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The neurofilament antibody RT97 recognises a developmentally regulated phosphorylation epitope on microtubule-associated protein 1B

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ABSTRACT

Microtubules are important for the growth and maintenance of stable neuronal processes and their organisation is controlled partly by microtubule-associated proteins (MAPs). MAP 1B is the first MAP to be expressed in neurons and plays an important role in neurite outgrowth. MAP 1B is phosphorylated at multiple sites and it is believed that the function of the protein is regulated by its phosphorylation state. We have shown that the monoclonal antibody (mAb) RT97, which recognises phosphorylated epitopes on neurofilament proteins, fetal tau, and on Alzheimer's paired helical filament-tau, also recognises a developmentally regulated phosphorylation epitope on MAP 1B. In the rat cerebellum, Western blot analysis shows that mAb RT97 recognises the upper band of the MAP 1B doublet and that the amount of this epitope peaks very early postnatally and decreases with increasing age so that it is absent in the adult, despite the continued expression of MAP 1B in the adult. We confirmed that mAb RT97 binds to MAP 1B by showing that it recognises MAP 1B immunoprecipitated from postnatal rat cerebellum using polyclonal antibodies to recombinant MAP 1B proteins. We established that the RT97 epitope on MAP 1B is phosphorylated by showing that antibody binding was abolished by alkaline phosphatase treatment of immunoblots. Epitope mapping experiments suggest that the mAb RT97 site on MAP 1B is near the N-terminus of the molecule. Despite our immunoblotting data, immunostaining of sections of postnatal rat cerebellum with mAb RT97 shows a staining pattern typical of neurofilaments with no apparent staining of MAP 1B. For instance, basket cell axons and axons in the granule cell layer and white matter stained, whereas parallel fibres did not. These results suggest that the MAP 1B epitope is masked or lost under the immunocytochemical conditions in which the cerebellar sections are prepared. The upper band of the MAP 1B doublet is believed to be predominantly phosphorylated by proline-directed protein kinases (PDPKs). PDPKs are also good candidates for phosphorylating neurofilament proteins and tau and therefore we postulate that the sites recognised by RT97 on these neuronal cytoskeletal proteins may be phosphorylated by similar kinases. Important goals are to determine the precise location of the RT97 epitope on MAP 1B and the kinase responsible.

Key words: Cerebellum; axonal growth; tau protein; Alzheimer's disease.

INTRODUCTION

Microtubule-associated protein (MAP) 1B is a high molecular weight structural MAP which stabilises microtubules (reviewed in Müller et al. 1994). Although MAP 1B is expressed in nonneuronal cells, neurons express particularly high levels (reviewed by Tucker, 1990; Müller et al. 1994; Bush et al. 1996*a*). Both MAP 1B protein and its mRNA are down-regulated with increasing developmental age in the

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CNS (Riederer et al. 1986; Safaei & Fischer, 1989; Fischer & Romano-Clarke, 1990; Garner et al. 1990) except in a few regions such as the olfactory system where axonogenesis continues throughout life (Viereck et al. 1989; Gordon-Weeks et al. 1993). MAP 1B has been implicated in axonal growth for the following reasons. It is the first structural MAP to be expressed in the developing brain (Tucker et al. 1988), and its expression correlates with axonogenesis, especially its phosphorylated isoforms (Bloom et al. 1985; Calvert & Anderton, 1985; Riederer et al. 1986, 1990; Schoenfeld et al. 1989; Fischer & Romano-Clarke, 1990). The presence of MAP 1B in growth cones is also consistent with a role in axon outgrowth (Mansfield et al. 1991; Black et al. 1994; Bush & Gordon-Weeks, 1994; Bush et al. 1996*b*). More direct evidence supporting a role for MAP 1B in axonogenesis comes from experiments with PC12 and neuroblastoma cells, in which it is the most abundant MAP (Brugg & Matus, 1988; Díaz-Nido et al. 1991). When these cells are induced to grow neurites, both MAP 1B and its mRNA are upregulated (Greene et al. 1983; Brugg & Matus, 1988; Zauner et al. 1992) and phosphorylated isoforms of MAP 1B are rapidly generated (Aletta et al. 1988; Díz-Nido et al. 1992). Furthermore, antisense oligodeoxynucleotides to MAP 1B reversibly block MAP 1B expression and neurite outgrowth by PC12 cells (Brugg et al. 1993) and cerebellar macroneurons (DiTella et al. 1996). Most recently, a transgenic MAP 1B 'knockout' mouse has been produced which shows widespread neurological disorders (Edelmann et al. 1996). Collectively these results suggest that MAP 1B plays a pivotal role in neuronal development.

The differential phosphorylation of MAPs can affect their microtubule binding properties (Murthy & Flavin, 1983; Lindwall & Cole, 1984; Brugg & Matus, 1988) and is therefore an important modulator of MAP function. Phosphorylated isoforms of MAP 1B have generally been recognised by several mAbs (for review see Bush et al. 1996*c*) but the location of the epitopes on MAP 1B recognised by these mAbs, and therefore their relation to each other, have yet to be determined. MAP 1B can be resolved into at least 2 bands by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (Aletta et al. 1988; Hasegawa et al. 1990; Ulloa et al. 1993*a*) and it was suggested by Ulloa et al. (1993*a*) that phosphorylation at mode I sites generates the higher molecular weight band, whilst phosphorylation at mode II sites has little effect on the electrophoretic mobility. The phosphorylation of MAP 1B is developmentally regulated (Fischer & Romano-Clarke, 1990; Riederer et al. 1990; Gordon-Weeks et al. 1993; Bush & Gordon-Weeks, 1994) and recently the mode I phosphorylated isoforms were shown to be downregulated in the adult, while the mode II sites were not (Ulloa et al. 1993*a*). It was suggested that proline-directed protein kinases phosphorylate mode I sites, and that casein kinase II (CKII) is responsible for phosphorylating mode II sites (Díaz-Nido et al. 1988; Ulloa et al. 1993*a*, *b*).

Phosphorylated brain MAP 1B, as well as MAP 1B phosphorylated by CKII in vitro, can coassemble with microtubules (Díaz-Nido et al. 1988, 1990). Furthermore, treatment of neuroblastoma cells with CKII antisense results in a reduced binding of MAP 1B to microtubules (Ulloa et al. 1993*b*). These results suggest that the phosphorylated mode II sites on MAP 1B generated by CKII activity enhance the microtubule binding of MAP 1B (Ulloa et al. 1993*b*). The properties of mode I phosphorylated isoforms of MAP 1B were not studied.

Mode I phosphorylated MAP 1B is enriched in growing axons and growth cones in culture (Mansfield et al. 1991; Black et al. 1994; Ulloa et al. 1994; Boyne et al. 1995; Bush et al. 1996*b*) and in vivo (Bloom et al. 1985; Schoenfeld et al. 1989; Gordon-Weeks et al. 1993; Bush & Gordon-Weeks, 1994). In contrast, the somatodendritic compartment contains mainly mode II and nonphosphorylated MAP 1B (Sato-Yoshitake et al. 1989; Fischer & Romano-Clarke, 1990; Riederer et al. 1990; Viereck & Matus, 1990; Gordon-Weeks et al. 1993). The concentration of MAP 1B at the distal ends of growing axons and their growth cones suggests that MAP 1B is associated with dynamic microtubules and may play a role in axonogenesis (reviewed in Gordon-Weeks, 1993; Bush et al. 1996*c*).

In this study we show that mAb RT97, a well characterised mAb that recognises phosphorylated neurofilament protein and tau (Anderton et al. 1982; Haugh et al. 1986; Miller et al. 1986; Ksiezak-Reding et al. 1987; Lee et al. 1987; Nukina et al. 1987; Coleman & Anderton, 1990), also recognises a developmentally regulated, phosphorylation epitope on MAP 1B. A preliminary report in abstract form of part of this work has already appeared (Johnstone & Gordon-Weeks, 1996).

MATERIALS AND METHODS

Preparation of recombinant MAP 1B protein and production of polyclonal antibodies

Rabbit polyclonal antibodies were raised to 2 recombinant MAP 1B proteins derived from a C-terminal

region of MAP 1B. One antibody (αMAP 1B–C1) was raised against a recombinant protein derived from a cDNA encoding the C-terminal region (neuraxin) of MAP 1B (Rienitz et al. 1989; Kirsch et al. 1990) and the other (αMAP 1B–C2) against a recombinant rat MAP 1B fusion protein encoded by a cDNA generated by RT-PCR.

The neuraxin cDNA (a kind gift from Prof. H. Betz; Rienitz et al. 1989) was amplified by polymerase chain reaction using a sense primer starting at nucleotide 384 of the neuraxin sequence (5'-A CAT ATG CTT CAC ATC ACC GAA AAT GGG CCA ACT-3′) and an antisense primer at nucleotide 1677 (5′-TT CGA AGT TCC CAA ACC TAA TTA CTT CCT TCC ACT CTC GAG CCA GAA-3′). The primers contained a *Nde* I and *Hind* III site respectively, for directional subcloning into pMW172K (Way et al. 1990) based on the original pET vectors of Studier (Studier et al. 1990). Following gel purification, the 1293 bp PCR generated fragment was digested with *Nde* I and *Hind* III, gel purified and ligated to *Nde* I–*Hind* III digested pMW172K. The ligation products were then transformed into *E. coli* strain BL21(DE3) (Studier & Moffat, 1986). The recombinant protein corresponded to amino acids 1691 to 2102 of the mouse MAP 1B sequence. Cultures (400 ml) of BL21(DE3) cells containing the cDNA construct were grown at 37 °C, induced by the addition of 1 mM IPTG (Novabiochem), harvested 4 h postinduction and frozen at −70 °C until use. Frozen pellets were resuspended in 10 ml of ice-cold Tris-buffered saline (TBS) containing 1 mM of PMSF and 1 mg/ml lysozyme (from chicken egg white, Sigma), left at room temperature for 30 min and extensively sonicated in the presence of 1 mM EDTA. Triton X-100 was added to 1% and then the mixture was centrifuged at 15000 g_{max} for 30 min to pellet the bacterial cell debris and aggregates. The supernatant was then heated to 100 °C for 5 min and centrifuged at 15000 g_{max} to pellet heat labile bacterial proteins. The recombinant MAP 1B protein derived from neuraxin was found to be heat stable and this provided an effective first step purification. Heat-stable proteins were separated on a preparative 4–12% gradient gel and stained with Coomassie Blue R250. A 70 kDa band was excised from the gel and eluted using an Amicon 'Centrilutor' Micro-eluter. Eluted protein was stored at −20 °C until use. Approximately 125 μg of the eluted recombinant protein was emulsified with complete Freund's adjuvant and injected subcutaneously into rabbits (New Zealand White), followed by 2 boosters with incomplete adjuvant and then the animals were exsanguinated. Immunoglobulin was obtained from sera by ammonium sulphate precipitation and further purified on Affi-Gel Blue columns (BioRad) according to the manufacturer's instructions.

αMAP 1B–C2 antibody was raised to a recombinant MAP 1B fusion protein. The cDNA was prepared by RT-PCR using rat brain polyA$^+$ RNA. The sense primer starting at nucleotide 5209 of the rat MAP 1B sequence was 5′-CGG GAT CCA TGC AGC ATC ACC TGG CC-3′ and the antisense primer at nucleotide 5931 was 5′-CGG AAT TCG TCA ACA TCC TGG CGT GC-3′. The primers contain a *Bam* H 1 and *Eco* R 1 site respectively for directional subcloning into pGEX-2T (Pharmacia). Following gel purification and ligation of the 722 bp fragment into pGEX-2T the plasmid was transformed into XL1-Blue cells. The fusion protein was induced by IPTG and affinity purified by glutathione-agarose columns (Sigma). The recombinant protein corresponded to amino acids 1836–2076 of mouse MAP 1B. Approximately 200 μg of the fusion protein was emulsified with complete Freund's adjuvant and injected subcutaneously into rabbits (New Zealand White), followed by 3 boosters with incomplete adjuvant and then the animals were exsanguinated.

A rabbit polyclonal antibody to a synthetic peptide encoding MAP 1B amino acids 1244–1246 was prepared as described (Johnstone et al. 1997) and rabbit polyclonal antibodies against synthetic peptides encoding amino acids 1–15 and 442–457 were a kind gift of Peter Brophy, Department of Veterinary Science, Edinburgh University.

All DNA manipulations were performed according to standard procedures (Maniatis et al. 1989).

Immunoprecipitation

To prepare tissue samples, whole cerebella were dissected from postnatal Wistar rats of various ages, immediately frozen in liquid nitrogen and stored at −80 °C. Frozen cerebella from postnatal day 4 (P4) Wistar rats were ground into a fine powder with a pestle and mortar kept on dry ice and then homogenised in homogenisation buffer [HB: 10 mM Tris HCl (pH 7.4) containing 5 mM EGTA, protease (1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and phosphatase inhibitors (20 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium molybdate, 0.25 mM sodium orthovanadate)] at 4 °C in a tight-fitting glass-glass homogeniser using 2 ml of buffer per gm wet weight of tissue. The homogenate was centrifuged at 15000 g_{max} for 30 min and the

supernatant (500 µl, 20 mg/ml) was incubated with 10 µl of preimmune rabbit serum and 200 µl of Protein A agarose (Sigma) overnight at 4 °C to remove nonspecifically bound proteins. The Protein A agarose was removed by centrifuging at 10000 g_{max} for 10 min. The rabbit polyclonal antibody to MAP 1B (10 µl) was then added to the precleared supernatant and incubated for 4 h at 4 °C. Protein A agarose (200 µl) was then added to the supernatant and incubated for a further 2 h at 4 °C and then washed by centrifugation 5 times with HB. The pellet was resuspended in 50 ml of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min.

Purification of MAP1B and NTCB digestion. An extract enriched in MAP 1B was obtained by poly-L-aspartic acid (PLAA) extraction of taxol microtubules as described by Fujii et al. (1990) with the following exceptions. Neonatal rat brains (P4–P8) were used and microtubules were extracted with 20 µg/ml of PLAA. The enriched extract was applied to a 1 ml Resource Q column (Pharmacia), previously equilibrated into column buffer (10 mM Mes, pH 6.5, 150 mM NaCl, 0.5 mM $Mg(CH_3OO)_2$, 0.1 mM EGTA, 1 mM DTT and 1 mM PMSF), at 1 ml/min. The column was eluted with a NaCl gradient between 150–1000 mM. MAP 1B eluted between 400–450 mM NaCl. The peak fractions were approximately 95% pure, as judged by SDS-PAGE. Typical yields were between 30–40 µg of MAP 1B per gram of brain tissue.

Purified MAP 1B fractions were precipitated with trichloroacetic acid and resuspended in cleavage buffer (200 mM Tris, pH 8.0, 7.5 M guanidine hydrochloride and 0.1 mM EDTA). Cysteine specific cleavage of MAP 1B was carried out using 2-nitro-5-thiocyanobenzoic acid (NTCB) as described by Petrucci & Morrow (1987). Cleaved products were dialysed into TBS and prepared for SDS-PAGE.

SDS-PAGE and immunoblotting

Protein was quantified using the Bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin as a standard according to manufacturer's instructions.

Protein samples were subjected to SDS-PAGE according to the procedure of Laemmli (1970) using polyacrylamide (4–10% gradient) gels. Gels were calibrated with molecular weight marker kits (Sigma, BioRad) and stained with Coomassie Brilliant Blue R250.

Protein samples, separated by SDS-PAGE, were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) at a constant voltage of 80 V for 1.5 h, in a wet blotting system (Bio-Rad). Nitrocellulose strips were incubated in blocking buffer [5% (w/v) nonfat milk proteins (Marvel) in TBS, pH 7.4] at room temperature for 2 h to block nonspecific binding. The blots were then incubated in mAb RT97 (1:500) or mAb AA6 (1:500; Riederer et al. 1986) in 1% (w/v) Marvel in TBS, pH 7.4, for 4 h at room temperature, washed for 20 min each in 0.05% Triton X-100 in TBS, pH 7.4, followed by 0.5 M NaCl, 0.05% Triton-X100 in TBS, pH 7.4 and a final wash in 0.05% Triton X-100 in TBS, pH 7.4. The blots were then incubated with the appropriate peroxidase-conjugated secondary antibody (Sigma) diluted in 1% (w/v) Marvel in phosphate-buffered saline (PBS) for 1 h at room temperature, washed again for 1 h in the same 3×20 min washes as above and developed with ECL kits (Amersham) as described in the manufacturer's instructions.

Alkaline phosphatase treatment of blots

Following blotting, nitrocellulose strips were blocked in 5% (w/v) Marvel in TBS, pH 7.4, for 2 h at room temperature, washed briefly in TBS, pH 7.4, and then incubated in alkaline phosphatase (Sigma Type VII-S, 205 IU/ml) in Tris-HCl, pH 8, containing 1 mM PMSF, 10 µg/ml pepstatin A, 10 µg/ml leupeptin and 10 µg/ml of aprotinin at 32 °C in a shaking water bath; controls lacked the alkaline phosphatase or included 100 mM sodium pyrophosphate in the incubation buffer. The reaction was stopped with 1 mM sodium fluoride and 100 mM sodium pyrophosphate-HCl, pH 8, for 5 min at room temperature and blots were then washed in borate-saline (100 mM boric acid, 25 mM sodium borate, 75 mM sodium chloride, pH 8.3), and immunostained with mAb RT97 as described above.

Immunostaining of sections

Neonatal and adult rats (Wistar) were anaesthetised with Sagatal (pentobarbitone) by intraperitoneal injection and killed by decapitation. Tissues were fixed by perfusion with 3% (w/v) formaldehyde/0.2% (v/v) glutaraldehyde, or 3% (w/v) formaldehyde alone, in PBS (pH 7.4) at 37 °C, washed in PBS and embedded in a solution of albumin (0.3 g/ml), gelatine (5 mg/ml) and sucrose (0.2 mg/ml) poly-

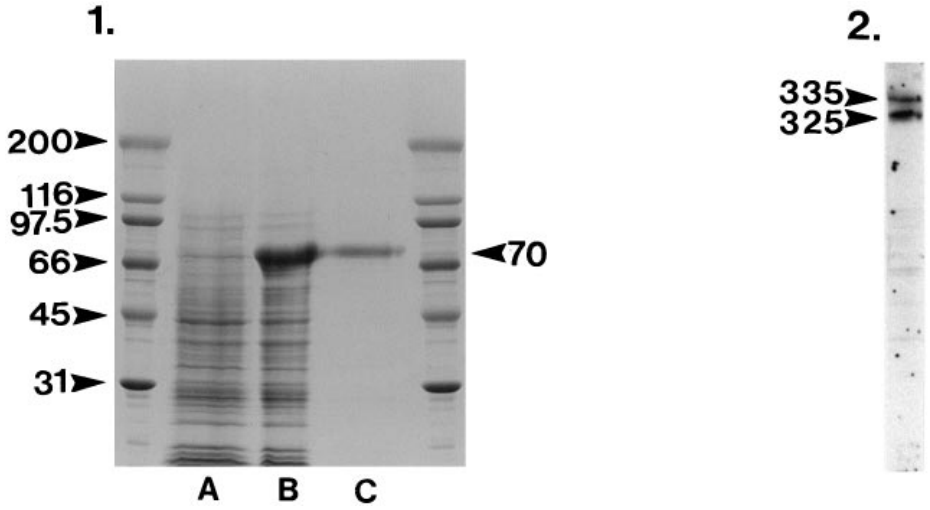

Fig. 1. Characterisation of polyclonal αMAP 1B–C1 antibody to MAP 1B. (1) Coomassie Blue stained gel showing the purification of the recombinant MAP 1B immunogen. Lane A, soluble proteins from control bacterial culture; lane B, soluble protein from MAP 1B transformed bacterial culture showing presence of a band at 70 kDa which is absent from control; lane C, heat-treated, gel purified neuraxin protein. (2) Immunoblot of P4 cerebellum probed with polyclonal αMAP 1B–C1 antibody. Both lower and upper bands of MAP 1B are recognised. Molecular weights are in kDa.

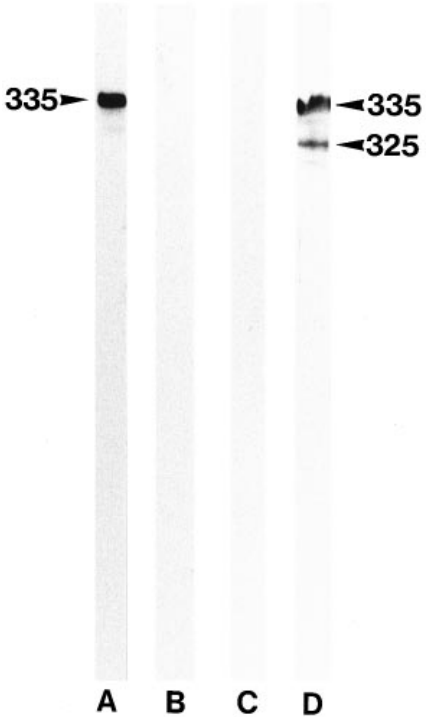

Fig. 2. Monoclonal antibody RT97 recognises immunoprecipitated MAP 1B. (*A*) Immunoblot of MAP 1B from P4 cerebellum immunoprecipitated with polyclonal antibody αMAP 1B–C1 and probed with mAb RT97. (*B*) Control showing a similar immunoblot to A without the primary antibody. (*C*) Control for nonspecifically binding proteins. Immunoblot of the P4 rat cerebellar proteins nonspecifically binding to Protein A agarose probed with mAb RT97. (*D*) Immunoblot of immunoprecipitated MAP 1B as in A but instead probed with anti-MAP 1B monoclonal antibody (clone AA6, Riederer et al. 1986). Note that mAb RT97 recognises the upper, mode I phosphorylated band of the MAP 1B doublet. There was no reaction when the primary antibody was omitted or when the blot in C was reprobed with AA6 (not shown). Molecular weights are in kDa.

merised with glutaraldehyde (2.5%). Sections (50 μm) were cut with a Vibroslice (Campden Instruments) and transferred to blocking buffer (BB; 5% (v/v) normal horse serum, 5% (v/v) normal goat serum, 50 mM L-lysine and 0.2% (v/v) Triton X-100 in PBS (pH 7.4) for a minimum of 2 h at room temperature or overnight at 4 °C. Free-floating sections were then incubated sequentially for 2 h or overnight with the following antibodies diluted in BB: monoclonal antibody (mAb) 150 (1:300) and peroxidase-conjugated goat antimouse, μ-chain specific (Sigma, 1:200), mAb SMI-31 (Affiniti, 1:24000), mAb RT97 (1:500), mAb RMO108 (1:1000) and goat anti-mouse IgG, rat serum adsorbed (Sigma, 1:50) and mouse PAP (Sigma, 1:100). Varying the dilution of these antibodies altered the intensity of the staining but did not alter the pattern. Sections was washed between each step for 30 min with PBS and the antibody complex visualised with diaminobenzidine (0.5 mg/ml) in Tris-buffered saline containing 0.01% H_2O_2. Controls, in which the primary antibody was replaced with BB, were developed in parallel. Sections were washed in Tris-buffered saline, air-dried onto gelatine-coated microscope slides, dehydrated in alcohol and mounted in DPX. Sections were viewed in an Olympus BH2 microscope equipped with Nomarski optics and photographed on Kodak T-Max 100 film.

For immunofluorescence, P 11 rat brains were frozen on dry ice and stored at −20 °C. Cryostat sections (20 μm) were cut at −18 °C and mounted onto gelatine-coated microscope slides as above. Sections were fixed in methanol at −20 °C for 10 min,

washed with TBS at room temperature and then immunolabelled as above, except that secondary antibodies were conjugated with fluorescein (Sigma). Sections were mounted in Citifluor (Citifluor Ltd, City University) and viewed with fluorescence optics using an Olympus BX50 microscope and photographed on Kodak T-Max 400 film.

RESULTS

mAb RT97 recognises a developmentally regulated phosphorylated epitope on MAP 1B

We obtained high levels of expression of both recombinant proteins and found that the recombinant protein derived from neuraxin was heat-stable (Fig. 1.1). Western blot analysis showed that antibody αMAP 1B-C1 (Fig. 1.2) and antibody αMAP 1B–C2 (not shown), produced from these recombinant proteins, specifically recognised MAP 1B in P4 rat cerebellum (Fig. 1.2) as well as their corresponding recombinant proteins (not shown).

We used antibody αMAP 1B–C1 to immunoprecipitate MAP 1B from P4 cerebellum. When blots of immunoprecipitated MAP 1B were probed with mAb RT97, the antibody recognised the upper band of the MAP 1B doublet (Fig. 2). We confirmed that antibody α-MAP 1B–C1 had immunoprecipitated MAP 1B by probing blots with a well-characterised monoclonal antibody to MAP 1B (clone AA6, Riederer et al. 1986; Fig. 2). Similar results were obtained with αMAP 1B–C2 antibody (not shown).

The epitope recognised by mAb RT97 on MAP 1B is phosphorylation dependent, as indicated by its complete abolition following treatment of the blots with alkaline phosphatase; an enzyme which removes phosphate from this protein (Fig. 3; Gordon-Weeks et al. 1993; Ulloa et al. 1993*a*). This effect is specific, and not due, for instance, to proteolysis of MAP 1B, since it can be prevented by inhibiting alkaline phosphatase with sodium pyrophosphate, a specific inhibitor (Fig. 3).

To map the epitope recognised by mAb RT97 on MAP 1B we purified native MAP 1B from neonatal rat brain and fragmented the molecule by chemical cleavage with the cysteine-specific reagent NTCB. Purified MAP 1B from neonatal rat brain consisted of 2 high molecular weight heavy chains (325 and 335 kDa) and a 34 kDa light chain (Fig. 4*a*, see also Pedrotti & Islam, 1995). Chemical cleavage of purified MAP 1B with NTCB generated a wide range of fragments (Fig. 4*a*). When these fragments were electroblotted and probed with site-specific polyclonal

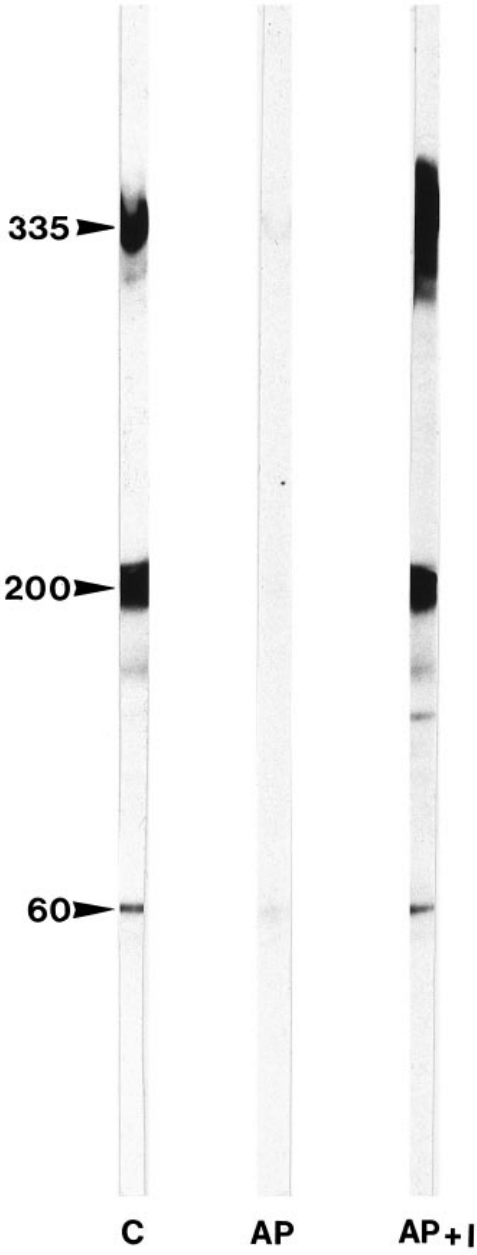

Fig. 3. Monoclonal antibody RT97 recognises a phosphorylation sensitive epitope on MAP 1B. Immunoblots of P4 rat cerebellum probed with mAb RT97. C, control; AP, alkaline phosphatase-treated; AP + I, alkaline phosphatase-treated in the presence of the inhibitor sodium pyrophosphate (100 mM). In control, mAb RT97 recognises MAP 1B (335 kDa band), high molecular weight neurofilament protein (200 kDa) and tau (60 kDa). Alkaline phosphatase treatment of the blots before immunolabelling completely abolishes binding of mAb RT97. Addition of the phosphatase inhibitor prevents removal of the phosphorylated epitope. Molecular weights are in kDa.

antibodies, 2 major, high-molecular weight fragments were identified of 215 kDa and 160 kDa (Fig. 4*b*). Both fragments were recognised by a polyclonal antibody to amino acids 442–457 and 1244–1264 but not by a polyclonal antibody to amino acids 1691–2102 whereas only the 215 kDa fragment was recog-

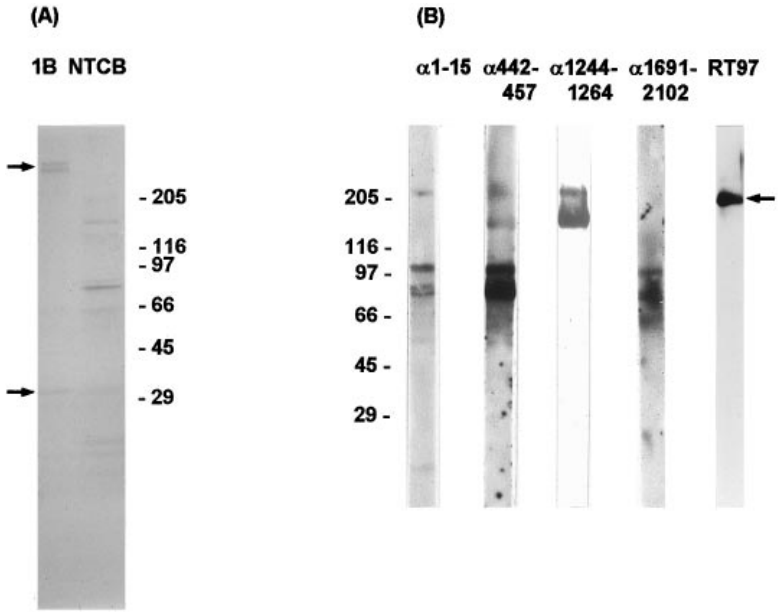

Fig. 4. (*A*) Coomassie blue stained gel of purified, native MAP 1B from neonatal rat brain showing the heavy chain doublet (1B, upper arrow) and the light chain (lower arrow) and the NTCB digest fragments (NTCB). (*B*) MAP 1B fragments generated by NTCB cleavage at the amino terminal side of cysteine residues were analysed by probing Western blots with site specific polyclonal antibodies and mAb RT97. A fragment of 215 kDa was recognised by mAb RT97 (arrow).

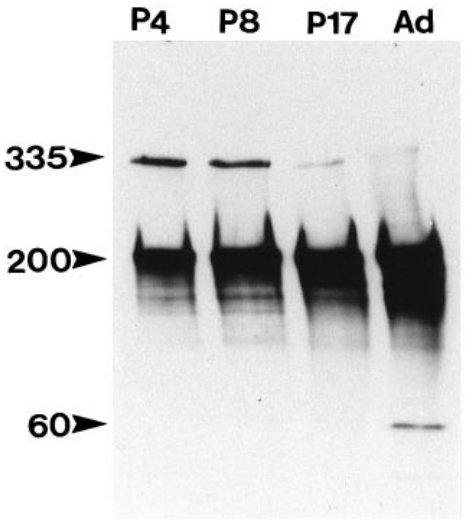

Fig. 5. Monoclonal antibody RT97 recognises a developmentally regulated phosphorylation epitope on MAP 1B in the developing cerebellum. Immunoblots of rat cerebellum at the postnatal (P) ages indicated probed with mAb RT97. mAb RT97 recognises immunoreactive bands at 335 kDa (MAP 1B), 200 kDa (NF-H) and 60 kDa (tau). The immunoreactive band at 33 kDa (MAP 1B) decreases in intensity from P4 to P17 cerebellum and is barely detectable in the adult. In contrast, the neurofilament immunoreactive band (200 kDa) increases in intensity from P4 to P17 and peaks in adult cerebellum. The tau epitope (60 kDa) appears only at the adult stage. Equal amounts of protein (40 μg) were loaded in each lane. Molecular weights are in kDa.

nised by a polyclonal antibody to the first 15 amino acids of MAP 1B (Fig. 4*b*). This suggests that the 160 kDa fragment is derived from the 215 kDa fragment by removal of the N-terminus. Examination of positions of cysteine residues along the MAP 1B sequence indicates that the 215 kDa fragment extends from the N-terminus to amino acid 1603. The location of the NTCB cleavage sites on MAP 1B further suggests that the 160 kDa fragment extends from amino acid 381 to 1603. Monoclonal antibody RT97 only recognised the 215 kDa fragment, suggesting that the epitope for this mAb lies in the first 380 amino acids of MAP 1B (Fig. 4*b*).

To determine the developmental expression of the phosphorylation epitope on MAP 1B recognised by mAb RT97, we immunoblotted samples of cerebellum from rats of different ages. During the first postnatal week, the MAP 1B epitope recognised by mAb RT97 was strongly expressed in the cerebellum but declined thereafter so that by P17 it was greatly reduced and in the adult it was absent (Fig. 5). In contrast, mAb RT97 immunoreactivity for neurofilament proteins increased during development and was highest in the adult (Fig. 5). The rapid decline in the expression of the mAb RT97 epitope on MAP 1B is much more dramatic than the down regulation of the total levels of MAP 1B in the cerebellum because the expression of the protein is maintained into adulthood, albeit at reduced levels, as indicated by blotting with antibodies against phosphorylation-independent epitopes of MAP 1B (not shown; see also Sato-Yoshitake et al. 1989).

These results show that mAb RT97 recognises a

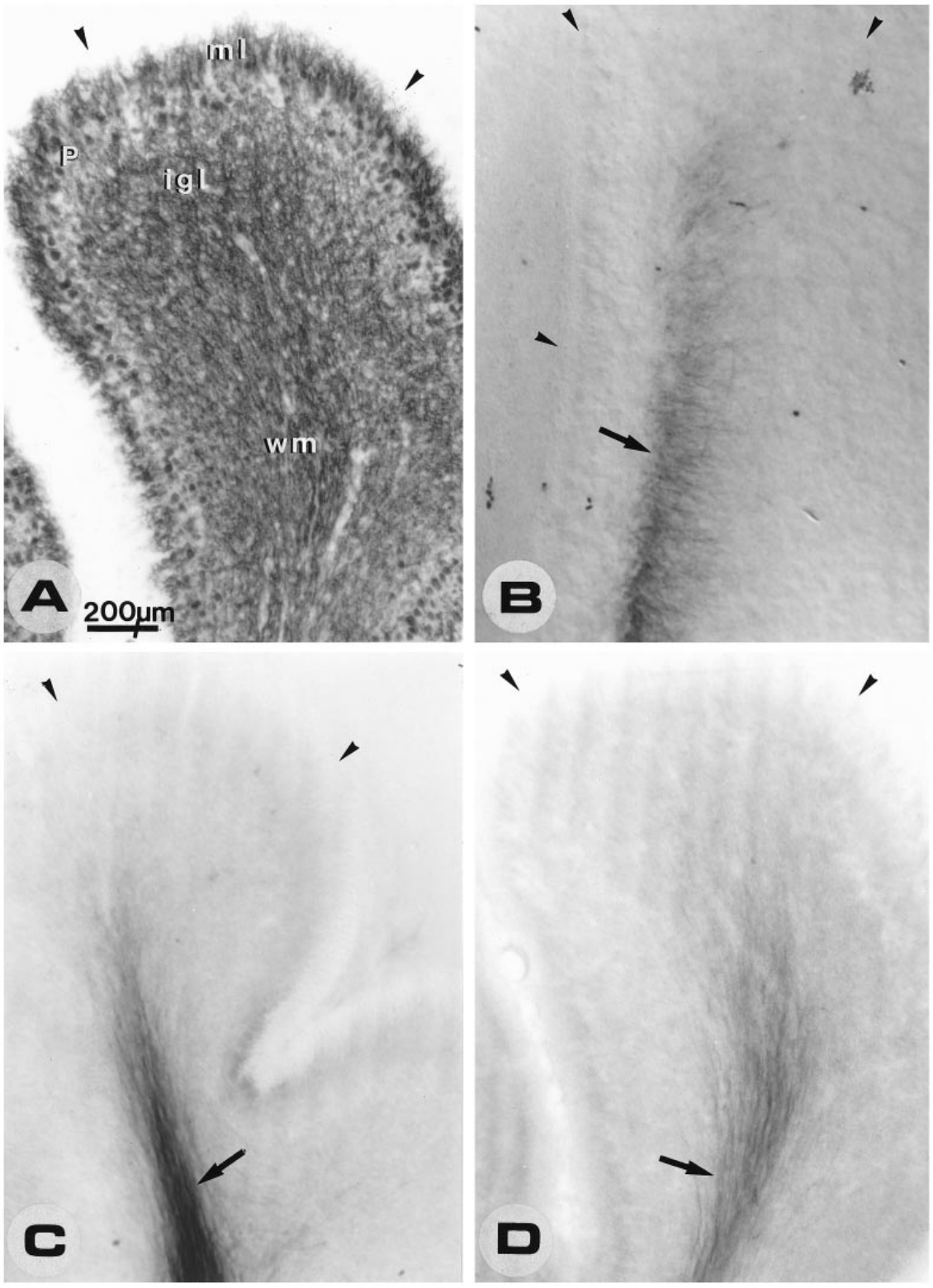

Fig. 6. Parasagittal vibroslice sections (~ 50 μm) of P5 rat cerebellum immunolabelled with mAb 150 (*A*), mAb RT97 (*B*), mAb SMI-31 (*C*) and mAb RMO108 (*D*) and viewed with Nomarski optics. mAb 150 (*A*) strongly labels growing axons throughout the cerebellum, including the parallel fibres in the developing molecular layer (ml), axons in the internal granule cell layer (igl) and the white matter (wm). mAb 150 also cross-reacts with a nuclear epitope present in some cells including Purkinje cells (P). mAb RT97 (*B*) SMI-31 (*C*) and RMO108 (*D*) stain axons in the white matter (arrows in *B*, *C* and *D*). In all micrographs, the pia and external granule cell layer, delimited by arrowheads, are unstained. Magnification in *B–D* as in *A*.

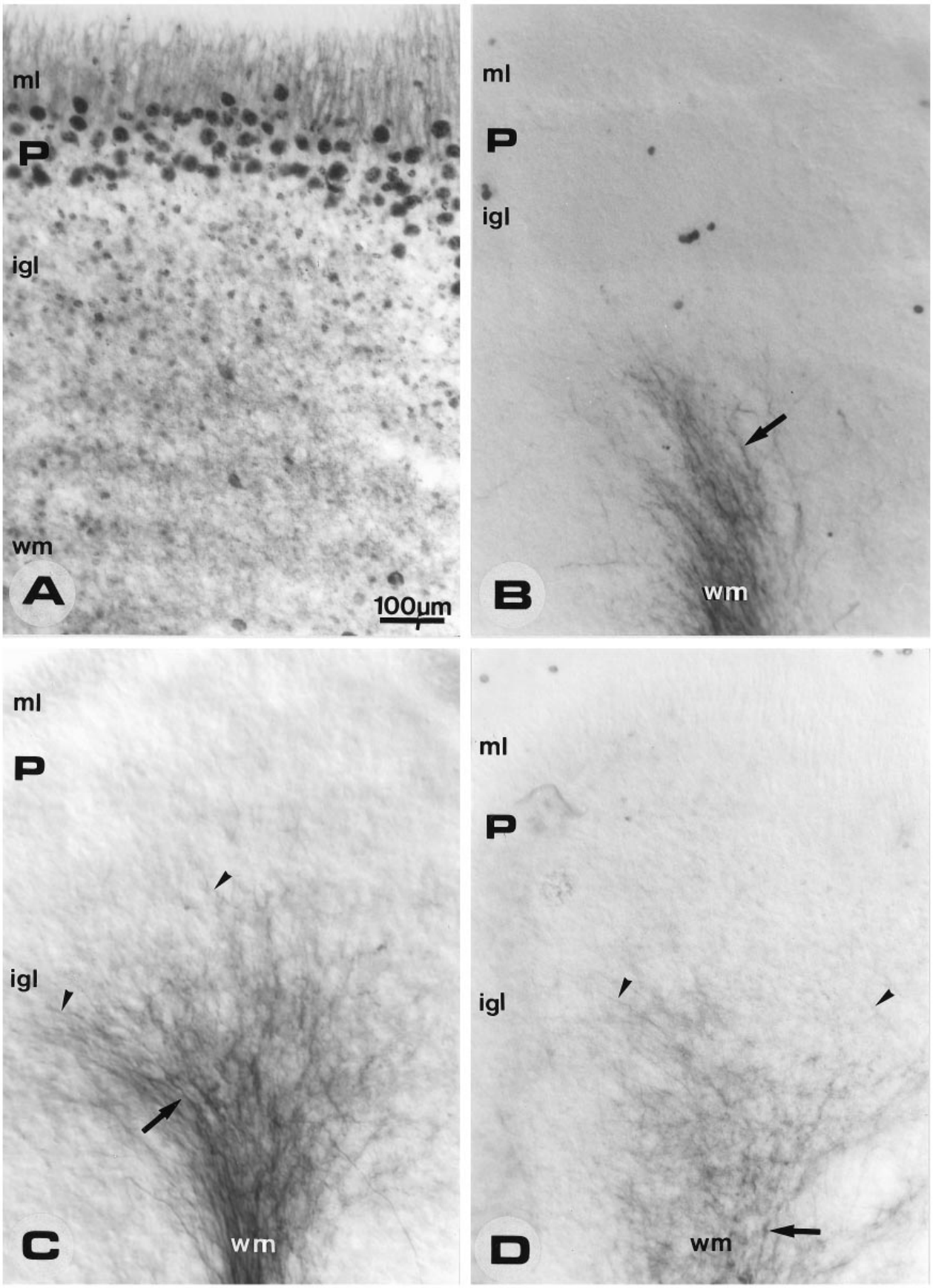

Fig. 7. Parasagittal vibroslice sections (~ 50 μm) of P7–P8 rat cerebellum immunolabelled with mAb 150 (*A*), mAb RT97 (*B*), mAb SMI-31 (*C*) and mAb RMO108 (*D*) and viewed with Nomarski optics. mAb 150 continues to label strongly the growing parallel fibres in the molecular layer (ml) and axons in the internal granule cell layer (igl) and white matter (wm). Note also the staining of the nuclear epitope in some cells, particular the Purkinje neurons (P). mAb RT97 labels axons in the white matter at this stage (arrows in *B*), whereas mAb SMI-31 (*C*) and mAb RMO108 (*D*) label axons in the white matter (arrows) and in the overlying internal granule cell layer (arrowheads; igl). Magnification in *B–D* as in *A*.

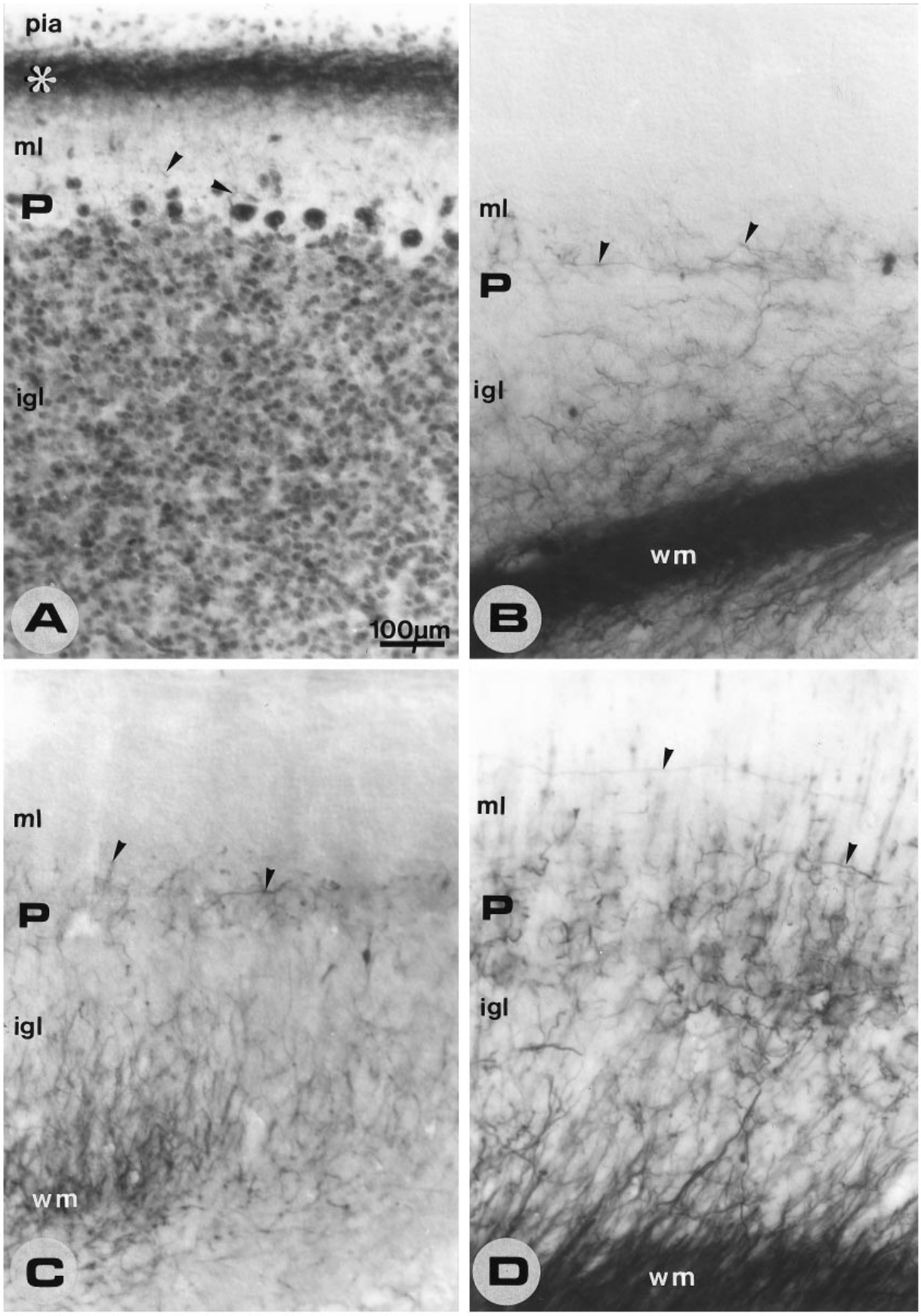

Fig. 8. Vibroslice parasagittal sections (~ 50 µm) of P14–P15 rat cerebellum immunolabelled with mAb 150 (*A*), mAb RT97 (*B*), mAb SMI-31 (*C*) and mAb RMO108 (*D*) and viewed with Nomarski optics. By the beginning of the third postnatal week, mAb 150 staining in the cerebellum is beginning to decline. In the molecular layer (ml) staining is restricted to an upper band of parallel fibres that are still growing

phosphorylation epitope on MAP 1B that is strongly regulated during development.

Map 1B and neurofilament antibody staining patterns in postnatal rat cerebellum

We compared the staining patterns of mAb RT97 with that of mAb 150, which recognises a developmentally regulated phosphorylation epitope on MAP 1B (Mansfield et al. 1991; Gordon-Weeks et al. 1993), mAb SMI-31, which recognises phosphorylation epitopes on MAP 1B, neurofilament proteins and tau (Goldstein et al. 1983; Sternberger & Sternberger, 1983; Fischer & Romano-Clarke, 1990; Sloan et al. 1991; Lichtenberg-Kraag et al. 1992; Bush & Gordon-Weeks, 1994), and mAb RMO108 which recognises a phosphorylated epitope on high molecular weight neurofilament protein (Carden et al. 1987).

First postnatal week. Monoclonal antibody 150 strongly stained axons throughout the rat cerebellar cortex and the white matter during the first postnatal week, as previously reported (Gordon-Weeks et al. 1993). (Fig. 6*a*). The parallel fibres in the developing molecular layer and axons in the internal granule cell layer and white matter were particularly strongly stained. This antibody cross-reacts with a nuclear epitope present in some cells, including Purkinje neurons, at this time (Gordon-Weeks et al. 1993). The nature of the nuclear epitope is unknown although it is phosphorylated (Gordon-Weeks et al. 1993; Riederer et al. 1993; Ulloa et al. 1994). In contrast, mAbs RT97, SMI-31 and RMO108 only stained axons in the white matter in the first week of postnatal life (Fig. 6*b–d*).

Second postnatal week. In the second postnatal week, mAb 150 continued to stain axons in the cerebellar cortex, particularly the growing parallel fibres (Fig. 7*a*). The nuclear epitope persisted and was particularly strong in Purkinje cells. mAb RT97 strongly stained axons in the white matter during the second postnatal week (Fig. 7*b*), as did mAb SMI-31 and RMO108. However, these last 2 antibodies also stained axons in the lower regions of the internal granule cell layer (Fig. 7*c–d*).

Third postnatal week. By the beginning of the third postnatal week, mAb 150 staining of the cerebellum started to decline. In the molecular layer, axonal staining was restricted to the superficial half (Fig. 8*a*). In the lower half of the molecular layer, basket cell axons were strongly stained while the parallel fibres were unstained (Fig. 8*a*). At P15, the basket cell axons stained by mAb 150 were varicose in outline, whereas at later ages they were smooth. mAbs RT97, SMI-31 and RMO108 also stained basket cell axons at these times but the axons were always smooth (Fig. 8*b–d*). These antibodies also continued to stain axons in the white matter and the internal granule cell layer although at no time during development did they stain the parallel fibres.

Adult. In the adult cerebellum, mAb 150 stained basket cell axons in the molecular layer and the dense accumulation of basket cell axons around the initial segment of the Purkinje cell axon (ponceau) and, occasionally, axons in the white matter (Fig. 9*a*). Parallel fibres were no longer stained nor were axons in the granule cell layer. Most of the Purkinje cell nuclei were no longer stained by mAb 150 whereas staining of nuclei in the internal granule cell layer persisted (Fig. 9*a*). mAbs RT97, SMI-31 and RMO108 all stained basket cell axons, including the ponceau, strongly in the adult (Fig. 9*b–d*). These antibodies also stained axons in the granule cell layer and the white matter. In the deep cerebellar nuclei, RMO108 but not RT97 and SMI-31, stained neurofilaments in neuronal cell bodies (not shown).

The lack of staining of the parallel fibres by mAbs RT97 and SMI-31, which contain developmentally-regulated phosphorylated isoforms of MAP 1B, as indicated by the mAb 150 staining, led us to investigate alternative fixation methods. We prepared frozen sections of unfixed cerebellum and fixed the sections in methanol. However, immunofluorescence with mAbs RT97 and SMI-31 revealed an identical staining pattern to that seen with aldehyde fixed material (not shown).

DISCUSSION

The mAb RT97 recognises a developmentally-regulated, phosphorylation epitope on MAP 1B

We have shown by Western blot analysis that the mAb RT97 recognises a protein that comigrates with the upper band of the MAP 1B doublet in postnatal rat cerebellum. Immunoprecipitation with 2 polyclonal antibodies to recombinant fragments of MAP 1B confirmed the identity of the protein recognised by RT97 as MAP 1B. Furthermore,

(asterisk) while the lower part of the molecular layer, where parallel fibres have formed synapses, is unstained, except for basket cell axons (arrowheads). The Purkinje cell nuclei still stain (P) and there is a resurgence in the staining of nuclei in the internal granule cell layer. mAb RT97, SMI-31 and RMO108, continue to stain axons in the white matter (wm) and, in addition, now stain many axons in the internal granule cell layer (igl) and basket cell axons (arrowheads) in the molecular layer (ml). Magnification in *B–D* as in *A*.

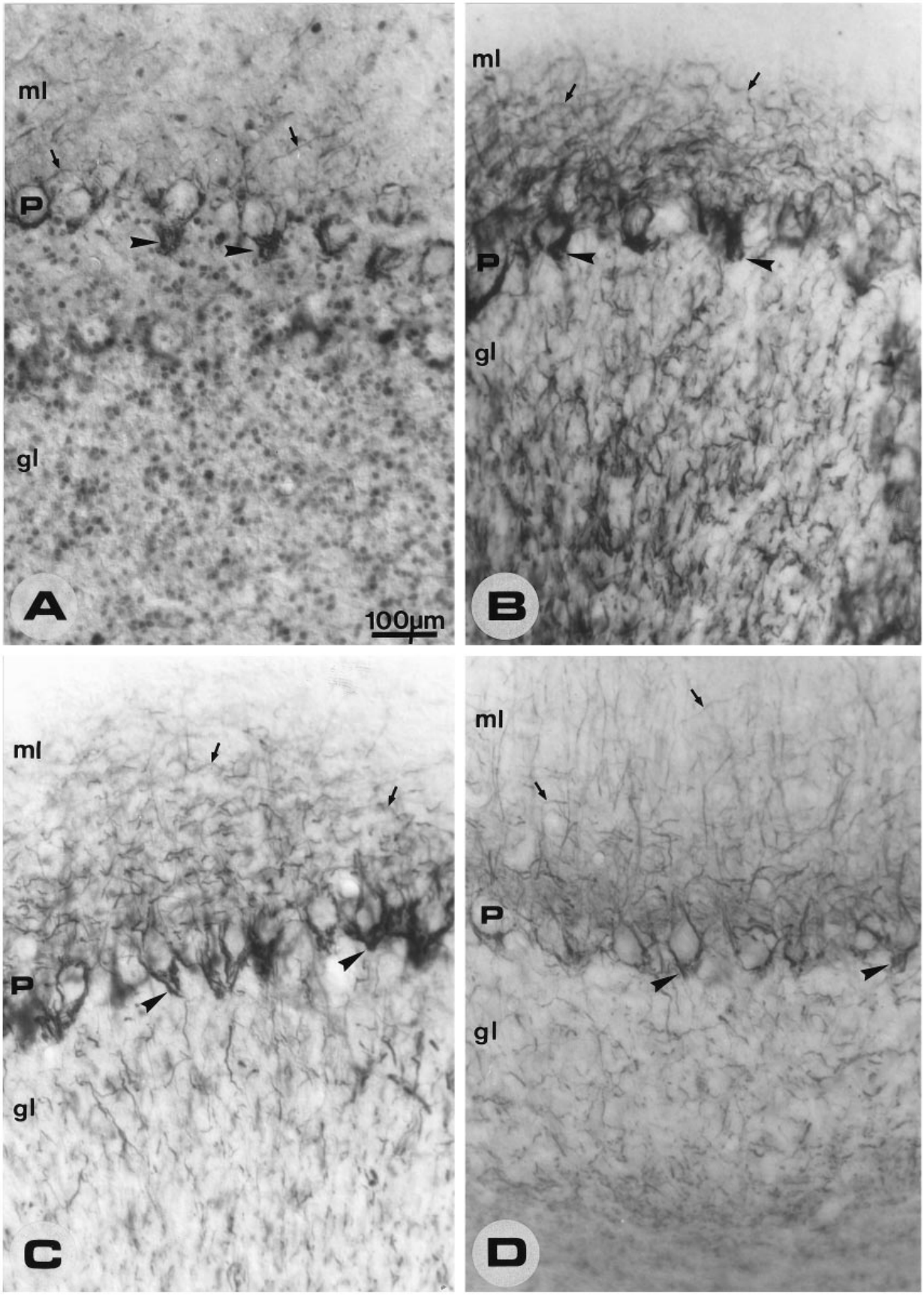

Fig. 9. Parasagittal vibroslice sections (~ 50 μm) of adult (~ P90) rat cerebellum immunolabelled with mAb 150 (*A*), mAb RT97 (*B*), mAb SMI-3 (*C*) and mAb RMO108 (*D*) and viewed with Nomarski optics. Parallel fibres in the molecular layer no longer stain with mAb 150. The staining of basket cell axons (arrows) persists in the molecular layer (ml) and particularly the ponceau around the Purkinje (P) cell body and initial axons (arrowheads). There is also staining of a small proportion of axons in the white matter (not shown). The Purkinje cell nuclear staining has gone, but not the nuclear staining in the granule cell layer (gl). Magnification in *B–D* as in *A*.

mAb RT97 recognised a chemical cleavage fragment of purified, native MAP 1B. The epitope is phosphorylated, as indicated by its sensitivity to alkaline phosphatase, and developmentally regulated. This is the first report of a crossreactivity of mAb RT97 with MAP 1B. Monoclonal antibody RT97 had previously been shown to recognise a phosphorylation epitope on high molecular weigh neurofilament protein (NF-H) and medium molecular weight neurofilament protein (NF-M) (Anderton et al. 1982; Haugh et al. 1986; Miller et al. 1986). The sequence recognised is Lysine-Serine-Proline (KSP in the one letter code) in the carboxy-terminal domain of NF-H and NF-M (Coleman & Anderton, 1990) which suggests that the kinase responsible belongs to the class of proline-directed serine kinases. Monoclonal antibody RT97 also recognises phosphorylated KSP in tau of Alzheimer neurofibrillary tangles (Anderton et al. 1982; Ksiezak-Reding et al. 1987; Lee et al. 1987; Nukina et al. 1987). Peptide mapping of MAP 1B fragments derived from cysteine specific cleavage using NTCB shows that the mAb RT97 epitope lies within the region between the N-terminus and residue 380. There are only 4 Serine-Proline sites in this region of rat MAP 1B and no Lysine-Serine-Proline sites. The kinase(s) responsible for phosphorylating neurofilament proteins and tau at the RT97 epitope has not been identified. In PC12 cells, the proline-directed serine kinase MAP kinase (ERK1/2) phosphorylates MAP 1B (Tsao et al. 1990; Loeb et al. 1992) and is therefore a candidate for generating the RT97 epitope on MAP 1B. Furthermore, this kinase is present in growth cones (Rocha & Avila, 1995). A serine/threonine kinase phosphorylates MAP 1B in fibroblasts (Hoshi et al. 1990) but it is not known if this kinase is expressed in neurons.

In many respects the epitope recognised by mAb RT97 on MAP 1B is similar to that recognised by mAb SMI-31. Like mAb RT97, mAb SMI-31 recognises phosphorylation epitopes on tau and the high and medium molecular weight proteins of neurofilaments (Sternberger & Sternberger, 1983; Fischer & Romano-Clarke, 1990; Lichtenberg-Kraag et al. 1992; Ulloa et al. 1993*a*). The SMI-31 epitope in tau involves the sequence Ser^{396} and Ser^{404}, both serine residues occur immediately upstream of prolines and both serines must be phosphorylated for mAb SMI-31 to bind (Lichtenberg-Kraag et al. 1992). Both epitopes on MAP 1B are down-regulated during neuronal development and both epitopes are lost or masked during the preparation of tissue sections (see below). However, we have recently located the mAb SMI-31 epitope on MAP 1B and it is on a different part of the molecule from the epitope recognised mAb RT97 (Johnstone et al. 1997).

Expression and distribution of mAb RT97 epitopes in vivo

The laminar organisation of the cerebellum, its relatively small cohort of neuronal cell types and the wealth of information about its development make it an attractive brain region to study neuronal differentiation. It is particularly appropriate for the present study because, although the parallel fibres of the granule cells, which comprise a major portion of the axons in the cerebellar cortex, contain neurofilaments (Peters et al. 1991), they are not composed of conventional neurofilament triplet proteins (Shaw et al. 1981; Goldstein et al. 1982; Sternberger & Sternberger, 1983). Neurofilaments in parallel fibres are composed of α-internexin, a protein related to the conventional neurofilament triplet proteins (Chiu et al. 1989; Kaplan et al. 1990; Fliegner et al. 1994). Parallel fibres do, however, express MAP 1B, including the developmentally-regulated phosphorylated isoforms (Calvert & Anderton, 1985; Sato-Yoshitake et al. 1989; Gordon-Weeks et al. 1993). In this study we found that the staining pattern in the rat cerebellum of 2 mAbs, RT97 and SMI-31, that recognise phosphorylation epitopes on MAP 1B in immunoblots, was closely similar to that of neurofilament expression, as seen with mAb RMO108, and that neither of these antibodies stained the parallel fibres in the molecular layer. As previously reported (Gordon-Weeks et al. 1993), mAb 150, which also recognises a developmentally regulated phosphorylation epitope on MAP 1B, stained the parallel fibres in the developing cerebellum. These results suggest that the epitopes recognised by mAbs RT97 and SMI-31 on MAP 1B are masked or lost in fixed material and that these epitopes are distinct from that recognised by mAb 150.

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