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# Localisation of Microtubule-Associated Protein 1B Phosphorylation Sites Recognised by Monoclonal Antibody SMI-31

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**Abstract:** MAP 1B is a microtubule-associated phosphoprotein that is expressed early in neurons and plays a role in axon growth. MAP 1B has two types of phosphoisoforms, one of which is developmentally down-regulated after neuronal maturation and one of which persists into adulthood. Because phosphorylation regulates MAP 1B binding activity, characterisation of the phosphorylation sites and identification of the corresponding kinases/phosphatases are important goals. We have characterised the developmentally down-regulated phosphorylation sites recognised by monoclonal antibody (mAb) SMI-31. We purified MAP 1B from neonatal rat brain and mapped the mAb SMI-31 sites to specific MAP 1B fragments after chemical cleavage. We then developed an in vitro kinase assay by using a high-speed spin supernatant from neonatal rat brain in the presence of ATP and recombinant proteins encoding selective regions of the MAP 1B molecule. Phosphorylation of the recombinant protein was detected on western blots using mAb SMI-31. This analysis showed that mAb SMI-31 recognises two recombinant proteins corresponding to residues 1,109–1,360 and 1,836–2,076 of rat MAP 1B after in vitro phosphorylation. The former phosphorylation site was further defined in the in vitro kinase assay by inhibition with peptides and antibodies from candidate regions of the MAP 1B sequence. This approach identified a region of 20 amino acids, from 1,244 to 1,264, characterised by a high concentration of serines immediately upstream of prolines, indicating that the kinase responsible is a proline-directed serine kinase. **Key Words:** Growth cone—Proline-directed kinase—Microtubule.
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The microtubule-associated protein (MAP) 1B is a developmentally regulated phosphoprotein that is expressed at high levels in growing neurons (for review, see Müller et al., 1994). Although the precise function of MAP 1B is unclear, indirect evidence strongly suggests that the molecule plays an important role in axon growth and possibly in growth cone structure and dynamics (for review, see Bush et al., 1996). For instance, the expression of MAP 1B, in particular its phosphorylated isoforms, correlates with axon growth (Bloom et al., 1985; Calvert and Anderton, 1985; Riederer et al., 1986; Schoenfeld et al., 1989; Fischer and Romano-Clarke, 1991). It is the first structural MAP to be expressed in developing neurons (Tucker et al., 1988) and inhibition of expression with antisense oligodeoxynucleotides blocks axon growth (Brugg et al., 1993; DiTella et al., 1996). More recently, a transgenic MAP 1B "knock-out" mouse has been produced in which the homozygotes die in utero and the heterozygotes show widespread neurological disorders (Edelmann et al., 1996). There are two phosphorylated isoforms; one is developmentally down-regulated, expressed only in growing axons and probably generated by proline-directed kinases, although which ones is not known; the second isoform is expressed throughout the neuron, maintained into adulthood and probably generated by casein kinase II (for review, see Bush et al., 1996). The developmentally regulated phosphorylated isoforms are particularly concentrated in growth cones (Mansfield et al., 1991; Black et al., 1994; Bush and Gordon-Weeks, 1994; DiTella et al., 1996), an observation that further supports their role in axon growth.

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*Abbreviations used:* BB, blocking buffer; DTT, dithiothreitol; GST, glutathione *S*-transferase; αGST, polyclonal antibody to GST; KB, kinase buffer; mAb, monoclonal antibody; MAP, microtubule-associated protein; NTCB, 2-nitro-5-thiocyanobenzoic acid; P, postnatal day; pAb, polyclonal antibody; PLAA, poly-L-aspartic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

Although the precise effect of phosphorylation on MAP 1B function is not known, it is clear that phosphorylated isoforms bind microtubules more effectively than nonphosphorylated isoforms (Díaz-Nido et al., 1988). The interaction of MAP 1B with actin filaments also appears to be regulated by phosphorylation, at least in vitro (Pedrotti and Islam, 1996). Several monoclonal antibodies (mAbs) recognise developmentally regulated phosphorylation epitopes on MAP 1B, including 1B6 (Sato-Yoshitake et al., 1989), 150 (Mansfield et al., 1991; Gordon-Weeks et al., 1993; Ulloa et al., 1993), 1BP (Black et al., 1994), SMI-31 (Fischer and Romano-Clarke, 1990; Bush and Gordon-Weeks, 1994), and RT97 (Johnstone et al., 1997). These antibodies have proved useful in mapping the developmental expression of the different isoforms. Here, we present data on the localisation of the sites on MAP 1B recognised by mAb SMI-31. One of them is characterised by a remarkably high concentration of serines followed, immediately downstream, by a proline, suggesting that the kinase responsible is a proline-directed serine kinase. This is a first step in determining the effect of phosphorylation at this site on the function of MAP 1B and in identifying the kinase responsible.

## EXPERIMENTAL PROCEDURES

### Purification of MAP 1B and 2-nitro-5-thiocyanobenzoic acid (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 (postnatal days 4–8, 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 buffer B [10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, 150 m*M* NaCl, 0.5 m*M* magnesium acetate, 0.1 m*M* EGTA, 1 m*M* dithiothreitol (DTT), and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)], at 1 ml/min. The column was eluted with a NaCl gradient of 150–1,000 m*M*. MAP 1B eluted between 400 and 450 m*M* NaCl. The peak fractions were ~95% pure, as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Typical yields were 30–40 μg of MAP 1B/g of brain tissue.

Purified MAP 1B fractions were precipitated with trichloroacetic acid and resuspended in cleavage buffer (200 m*M* Tris, pH 8.0, 7.5 *M* guanidine hydrochloride, and 0.1 m*M* EDTA). Cysteine-specific cleavage of MAP 1B was performed by using NTCB as described by Petrucci and Morrow (1987). Cleaved products were dialysed into Tris-buffered saline (TBS) and prepared for SDS-PAGE.

### Production and purification of glutathione *S*-transferase (GST)-MAP 1B fusion proteins

A library of GST-MAP 1B fusion proteins was produced covering the entire MAP 1B sequence. We produced GST-MAP 1B fusion proteins from amino acids 1–567, 568–1,109, 1,110–1,688, 1,674–2,102, and 1,690–2,464 by subcloning these regions of the mouse MAP 1B cDNA into the prokaryotic expression vector pGEX-2T, transformed into *E. coli* XL-1 Blue cells. In addition, two further GST-MAP 1B fusion proteins, GST-1B750 and GST-1BNR, were prepared by subcloning the rat MAP 1B sequence (accession no. X60550) from amino acid 1,109 to 1,360 and 1,836 to 2,076 (based on the numbering system of the murine sequence; accession no. X51396), respectively, into pGEX-2T, transformed into *E. coli* XL-1 Blue cells. All of the expressed fusion proteins were soluble and were purified from lysed bacterial cells under nondenaturing conditions by absorption with *S*-linked glutathione–agarose beads (Sigma). Recombinant proteins were quantified for use in the kinase assay (see below) by gel electrophoresis using bovine serum albumin as standard. The gels were stained with Coomassie Brilliant Blue R-250 and scanned with a Molecular Dynamics image analyser or a flat-bed scanner (Hewlett-Packard ScanJet 4C) and analysed using Phoretix 1-D gel analysis software to determine the integrated peak areas of the protein bands.

### Phosphorylation of recombinant MAP 1B protein in vitro by neonatal rat brain extract

Early postnatal (P1–P5) rat brain extracts were prepared by homogenising rat brain in 10 m*M* Tris-HCl, pH 7.4, containing 5 m*M* EGTA, 2 m*M* DTT, protease inhibitors (1 m*M* PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and phosphatase inhibitors [50 m*M* sodium fluoride and 1 m*M* okadaic acid (LC Laboratories)]. The homogenisation was performed using 2.5 ml of buffer/g wet weight of tissue. Homogenates were centrifuged at 100,000 $g_{max}$ for 1 h at 4°C and the supernatant was collected giving a cytosolic extract ($S_1$). Endogenous microtubule protein was removed from the supernatant by the addition of GTP (1 m*M*) and taxol (20 μ*M*), incubation at 37°C for 30 min and centrifugation at 100,000 $g_{max}$ for 30 min at 26°C, giving a postmicrotubule supernatant ($S_2$).

Phosphorylation reactions were performed in kinase buffer [KB; 40 m*M* HEPES, pH 7.4, 2 m*M* ATP, 5 m*M* EGTA, 2 m*M* $MgCl_2$, 2 m*M* DTT, 50 m*M* sodium fluoride, 1 m*M* okadaic acid, and protease inhibitors (1 m*M* PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin)] containing 0.1 mg/ml fusion protein attached to glutathione–agarose beads. Reactions were initiated by the addition of rat brain extract (1 μl $S_1$ or 2.5 μl $S_2$ in 50 μl of reaction buffer) and incubated at 37°C in a gently shaking incubator.

Samples were pipetted from the assay at various time points and the beads were pelleted by spinning at 500 $g_{max}$ for 1 min in a bench-top centrifuge. The supernatant was aspirated off and the beads were washed in 500 μl of KB. Recombinant protein was then eluted by resuspending the beads in SDS sample buffer and boiling for 5 min. The samples were centrifuged at 500 $g_{max}$ for 1 min, and the supernatants were loaded directly onto SDS-polyacrylamide gels (gradient of 4–15% acrylamide). After SDS-PAGE, proteins were transferred to nitrocellulose membranes for immunoblotting. The extent of phosphorylation was monitored by probing the blots with mAb SMI-31; levels of GST-fusion proteins were monitored by probing the stripped blots with a rabbit pAb directed against GST (αGST; Sigma).

### Peptides and antibodies

The following synthetic peptides were synthesised: GDW-KNSNLDRHNLQ and pQRLSPAKSPSLSPSPPSPIEKa,

corresponding to amino acids 194–207 and 1,244–1,264 of mouse MAP 1B, respectively, and pQPRLPSAKPSSLPSSSIPEPKa, which is a scrambled version of peptide 1,244–1,264. An antibody to the second peptide was made by conjugating the peptide to keyhole limpet haemocyanin (Sigma) using the carboxyl reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (Pierce). The conjugated peptide (125 μg) was emulsified with complete Freund's adjuvant and injected subcutaneously into a rabbit (New Zealand white), followed by two boosters with incomplete adjuvant, and then the animal was exsanguinated. Immunoglobulin was obtained from sera by ammonium sulphate precipitation and further purified on Affi-Gel Blue columns (Bio-Rad) according to the manufacturer's instructions. This polyclonal antibody (pAb) is referred to as α1,244–1,264. A pAb raised against a MAP 1B recombinant protein encoding amino acids 1,691–2,102 has been described previously (Johnstone et al., 1997) and pAbs against synthetic peptides encoding MAP 1B amino acids 1–15 and 442–457 were a kind gift of Peter Brophy, Department of Preclinical Veterinary Sciences, Edinburgh University (Vouyiouklis and Brophy, 1993). A rabbit pAb was raised against the extracellular matrix protein laminin (Gordon-Weeks et al., 1989), and rabbit pAbs to GST- and peroxidase-conjugated secondary antibodies were purchased from Sigma.

### Characterisation of α1,244–1,264

$S_1$ (5 μg) and purified GST-1B750 (0.25 μg), prepared as described above, were separated by SDS-PAGE and immunoblotted with α1,244–1,264 (diluted 1:200). In some experiments, peptide 1,244–1,264 or a scrambled version of this (see above) were included in primary antibody incubations at 2.5 μg/ml. The blots were then washed, probed with anti-rabbit peroxidase-conjugated antibodies, and developed as described below.

### Gel electrophoresis and immunoblotting

Protein samples were subjected to SDS gradient (4–15% or 4–20%) gel electrophoresis using Laemmli (1970) buffers and western blotted onto nitrocellulose membrane according to Towbin et al. (1979). To block nonspecific binding, nitrocellulose blots were incubated in blocking buffer 1 [BB1; 5% (wt/vol) nonfat milk solids (Marvel) in TBS, pH 7.4] at room temperature for 1 h or at 4°C overnight. The blots were then incubated in the appropriate primary mAb or pAb in blocking buffer 2 [BB2; 1% (wt/vol) nonfat milk solids (Marvel) in TBS, pH 7.4], for 4 h at room temperature. Blots were then washed for 20 min each in 0.05% (vol/vol) Triton X-100 in TBS, pH 7.4, followed by 0.5 *M* NaCl, 0.05% (vol/vol) Triton X-100 in TBS, pH 7.4, and a final wash in 0.05% (vol/vol) Triton X-100 in TBS, pH 7.4. Blots were then incubated in the appropriate peroxidase-conjugated secondary antibody (Sigma) diluted 1:500 in BB2 for 1 h at room temperature. The blots were then washed as above, developed with ECL chemiluminescent kit (Amersham) and quantitated using a Bio-Rad molecular imager system (Model GS-363) linked to a Macintosh Power computer, or scanned using a flat-bed scanner (Hewlett-Packard ScanJet 4C) and analysed using Phoretix 1-D gel analysis software.

### Addition of synthetic peptides or antiserum to the kinase assay

In separate experiments, various synthetic peptides or antiserum were included in the in vitro kinase assay. Peptides were added at up to a 750× molar excess to the recombinant protein. Antibodies (250 μg) were incubated with recombinant protein (50 μg) attached to beads in the presence or absence of 250 μg of synthetic peptide at room temperature for 60 min. The recombinant protein was washed four times with KB and included in the kinase assay as described above.

## RESULTS

### Cysteine cleavage of purified MAP 1B from neonatal rat brain generates two mAb SMI-31 binding fragments

Purified MAP 1B from neonatal rat brain consisted of two, high-molecular mass heavy chains (325 and 335 kDa) and a 34-kDa light chain (not shown; see, also, Pedrotti and Islam, 1995). Chemical cleavage of purified MAP 1B, using the cysteine-specific reagent NTCB, generated a wide range of fragments (not shown). When these fragments were electroblotted and probed with site-specific pAbs (α1–15, α442–457, α1,244–1,264, and α1,691–2,102), two major, high-molecular mass fragments of 215 and 160 kDa were identified (Fig. 1). Both fragments were recognised by a pAb to amino acids 442–457 and 1,244–1,264 but not by a pAb to amino acids 1,691–2,102, whereas only the 215-kDa fragment was recognised by a pAb to the first 15 amino acids of MAP 1B (Fig. 1). This suggests that the 160-kDa fragment is derived from the 215-kDa fragment by removal of the N-terminus. The location of the NTCB cleavage sites of cysteines on MAP 1B further suggests that the 160-kDa fragment

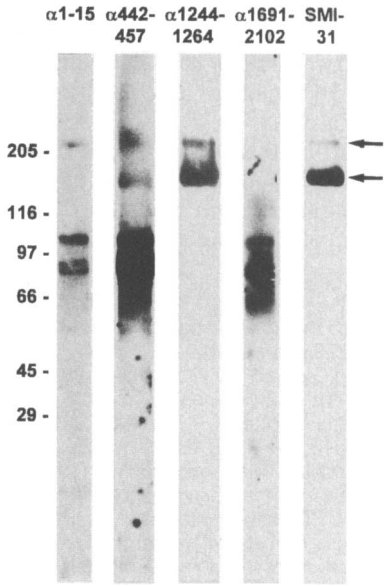


**FIG. 1.** Cysteine-specific cleavage of native MAP 1B purified from neonatal rat brain. MAP 1B fragments generated by NTCB cleavage at the amino-terminal side of cysteine residues were analysed by probing western blots with site-specific pAbs and mAb SMI-31. mAb SMI-31 recognised fragments of 215 and 160 kDa (arrows).

extends from amino acid 381 to 1,603. mAb SMI-31 recognised these two fragments, suggesting that there is an epitope for this mAb within this region of MAP 1B (Fig. 1). No other fragments were recognised by mAb SMI-31.

### mAb SMI-31 recognises phosphorylated MAP 1B fusion proteins GST-1B750 and GST-1BNR

We produced a series of GST-MAP 1B fusion proteins covering the entire MAP 1B molecule and screened these proteins in our in vitro kinase assay to see if we could reconstitute the mAb SMI-31 epitope. The NTCB digest data suggest that the mAb SMI-31 epitope is located approximately in the middle region of MAP 1B. One recombinant protein, GST-1B750, encoding amino acids 1,109–1,360, became mAb SMI-31 positive between 6 and 12 h in the kinase assay (Fig. 2A). A second recombinant protein, GST-1BNR, encoding amino acids 1,836–2,076, also became mAb SMI-31 positive between 6 and 12 h in the kinase assay (Fig. 2B). Control GST protein alone was not mAb SMI-31 positive in the kinase assay (not shown).

### Specific inhibition of the phosphorylation of GST-1B750 with a synthetic peptide

Analysis of the MAP 1B sequence encoding GST-1B750 revealed a region that contained a high concentration of serines followed immediately by a proline. Because MAP 1B is thought to be a substrate for proline-directed serine/threonine kinases, we decided to study this region further. Inclusion in the kinase assay of 100-fold molar excess of a synthetic peptide encoding this region (amino acids 1,244–1,264) inhibited phosphorylation of the recombinant protein at the mAb SMI-31 site, presumably by competing for the kinase(s) (Fig. 3A). Quantitative densitometric analysis from three data points from three independent experiments showed that there was 30 ± 2% (mean ± SD) inhibition at 12 h and 40 ± 1.5% (mean ± SD) at 24 h. In a separate experiment, addition of increasing concentrations of peptide, between 0- and 750-fold molar excess of peptide, resulted in progressive decrease in phosphorylation at the mAb SMI-31 site (Fig. 3B). In agreement with the previous data, inclusion of a 94-fold excess of peptide resulted in a 30% reduction in phosphorylation after 16 h. Inhibition of up to 70% was observed after 16 h when a 750-fold molar excess of peptide was included in the kinase assay (Fig. 3B).

The specificity of this inhibition was analysed by using control peptides. An unrelated peptide encoding a different region of MAP 1B and a scrambled version of peptide 1,244–1,264, having the same mass and charge but in which there were no prolines immediately downstream of serines, were added to the same molar excess as the peptide 1,244–1,264. Neither peptide had detectable effects on phosphorylation at the mAb SMI-31 site (Fig. 3A and B).

### Inhibition of the phosphorylation of GST-1B750 with an antiserum to peptide 1,244–1,264

To independently confirm the identity of the phosphorylated region, we raised a rabbit pAb to the inhibitory peptide ($\alpha$1,244–1,264) and included the pAb in the kinase assay. We found that the antibody recognised the GST-1B750 recombinant protein and native MAP 1B on blots (Fig. 4). A broad band corresponding to the molecular weight of MAP 1B is recognised in brain extracts by $\alpha$1,244–1,264. This could be resolved into a doublet when similar blots were developed with chloronaphthol, indicating that both phosphorylated isoforms of MAP 1B are recognised (not shown). Addition of peptide 1,244–1,264 (2.5 $\mu$g/ml) to the primary antibody incubation blocked binding to MAP 1B and GST-1B750 (Fig. 4). A scrambled version of this peptide had no effect (Fig. 4), indicating that the antibody is directed specifically against residues 1,244–1,264 of MAP 1B.

Preincubation of GST-1B750 with 2.5-fold molar excess of $\alpha$1,244–1,264 before inclusion in the kinase assay inhibited the phosphorylation of the recombinant protein (Fig. 5). Densitometric scanning of blots of

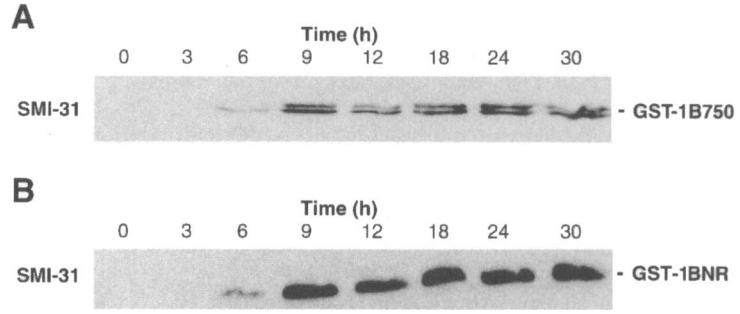


**FIG. 2. A:** In vitro phosphorylation of MAP 1B fusion protein GST-1B750 at the mAb SMI-31 site. The MAP 1B fusion protein GST-1B750 was phosphorylated in vitro as described in Experimental Procedures and the proteins immunoblotted with mAb SMI-31. mAb SMI-31 recognised the GST-1B750 fusion protein after 6 h in the assay, but it did not recognise the unphosphorylated fusion protein present at zero time or the control GST protein phosphorylated under identical conditions (not shown). Reprobing the blots with $\alpha$GST showed that the loading levels were similar (not shown). **B:** In vitro phosphorylation of MAP 1B fusion protein GST-1BNR at the mAb SMI-31 site. The MAP 1B fusion protein GST-1BNR was phosphorylated in vitro as described in Experimental Procedures and the proteins immunoblotted with mAb SMI-31. mAb SMI-31 recognised the GST-1BNR fusion protein after 6 h in the assay, but it did not recognise the unphosphorylated fusion protein present at zero time or the control GST protein phosphorylated under identical conditions (not shown). Reprobing the blots with $\alpha$GST showed that the loading levels were similar (not shown).

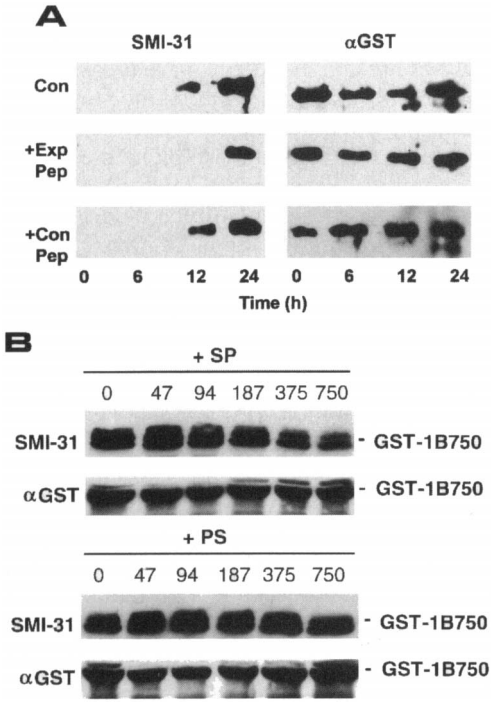


**FIG. 3.** Inhibition of the phosphorylation of GST-1B750 at the mAb SMI-31 site by a MAP 1B synthetic peptide corresponding to amino acids 1,244–1,264. **A:** The MAP 1B fusion protein GST-1B750 was phosphorylated in vitro, as described in Experimental Procedures, in the presence of 100-fold molar excess of a synthetic peptide, corresponding to amino acids 1,244–1,264 (+Exp Pep). Addition of 100-fold molar excess of a synthetic peptide, corresponding to amino acids 194–207 of MAP 1B, did not inhibit the phosphorylation of the GST-1B750 recombinant protein (+Con Pep). Samples were prepared for SDS-PAGE and immunoblotted with mAb SMI-31 and reprobed with αGST antibodies. Reprobing the blots with αGST confirmed the specificity of the inhibition. **B:** The MAP 1B fusion protein GST-1B750 was phosphorylated in vitro in the presence of various concentrations of a synthetic peptide, corresponding to amino acids 1,244–1,264 (+SP), or a scrambled peptide of the same amino acid composition that lacked any SP motifs (+PS) for 16 h as described in Experimental Procedures. Samples were prepared for SDS-PAGE and immunoblotted with mAb SMI-31 and reprobed with αGST antibodies. Addition of increasing concentrations of peptide (a range between 0- and 750-fold molar excess of peptide) resulted in progressive decrease in phosphorylation at the mAb SMI-31 site. Inclusion of the scrambled peptide had no effect at any concentration tested.

four data points from two independent experiments showed that phosphorylation was reduced to 10% of control levels. The inhibition was abrogated if the peptide was used to block specific binding of pAb to GST-1B750 (Fig. 5). No inhibition was seen with an unrelated (anti-laminin) antibody (not shown).

## DISCUSSION

The phosphorylation of MAPs affects their ability to bind to microtubules and provides a mechanism to

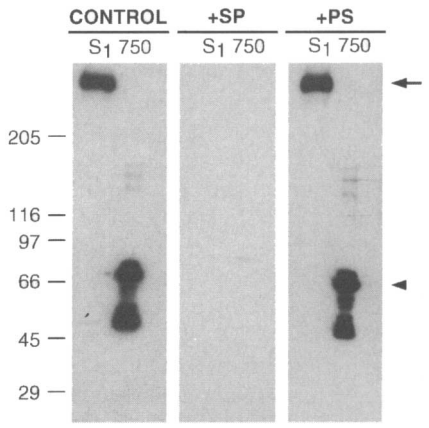


**FIG. 4.** Specific peptide inhibition of α1,244–1,264 binding to MAP 1B and GST-1B750. Cytosolic extracts from neonatal rat brain containing MAP 1B ($S_1$) and purified GST-1B750 (750) were immunoblotted with α1,244–1,264 in the presence (2.5 μg/ml as indicated) or absence of peptide 1,244–1,264 (+SP) and a scrambled version of this peptide (+PS). In the absence of added peptide, a broad band corresponding to the molecular weight of MAP 1B is recognised in brain extracts by α1,244–1,264 (arrow). GST-1B750 is also recognised by α1,244–1,264 (arrowhead). Inhibition of binding by peptide 1,244–1,264 but not by a scrambled version of this peptide indicates that α1,244–1,264 recognises this region of MAP 1B.

modulate the properties of microtubules in terms of stability, bundling, and associations with other filaments. MAP 1B has multiple phosphorylation sites that are differentially regulated during development of the nervous system and exhibit distinct distributions within neurones. Several phosphorylation sites of MAP 1B can be recognised by specific mAbs, providing a useful tool in studying the expression and regulation of the MAP 1B isoforms.

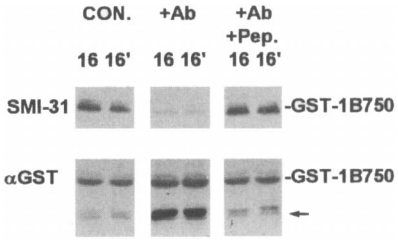


**FIG. 5.** An antibody specific for residues 1,244–1,264 of MAP 1B inhibits in vitro phosphorylation of mAb SMI-31 site in the recombinant MAP 1B fusion protein GST-1B750. GST-1B750 was incubated with α1,244–1,264 in the presence or absence of a synthetic peptide corresponding to residues 1,244–1,264 of MAP 1B and phosphorylated in vitro as described in Experimental Procedures for 16 h. Specific binding of α1,244–1,264 to GST-1B750 reduced phosphorylation at the mAb SMI-31 site to 10% of control levels. Binding of α1,244–1,264 to GST-1B750 was indicated by the presence of the antibody heavy chain in the sample, visualised by the anti-rabbit IgG peroxide-conjugated antibody used to detect GST (arrow). Inclusion of excess peptide abolished this binding indicating the specificity of antibody–GST-1B750 interaction.

The phosphorylation epitope on MAP 1B recognised by mAb SMI-31 is down-regulated during development and presumably plays some role in regulating the function of MAP 1B during axon growth, possibly by altering microtubule or actin filament binding (Bush et al., 1996). We have mapped mAb SMI-31 sites to two regions of the molecule from amino acids 1,109–1,360 and 1,836–2,076. Further analysis of the former region identified a 20-residue stretch from amino acid 1,244–1,264 in which there is a high concentration of serine–proline motifs. Our evidence for the localisation of a mAb SMI-31 site to this region on MAP 1B is fourfold; i.e., mapping of NTCB cleavage fragments from native MAP 1B showed that the site was between amino acids 381 and 1,603; the site was reconstituted on a recombinant protein encoding regions 1,109–1,360 after in vitro phosphorylation with a neonatal cytosolic brain extract; a synthetic peptide encoding amino acids 1,244–1,264 and an antibody specific for this peptide both inhibited in vitro phosphorylation of the mAb SMI-31 site.

The chemical cleavage mapping is based on identification of MAP 1B fragments on western blots by antibodies prepared against specific domains of the protein combined with location of cysteine residues at that region. The results clearly indicate that a 160-kDa fragment corresponding to amino acids 381–1,603 is recognised by mAb SMI-31. The next level of mapping, performed by in vitro phosphorylation assays with various recombinant proteins within this region, show that phosphorylated GST-1B750 and GST-1BNR, corresponding to residues 1,109–1,360 and 1,836–2,076, are recognised by mAb SMI-31. The most detailed mapping used competition experiments with a synthetic peptide encoding amino acids 1,244–1,264 and binding experiments with the antibody generated by this peptide. It is not clear why the NTCB digest did not also identify the 1,836–2,076 region. However, included in the GST-1BNR sequence are six cysteine residues, indicating six possible NTCB cleavage sites. Therefore, it is possible that the mAb SMI-31 epitope was disrupted by NTCB cleavage. A conformational component to the mAb SMI-31 epitope present on tau has been reported (Lichtenberg-Kraag et al., 1992), suggesting that a cleavage site need not cut through the epitope to affect antibody recognition. In contrast, the GST-1B750 sequence contains only one cysteine residue, distant from the site localised as the mAb SMI-31 epitope in this report, making disruption of this site less likely.

It is clear that peptide 1,244–1,264 is not a very effective inhibitor of GST-1B750 phosphorylation (a 750-fold molar excess of peptide failed to completely abolish phosphorylation). However, the specificity of the inhibition is demonstrated with a variety of control peptides that included an unrelated peptide and a peptide with an identical, but scrambled, sequence that had no prolines next to the serine residues. It is likely that phosphorylation of the inhibitory peptide reduces its inhibitory activity; but because it is also likely that phosphatases are active in our kinase assay, despite the inclusion of phosphatase inhibitors, the phosphorylated peptide may become dephosphorylated and recover its inhibitory activity. This may explain the increase in peptide inhibition seen at the later time point. Our data also indicate a proline-directed serine kinase is responsible for generating the mAb SMI-31 epitope on MAP 1B. mAb SMI-31 also recognises phosphorylation epitopes on tau and the high and intermediate weight proteins of neurofilaments (Sternberger and Sternberger, 1983; Lichtenberg-Kraag et al., 1992; Ulloa et al., 1993) and these epitopes contain serine/proline sites (Lichtenberg-Kraag et al., 1992; Doroudchi and Durham, 1996).

The inhibitory peptide, but not a scrambled version, blocked $\alpha$1,244–1,264 binding to the native protein and GST-1B750 protein on blots (Figs. 4 and 5), indicating that $\alpha$1,244–1,264 binds specifically to residues 1,244–1,264. This observation, and the lack of inhibition by control antibodies, indicates that it is antibody binding to this region of GST-1B750 that causes a decrease in phosphorylation. Presumably $\alpha$1,244–1,264 inhibits noncompetitively by blocking access of the kinase to the GST-1B750 protein, whereas the inhibitory peptide acts as a competitive inhibitor by competing with the recombinant protein for kinase activity. This may explain the greater effectiveness of $\alpha$1,244–1,264, compared with the peptide, in inhibiting the kinase.

The highest concentration of serine–proline motifs (five) in the MAP 1B sequence are found in the 1,244–1,264 region. It is significant that there is complete homology of this region of MAP 1B between rat, mouse, and human genes (Noble et al., 1989; Zauner et al., 1992; Lien et al., 1994).

The localisation of the mAb SMI-31 site to a region highly enriched in serine–proline motifs suggests that the kinase responsible is a proline-directed serine/threonine kinase. These conclusions are consistent with previous studies on the properties of the mAb SMI-31 epitope in neurofilaments and tau proteins (Lichtenberg-Kraag et al., 1992; Doroudchi and Durham, 1996). For example, in neurofilaments, mAb SMI-31 recognises phosphorylated epitopes at KSP sites on the C-terminal domain (Doroudchi and Durham, 1996). In tau, mAb SMI-31 binds between $Ser^{396}$ (in the second KSP motif) and $Ser^{404}$, both of which must be phosphorylated (Lichtenberg-Kraag et al., 1992). In addition, western blot analysis showed that tau epitopes phosphorylated by cdc2 kinase are recognised by mAb SMI-31 (Hosoi et al., 1995). Other candidate kinases for proline-directed serine/threonine phosphorylation include cdk5, ERK 1 and 2, and GSK3$\beta$, previously identified as kinases phosphorylating other cytoskeletal proteins (Avila et al., 1994). This is the first report localising a developmentally regulated phosphoryla-

tion site on MAP 1B, and we are now using our kinase assay to identify the kinase responsible.

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## REFERENCES

Avila J., Dominguez J., and Díaz-Nido J. (1994) Regulation of microtubule dynamics by microtubule-associated proteins: expression and phosphorylation during neuronal development. *Int. J. Dev. Biol.* **38,** 13–25.

Black M. M., Slaughter T., and Fischer I. (1994) Microtubule-associated protein 1b (MAP 1B) is concentrated in the distal region of growing axons. *J. Neurosci.* **14,** 857–870.

Bloom G. S., Luca F. C., and Vallee R. B. (1985) Microtubule-associated protein 1B: identification of a major component of the neuronal cytoskeleton. *Proc. Natl. Acad. Sci. USA* **82,** 5404–5408.

Brugg B., Reddy D., and Matus A. (1993) Attenuation of microtubule-associated protein 1B expression by antisense oligodeoxynucleotides inhibits initiation of neurite outgrowth. *Neuroscience* **52,** 489–496.

Bush M. S. and Gordon-Weeks P. R. (1994) Distribution and expression of developmentally regulated epitopes on MAP 1B and neurofilament proteins in the developing rat spinal cord. *J. Neurocytol.* **23,** 682–698.

Bush M., Johnstone M., and Gordon-Weeks P. R. (1996) The rôle of microtubules in axonal growth cones, in *Nerve Growth and Guidance, Frontiers in Neurobiology 3* (McCaig C. D., ed), pp. 35–53. Portland Press Ltd., London.

Calvert R. and Anderton B. H. (1985) A microtubule associated protein MAP 1 which is expressed at elevated levels during development of rat cerebellum. *EMBO J.* **4,** 1171–1176.

Díaz-Nido J., Serrano L., Méndez E., and Avila J. (1988) A casein kinase II-related activity is involved in phosphorylation of microtubule-associated protein MAP 1B during neuroblastoma cell differentiation. *J. Cell Biol.* **106,** 2057–2065.

DiTella M. C., Feiguin F., Carri N., Kosik K. S., and Cáceres A. (1996) MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth. *J. Cell Sci.* **109,** 467–477.

Doroudchi M. M. and Durham H. D. (1996) Activation of protein kinase c induces neurofilament fragmentation hyperphosphorylation of perikaryal neurofilaments and proximal dendritic swellings in cultured motor neurons. *J. Neuropathol. Exp. Neurol.* **55,** 246–256.

Edelmann W., Zervas M., Costello P., Roback L., Fischer I., Hammarback J. A., Cowan J., Davies P., Wainer B., and Kucherlapati R. (1996) Neuronal abnormalities in microtubule-associated protein 1B mutant mice. *Proc. Natl. Acad. Sci. USA* **93,** 1270–1275.

Fischer I. and Romano-Clarke G. (1990) Changes in microtubule-associated protein MAP1B phosphorylation during rat brain development. *J. Neurochem.* **55,** 328–333.

Fischer I. and Romano-Clarke G. (1991) Association of microtubule associated protein (MAP 1B) with growing axons in cultured hippocampal neurons. *Mol. Cell. Neurosci.* **2,** 39–51.

Fujii T., Nakamura A., Ogama Y., Kondo Y., and Arai T. (1990) Selective purification of microtubule associated proteins 1 and 2 from rat brain using poly(L-aspartic acid). *Anal. Biochem.* **184,** 268–273.

Gordon-Weeks P. R., Giffin N., Weekes C. S. E., and Barben C. (1989) Transient expression of laminin immunoreactivity in the developing rat hippocampus. *J. Neurocytol.* **18,** 451–463.

Gordon-Weeks P. R., Mansfield S. G., Alberto C., Johnstone M., and Moya F. (1993) Distribution and expression of a phosphorylation epitope on MAP 1B that is transiently expressed in growing axons in the developing rat nervous system. *Eur. J. Neurosci.* **5,** 1302–1311.

Hosoi T., Uchiyama M., Okumura E., Saito T., Ishiguro K., Uchida T. P., Okuyama A., Kishimoto T., and Hisanaga S. (1995) Evidence for cdk5 as a major activity phosphorylating tau protein in porcine brain extract. *J. Biochem. (Tokyo)* **117,** 741–749.

Johnstone M., Goold R. G., Fischer I., and Gordon-Weeks P. R. (1997) The neurofilament antibody RT97 recognises a developmentally regulated phosphorylation epitope on microtubule-associated protein 1B. *J. Anat.* (in press).

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lichtenberg-Kraag B., Mandelkow E. M., Biernat J., Steiner B., Schroter C., Gustke N., Meyer H. E., and Mandelkow E. (1992) Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer's tau. *Proc. Natl. Acad. Sci. USA* **89,** 5384–5388.

Lien L. L., Feener C. A., Fischbach N., and Kunkel L. M. (1994) Cloning of human microtubule-associated protein 1B and the identification of a related gene on chromosome-15. *Genomics* **22,** 273–280.

Mansfield S. G., Díaz-Nido J., Gordon-Weeks P. R., and Avila J. (1991) The distribution and phosphorylation of the microtubule-associated protein MAP 1B in growth cones. *J. Neurocytol.* **21,** 1007–1022.

Müller R., Kindler S., and Garner C. C. (1994) The MAP1 family, in *Microtubules* (Hyams J. S. and Lloyd C. W., eds), pp. 141–154. Wiley-Liss, New York.

Noble M., Lewis S. A., and Cowan J. (1989) The microtubule binding domain of the microtubule-associated protein MAP-1B contains a repeated sequence motif unrelated to that of MAP-2 and tau. *J. Cell Biol.* **109,** 3367–3376.

Pedrotti B. and Islam K. (1995) Purification of microtubule associated protein MAP 1B from bovine brain: MAP 1B binds to microtubules but not to microfilaments. *Cell Motil. Cytoskeleton* **30,** 301–309.

Pedrotti B. and Islam K. (1996) Dephosphorylated but not phosphorylated microtubule associated protein MAP 1B binds to microfilaments. *FEBS Lett.* **388,** 131–133.

Petrucci T. C. and Morrow J. S. (1987) Synapsin I—an actin bundling protein under phosphorylation control. *J. Cell Biol.* **105,** 1355–1363.

Riederer B., Cohen R., and Matus A. (1986) MAP 5: a novel microtubule associated protein under strong developmental regulation. *J. Neurocytol.* **15,** 763–775.

Rienitz A., Grenningloh G., Hermans-Borgmeyer I., Kirsch J., Littauer U. Z., Prior P., Gundelfinger E. D., Schmitt B., and Betz H. (1989) Neuraxin, a novel putative structural protein of the rat central nervous system that is immunologically related to microtubule-associated protein 5. *EMBO J.* **8,** 2879–2888.

Sato-Yoshitake R., Shiomura Y., Miyasaka H., and Hirokawa N. (1989) Microtubule-associated protein 1B: molecular structure, localization, and phosphorylation-dependent expression in developing neurons. *Neuron* **3,** 229–238.

Schoenfeld T. A., McKerracher L., Obar R., and Vallee R. B. (1989) MAP1A and MAP 1B are structurally related microtubule associated proteins with distinct developmental patterns in the CNS. *J. Neurosci.* **9,** 1712–1730.

Sternberger L. A. and Sternberger N. H. (1983) Monoclonal anti-

bodies distinguish between phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA* **80,** 6126–6130.

Takemura R., Okabe S., Umeyama T., Kanai Y., Cowan N. J., and Hirokawa N. (1992) Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP-1B, MAP2 and tau. *J. Cell Sci.* **103,** 953–964.

Towbin H., Staehelin T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

Tucker R. P., Binder L. I., and Matus A. (1988) Neuronal microtubule-associated proteins in the embryonic avian spinal cord. *J. Comp. Neurol.* **271,** 44–55.

Ulloa L., Avila J., and Díaz-Nido J. (1993) Heterogeneity in the phosphorylation of microtubule-associated protein MAP1B during rat brain development. *J. Neurochem.* **61,** 961–972.

Vouyiouklis D. A. and Brophy P. J. (1993) Microtubule-associated protein 1B expression precedes the morphological differentiation of oligodendrocytes. *J. Neurosci. Res.* **35,** 257–267.

Zauner W., Kratz J., Staunton J., Feick P., and Wiche G. (1992) Identification of two distinct microtubule binding domains on recombinant rat MAP 1B. *Eur. J. Cell Biol.* **57,** 66–74.