule to elucidate the ontogeny, development, and pathophysiological functions of astrocyte-lineage cells. © 2008 Wiley-Liss, Inc.

Key words: immunohistochemical marker; neurogenesis; neural stem cells; astroglia

Large numbers of new neural stem cells (NSCs) are generated in the postnatal subventricular zone (SVZ; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Alvarez-Buylla and Garcia-Verdugo, 2002; Bedard and Parent, 2004). The phenotypes of these stem cells have been the subject of intense research, and it is well accepted that some glial fibrillary acidic protein (GFAP)-expressing cells are stem cells in rodent and human SVZ (Doetsch et al., 1999; Magavi et al., 2000; Laywell et al., 2000; Alvarez-Buylla et al., 2001; Skogh et al., 2001; Capela and Temple, 2002; Imura et al., 2003; Garcia et al., 2004; Liu et al., 2006). Although these GFAP-expressing cells have several features in common with astrocytes, it remains to be determined whether these stem cells are astrocyte-lineage cells, a new class of cells, or cells in a transitional stage from radial glia (Alvarez-Buylla et al., 2001; Ganat et al., 2006; Liu et al., 2006). Furthermore, recent studies of embryonic and adult neurogenesis in mammalian central nervous system have suggested unexpected plasticity and complexity of neural cell ontogenesis (Sohur et al., 2006). Redefinition and reconstitution of cell classification and lineage relationships, especially between glial and neural precursors, is an urgent and crucial concern.

The problem of astrocyte identity is quite confusing, because astrocytes are unusually multifunctional (D'Ambrosio et al., 1998; Matthias et al., 2003; Kimelberg, 2004; Wallraff et al., 2004; Zhou et al., 2006).

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Although GFAP is generally accepted as a marker for astrocytes, GFAP-negative populations are believed to exist in astrocytes (Liu et al., 2006; Sergent-Tanguy et al., 2006). As described above, some GFAP-positive neuronal stem cells are clearly discernible from conventional astrocytes, whereas young astrocytes have a neurogenetic property (Laywell et al., 2000). There are also limitations in the specificity and usability of other astroglial markers, including S100β, GLAST, GLT1, glutamate synthetase, and aquaporin-4 (Kimelberg, 2004).

Here we describe a new monoclonal antibody, A3B10, which recognizes a majority of cells in ependyma in neonatal and adult rats. Although detailed analysis of specificity and usability of this antibody will be performed in the future, it seems to label a wide array of astrocytic-lineage cells. Screening a cDNA library of rat embryonic brain has revealed that the antibody recognizes calmodulin-regulated spectrin-associated protein 1 (Camsap1). Thus this antibody might provide not only a new marker to identify astrocyte-lineage cells but also a new target molecule to elucidate the ontogeny, development, and pathophysiological functions of astrocyte-lineage cells.

MATERIALS AND METHODS

Animals

Pregnant and normal Wistar rats of both sexes at different ages (Japan SLC, Shizuoka) were used. The day of birth was defined as postnatal day zero (P0). The day of conception, E0, was estimated by the presence of a vaginal sperm plug. GFAP-deficient mice (Gomi et al., 1995) and C57BL/6 wild-type mice were obtained from Riken BioResource Center (Tsukuba, Ibaraki, Japan) and Japan SLC, respectively. The experimental protocol was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Tokyo Metropolitan Institute of Gerontology.

Cell Culture

The cerebral hemispheres from E17 rat embryos were dissected and dissociated enzymatically in a solution of dispase II (0.3 mg/ml; Boehringer Mannheim, Mannheim, Germany) and 0.05% DNase (Boehringer Mannheim) in Dulbecco's modified Eagle's medium (DMEM; BRL, Rockville, MD). After being washed with DMEM, the dissociated cells were sieved through nylon mesh with a 70-μm pore size (No. 2350; Becton Dickinson, Franklin, NJ) and seeded onto poly-L-lysine-coated culture dishes (1.4×10^7 cells/60-mm dish) in DMEM containing 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS). After 7 days of culture, the cells were passaged with 0.25% trypsin in PBS, centrifuged for 10 min at 500*g* at 4°C, and seeded in tissue culture dishes at a density of 1×10^6 cell/dish. The resultant culture contained predominantly astrocytes, with a small number of oligodendrocyte progenitor cells (OPCs) and neurons. These cells were used for antigen preparation and for immunocytochemistry. For antigen preparation, the membrane fraction was prepared as described previously (Yoshimura et al., 1996).

Production of Monoclonal Antibody A3B10

The monoclonal antibodies (mAb) were produced as previously described (Yoshimura et al., 1996). BALB/c mice were injected intravenously three or four times at 2-week intervals with the membrane fraction prepared as described above (under Cell culture). The splenocytes isolated from immunized animals were fused with a myeloma cell line NS1 (American Type Culture Collection, Rockville, MD) and the clonal specificity for certain brain loci, including epndeyma, were detected by immunohistochemical screening of the culture supernatant against the rat brain section. Positive clones were subcloned further by limiting dilution to ensure monoclonality. Based on its specific immunohistochemical pattern, a clone, A3B10, was chosen for detailed characterization. The A3B10 antibody was found to be an IgM antibody by using a mouse monoclonal antibody isotyping kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

Immunocytochemistry and Microscopy

Wistar rats were anesthetized with pentobarbital (40 mg/kg) and subjected to perfusion with lactating Ringer's solution and phosphate-buffered saline (PBS; 100 ml/100 g body weight) containing 4% paraformaldehyde via the left cardiac ventricle to flush out blood. Brain sections obtained from the rats were fixed in 4% paraformaldehyde-PBS solution. The samples were processed for immunohistochemistry as previously described (Yoshimura et al., 1998, 2001). Antibodies used were monoclonal antibodies against A3B10, GFAP (clone 6F2; Dako Japan, Tokyo, Japan), and vimentin (clone V9; Sigma Aldrich Japan, Tokyo, Japan) and anti-GFAP rabbit polyclonal antibody. Secondary antibodies, including goat anti-mouse IgG or anti-mouse IgM, and anti-rabbit IgG conjugated with rhodamine, fluorescein, or horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Fluorescent sections were examined under an Olympus BH-2 fluorescent microscope (Olympus, Tokyo, Japan) or by confocal laser scanning microscopy (CLSM; Radiance 2000; Bio-Rad, Hercules, CA). The sections labeled with peroxidase-conjugated antibodies were incubated in 3,3-diaminobenzidine and H_2O_2 to provide the chromogen, washed in running tap water, and lightly counterstained in methyl green. The specificity of all antibodies immunoreactions was confirmed by evaluating control sections that were processed without primary antibodies.

Immunoblot Analysis

Immunoblotting was performed according to a method previously described (Asou et al., 1996). Rat brain tissues were homogenized in a tenfold volume of 0.5% Noidet P-40/0.15 M NaCl/20 mM/Tris-HCl (pH7.4). The homogenate was kept on ice for 60 min and centrifuged at 40,000*g* for 60 min at 4°C. The protein concentration of each lysate was measured using micro-BCA protein assay reagent (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 6% polyacrylamide gel (Dauchi-Chem, Inc., Tokyo, Japan). Protein bands were electroblotted onto PVDF membranes and further processed according to the standard procedure. After blocking

with 5% nonfat skim milk in TTBS (10 mmol/liter Tris-HCl, pH 7.5, 140 mmol/liter NaCl, 0.05% Tween 20) for 1 hr at room temperature, the membrane was incubated overnight at 4°C with primary antibody in TTBS containing 1% normal horse serum. The membrane was then washed with TTBS and incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG or IgM and anti-rabbit IgG; see above). After washing with TTBS, the blot was developed using an ECL detection kit (Amersham Life Science, Tokyo, Japan) and Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY).

Immunoelectron Micrography

Primary and secondary antibody incubation was done for 2 days at 4°C each. For single staining, anti-GFAP rabbit IgG or A-3B10 antibody was used as the primary antibody, and anti-rabbit Ig or anti-mouse Ig antibody conjugated to 1.4-nm gold particles (1/100 each; Nanoprobes, New York, NY) was used as the secondary antibody, respectively. Sections were subjected to a silver-enhancement technique using gold particles (Kitada et al., 2001). For double staining, sections were incubated with anti-GFAP rabbit IgG and A3B10 mouse IgM antibodies, followed by anti-rabbit IgG conjugated to horseradish peroxidase (HRP; 1/200) and anti-mouse IgM conjugated to 1.4-nm gold particles (1/100; Nanoprobes). Sections were treated with the silver-enhancement technique, and then the HRP reaction was carried out with 3,3′-diaminobenzidine (DAB; Kitada et al., 2001). For embedding on epoxy resin, sections were postfixed with 1% osmium tetroxide in 0.1 M PB, dehydrated with sequential concentrations of ethanol, and embedded on Epon (Nacalai). Ultrathin sections were cut and stained with lead citrate and observed using an electron microscope (JEM 1200 EX; JEOL). Detailed protocols are available upon request.

Immunoscreening, Cloning, and Characterization of A3B10 Antigen

The cDNA library was constructed from E18 rat brain using λgt11 according to the manufacturer's protocol. Immunoscreening was performed according to a published protocol (Yoshimura et al., 1996, 1998, 2001), with minor modifications. The library was plated out at a density of 75,000 plaques/150-mm plate using *Escherichia coli* XL1 blue, grown at 42°C, overlaid with nitrocellulose membranes (Amersham Life Science) impregnated with 1 mM isopropyl β-D-thiogalactopyranaside (IPTG; Sigma) and grown at 37°C for 4 hr. The membranes were removed and blocked with BlockAce (Dainippon Chemicals, Osaka, Japan). The membranes were then incubated with A3B10 mAb. Alkaline phosphatase-conjugated anti-mouse IgM was used as the secondary antibody and nitroblue tetrazorium (NBT) and 5-bromo-4-chrolo-3-indolyl phosphpate (BCIP) were used for visualization. Only the clones reacting specifically with A3B10 mAb were selected and rescreened twice at a lower density. PCR was performed on the positive clone 3B10-13, and the PCR product was cloned directly into the pCR T7 TOPO TA expression vector (Invitrogen, Carlsbad, CA). The cDNA library was screened using the 3B10-13 DNA sequence as a DNA probe, and four additional clones were obtained. The PCR products of these clones were sequenced using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). These clones have overlapping sequences, and reconstitution of the sequences gave a 5,010-nucleotide sequence. This sequence was examined for homology with sequences in the GenomeNet protein and nucleic acid databases (http://www.genome.jp/en/) using the BLAST program. Searches for gene information, expression profiles, and amino acid motifs were performed using EntreGenes (NCBI; http://www.ncbi.nlm.nih.gov/sites/entrez), EST profile viewer (NCBI), and INTERPRO (EMBL-EBI; http://www.ebi.ac.uk/interpro/index.html), respectively.

RESULTS

Immunohistochemistry of SVZ Tissue by A3B10

Immunohistochemical localization of A3B10 reactive antigen in the subventricular zone (SVZ) facing the dorsal aspect of the lateral ventricle and corpus callosum is illustrated in Figures 1 and 2. The SVZ of the adult rat brain is a discontinuous layer containing several types of cells next to the ependymal cell lining. Almost all regions in the SVZ, including the ependymal layer, appeared stained by the antibody, whereas, in the corpus callosum, the antigen was scattered diffusely, and staining of both areas was in a fibrous pattern. Double-immunofluorescent staining using A3B10 and anti-GFAP (Fig. 1) and/or anti-S100β (Fig. 2) Abs revealed that, both in the ependyma and in the corpus callosum, the fibrous texture positive for A3B10 contained almost all of the GFAP-positive cells and some of the S100β-positive cells. However, the A3B10 antigen was also localized in the cells negative for GFAP staining, especially in the ependymal cell layer. The corpus callosum apparently contained no A3B10-positive, GFAP-negative population. It is supposed that the ependyma contains a vast number of progenitors, precursors, and mature cells of astrocyte lineage, whereas the corpus callosum contains predominantly relatively differentiated astrocytes. These data addressed the possibility that the A3B10 mAb detects mature, GFAP-positive astrocytes and their precursors, as well as closely related cell types such as ependymal cells and NSCs.

Immunoblot Analysis of Developmental, Neonatal, Postnatal, and Adult Brains

Western blot analysis using tissue homogenate prepared from E18 to adult rat brains was carried out to clarify the expression pattern of A3B10 antigen during growth and development of the CNS (Fig. 3). The A3B10 mAb identified a single band of approximately 250 kDa. The expression of the antigen was first shown at E18, and it increased with growth and development in spite of a temporal decrease on P14. The antigen was still expressed in adult animals, in both the cerebrum and the cerebellum, although the expression level appeared lower in adults than in younger animals.

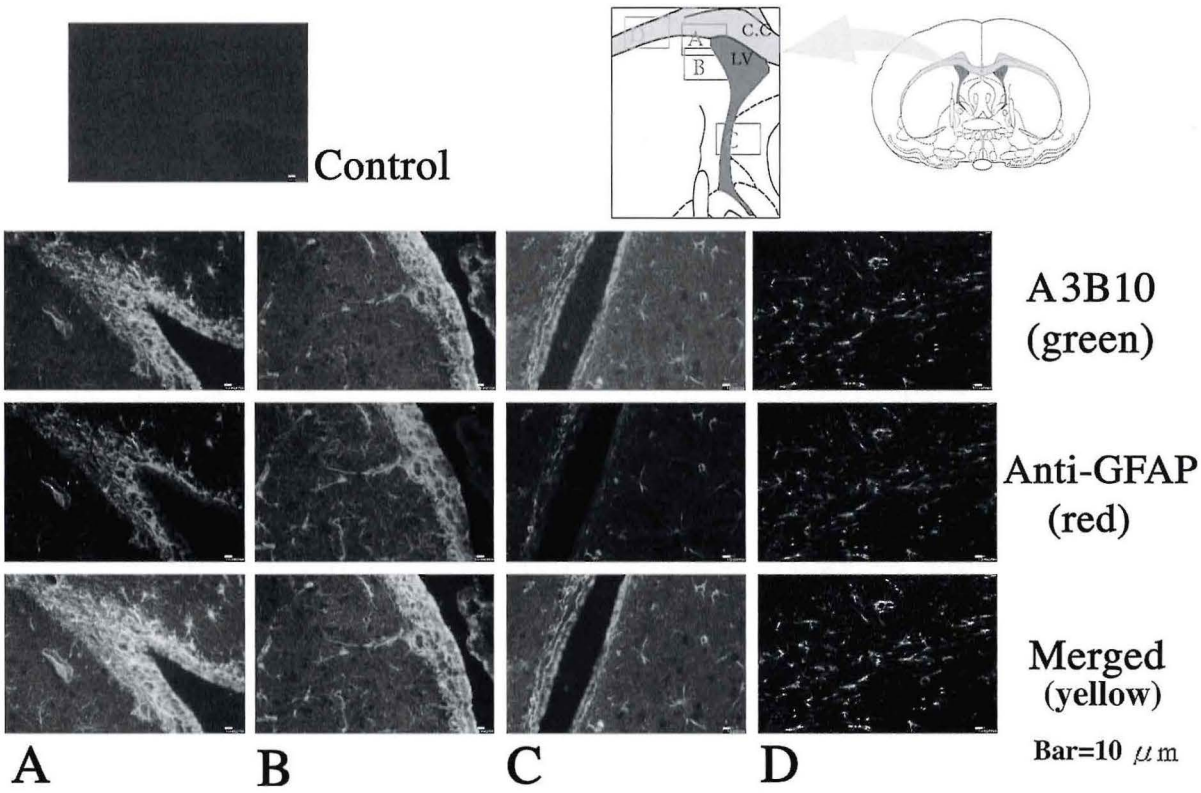

Fig. 1. Immunohistochemical localization of A3B10 (green)- and anti-GFAP (red)-reactive antigens in the subventricular zone (SVZ; **A–C**) and corpus callosum (**D**) in rats. Control with no primary antibody treatment gave no signal. The majority of cells in the ependyma, including ependymal cells and almost all of the $GFAP^+$ cells, were stained with the A3B10 antibody. In the corpus callosum, anti-GFAP and A3B10 antibodies appear to stain the same cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Immunocytochemistry of In Vitro Cultured Neural Cells

To determine the cellular specificity of A3B10 binding in vitro, primary mixed cell cultures from the cerebral hemispheres of E17 rat embryos were labeled with A3B10 and either anti-GFAP antibody. Phase-contrast micrographs show the discrete presence of neurons and oligodendrocyte precursor cells (OPCs) on a layer of astrocytes, as previously reported (Yoshimura et al., 1998). The A3B10 antibody stained the astrocyte layer but not the neuron or OPCs (Fig. 4). Double immunostaining with A3B10 and anti-GFAP antibodies indicated that the subcellular localizations of GFAP and A3B10 antigen partially coincide (Fig. 5).

Immunohistochemisty of GFAP KO Mice by A3B10

The SVZs of GFAP-deficient mice were immunostained with either the A3B10 or the anti-GFAP antibody. In accordance with the above-mentioned data, GFAP-deficient mice lacked GFAP immunoreactivity, whereasd A3B10 antibody stained ependymal cells in both GFAP +/+ and −/− mice (Fig. 6).

Immunoelectron Microscopy of A3B10 Antigen

As previously described, although A3B10 and GFAP immunoreactivity did not coincide completely, the apparent colocalization of A3B10 and GFAP is a predominant characteristic of the central nervous system. This suggests that there is an extremely close relationship between the A3B10 antigen and GFAP. Examination of several loci revealed that the most specific A3B10 labeling pattern was observed in the ependymal cells of the adult spinal cord. GFAP immunoreactivity was detected in the adult spinal cord parenchyma but not in the ependymal cells (Fig. 7A,C). Immunohistochemistry showed A3B10 immunoreactivity on the ependymal cells (Fig. 7A,B,D,E). The ependymal cells in the adult spinal cord were positive for vimentin (Fig. 7A,C), and they had cellular processes extending into the parenchyma, which occasionally reached the surface of the capillary vessels (Fig. 7A,C). Figure 7D–F shows colocalization of A3B10 antigen and vimentin. Not all vimentin-positive structures were also recognized by A3B10 antigen. One possible explanation of this is that endothelial cells also possess vimentin. More detailed examination of the localization of A3B10 antigen was performed by immunoelectron microscopy of the adult spinal cord

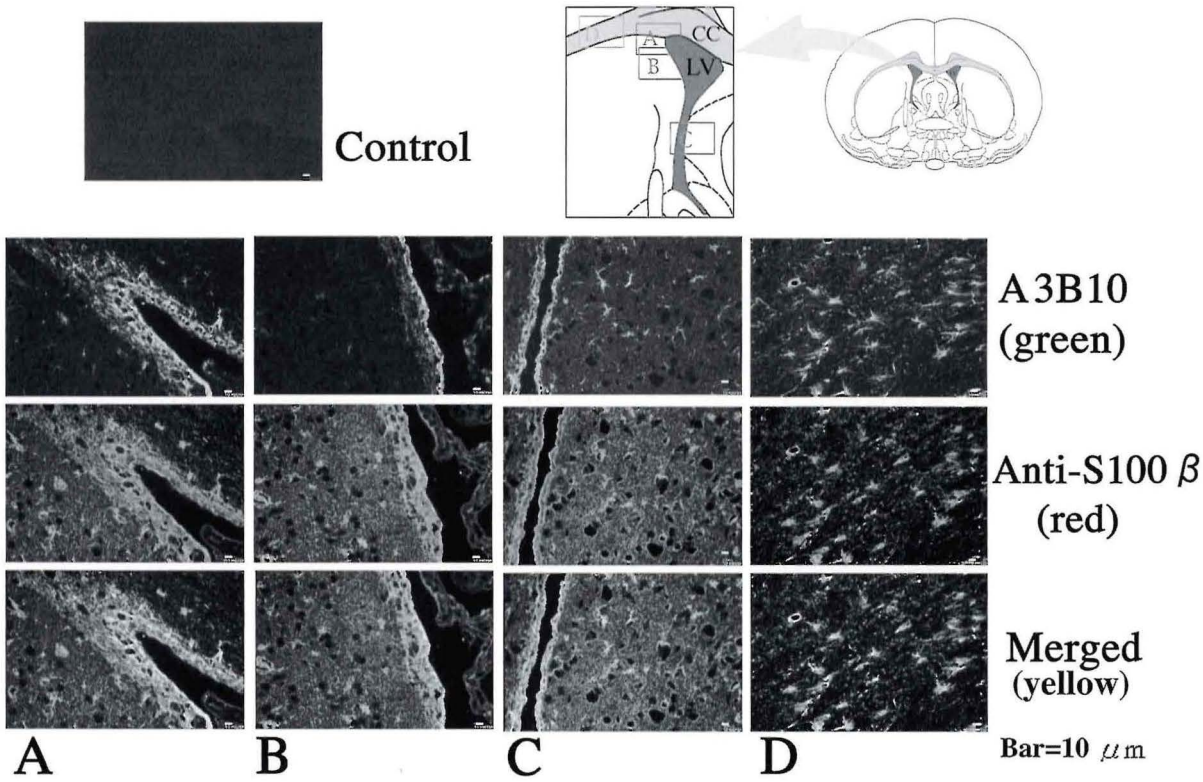

Fig. 2. Immunohistochemical localization of A3B10 (green)- and anti-S100β (red)-reactive antigens in the subventricular zone (SVZ; **A–C**) and the corpus callosum (**D**) in rats. Controls with no primary antibody treatment gave no signal. Both in the SVZ and in the corpus callosum, partial colocalization of A3B10 and S100β immunosignals was observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 7G–I). A3B10 antigen was visualized using silver-enhanced gold particles. In the spinal cord parenchyma, in which A3B10 antigen was colocalized with GFAP, gold particles were found on the intermediate filament bundles (Fig. 7G). Next, we tried to label GFAP and A3B10 antigen. By using a double-staining method, GFAP and A3B10 antigen were visualized by DAB and gold particles. The spinal cord tissue, including the ependymal cells, was processed and observed under the electron microscope. An accumulation of gold particles was seen (Fig. 7H,I, arrows). A magnified view of the electron micrograph showed that gold particles were on the intermediate filament bundles, which were negative for DAB structure, and this indicated that the accumulation might be on the transverse region of intermediate filament bundles (Fig. 7I, arrows). These findings suggest that the A3B10 antigen may be used to construct intermediate filament bundles in vivo, together with GFAP or vimentin.

Identification of A3B10 Antigen

To identify the A3B10 antigen, the cDNA library prepared from E18 rat brains was screened for immunostaining by A3B10. Four A3B10-positive clones were obtained and subjected to DNA sequence analysis. These four clones had overlapping DNA sequences, and a single DNA sequences of 5,010 bp was thus deduced. The DNA sequence gave a 1,458-amino-acid sequence, which is supposed to be partial, because the sequence has a stop codon but not a start codon. The result of a BLAST search of this sequence is shown in Figure 8. In spite of an 11% deletion, A3B10 is identified as "similar to calmodulin regulated spectrin-associated protein 1" (official symbol RGD1565022_predicted, gi: 296580O), a rat ortholog of the human CAMSAP1 gene.

DISCUSSION

Screening of a cDNA library generated from an enriched astrocyte culture was carried out using a new monoclonal antibody, A3B10. This antibody recognized a majority of SVZ cells and astrocytes. SVZ has been reported to contain at least six cell types: neuroblasts ("type A"), NSCs ("B1"), astrocytes ("B2"), putative oligodendrocyte/neuron precursor cells ("C"), tancytes ("D"), and ependymal cells ("E"; Doetsch et al., 1997). It has not been determined whether A3B10 stains all of these cell types, but the present results suggest that the antibody stains at least type B1, B2 (both GFAP-positive), and E cells. Although type E ependymal cells of rodents are GFAP negative, other mammalian ependymal cells (e.g., human) have been shown to express the

GFAP protein. In the corpus callosum, the immunosignal of A3B10$^+$ regions is a bit wider than that of the GFAP$^+$ regions, and it is narrower than that of the S100β$^+$ region. Furthermore, in in vitro mixed cell cultures, A3B10 stained astrocytes but not neurons, oligodendrocytes, or OPCs. These data suggest that A3B10 may recognize not only mature (GFAP$^+$) cells but also immature and/or GFAP$^-$ astrocytes.

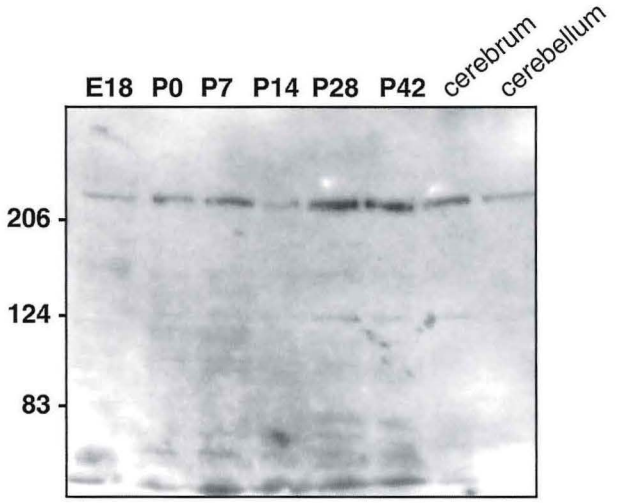

Fig. 3. Western blot analysis of tissue homogenates from E18 to adult rat brains. Proteins were separated by 6% SDS-PAGE. Each lane was loaded with 200 μg total protein. A3B10 antibody identified a single band of approximately 250 kDa. Expression of the antigen was detected at E18 and increased with growth and development, despite a temporal decrease on P14. The antigen still was expressed in adult animals, both in the cerebrum and in the cerebellum.

These data address the possibility that A3B10 may be used as a new marker for labeling a broad range of astrocytic-lineage cells. Currently, several markers for astroglial markers other than GFAP are available, such as nestin (Lendahl et al., 1990; Yu et al., 2006), S100β (Sen and Belli, 2007), GLAST, GLT1 (Vitellaro-Zuccarello et al., 2005; Williams et al., 2005), glutamate synthetase (GS; Cammer, 1990), aquaporin-4 and -9 (Cavazzin et al., 2006), and A2B5 antigen (Seidenfaden et al., 2006), although they have some limitations. The expression of nestin, an intermediate filament protein expressed in glial progenitor cells, disappears and is replaced by GFAP protein during the process of astrocyte differentiation (Chanas-Sacre et al., 2000). The expression of A2B5 is limited to relatively narrow stages of differentiation. The glutamate transporters GLAST and GLT1 have recently been used as radial glia-astrocytic-lineage cell markers. However, the expression of transporters is largely affected by their environment (Dallas et al., 2007; Regan et al., 2007). S100β, GS, and aquaporins have insufficient specificity as astrocyte markers. Thus, it remains very important to find reliable immunohistochemical markers for characterization and classification of astrocytic-lineage cells.

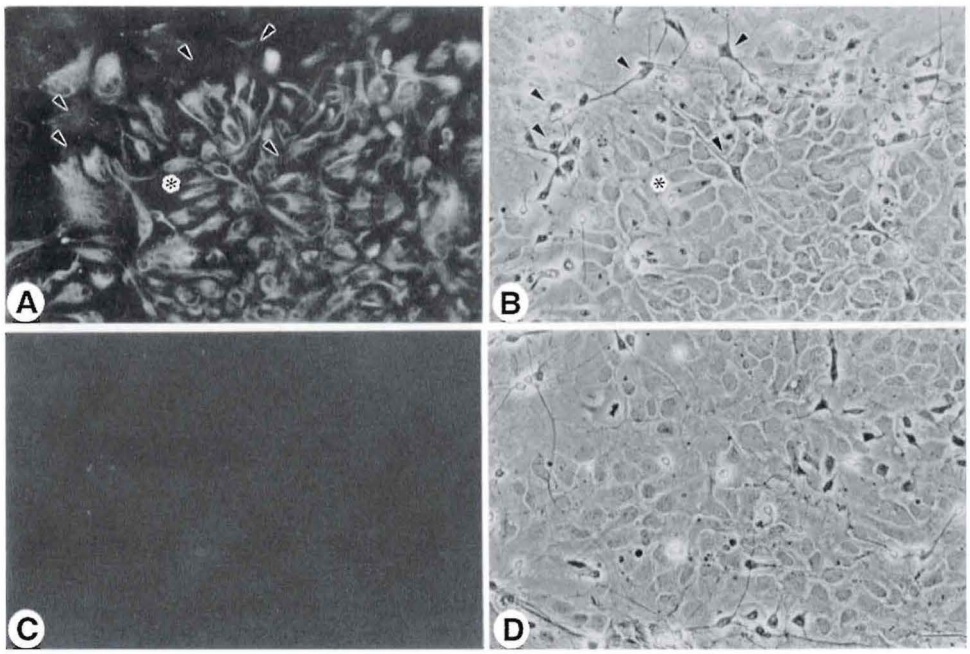

Fig. 4. Immunocytochemistry using the A3B10 antibody to stain in vitro neural cell cultures. First-passage cells from 7-day cultures derived from the cerebral hemispheres of E17 rat embryo were stained with (**A**) or without (**C**) A3B10 antibody. The corresponding phase-contrast micrographs of A and C are shown in **B** and **D**, respectively. Astrocytes, but not neurons (arrowheads) or oligodendrocyte precursor cells (OPCs; asterisk), were stained with A3B10 antibody. Neurons and OPCs had been identified by the antibodies to neurofilament, MAP2, and Tuj1 for neurons and NG2, Olig2, A2B5, and PDGF-α receptor for OPCs, respectively (data not shown). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

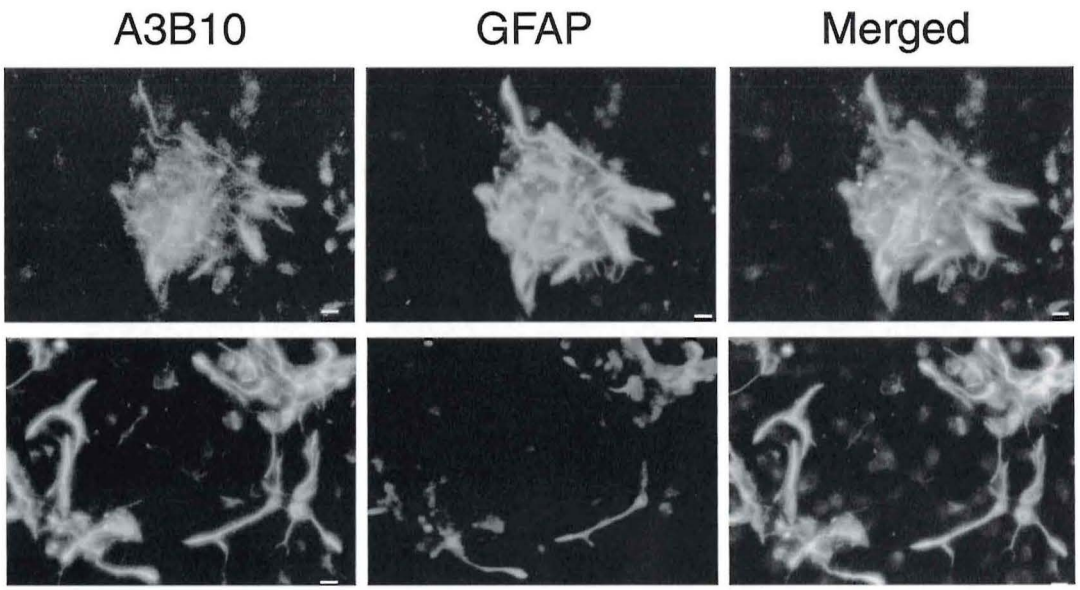

Fig. 5. Double immunostaining by A3B10 and anti-GFAP antibody of in vitro neural cell cultures. First-passage cells of 7-day cultures derived from the cerebral hemispheres of E17 rat embryo were stained with A3B10 (left, green) or anti-GFAP (center, red) antibodies. Merged images (yellow) are shown in the right column. A3B10 antibody stained $GFAP^-$ fibers in addition to $GFAP^+$ fibers of astrocytes. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

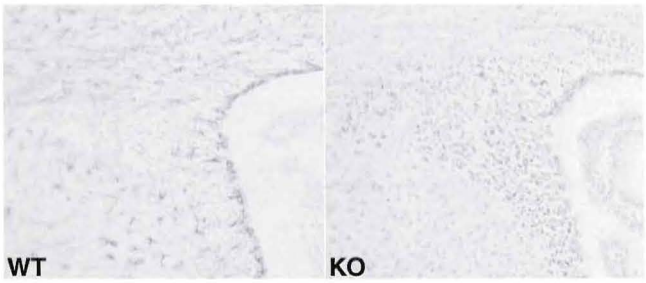

Fig. 6. Immunohistochemical staining by A3B10 of SVZ in GFAP-deficient and wild type mice. A similar staining pattern of ependyma by A3B10 antibody was seen in mice (**left**). Furthermore, ependymal cells of GFAP-deficient mice were stained clearly by the antibody (**right**). DNA was counterstained by methyl green. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Introduction of the A3B10 antibody as a marker for use in neuroscience research may provide a useful tool for elucidation of developmental and adult neurogenesis. In recent years, much evidence has confirmed that neurogenesis occurs in the adult brain and that NSCs reside in mainly two areas of the adult brain, the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ; Gage, 2000; Temple, 2001). The NSCs in both DG and SVZ express GFAP and have several features in common with astrocytes (Gage, 2000). Cells in the adult ependyma, type B1 neuronal stem cells, and possibly type E ependymal cells have been supposed to function as NSCs to generate neuron, astrocytes, and oligodendrocytes (Alvarez-Buylla and Garcia-Verdugo, 2002). These NSCs also express several features in common with radial glia (Alvarez-Buylla et al., 2001; Seri et al., 2001). Radial glia are considered to be multipotent NSCs in the developing mammal neocortex. Derivad from neuroepithelial cells that surround the neural tube, radial glial cells in most regions of the mammalian brain disappear or transform into astrocytes when neuronal generation and migration are complete (Misson et al., 1991; Alvarez-Buylla et al., 2001; Seri et al., 2001; Malatesta et al., 2003). Furthermore, young astrocytes, which are derived from radial glia during development, function as the primary precursors for new granule neurons in DGs (Craig et al., 1996; Kuhn et al., 1997; Tropepe et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002). Together, these findings have led to the proposition that NSCs lie within the neuroepithelial-radial glia-astrocyte lineage, although this conclusion is still controversial (Alvarez-Buylla et al., 2001; Doetsch et al., 2002). Therefore, the identification of reliable molecular markers to distinguish terminally differentiated astrocytes from those that can function as NSCs will be quite important for future research. Several immunohistochemical markers, such as nestin, GLAST, NeuN, DCX, SOX-2, brain lipid-binding protein, LeX, and GFAP (Sohur et al., 2006), have been used as markers either alone or in combination. However, they appear to be only partially specific or selective for unknown subpopulations of NSCs. The A3B10 antibody appears to stain a wide variety of radial glia-astrocytic-lineage cells from embryonic to adult stages, including GFAP cells in adult

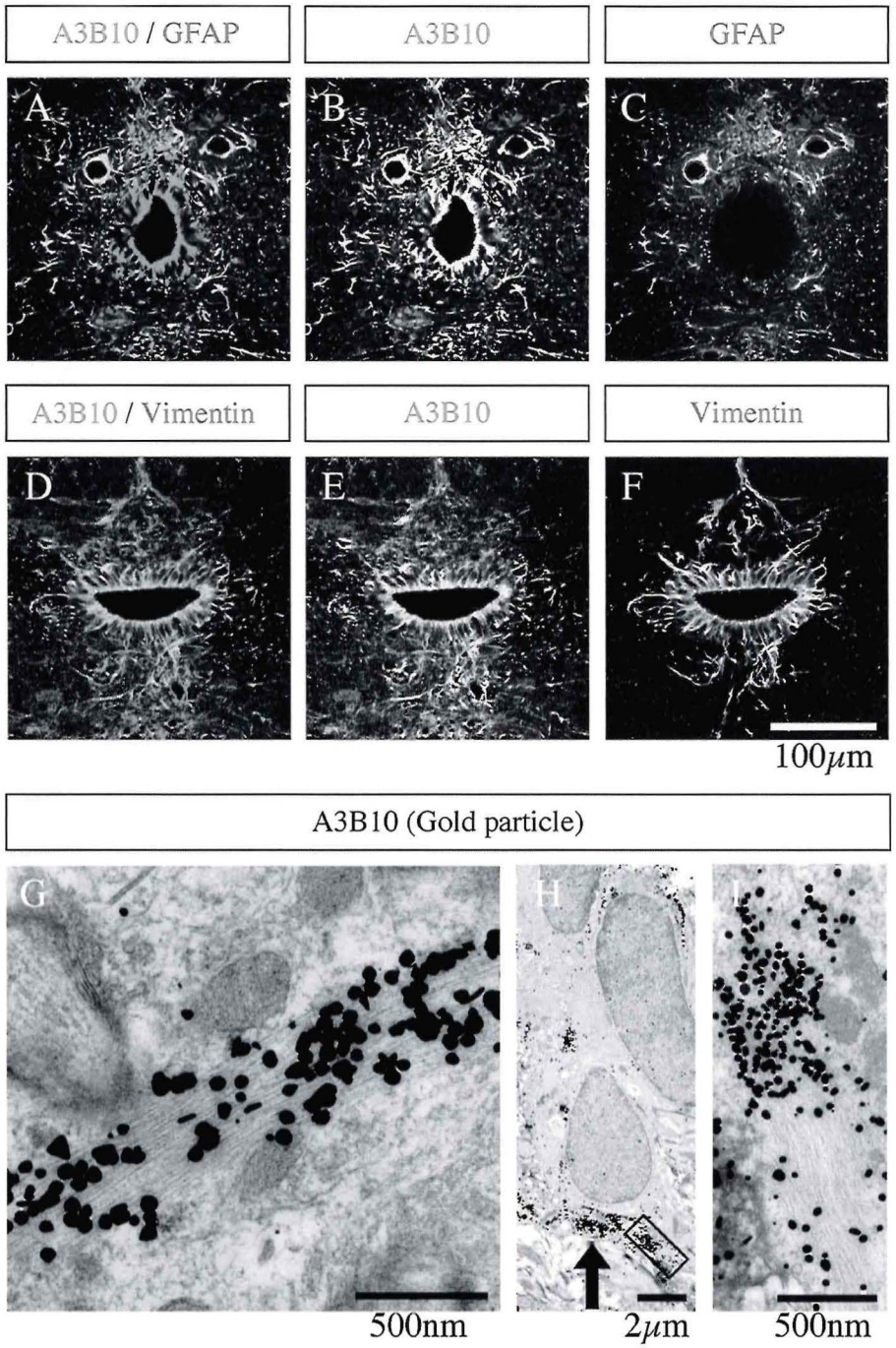

Fig. 7. **A–C:** Distinct labeling of A3B10 and GFAP in the ependymal cells in the adult spinal cord. A3B10 antigen (green) localized to the ependymal cells, but GFAP (red) did not. **D–F:** Colocalization of A3B10 antigen and vimentin in the ependymal cells. The ependymal cells were positive for vimentin (red), as expected. A3B10-positive (green) cellular processes of the ependymal cells were sometimes colocalized to vimentin-positive structures. **G:** Single immunoelectron microscopy using the A3B10 antibody, visualized by gold particles. Gold particles were found on the intermediate filament bundles in the adult spinal cord parenchyma, in which localization of A3B10 antigen was almost the same as that seen for GFAP. **H,I:** Double immunoelectron microscopy using A3B10 and anti-GFAP antibodies, indicated by gold particles and DAB-positive structures. Accumulation of gold particles was found in the cellular processes of the ependymal cells (arrows). A magnified view (I) shows that gold particles were on intermediate filament bundles, and accumulation of gold particles was observed at the apparent ends of intermediate filament bundles. Scale bars = 100 μm in F (applies to A–F); 500 nm in G; 2 μm in H; 500 nm in I. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

```
>rno:296580 RGD1565022_predicted; similar to calmodulin regulated
            spectrin-associated protein 1 (predicted)
          Length = 1604

 Score = 2830 bits (7335), Expect = 0.0
 Identities = 1429/1604 (89%), Positives = 1443/1604 (89%), Gaps = 20/1604 (1%)

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Sbjct: 1    MVDAGGRSAAEGWRRMEAPPEGADLVPLDRYDAARAKIAANLQWICAKAYGLDNIPEDLR 60

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Sbjct: 61   DPFYIDQYEQEHIKPPVIKLLLSSELYCRVCSLILKGDQAATLQGHQSVIQALSRKGIYV 120

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Sbjct: 181  PYDLEDAMVFWINKVNLKMREITEKEVKLKQQPLESPAHQKPGLEHAVMHCMLEPVDFAR 240

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Sbjct: 241  VVRYRREHLSARQSPYFPLLEDLMRDGSDGAALLAVVHYYCPEQMKLDDICLKEVPSMAD 300

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            SLYNIRLLREFSNEHLNKCFYLTLEDMLYAPLVLKPNVMVFIAELFWWFENVKPDFVQPR
Sbjct: 301  SLYNIRLLREFSNEHLNKCFYLTLEDMLYAPLVLKPNVMVFIAELFWWFENVKPDFVQPR 360

Query: 357  DIQELKDAKTVLQQKSSRPPVPISNATKRSFLGSPAAMSPADLPPSTQPLTEGSHRYHLH 416
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Sbjct: 361  DIQELKDAKTVLQQKSSRPPVPISNATKRSFLGSPAAMSPADLPPSTQPLTEGSHRYHLH 420

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```

Fig. 8. Results of a BLAST search of the A3B10 peptide sequence. The amino acid sequence of A3B10 antigen deduced from the nucleotide sequence (top row) is aligned with that of "similar to calmodulin regulated spectrin-associated protein 1" (official symbol RGD1565022_predicted, gi: 296580O), a rat ortholog of the human CAMSAP1 gene (bottom row).

SVZ ependyma. These data address the possibility that the antibody may stain at least subpopulations of NSCs.

The exact specificity and sensitivity of the A3B10 antibody as a marker for the radial glial-astrocytic cell lineage, including NSCs, must be further clarified. Does the antibody stain radial glial cells in the developmental and adult stages (e.g., Bergman glia and Muller cells)? Is A3B10 useful for detection of NSCs in the DG of the hippocampus? Is the A3B10 antigen expressed in reactive astrocytes or their precursors? What is the relationship among A3B10 and other astroglial cells and/or NSC markers? What are the functions and biological significance of the A3B10 antigen, calmodulin-regulated spectrin-associated protein 1 (CAMSAP1)? The nucleotide sequence of human CAMSAP1 was originally deposited to GeneBank databases (AJ519841, gi:38636482) by A.J. Baines and his colleagues, who have been publishing the papers focusing on spectrin and related proteins. However, as far as we know, experimental data concerning biological activities of CAMSAP1, for example, its association with spectrin or possible changes in its expression induced by calmodulin, have not been published yet. A protein motif search using InterProScan (EMBL-EBI) has shown that the present amino acid sequence contains a calponin-like actin-binding site (IPR001715). The cytoskeletal spectrin network, in association with actin filaments, is known to play an extraordinarily important role in physiological and pathological neuritogenesis involving neurons and astrocytes (Lencesova et al., 2004; Czogalla and Sikorski, 2005). Acidic calponin, an actin- and calmodulin-binding protein, is enriched in neurons and glial cells in vivo, including radial glia, Bergmann glia, and mature astrocytes, and ex vivo, where acidic calponin strongly colocalizes with intermediate GFAP and vimentin filaments (Plantier et al., 1999; Agassandian et al., 2000; Egnaczyk et al., 2003). In the present study,

immunoelectron micrography suggested that A3B10 antigen may be involved in construction of intermediate filament bundles, along with GFAP and/or vimentin. By altering their morphology, astrocytes, including those involved in the maintenance and plasticity of neurons and in clearance of transmitters, play important roles in synaptic transmission. Morphological plasticity characteristic of astroglial cells, and therefore the regulation of cytoskeletal proteins, is thus suggested to play an extraordinarily important role. Accordingly, it must be noted that the conventional markers for glial cells, GFAP, nestin, RC1 (Edwards et al., 1990), and RC2 (Chanas-Sacre et al., 2000), are intermediate filament proteins involved in modulation of astroglial morphology. Further elucidation of biological activities and functions of CAMSAP1 may also contribute to improved understanding of ontogeny, differentiation, and pathophysiological roles of astroglial cells.

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