J. Biochem. 125, 443-448 (1999)

A Monoclonal Antibody, 3A10, Recognizes a Specific Amino Acid Sequence Present on a Series of Developmentally Expressed Brain Proteins

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Received September 16, 1998; accepted November 16, 1998

Immunoblotting showed that a monoclonal antibody, 3A10, binds to a series of rat brain-specific antigens with molecular masses of 150-, 120-, 118-, 106-, 104-, 79-, and 77-kDa. The expression of 3A10 antigens is dependent on the developmental stage of the brain; only the 106-kDa antigen is detected during embryonic stages of rat brain development, while the expression of the remaining 6 antigens starts after birth and reaches a maximum during postnatal days 15-21. Detection of the 3A10 antigens in cultured neuronal and glial cells derived from cerebral cortices of rat brain at embryonic day 18 showed that the 77-, 79-, 106-, and 150-kDa antigens are specifically expressed in neuronal cells. The 77-kDa antigen was purified and identified as synapsin I by amino acid sequence analyses of the peptide fragments isolated after *Achromobacter* protease I treatment. During the isolation of 3A10-reactive proteins by immunological screening of cDNA libraries constructed from adult rat brain, we found that all of the 3A10-reactive clones contain nucleotide sequences encoding the unique amino acid sequence TRSP(S,R,G)P. Analyses of 3A10-binding to various synthetic peptides showed that the monoclonal antibody recognizes a specific conformational structure formed by either the TRSPXP sequence or similar amino acid sequences that are expressed on a series of developmentally expressed brain proteins.

Key words: brain, development, monoclonal antibody, neuron, synapsin I.

The complex and diverse functions of the mature nervous system depend on the precise interconnections formed by a great number of neural cell types. A mature pattern of neuronal connections is not fully developed at birth, but is established during the early postnatal stages. The neuronal connectivity in rat cerebral cortex increases 10-fold between days 12 and 30 after birth (*1*), and in the molecular layer of the rat hippocampal dentate gyrus, less than 1% of the adult number of synapses are present 4 days after birth, but this number doubles daily, reaching more than 90% of the adult value 30 days after birth (*2*). A major question is how maturation of the nervous system is achieved by factors acting early in neurogenesis or by epigenic cues present in the subsequent environment of developing neurons. One approach to understanding the molecular mechanisms of these neurogenic processes is to identify the molecules that play crucial roles in the development of the central nervous system. Although several molecules such as cell adhesion molecules (*3*), trophic factors (*4-7*), cytoskeletal proteins (*8-10*), receptors (*11, 12*), and signal transducing molecules (*13-15*) play roles in forming the mature pattern of neuronal connections, these studies have just begun and information remains limited.

We used monoclonal antibodies (mAbs) to identify molecules that are specifically expressed in neural tissues during the early postnatal stages. The mAb, 3A10, recognizes a series of brain-specific antigens that are expressed from the early postnatal stages. These findings raise questions regarding the functional roles of these antigens and how 3A10 recognizes such a variety of molecules. In this study, we analyzed the cell-type specific expression of 3A10 antigens and identified one of them as synapsin I. We also showed that 3A10 recognizes a particular conformational structure formed by either a novel 6-amino acid TRSPXP sequence or similar amino acid sequences that are expressed on a series of developmentally expressed brain proteins.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; mAb, monoclonal antibody; NSE, neuron specific enolase.

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MATERIALS AND METHODS

Antibodies—We established a monoclonal antibody (mAb), 3A10, as described (*16*). In brief, a crude membrane fraction from the near-diploid mouse embryonic cell line m5S/1M that has been shown to express neural cell adhesion molecules (NCAM) abundantly was injected into 8-week-old female Wistar rats by the intra-splenic immunization method as described previously (*17*). Spleen cells (5×10^8) from an immunized rat were fused with 5×10^7 P3X63-Ag8.653 myeloma cells (*18*). Antibody secreting hybridoma cells were screened by immunoblotting and 3A10 was selected as it recognizes a series of rat brain proteins. Anti-GFAP mAb was purchased from Amersham International plc (Little Chalfont, UK), anti-NSE polyclonal antibody from Wako Pure Chemical Industries (Tokyo), anti-neurofilament mAb from Affiniti Research Products (Mamhead Castle, UK), and anti-synapsin I polyclonal antibody from COSMO BIO (Tokyo).

Cell Culture—Pregnant mice were sacrificed by cervical dislocation on the 18th-day of gestation. Embryos were isolated from the uterus, then the outer layer of the pelvis was cut to remove the fetal meninges, and the cerebral cortices were placed in Dulbecco's modified minimum essential medium (DMEM, Gibco BRL, Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS, Gibco BRL). Following mechanical dissociation and passage through #100 mesh, the cells were suspended in DMEM containing 10% FBS and 292 μg/ml L-glutamine for glial cell culture. Neuronal cells were suspended in neurobasal medium (Gibco BRL) containing 2% B27 supplement (Gibco BRL), 74 μg/ml L-glutamine, and 25 μM L-glutamate, then plated in culture dishes coated with poly-L-lysine (100 μg/ml), and incubated in an atmosphere of 95% air and 5% CO_2 at 36°C. The medium in these cultures was replaced on the 4th day with the same supplemented medium without L-glutamate. Thereafter, the medium was changed every 6 days. The medium in which the glial cells were cultured was changed every 4 days and the cells were passaged once with trypsin-EDTA. We estimated that our neuronal and glial cultures consisted of 80% neurons and over 95% glia, respectively, based on the immunochemical criteria for the expression of the markers, neurofilament protein and GFAP.

Immunoblotting Analysis—Organs were isolated from Wistar rats, then homogenized in 10 w/v SET buffer (10 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose, 5 mM *N*-ethylmaleimide, 1 mM phenylmethanesulforyl fluoride) at 4°C. Protein levels in the homogenates were determined using the BCA system (Pierce Chemical, Rockford, IL). The protein concentrations of the samples were balanced and the samples were placed in reducing sample buffer. Immunoblotting was performed as described (*19*). In brief, proteins from various sources were separated by SDS-PAGE in 7.5% (w/v) acrylamide gels (*20*) and blotted onto nitrocellulose membranes at 4°C using a protein transfer system (Bio-Rad Laboratories, Richmond, CA) (*21*), as described (*19*). The membranes were blocked by incubation in Tris-buffered saline (TBS, 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl) containing 30 mg/ml bovine serum albumin (3% BSA-TBS) at room temperature for 2 h with gentle shaking, and then incubated with 3A10 (2 μg/ml) in TBS containing 10 mg/ml BSA and 0.05% Tween 20 (1% BSA-TBS-T) for 2 h at room temperature. Thereafter, the membranes were immersed in peroxidase-conjugated goat anti-mouse IgG (GAM•HRP, Bio-Rad) diluted with 1% BSA-TBS-T. Antibody bound to the membranes was detected by color development using 4-chloro-1-naphthol as the substrate. GFAP and NSE were detected as described above with the following modifications. Nitrocellulose membranes with blotted proteins were blocked with TBS containing 5% skim milk (Difco Laboratories, Detroit, MI), then incubated with either anti-GFAP mAb (1/400 dilution with 1% BSA-TBS-T) or anti-NSE polyclonal antibody (1/200 dilution with 1% BSA-TBS-T). Bound antibodies were detected with either HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG (1/1,000 dilution with 1% BSA-TBS-T) (Amersham) using an ECL Western blotting detection kit (Amersham).

Isolation and Amino Acid Sequence Analysis of the 77-kDa Antigen—Total brain homogenates were centrifuged at $1,500\times g$ for 20 min at 4°C and the precipitates were suspended in buffer containing 20 mM Hepes-KOH (pH 7.9), 0.33 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulforyl fluoride, 14 μg aprotinin, and 10% glycerol. The suspensions were then centrifuged at $9,000\times g$ for 15 min at 4°C, and the resulting supernatants were subjected to preparative SDS-PAGE. The 3A10-reactive, 77-kDa protein was electrophoretically eluted as described previously (*16*), and separated further by SDS-PAGE in gels containing 6 M urea (*22*). The purified protein was subjected to enzymatic digestion with *Achromobacter* protease I (API; *23*), a gift from Dr. Masaki, Ibaraki University, in gels containing 0.1% SDS. The extracted peptides were separated essentially according to the method of Kawasaki and Suzuki (*24*) by RP-HPLC on tandemly connected DEAE-5PW (2×20 mm, Tosoh) and Mightysil RP-18 (2×50 mm, Kanto Chemical) columns eluted with a linear gradient of 0.09% aqueous trifluoroacetic acid to 0.08% trifluoroacetic acid/60% acetonitrile (v/v) in 60 min at a flow rate of 0.1 ml/min using a Hewlett Packard Liquid Chromatograph model 1100. The collected fractions were subjected to MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry) on a Bruker REFLEX mass spectrometer with α-cyano-4-hydroxy cinnamic acid (CHCA; obtained from Aldrich) as a matrix. The isolated peptides were analyzed by automated Edman degradation on an Applied Biosystems Protein Sequencer model 477A connected in line to a PTH Analyzer model 120A using an in-house generated gas-phase program.

Immunological Screening of a cDNA Library—A λgt11 cDNA library was constructed from the brain of an 8-week-old female Wistar rat. Poly(A)$^+$ RNA was prepared using Oligotex-dT 30 (Takara Shuzo, Otsu) and cDNAs were constructed using a cDNA Synthesis System Plus (Amersham). The cDNA library was constructed using a LAMBDA gt11 cloning kit (Stratagene Cloning Systems, La Jolla, CA). The recombinant phages were screened by immunoassay with the 3A10 antibody (*25*). Briefly, nitrocellulose membranes (Millipore Corporation, Bedford, MA) with blotted recombinant proteins were blocked with 3% gelatin in TBS, incubated with 3A10 antibody in TBS containing 1% gelatin and 0.05% Tween 20 for 2 h. The

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membranes were washed with TBS containing 0.05% Tween 20, incubated with HRP-conjugated goat anti-mouse IgG (GAM-HRP, Bio-Rad) for 1 h, and washed with TBS containing 0.05% Tween 20. Color was detected using a Konica Immunostaining HRP-1000 (Konica, Tokyo). Phage DNA was digested with *Eco*RI, and the inserts were subcloned into pBluescript vector (Stratagene). DNA was sequenced on an ALFred DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) using an AutoCycle Sequencing kit (Pharmacia).

Analyses of 3A10 Binding to Synthetic Peptides—Peptides were synthesized with an automated peptide synthesizer (Advanced Chemtech, model 396 MPS) and an extra cysteine residue was added to the C-terminal end of each peptide (*26*). The peptides were purified by reverse-phase HPLC on a PEGASIL-300 C4 column (Senshu, Tokyo) eluted with a 5-60% (v/v) linear acetonitrile gradient containing 0.1% (v/v) trifluoroacetic acid. The sequence of each peptide was confirmed with an amino acid sequencer and the molecular mass with a JMS-HX110 double focusing mass spectrometer equipped with a fast atom bombardment ion source and a JMA-DA5000 data system (JEOL, Tokyo). The synthetic peptides, TRSPSPGGGC and PSPSRTGGGC, were conjugated to maleimide-activated horseradish peroxidase (HRP) (Pierce Chemical) *via* their cysteine residues according to the manufacturer's protocol. The binding of 3A10 to the synthetic peptides was examined by ELISA as described (*27*). In brief, the wells of microtiter plates (Immulon 2, Dynatech Laboratories, Alexandria, VA) were coated with 300 ng/well 3A10 in Tris-buffered saline (TBS, 10 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl) for 16 h at 4°C, and then blocked with 3% BSA-TBS for 2 h at room temperature. The wells were washed with TBS, and then incubated with various amounts of synthetic peptides conjugated to HRP in TBS containing 10 mg/ml BSA (1% BSA-TBS) for 2 h at room temperature. The bound peptide-HRP conjugates were detected colorimetrically with *o*-phenylenediamine as the substrate. To determine the amino acid residues involved in the interaction with 3A10, various synthetic peptides were coated onto the wells of microtiter plates and 3A10 binding was examined by ELISA. Briefly, various amounts of synthetic peptides were coated onto the wells of microtiter plates for 12 h at 37°C. The wells were then incubated with 2 μg/ml of 3A10 diluted in 1% BSA-TBS for 2 h, biotinylated anti-mouse IgG (Zymed Laboratories, San Francisco, CA) diluted in 1% BSA-TBS for 1.5 h at room temperature, and with HRP-conjugated streptavidin for 45 min at room temperature. The bound 3A10 was evaluated colorimetrically using *o*-phenylenediamine as the substrate.

RESULTS AND DISCUSSION

Developmentally Regulated Expression of 3A10 Antigens—The expression of 3A10 antigen in adult rat tissues was examined by immunoblotting (Fig. 1a). Protein bands with molecular masses of 150-, 120-, 118-, 106-, 104-, 79-, and 77-kDa were detected in the brain. A 106-kDa protein band was also detected in the spleen, thymus, and liver, while a faint 106-kDa band was evident in the lung. The expression of 3A10 antigens was dependent on the developmental stage of the brain. Only the 106-kDa protein was detected during embryonic stages, and the remaining 6 bands were expressed during the first postnatal week, peaking during postnatal days 15-21 (Fig. 1b). The reactivity profiles of the 3A10 antigens with adult brain proteins were unaffected by disulfide bond reduction with β-mercaptoethanol, suggesting that the 3A10 antigens are composed of single polypeptides. The seven bands with molecular masses similar to those of the rat brain peptides, were also detected in homogenates of mouse, rabbit, bovine, and human brain, suggesting that the 3A10 antigens are conserved among mammals (data not shown).

Expression of 3A10 Antigens in Cultured Neuronal and Glial Cells—We used immunoblotting to determine whether the 3A10 antigens are expressed in neuronal or glial cells from E18 rat brain. Primary cultures of neuronal and glial cells were established as described in "MATERIALS AND METHODS." Figure 2a shows that an embryonic form of the antigen with a molecular mass of 106-kDa was expressed abundantly in cultured neuronal cells but only weakly in glial cells. The level of expression of the 106-kDa antigen in neuronal cells decreased during the culture period, whereas the 150- and 77-kDa antigens became detectable on the 14th day of neuronal cell cultivation, but never in glial cells. The faint 79-kDa band also became detectable on the 23rd day of neuronal cell cultivation. The levels of NSE expression, which correlate closely with

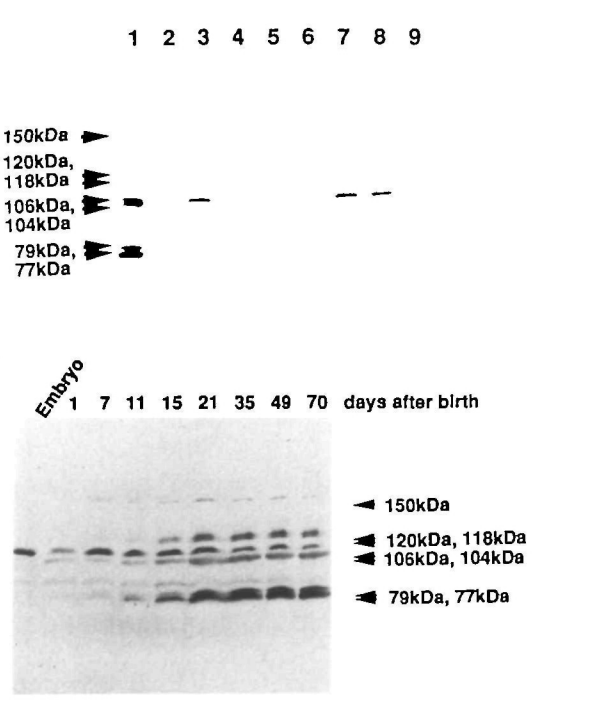

Fig. 1. **Immunoblots of various tissue homogenates with mAb 3A10.** Tissue homogenates were solubilized and samples (20 μg proteins) were separated by 7.5% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose membranes and immunoblotted with 3A10 (2 μg/ml). The bound mAb was detected using peroxidase-conjugated goat anti-mouse IgG. Panel a: lane 1, brain; lane 2, lung; lane 3, liver; lane 4, kidney; lane 5, heart; lane 6, small intestine; lane 7, spleen; lane 8, thymus; lane 9, skeletal muscle. Panel b: Brain homogenates at various developmental stages were solubilized and samples (20 μg proteins) were immunoblotted with 3A10 as described above.

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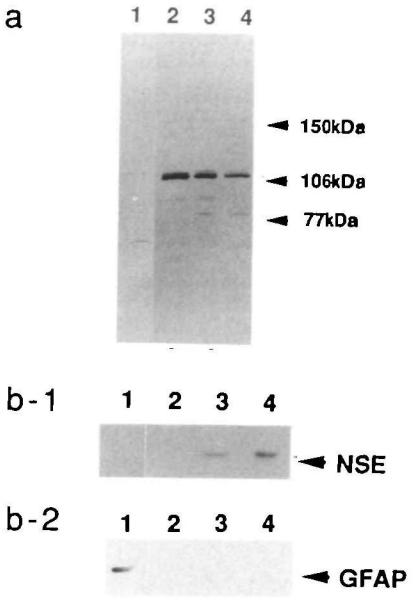

Fig. 2. **Immunoblots of cultured neuronal and glial cells.** Cell homogenates (15 μg proteins) were solubilized and separated by 7.5% SDS-PAGE. Binding of either 3A10 (panel a), anti-neuron-specific enolase (NSE) polyclonal antibody (panel b-1), or anti-glial fibrillary acidic protein (GFAP) mAb (panel b-2) to the separated protein bands was examined by immunoblotting as described in "MATERIALS AND METHODS." Lane 1, glial cells cultured for 16 days; lane 2, neuronal cells cultured for 5 days; lane 3, neuronal cells cultured for 14 days; lane 4, neuronal cells cultured for 23 days.

neuronal differentiation (*8, 28*), increased during the culture period in neuronal cells, while NSE was undetectable in glial cells (Fig. 2b-1). GFAP, which is expressed only in astroglia, was detectable in cultured glial cells but not in neuronal cells (Fig. 2b-2). These results suggest that the 106-, 150-, 79-, and 77-kDa antigens are expressed in neuronal, but not in glial cells, and that their expression is developmentally regulated.

Isolation of 3A10-Reactive Proteins—To identify the 3A10 antigens, we used two approaches; one to purify the 3A10-reactive proteins and the other to isolate cDNAs that encode the 3A10-reactive proteins by immunological screening of expression libraries. We isolated the 77-kDa antigen, which was shown to be expressed specifically in neuronal cells, by sequential electroelution of the electrophoretically separated 77-kDa 3A10-reactive protein. The 77-kDa protein was fragmented by enzymatic digestion with *Achromobacter* protease I (API) and the resulting peptides were separated on a reverse-phase HPLC column. Edman degradation of the peptides yielded five fragmental sequences, IHGEid-, klgtxxfplidq-, EMLS-, VDNQHDFQDi-, and AYMRTSVSGNwK, where the lower case letters indicate weakly assigned residues. Database search with these sequences identified the 77-kDa protein as synapsin I (*29*). Matching the 8 peptide mass values expected from synapsin I, the MALDI-TOF MS results supported this identification (data not shown). Synapsin I was first identified as a phosphoprotein present in neuronal cells and was later shown to play a pivotal role in the modulation of neurotransmitter release (*30, 31*). Differential splicing of primary synapsin I transcripts generates two isoforms, synapsins Ia and Ib, which are collectively referred to as synapsin I (*29*). In the rat, the two isoforms are composed of 704 (synapsin Ia) and 668 (synapsin Ib) amino acids and are found in molar ratios of about 1:2 in most brain areas (*30*). The identification of the 77-kDa antigen as synapsin I was further confirmed by immunoblotting analysis using a polyclonal anti-synapsin I antibody (data not shown). The anti-synapsin I antibody bound significantly to the isolated 77-kDa 3A10 antigen and also to both the 77- and 79-kDa bands when brain homogenates were examined by immunoblot analysis. The binding profiles of 3A10 to the 77- and 79-kDa proteins were identical to those observed with the anti-synapsin I antibody when brain homogenates were electrophoresed under various conditions as described in "MATERIALS AND METHODS." These results indicate that the 79- and 77-kDa 3A10 antigens correspond to synapsins Ia and Ib, respectively. The expression of synapsin I became significant during the early postnatal stages of brain development, a time that correlates with synapse formation (*30-32*). Also the time course for the expression of the 77- and 79-kDa 3A10 antigens observed in this study (Fig. 1b) is consistent with that observed for synapsin I (*32*). So far, the isolation and identification of other 3A10-reactive proteins have not been successful because of their very small quantities in rat brain.

We immunologically screened expression libraries constructed from adult rat brain to identify the 3A10 antigens. We isolated ten 3A10-reactive clones, five of which encoded novel cDNAs. Northern blotting showed the expression of these five clones to be highly specific to the brain (data not shown). The nucleotide sequences of the cDNA clones were deposited in the GenBank database [accession numbers, AF022963, AF022964, AF022965, AF022966, AF033032]. Although 3A10 bound effectively to the polypeptides expressed by these cDNA clones, it remains to be determined whether or not these cDNA clones encode protein that are 3A10-reactive upon immunoblotting.

Identification of the 3A10-Reactive Amino Acid Sequence—Since 3A10 bound to a series of proteins, it is likely that the mAb recognizes either a particular amino acid sequence or a conformational structure commonly expressed on these 3A10-reactive proteins. Among the ten 3A10-reactive clones, five encoded short polypeptides resulting from an inappropriate transcription frame: clone 1, ribosomal RNA (GenBank database accession number, X00722); clone 2, Fn54 mRNA (GenBank database accession number, AF001533); clone 3, transforming growth factor β-1 (GenBank database accession number, X52498); clone 4, unknown sequence; clone 5, heat shock cognate protein 70-ps1 (GenBank database accession number, X70065) (Fig. 3a). Comparison of the amino acid sequences of the peptides deduced from the cloned cDNAs revealed that all positive clones contained the unique amino acid sequence TRSPXP (single-letter amino acid code), suggesting that 3A10 recognizes this consensus sequence (Fig. 3a).

To test whether or not 3A10 recognizes the consensus amino acid sequence, we synthesized various peptides and examined their reactivities with the mAb. The synthetic peptides TRSPSPGGGC and PSPSRTGGGC were conjugated to maleimide-activated HRP *via* their cysteine residues, and then their binding to plates coated with 3A10 was examined by ELISA. Figure 3b shows that the TRSPSPGGGC peptide-HRP conjugate bound 3A10 and that the binding was inhibited by soluble TRSPSPGGGC peptide.

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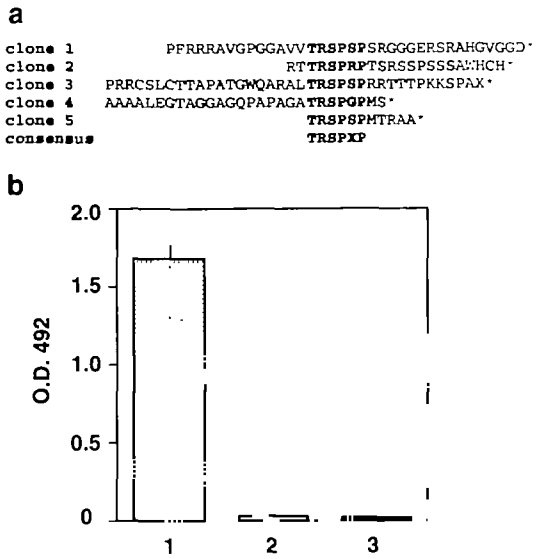

Fig. 3. **Binding of a synthetic decapeptide to 3A10.** a: Amino acid sequences deduced from the cDNA of 3A10-reactive clones (clones 1-5) and conserved amino acid residues are indicated in boldface letters. Asterisks (*) represent the end of translation due to stop codons in the 3A10-reactive clones. b: Reactivity of synthetic peptides to 3A10. The monoclonal antibody 3A10 (300 ng/well) was coated onto the wells of microtiter plates and incubated with HRP-conjugated synthetic peptides (0.1 μg/ml), TRSPSPGGGC (columns 1 and 2), and SPSPRTGGGC (column 3). Column 2: The HRP-conjugated TRSPSPGGGC peptide was incubated with 3A10 coated onto plates in the presence of unconjugated TRSPSPGGGC (20 μg/ml).

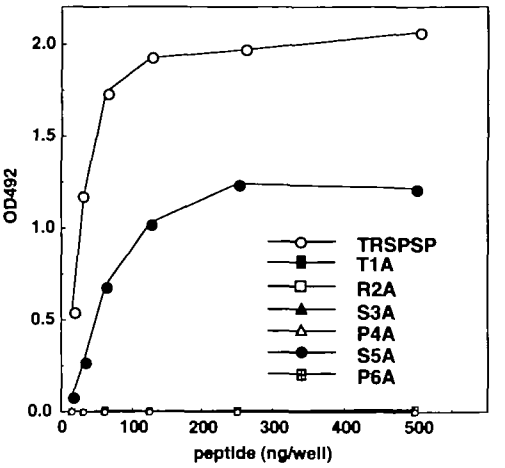

Fig. 4. **Amino acid residues indispensable for 3A10 binding to the TRSPSP peptide sequence.** Each amino acid was individually replaced with alanine and 3A10-binding to each synthetic peptide was examined by ELISA. Various amounts of synthetic peptides were coated onto microtiter plates for 12 h at 37°C, then incubated with 3A10 (2 μg/ml). Bound 3A10 was determined using biotinylated anti-mouse IgG and peroxidase-conjugated streptavidin. T1A, ARSPSPGGGC; R2A, TASPSPGGGC; S3A, TRAPSPGGGC; P4A, TRSASPGGGC; S5A, TRSPAPGGGC; P6A, TRSPSAGGGC.

The reversed sequence peptide, PSPSRTGGGC, conjugated to HRP, did not bind 3A10. To specify the residues involved in binding, peptides were prepared in which each amino acid was individually replaced with alanine, and 3A10-binding to each synthetic peptide was examined. Replacement of the fifth serine residue with alanine reduced the binding slightly, whereas other replacements all prevented binding (Fig. 4). These results indicate that 3A10 recognizes peptides with the consensus TRSPXP sequence.

When we searched for proteins with the consensus TRSPXP sequence using the computer-aided homology search program, BLAST (*33*), we found various mammalian proteins that contain the consensus sequence (data not shown). Since 3A10 bound to a limited number of brain-specific proteins in the immunoblotting analyses, it is likely that the mAb recognizes a particular conformational structure formed by the TRSPXP sequence, and that adjacent polypeptide chains may also be involved in the formation of the polypeptide epitope to 3A10. Our preliminary observations indicated that 3A10 bound only weakly to the synthetic peptide CGTGTSPKPSTRSPSPP, derived from one of the 3A10-reactive clones (accession number A022966), again suggesting that 3A10 recognizes a conformational structure associated with TRSPXP. In immunoblotting experiments, 3A10-binding to the 150-, 120-, 118-, 106-, 104-, 79-, and 77-kDa brain protein bands was abolished by preincubating the 3A10 with the synthetic peptide TRSPSPGGGC, suggesting that the mAb recognizes the consensus sequence present on these proteins (data not shown). Although an amino acid sequence similar to the TRSPXP sequence was found in both synapsins Ia and Ib (QRLPSP, amino acids 504-509 of rat brain synapsins I), it remains to be confirmed whether the region is responsible for the interaction with 3A10.

Recent studies have shown that conserved peptide motifs play crucial regulatory roles in cell-cell, protein-protein, and protein-lipid interactions (*27, 34, 35*). Our preliminary experiments show that the TRSPSPGGGC peptide conjugated to HRP specifically binds to a brain-specific 250 kDa protein, suggesting that the amino acids in this particular sequence play a role in cellular protein-protein interactions. Further studies are required to determine the functional significance of the consensus sequence recognized by 3A10.

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