IDENTIFICATION AND CHARACTERIZATION OF A MITOSIS-ASSOCIATED PHOSPHOEPITOPE UP-REGULATED DURING NEURONAL DIFFERENTIATION

DISSERTATION

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By

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Protein components of the neuronal cytoskeleton are fundamentally important for architectural support and organelle transport in axons and dendrites. Among them, microtubules (MTs) and microtubule-associated proteins (MAPs) are major components involved in neuronal differentiation and neurodegenerative diseases. Neuronal MTs are relatively stable, yet they are not static structures. A fine-tuned balance in MT stability is required to assure proper plasticity of neuronal processes. MAPs are key players in regulating the stability of MTs, and MAP function is in turn regulated by phosphorylation. It was found in this study that a dramatic increase in MAP1B phosphorylation occurred as PC12 cells developed neurites in response to nerve growth factor. The phosphorylation of MAP1B was identified by a monoclonal antibody MPM2, which identifies a mitosis-associated phosphoepitope. The increase in MPM2 reactivity of MAP1B was also observed in synchronized mitotic PC12 cells, suggesting that related kinase activities are involved in pathways inducing cell division and the differentiation of postmitotic neuronal cells. MPM2-reactive MAP1B was localized to neurites of PC12 cells, but not cell bodies by indirect immunofluorescence and immunogold electron
microscopy. In order to understand the functional significance of the phosphorylation of MAP1B, the identification of the MPM2 epitope was required. Through proteolytic digestion and peptide microsequencing the epitope was shown to be located in the N-terminal 40 kD of MAP1B. A potential phosphothreonine site fitting a model of known MPM2 epitopes was identified, and peptides containing these sequences were synthesized in both phosphorylated and dephosphorylated forms. The selected phosphopeptide was highly reactive with the MPM2 antibody, while the dephosphopeptide was not. Furthermore, the phosphopeptide had the ability to compete for MPM2 antibody binding with both MAP1B and other phosphoproteins in mitotic cells. These results indicate that the phosphopeptide had all of the elements required for MPM2 antibody recognition. A phosphorylation state-specific antibody PMB1 was generated by using the phosphopeptide conjugated to keyhole limpet hemocyanin as immunogen. The purified antibody was shown to be specific to phosphorylated MAP1B. The phosphorylation site and antibody probe described here are expected to assist in identifying the kinase responsible for the modification on MAP1B and understanding the functional significance of this modification in neurogenesis.
To My Parents

Who Got Me Started

and

To My Husband and Daughter

Who Keep Me Going
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<tr>
<td>BCA</td>
<td>BiCinchoninic Acid</td>
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<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary cell line</td>
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<tr>
<td>DAPI</td>
<td>DiAmine Propidium Iodide</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
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<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
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<tr>
<td>EDTA</td>
<td>EthyleneDiamine-Tetraacetic Acid</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGTA</td>
<td>Ethylene Glycol-bis(-aminoethyl ether)-N,N,N',N'-Tetraacetic Acid</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FITC</td>
<td>Fluorescein Iso ThioCyanate</td>
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<td>HPMA</td>
<td>HydroxyPropylMethAcrylate</td>
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<td>kD</td>
<td>KiloDaltons</td>
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<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
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<td>MAP</td>
<td>Microtubule-Associated Protein</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>MPM2</td>
<td>Mitotic Phosphoprotein Monoclonal antibody 2</td>
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<td>MT</td>
<td>MicroTubule</td>
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<td>MTOC</td>
<td>MicroTubule Organization Center</td>
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<td>NFT</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PBSa</td>
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<td>PMSF</td>
<td>PhenylMethylSulfonyl Fluoride</td>
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<td>PVDF</td>
<td>PolyVinylidene DiFluoride</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
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INTRODUCTION

The evolution of increasingly complex and large animals has necessitated extensive systems of cellular communication and coordination. The nervous system carries out these indispensable tasks in vertebrates. Basic units of the nervous system are terminally-differentiated cells including both neurons and their supporting cells, the glial cells. Neurons are extremely polarized cells characterized by their long axonal and dendritic processes, which are fundamentally important for the neuronal circuit. Axons arise either from the nerve cell body or from the proximal part of a dendrite. They are slender cellular extensions having a uniform diameter and smooth contour. The area where axons emerge from the cell body or dendrite is termed the axon hillock. Axons are responsible for propagating the electrical signal from cell bodies to recipient cells. Dendrites are generally shorter than axons and taper away from cell bodies, but they branch repeatedly and their surface is studded with fine spines. Neurons obtain signals from adjacent cells and the environment through dendrites. The elaboration of dendritic surface area allows large neurons to receive as many as 100,000 separate axon terminals. Most neurons have one axon and multiple dendrites (Bunge, 1973). In cultured neuronal cells such as differentiated pheochromocytoma PC12 and neuroblastoma cells, however, it is difficult to distinguish axons from dendrites solely by their
morphologies. Therefore, these cellular processes are collectively called neurites. The distal tip of neurites is characterized with a thin and fan-shaped structure, termed the growth cone. The growth cone is actively involved in pathfinding, neurite extension, and synapse formation (Heidemann, 1996).

While neuronal processes are specialized in transmitting impulses, the cell body is the center of protein synthesis of the neuron. Because neurites lack protein synthetic machinery, the large amount of proteins necessary for growth, maintenance, and function of neurites must be transported from the cell body over a great distance down the neurite shaft after synthesis. These formidable architectural and transport needs are fulfilled by elements of the neuronal cytoskeleton (Maccioni and Cambiazo, 1995). There are three cytoskeletal members of interest: microfilaments, neurofilaments (NFs) and microtubules (MTs). Neuronal microfilaments are concentrated in the cytoplasmic cortex, filopodia of the neurite, and the peripheral region of the growth cone. They provide the mechanical support necessary for the maintenance of the axon and the extension of the leading edge of the growth cone. Neurofilaments extend along the length of the neurite and resist stress forces caused by motion, which would otherwise break the long neurite. The organization of the neurofilaments seems to depend on their interaction with MTs. Microtubules (MTs) are also a prominent member of the neuronal cytoskeleton and will be discussed in greater detail below.
Cell motility, vesicle transportation, and segregation of chromosomes during cell division as well as maintenance of cytoarchitecture are dependent upon MTs (Gelfand and Bershadsky, 1991). The basic units of the MT are heterodimers of \( \alpha \) and \( \beta \)-tubulins. Tubulin dimers polymerize to form cylindric MTs with a diameter of 25 nm. Each MT is composed of 13 linear protofilaments (Diagram 1). Because the tubulin dimers in protofilaments are arranged in the same orientation, MTs are polar structures. The end that exhibits more rapid growth characteristics due to a greater association rate for dimer binding is defined as the plus end. The opposite end of the MT, having slower growth characteristics is defined as the minus end (Bergen and Borisy, 1980). In most cells, MT plus ends project toward the periphery of the cell, and their minus ends are typically anchored within the microtubule organization center (MTOC) near the center of the cell. MTs are dynamic structures, with their free or plus ends undergoing both elongation and shrinkage. This behavior, given the name dynamic instability (Mitchison and Kirschner, 1984), allows MTs to reorganize within the cell and maintain networks that are specific to the morphology and polarity of different cell types. The stability of MTs is regulated within individual cells by the microtubule-associated proteins (MAPs). MAPs are proteins that binds to MTs, and stimulate assembly of tubulins in vitro. In vivo the binding of MAPs is thought to control, at least in part, the dynamic properties of MT assembly and disassembly.
In neuronal cells, the organization of MTs is unique (Diagram 2). Minus ends of MTs in dendrites and axons are free and not embedded within the MTOC, as seen in other cell types (Lyser, 1968; Bray and Bunge, 1981). MTs are nucleated at the neuronal centrosome and subsequently released allowing for migration into neurites (Yu et al., 1993). MTs are also organized differently in different cellular compartments of neurons. In axons, all MTs are oriented with their plus ends toward the distal part of the process (Heidemann et al., 1981), while in dendrites roughly equal numbers of MTs have either orientation (Baas et al., 1988; Burton, 1988). In both axons and dendrites, MT arrays run parallel to each other along the axis of the neurite shaft and form tight bundles. The formation of the MT bundles is considered to be an essential requirement for the elongation of neurites. The mechanisms involved in the unique organization of MTs in neuronal processes is not well-defined. One possible mechanism regulating neurite formation and structure may relate to the temporal and spatial distribution as well as the function of neuronal MAPs in axons and dendrites.

The organization of the neuronal cytoskeleton is well-balanced to ensure proper morphology and function. Microfilaments are abundant in growth cones and filopodia of neurites, where motion and response to the substrate occur actively. In comparison, the neurite shaft has relatively few microfilaments, but is enriched with MTs. However, both cytoskeletal elements are required for establishment and maintenance of the neurite, and disruption of one will affect the other. Hippocampal
neurons treated with microtubule-depolymerizing drugs lose their characteristic 
neurites, and only lateral filopodia are produced with the outline of these processes 
being irregular (Matus, 1988). Therefore, microfilaments are constrained from 
driving neuritic motility by the lack of influence by MTs in these treated neurites. 
However, if this balance is over-corrected, neurites become too rigid. For instance, 
when taxol, which stabilizes MTs, is applied to neurons growing in culture, MTs are 
formed in the growth cones and the growth cones lose their motility (Letomeau and 
Ressler, 1984). Therefore, functional dominance of microfilaments promote motility 
and morphological alteration, while dominance of MTs inhibits growth cone motility 
and arrests the neuritic structure. Within the population of MTs present in neurites, 
stability varies depending on their localization along the length of the neurite shaft. 
MT polymers in the growth cone are much more dynamic than MTs in the neurite 
shaft (Ahmad et al., 1993). The conversion from the plasticity of the growth cone to 
the relative rigidity of the neurite shaft seems to depend on a fine-tuned balance 
between neuronal cytoskeletal components. How is this balance maintained? A 
wide range of research data suggests that microtubule-associated proteins may be 
the key players in this complicated process, because MAPs also interact with 
microfilaments and neurofilaments (Selden and Pollard, 1983; Heimann et al., 1985; 
Sattilaro, 1986).

There are three types of proteins known to be associated with microtubules 
in vivo. The first type are the MT-motor proteins, including kinesin, dynein and their
superfamilies (Vallee and Shpetner, 1990; Skoufias and Scholey, 1993). They are
the force-generating enzymes required for organelle translocation and chromosome
segregation during cell division. Kinesin moves organelles in the anterograde
direction, toward the plus ends of MTs, while cytoplasmic dynein moves organelles
in the retrograde direction, toward the minus ends of MTs. The second type of
proteins are the conventional microtubule-associated proteins (MAPs), or structural
MAPs. These MAPs are a group of heterogeneous proteins, co-purifying with tubulins
during temperature-dependent assembly and disassembly cycles (Olmsted, 1986).
MAP1A, MAP1B (previously named MAP5, MAP1.2 or MAP1X), MAP2, MAP4 and
tau are the main members of this class of microtubule-associated proteins. The last
type of proteins associated with MTs are protein kinases and phosphatases.
Several reports have shown that a significant proportion of p34cdc2 (Rattner et al.,
1990; Tombes et al., 1991) and phosphatase 2A (Sontag et al., 1995) are
associated with MTs. These enzymes are capable of modifying motor proteins and
MAPs.

Based on their molecular structures, structural MAPs are classified into two
groups (Hirokawa, 1994) (Diagram 3). The first, MAP2, MAP4 and tau, all have their
MT-binding domain on their C-termini. The MT-binding domain in this class of MAPs
is composed of three or four imperfect repeats of 31 amino acids (Himmler et al.,
1988; Lewis et al., 1988; Aizawa et al., 1990). Between the MT-binding domain and
projection domain, there is a proline-enriched hinge region, having numerous
potential phosphorylation sites for proline-directed kinases. The MT-binding domain of the second group of MAPs, including MAP1A and MAP1B, is localized near their N-termini. Their MT-binding domains are highlighted with twenty-one repeats of four amino acids, with basic amino acids (lysine) enriched in all repeats (Noble et al., 1989; Langkopf et al., 1992). Because the MT-binding domain of MAPs can bind to multiple tubulin dimers simultaneously, MAPs speed up the nucleation of MTs and promote MT polymerization. Their binding also stabilizes polymerized MTs. The projection domain of MAPs is believed to both crosslink MTs and link MTs with other cellular structures. This domain also has the ability to bind kinases and phosphatases to MTs. Among all of the MAPs, MAP4 is the only MAP that mainly exists in non-neuronal cells. The remaining MAPs are distributed preferentially in neuronal tissues. MAP2 is considered to be a marker for dendrites, whereas MAP1A, MAP1B and tau are concentrated in axons. The distribution of MAPs may relate to the different MT organization in axons and dendrites.

The expression of MAPs is developmentally regulated. For instance, MAP1B, the target of this research, is highly expressed from late embryonic stages to postnatal day 10 in rat brains. It is down-regulated afterwards (Matus, 1988). In adult rat brain, high levels of MAP1B are only detected in the olfactory bulb, hippocampus and retina (Scheonfeld et al., 1989; Viereck et al., 1989), where growth and plasticity of neuronal connections continue throughout the life of the organism. The hippocampus is also the area responsible for memory and learning.
Similar results have also been found in avian and amphibian nervous systems (Tucker et al., 1988; Viereck and Matus, 1990). In developing neurons, MAP1B is concentrated in growing axons, while in mature neurons the expression of MAP1B is weak and not specific to cellular compartments (Riederer et al., 1986; Schoenfeld et al., 1989). In agreement with its change in expression levels, a recent report has shown that rat MAP1B gene has two alternative promoter regions. The upstream promoter is inducible during development, while the downstream promoter is constitutive, which allows low levels of expression in adult brain (Liu and Fischer, 1996b). Therefore, the expression of MAP1B is closely coupled to the neurogenesis characteristic of late embryonic development and in selected regions of the adult brain.

The temporal and spatial distribution of MAP1B suggests that MAP1B is important for neuronal development, though its exact role has not been completely defined. MAP1B has been found to form cross-bridges between MTs in vitro (Sato-Yoshitake et al., 1989), implying that MAP1B may stabilize and crosslink MTs in neuronal processes. Treatment of PC12 cells with MAP1B antisense eliminates the expression of MAP1B in these cells, and abolishes their ability to develop neurites in response to nerve growth factor (Brugg and Matus, 1993). The importance of MAP1B in neuronal development is further demonstrated by a MAP1B gene disruption study in mice, in which only a fragment with N-terminal 517 amino acids of MAP1B was allowed to express. Homozygotes with mutant phenotypes could not
survive the embryonic stage, and heterozygotes displayed a number of neuronal abnormalities, indicating that MAP1B protein is essential for development (Edelmann et al., 1996). Another group using shorter truncated form (N-terminal 11 amino acids) of MAP1B observed less severe defects in homozygotes, and heterozygotes in this study seemed to be normal (Takei et al., 1997).

The complete cDNA sequence of mouse MAP1B has been reported by Noble et al. (1989). Predicted from its primary sequence, mouse MAP1B protein has a molecular weight of 255,534. MAP1B was shown to possess a single MT-binding domain that is located near the amino-terminus (amino acid 517-848) and contain about 21 copies of the basic motif KKEE(I/V). This MT-binding motif is completely unrelated to that of MAP2, MAP4 and tau. The complete sequence of MAP1B from rat (Zauner et al., 1992; Liu and Fischer, 1996a) and human (Lien et al., 1994) has also been identified, and sequence analysis revealed >90% identical residues among the three species. There has also been a report demonstrating that MAP1B is encoded as a polyprotein, which is processed to form a complex of a heavy chain and two light chains (light chain 1 and light chain 3). Both the heavy and light chains are encoded within the sequence cloned by Noble et al. Light chain 1 (34 kD) is encoded within the 3' end of the heavy chain mRNA. Since internal initiation and termination of protein synthesis from the common mRNA were not observed, light chain 1 is supposedly produced by proteolysis of a MAP1B polyprotein precursor. Light chain 1 binds the N-terminus MT-binding domain of the heavy chain
(Hammarback et al., 1991). The light chain 3 (16.4 kD) sequence is not found in MAP1B/LC1 polyprotein cDNAs. It is encoded by a different gene. How light chain 3 binds to the heavy chain and/or MTs is not clear (Mann and Hammarback, 1994). The molecular shape of MAP1B protein has been observed by the quick-freeze, deep-etch technique. Affinity purified MAP1B was visualized as a long, filamentous molecule (186±38 nm) with a small globular head forming cross-bridges between MTs in vitro (Sato-Yoshitake et al., 1989).

Purification of MAP1B has been challenging because of its large size, low abundance, and sensitivity to heat and proteases. The lack of satisfactory purification procedures has limited biochemical studies with MAP1B. An early paper showed that MAP1 (including MAP1A and MAP1B) could be dissociated from taxol-stabilized MTs with poly(L-aspartic acid) and separated from other MAPs by the combination of ion exchanger and gel filtration (Fujii et al., 1990). However, because MAP1A shares molecular structure and biochemical properties with MAP1B (Langkopf et al., 1992), it has been difficult to separate MAP1B from MAP1A. Attempts to purify MAP1B by the immunoaffinity method have also been reported, but yields are poor (Reiderer et al., 1986; Diaz-Nido et al., 1988; Sato-Yoshitake et al., 1989). A more recent report (Pedrotti and Islam, 1995) showed that relatively pure MAP1B can be isolated by taking advantage of the difference in affinities of MAPs for MTs. These authors found that during the first MT polymerization cycle, the majority of MAP1B stays in the supernatant, while other
MAPs bind to and pellet with MTs. The MT-minus supernatant was taken as the source for purification of MAP1B, and was subjected to a sequence of ion exchangers. By using calf brain, instead of adult animal brains, this procedure typically recovers about 25-30 mg MAP1B/kg of brain tissue. To date this is the only method reported to prepare purified MAP1B with reasonable yields. Although young animal brains can be difficult to obtain, relatively large amounts of MAP1B are required for extensive biochemical and functional analysis of the protein making fetal or newborn brain samples the only suitable protein source.

By standard SDS-PAGE analysis, MAP1B runs at a position of 325 kD, which is much larger than the size predicted from its primary sequence. This has been true of most high molecular weight MAPs, and the retardation on SDS-PAGE may result from posttranslational modifications such as glycosylation and phosphorylation. Evidence has been presented that all MAPs are glycosylated (Ding and Vandré, 1996; Arnold et al., 1996) in vivo. A variety of data indicate that the function of MAPs are also regulated by the phosphorylation state of these proteins. In this context, phosphorylation of MAP4, MAP2, and tau diminishes their affinity for tubulin polymers (Lindwall and Cole, 1984; Drechsel et al., 1992; Faruki et al., 1992; Ookata et al., 1995). This effect is most obvious in the neurofibrillary tangles (NFT) of Alzheimer's disease brain. The main component of NFT are paired-helical filaments (PHF), which in turn consist primarily of
hyperphosphorylated tau. Hyperphosphorylated tau cannot bind to MTs and
dissociates from the MT lattice, where it is then capable of self-association resulting
in the formation of PHF.

Phosphorylation of MAP1B has been observed in a variety of neuronal cell
lines (Greene et al., 1983; Aletta et al., 1988; Diaz-Nido et al., 1988) and nerve
tissues (Riederer, 1992; Riederer, 1994; Bush et al., 1994; Mansfield et al., 1994).
MAP1B isolated from bovine or rat brain bears various amounts of phosphate
depending on the developmental stage (Ulloa et al., 1993; Pedrotti et al., 1996). In
developing rat brain and the adult olfactory bulb, MAP1B is phosphorylated at
numerous sites, while in adult rat brain it is largely dephosphorylated. This again
implies that phosphorylation of MAP1B is closely correlated with the plasticity of
neurites during neurogenesis. Despite these observations, the effect of MAP1B
phosphorylation on protein function has been controversial based on in vitro
experiments. An early report showed that brain MAP1B, when it was
phosphorylated by casein kinase II-like activity, had a similar or higher affinity for
MTs than unphosphorylated MAP1B (Diaz-Nido et al., 1988). However, more
recent data using more highly purified MAP1B isolated from newborn bovine brain
and alkaline phosphatase-treated MAP1B (1B-AP) obtained the opposite result
(Pedrotti et al., 1996). When the two forms of MAP1B were co-sedimented with
taxol-stabilized MTs, higher salt concentrations were required to dissociate
dephosphorylated 1B-AP from the MTs than the more heavily phosphorylated native
MAP1B. This result suggests that dephosphorylated MAP1B binds to MTs tighter
MAP1B. This result suggests that dephosphorylated MAP1B binds to MTs tighter than phosphorylated MAP1B. The different effects of phosphorylation obtained in these two studies may result from the difference in phosphorylation sites. In the two studies mentioned above, the phosphorylation sites were not known.

Studies attempting to correlate MAP1B phosphorylation sites with its function have also been reported (Dia-Nido et al., 1988; Ulloa et al., 1993a; Ulloa et al., 1993b; Pedrotti et al., 1996). In these studies, four antibodies specific to different phosphorylation states of MAP1B were used (Table 1). It has been found that MAP1B isolated from young rat brains reacts to monoclonal antibodies mAb150 and mAb125, which appear to recognize only phosphorylated forms of MAP1B. While the epitope recognized by mAb150 is sensitive to protein phosphatase 2A (PP2A) and 2B (PP2B), but not protein phosphatase 1 (PP1), the phosphoepitope recognized by mAb125 can be eliminated by PP1 and PP2A, but not PP2B. In comparison to the decreased reaction to mAb150 and mAb125 after phosphatase treatment of MAP1B, the reactivity of two polyclonal antibodies, poly-531 and poly-842, which recognize non-phosphorylated forms of MAP1B increases. Dephosphorylation of MAP1B by PP2A and PP2B causes decrease in reactivity to mAb150 as well as gain of reactivity to poly-531. PP1 and PP2A treatment leads to decrease in reactivity to mAb125 and gain of reactivity to poly-842 (Ulloa et al., 1993b). Poly-531 was raised against a 15 amino acid-synthetic peptide S*PAKS*PSLS*PS*PPS*P, corresponding to amino acid 1247~1261 of mouse
Poly-842 was raised against synthetic peptide ASTYS*YET*SD, corresponding to amino acid 2050-2059 of mouse MAP1B. This peptide sequence has two serine/threonine residues (marked by *) followed by acidic residues E or D, which are potential substrates for casein kinase II (CK II) (Ulloa et al., 1993a). Because MAP1B isolated from young rat brains reacts to poly-531 and poly-842 antibodies more heavily after phosphatase treatment, it has been proposed that the phosphorylation of MAP1B in vivo may be regulated by PDPK and CK II. In support of this hypothesis, a study has shown that MAP1B phosphorylated in vitro by purified CK II and in vivo phosphorylated MAP1B from neuroblastoma cells have identical phosphopeptide pattern (Diaz-Nido et al., 1988).

The phosphorylation state of MAP1B specified by mAb150 and poly-531 has been termed phosphorylation mode I. Mode I phosphorylation is possibly catalyzed by PDPK, and is characterized by an upward shift in electrophoretic mobility of the MAP1B protein band. The phosphorylation state of MAP1B specified by mAb125 and poly-842 has been termed phosphorylation mode II. Mode II phosphorylation is possibly catalyzed by CK II, and does not cause a mobility shift on SDS-PAGE gels (Ulloa et al., 1993a). MAP1B purified from calf brain has been found to be phosphorylated at mode II sites, but only partially phosphorylated at mode I sites (Pedrotti et al., 1996). The significance of different phosphorylation modes relating to MAP1B function is not well understood. The exact epitope recognized by
mAb150 and mAb125 has not been defined, thus their relationship to the polyclonal antibody recognition sites is not known. Therefore, these two antibodies provide limited information, and their utilization in functional analysis of MAP1B is also limited.

MAP1B has also been found in other tissues besides neuronal cells, including pituitary gland (Bloom et al., 1985), adrenal gland (Kotani et al., 1986), and heart and testis (Wiche et al., 1984). In liver, kidney, stomach, spleen, and thymus, MAP1B mRNA was detected, although at much lower levels than in brain (Lewis et al., 1986). MAP1B was also found to be associated with mitotic Chinese hamster ovary (CHO) cell spindle poles. This population of MAP1B was phosphorylated on an epitope identified by mitotic protein monoclonal antibody 2 (MPM2) (Vandré et al., 1986; Tombes et al., 1991). The MPM2 antibody was raised against a protein extract of mitotic HeLa cells, and selected by its preferential staining in mitotic cells versus interphase cells by indirect immunofluorescence (Davis et al., 1983). It stains mitotic spindle poles, spindle fibers, kinetochores and midbodies (Vandré et al., 1984; Vandré et al., 1991). Microinjection of MPM2 antibody into HeLa cells and Xenopus eggs blocks the completion of M-phase in those cells (Davis et al., 1989; Kuang et al., 1989), indicating that the modification identified by MPM2 antibody is an important regulatory step during the cell cycle. On immunoblot, MPM2 recognizes a number of protein bands. Most of these proteins are synthesized in interphase and phosphorylated at the onset of mitosis.
MPM2 antibody binding requires phosphorylation on threonine/serine, but not tyrosine residues (Zhao et al., 1989). To date, many MPM2 antigens have been identified: cdc25 protein phosphatase (Kuang et al., 1994), wee1 protein kinase (Mueller et al., 1995a), myt1 protein kinase (Mueller et al., 1995b), NIMA kinase in Aspergillus (Ye et al., 1995), p42\textsuperscript{mapk} (Taagepera et al., 1994), DNA topoisomerase II\textalpha{} and \textbeta{} (Taagepera et al., 1993b), MAP4 (Vandre et al., 1991), MAP1 (Vandre et al., 1986), and PHF-tau (Kondratick and Vandre, 1996). Recent studies have identified twenty more MPM2-reactive proteins, with most of them being unknown proteins (Matsumoto-Taniura et al., 1996; Stukenberg et al., 1997). Among these known MPM2-reactive proteins, most of them are key regulators of the cell cycle. The MPM2 epitopes on p42\textsuperscript{mapk} and DNA topoisomerase II have been proposed (Taagepera et al., 1993a; Taagepera et al., 1994). This study is intended to examine whether phosphorylated MAP1B in neuronal systems is recognized by the MPM2 antibody, and to identify the epitope site in MAP1B required for MPM2 antibody binding. Having established this phosphoepitope, the functional analysis of MAP1B phosphorylation at this epitope site during neuronal differentiation will be possible and will establish a role for MPM2-reactive phosphoproteins in neurogenesis.

In chapter 1, data are presented to delineate whether and when MAP1B becomes MPM2-reactive in differentiated PC12 cells. Immunoblot analysis against MPM2 and MAP1B-specific antibodies were performed to observe the
phosphorylated MAP1B in lysates of PC12 cells treated with different growth factors. Localization of MAP1B was examined by indirect immunofluorescence and immunogold labeling experiments. Labeling of cell bodies and neurites of differentiated PC12 cells were compared. While MPM2 antibody specifies a mitosis-associated phosphoepitope, the appearance and up-regulation of the epitope during neuronal differentiation is a novel phenomenon. It suggests that there exists a conserved pathway to phosphorylate MAP1B, and possibly other proteins, in cell division and differentiation of postmitotic neuronal cells. The specific localization of phosphorylated MAP1B in neurites also implies that MAP1B and phosphorylation of MAP1B may play an important role in neurite outgrowth in PC12 cells.

Chapter 2 is devoted to the MPM2 epitope analysis on MAP1B. MAP1B was subjected to proteolytic digestion, and products from the limited digestion were resolved on standard SDS-PAGE. Small fragments still remaining MPM2-reactive were traced by immunoblot analysis using MPM2 and region-specific MAP1B antibodies. These fragments were then recovered for N-terminal microsequencing. This narrowed down the epitope region from the whole molecule of 320 kD to the N-terminal 40 kD of MAP1B. Together with a working model of the MPM2 epitope determined from other MPM2 antigens, four potential MPM2 epitope sites were found in the primary sequence of MAP1B. The site which fits the MPM2 epitope model best, and which also localized within the N-terminal 40 kD was selected for analysis. MAP1B-derived peptides containing the predicted epitope sequence were synthesized in both threonine-phosphorylated and dephosphorylated forms. The
phosphopeptide TP-1B was highly reactive with MPM2 antibody, and it also competed for MPM2 antibody binding with phosphorylated MAP1B in PC12 cells and other phosphoproteins in mitotic cells. These results showed that the predicted sequences have all the elements required for MPM2 antibody recognition. Identification of the epitope on MAP1B should aid in understanding the functional properties of the MPM2 epitope on MAP1B and serve as a substrate to help identify the MAP1B-MPM2-epitope kinase.

Chapter 3 describes efforts to generate and characterize a site and phosphorylation state-specific antibody PMB1. The MAP1B phosphopeptide TP-MAP1B/KLH conjugate was used as an immunogen to immunize rabbits. The immune sera contain IgG specific to MAP1B. Antibody PMB1 was purified from immune sera by an affinity column coupled with synthetic peptide TP-MAP1B. PMB1 was shown to recognize the phosphorylated MAP1B in differentiated PC12 cells, but not the dephosphorylated form. This indicates that the predicted epitope site is highly likely to be phosphorylated in vivo. Furthermore, on immunoblots PMB1 shared recognition with a subset of phosphoproteins identified by the MPM2 antibody in mitotic cell lysates. By indirect immunofluorescence, PMB1 stained mitotic spindle poles, but not spindle fibers or kinetochores. Therefore, PMB1 is an MPM2-like phosphospecific antibody, but not identical to the MPM2 antibody. PMB1 is more selective than MPM2 antibody. This unique antibody is expected to assist in elucidating the functional significance of this posttranslational modification of MAP1B during neurogenesis.
Diagram 1

Microtubule structure
Diagram 2

Organization of microtubules in neuronal cells
Diagram 3. Structure of Microtubule-associated Proteins
<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Epitope</th>
<th>Putative kinase</th>
<th>Phosphatase sensitivity</th>
<th>Phosphorylation mode</th>
</tr>
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<tr>
<td>mAb 150 IgM¹</td>
<td>unknown</td>
<td></td>
<td>PP2A, PP2B but not PP1</td>
<td>I</td>
</tr>
<tr>
<td>mAb 125 IgM¹</td>
<td>unknown</td>
<td></td>
<td>PP1, PP2A but not PP2B</td>
<td>II</td>
</tr>
<tr>
<td>poly-531²</td>
<td>a.a.1247~1261</td>
<td>Proline-directed protein kinase</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S<em>PAKS</em>PSLS* PS<em>PPS</em>P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>poly-842²</td>
<td>a.a.2050~2059</td>
<td>casein kinase II</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>(ASTYS<em>YET</em>S D)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Residues marked with * are proposed phosphorylation sites for the corresponding kinase.
1. Ulloa et al., 1993b.
2. Ulloa et al., 1993a.

**Table 1. MAP1B phosphorylation state-specific antibodies.**
CHAPTER 1

The Mitotic Phosphoepitope Recognized by the MPM2 Antibody is Up-regulated During NGF Stimulated Differentiation of PC12 Cells

INTRODUCTION

Neuronal microtubules (MTs) are considered to be essential for the growth and maintenance of dendrites and axons (Drubin et al., 1985). The cellular functions of MTs are coordinated by their dynamic properties. Microtubule-associated proteins (MAPs), which copurify with tubulin during temperature-dependent assembly and disassembly cycles of MTs, play an important role in both promoting assembly and stability of MTs (Pryer et al., 1992). MAP1B, a high molecular weight MAP, is one of the earliest proteins expressed as neurons begin extending axons and dendrites. Expression of MAP1B is elevated throughout the late embryonic stage until postnatal day 10 in the rat brain, and is down-regulated afterward (Riederer et al., 1986). In adult brain, high levels of MAP1B are only detected in the olfactory bulb, hippocampus, and dendrites of Purkinje cells in the cerebellum, where neuronal outgrowth and plasticity continue throughout life (Viereck et al., 1989). In developing neurons, MAP1B is concentrated in areas of axonal growth, while in mature neurons, its expression is weak and widespread in
all cellular compartments (Riederer et al., 1986; Ulloa et al., 1993; Nothias et al., 1996). Therefore, both the differential expression of MAP1B in neuronal tissues and its localization in neuronal cells suggest an important role for MAP1B in neurogenesis. Treatment of PC12 cells with MAP1B antisense oligonucleotides abolished both MAP1B expression and the ability of the cells to extend neurites upon nerve growth factor (NGF) stimulation (Brugg et al., 1993). Recently, defects in neuronal development have been observed in MAP1B mutant mice (Edelmann et al., 1996). The homozygous mutant mice did not survive the late embryonic stage of development, indicating that MAP1B is an essential protein for normal development. The mutant MAP1B heterozygotes displayed a series of neuronal abnormalities, including motor system abnormalities, lack of visual acuity, and slow growth rate. Abnormal dendritic processes were found in Purkinje cells, in the olfactory bulb, hippocampus, and retina of heterozygous mutants. These findings strongly indicate that MAP1B is indispensable in neurogenesis.

Post-translational modifications such as the phosphorylation of MAP1B may affect the function of this MAP. MAP1B phosphorylation increased during neurite outgrowth in differentiated PC12 cells and neuroblastoma cells (Aletta et al., 1988; Diaz-Nido et al., 1991; Keating and Asai, 1994). Phosphorylated forms of MAP1B were observed in developing axons, whereas unphosphorylated forms were limited to cell bodies and dendrites (Sato-Yoshitake et al., 1989). Several kinases that are capable of phosphorylating MAP1B have been identified, including casein kinase
directed kinase (Ulloa et al., 1993; Tsao et al., 1990), and a mitotic spindle-associated kinase (Tombes et al., 1991). While the phosphorylation of MAP4, MAP2, and tau has been shown to reduce their abilities to stabilize MTs (Lindwall and Cole, 1984; Drechsel et al., 1992; Faruki et al., 1992; Ookata et al., 1995), the effect of phosphorylation on the function of MAP1B is still unclear. Phosphorylation of MAP1B by casein kinase II was suggested to increase its affinity to MTs (Diaz-Nido et al., 1988). However, a recent study (Pedrotti et al., 1996) using purified MAP1B, found that alkaline phosphatase-treated MAP1B bound to taxol-stabilized MTs more strongly than the phosphorylated MAP1B.

Several MAP1B phosphorylation state-specific antibodies have been reported (Noble et al., 1989; Ulloa et al., 1993a). Monoclonal antibodies 125 and 150 recognize MAP1B when it is phosphorylated by casein kinase II (CKII) and proline-directed protein kinase (PDPK), respectively. However, the sites these two antibodies specifically recognize are not known. It is highly likely that phosphorylation on additional sites may also affect the functional properties of MAP1B, therefore, these two antibodies provide only limited information, regarding the functional consequences of MAP1B phosphorylation. Polyclonal antibodies 842 and 531 have also been raised against synthetic peptides corresponding to MAP1B sequences 2050-2059 and 1247-1261, respectively, that contain potential phosphorylation sites (Ulloa et al., 1993a; Ulloa et al., 1993b). These two polyclonal antibodies only recognize MAP1B when it is not phosphorylated on the
antibodies only recognize MAP1B when it is not phosphorylated on the corresponding sequences. Using these antibodies, it has been reported that phosphorylation of MAP1B catalyzed by PDPK is elevated in newborn and early postnatal rat brains, whereas in adult brain, MAP1B is partially phosphorylated on the CKII site (Ulloa et al., 1993b). While these reports indicate that phosphorylation of MAP1B at PDPK and CKII sites correlates with different stages of neuronal development, the functional significance of the phosphorylation identified by antibodies 125 and 150 is not fully understood.

The MPM2 monoclonal antibody was originally raised against a protein extract of mitotic HeLa cells and recognizes a phosphoepitope enriched in mitotic cells (Davis et al., 1983). Not only have MPM2-reactive proteins been localized to a number of mitotic structures, but MPM2 antibody staining of proteins in brain extract and MTs of neuroblastoma cells has also been observed (Kondratick and Vandré, 1996; Feng and Vandré, 1996). Previous reports have shown that the MPM2 antibody recognized MAP1B in mitotic CHO cells (Vandré et al., 1991; Tombes et al., 1991). We have used the monoclonal antibody MPM2 to examine the phosphorylation of MAP1B during the differentiation of PC12 cells. We found that MPM2 antibody recognized phosphorylated MAP1B present in differentiated PC12 cells, and the level of MPM2 reactivity increased with extent of neurite outgrowth. MPM2-reactive MAP1B was localized to the neurite, but not the cell body as shown by both immunofluorescence and immunogold labeling. The
increase in MAP1B phosphorylation and MPM2 reactivity was exclusively associated with NGF and fibroblast growth factor (FGF)-induced neurite outgrowth, but was not associated with forskolin or epidermal growth factor (EGF)-treated cells. Thus, the mitotic-associated phosphoepitope recognized by the MPM2 antibody is also up-regulated during differentiation of neuronal PC12 cells, but only as a result of extensive neurite outgrowth.
Materials and Methods

Materials  Dulbecco's modified Eagle's medium and fibroblast growth factor were purchased from GIBCO (Grand Island, NY). Horse serum and iron-enriched calf serum were purchased from Intergen (Purchase, NY). Matrigel and epidermal growth factor were obtained from Collaborative Biomedical Products (Bedford, MA). Nerve growth factor was purchased from Boehringer Mannheim (Germany). MPM2 antibody was generously provided by Dr. Potu N. Rao (Department of Chemotherapy Research, The University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, TX). Anti-MAP1B-4 antibody was kindly provided by Dr. Richard B. Vallee (Worcester Foundation for Experimental Biology, Shrewsbury, MA). Tubulin antibodies DM1A and DM1B were purchased from ICN (Lisle, IL). FITC-conjugated goat anti-mouse antibody was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). AuroProbe EM goat anti-mouse IgG (Fc) G5 was purchased from Amersham (Arlington Heights, IL). Tannic acid (#1764) was obtained from Mallinckrodt. Inc. (Paris, KY). Prestained protein molecular weight standards were purchased from Novex Experimental Technology (San Diego, CA). Forskolin, and other chemicals were purchased from Sigma (St. Louis, MO).

Matrigel Coating of Culture Flasks and Coverslips

Glass coverslips were washed in 75% ethanol for 5 min, followed by flame drying. Before matrigel coating, all culture flasks, petri dishes with coverslips, and
pipettes were first chilled in a freezer. A sufficient amount of sterile matrigel solution (1%, V/V) in PBS (140 mM NaCl, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, 6.5 mM Na$_2$HPO$_4$, pH 7.4) was added to flasks and petri dishes to cover the culture surface. Culture vessels were immediately moved to refrigerator and stored at 4°C for 30 min, before they were allowed to stand at room temperature for 1 h. The matrigel solution was then aspirated, and vessels were rinsed twice with warm PBS.

**Cell Culture**

PC12 cells were maintained in DMEM with high glucose supplemented with 5% horse serum and 5% calf serum. For differentiation experiments, cells were cultured on matrigel-coated flasks or glass coverslips. To trigger differentiation, nerve growth factor was included 1 day after culture to a final concentration of 50 ng/ml. Differentiation was better induced if cells were refed with fresh NGF 2 days after the primary treatment. Extensive neurite networks were observed after NGF treatment of total 5–7 days. Three other reagents were also tested at following concentrations to induce differentiation: fibroblast growth factor (FGF 10 ng/ml), epidermal growth factor (EGF 10 ng/ml) and forskolin (10 μM).

Mitotic PC12 cells were obtained by synchronizing cells at M-phase with nocodazole (0.04 μg/ml) for 16 h. Mitotic cells were shaken off and collected to make whole cell lysates.
Immunofluorescence

Immunofluorescence staining was conducted as described by Vandré et al. (1984). Briefly, differentiated PC12 cells grown on matrigel-coated coverslips were rinsed with prewarmed PBSa (0.002% NaN₃ in PBS, pH 7.4). Cells were extracted with 0.1% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) for 60 seconds, and then fixed with 0.7% glutaraldehyde in PHEM for 15 min. Coverslips were washed with PBSa and the residual glutaraldehyde was reduced by incubation in freshly made NaBH₄ (1 mg/ml) in TBS (100 mM Tris, 0.9% NaCl, pH 7.4). Cells were then blocked in 4% normal goat serum for 30 min and incubated with the primary antibody for 2 h. Monoclonal antibody MPM-2 (1:500) or anti-tubulin antibody DM1A/1B (1:250) were used. Cells were washed with PBSa to eliminate the non-specific binding and then stained with secondary FITC-conjugated goat anti-mouse IgG (1:40). After washing, coverslips were mounted in mowiol medium containing 1 mg/ml of p-phenylenediamine and observed under an epifluorescence microscope (Zeiss).

Immunoelectron Microscopy

Differentiated PC12 cells grown on matrigel-coated glass coverslips were treated with 0.5% glutaraldehyde and 1% Triton X-100 in PHEM for 1 min, and then fixed in 0.5% glutaraldehyde in PHEM for 10 min. Cells were further permeabilized with 1% CHAPs (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in PHEM for 30 min. Incubation with the primary antibody MPM-2 (1:50) was carried
out at 37°C for 10 h. After rinsing with PBSa, cells were incubated with 5 nm gold-conjugated goat anti-mouse antibody (1:5) at 37°C for 10 h. Cautions were taken to avoid drying cells during the incubation. Cells were then postfixed with 1% glutaraldehyde and 0.2% tannic acid in PBSa for 30 min at room temperature to fix bound antibodies. Osmication was performed with 1% osmium tetroxide for 30 min, followed by dehydration in an ethanol series (30, 50, 80, 95, 100%, and 100%), for 5 min in each solution. Cells were incubated with hydroxypropylmethacrylate (HPMA) for 30 min each with two changes of HPMA and gentle shaking. Incubation was then carried out in resin LX112 with 2 changes to allow sufficient infiltration. Cells were then embedded in inverted BEEM capsules filled with resin. Resin was allowed to polymerize overnight at 60°C. Resin blocks were trimmed and ultrathin sections were made. Sections were stained with 2% aqueous uranyl acetate (15 min) and Reynolds lead citrate (3 min) (Reynolds, 1963), and observed using Philip’s CM-12 TEM.

Immunoblot Analysis

PC12 cells were first washed gently with cold PBS, and then lysed with cold RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 0.1 M NaH₂PO₄, pH 7.2, 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 0.1 mM sodium vanadate) supplemented with a protease inhibitor cocktail (aprotinin 100 KIU, PMSF 1 mM, leupeptin 1 μg/ml, pepstatin 1 μg/ml). The lysate was sonicated and quickly frozen. Protein concentration was determined by the BCA method (Pierce). For
immunoblots, 25 μg protein of each lysate was first resolved on a 4% urea/SDS-PAGE according to the method of Laemmli (1970) with slight modifications (Aletta et al., 1988), and then transferred to nitrocellulose membrane as described (Vandré et al., 1991). The membrane was blocked in 10 % heat-inactivated horse serum in TBS for 1 h and incubated with MPM2 (1:2000) or anti-MAP1B-4 (1:1000) antibody for 2 h. After washing, the blot was incubated with goat anti-mouse peroxidase-conjugated antibody for 1 h, followed by development with 4-chloro-1-napthol.
RESULTS

MPM2 recognition of the phosphorylated MAP1B in differentiated PC12 cells.

The recognition of MAP1B in mitotic cells by the MPM2 antibody suggested that phosphorylated forms of MAP1B associated with neurite outgrowth might also be MPM2-reactive. To investigate this possibility, we analyzed the phosphorylation state of MAP1B in PC12 cells undergoing differentiation. Upon exposure to NGF, PC12 cells display a number of features characteristic of sympathetic neurons, e.g. cessation of proliferation, neurite outgrowth, development of electrical excitability, and production of neurotransmitter-synthesizing enzymes (Greene and Tischler, 1976). Morphologically, in response to NGF treatment, PC12 cells initiated neurite outgrowth in 1-2 days, and establish long elaborate neurite networks after ~5 days of differentiation. These neurites have length many times of the diameter of the cell body, and they usually have growth cones on their tips. To examine changes in protein levels and the phosphorylation state of MAP1B during neurite outgrowth, PC12 cell lysates were prepared at different time points after the addition of NGF and examined by immunoblot. Undifferentiated control cells showed only very low basal levels of MAP1B (Figure 1, B). Following stimulation with NGF, the MAP1B level increased gradually. After 5 days of NGF treatment, significant amounts of MAP1B were detected. The dramatic increase in MAP1B level was coincident with the outgrowth of neurites (MS thesis by Colin Lowry). In a similar fashion, MPM2 antibody recognition of MAP1B in NGF-treated cells correlated with the
differentiation state of the PC12 cells (Figure 1, A). Almost no MPM2 reactivity was detected in undifferentiated PC12 cells, but MPM2 reaction levels increased dramatically following NGF treatment. Maximal levels of MPM2 reactivity were reached after 5 days of differentiation. The early appearance of MPM2 reactivity indicates that phosphorylation of this site is associated with growth factor stimulation and may be triggering MAP1B function during neurite outgrowth in PC12 cells. For comparison, synchronized mitotic PC12 cells were also tested for MPM2 reactivity. MPM2 antibody recognized multiple phosphoprotein bands, as it does in many non-neuronal mitotic cells. The MAP1B band comigrated with one of MPM2-reactive bands in mitotic PC12 cells. However, in differentiated cells the MPM2 antibody almost exclusively recognized phosphorylated MAP1B. In most immunoblots of differentiated cells, no proteins other than MAP1B reacted with the MPM2 antibody.

Phosphorylation of MAP1B on the MPM2 epitope is only associated with signaling pathways that induce neurite outgrowth.

To further examine whether up-regulation of the MPM2 epitope is specifically associated with the development of neurites, PC12 cells were treated with different growth factors. Several different stimulating factors have been used to differentiate PC12 cells in addition to NGF, including FGF (Kremer et al., 1991) and EGF (Qiu et al., 1991), the secondary messenger stimulator forskolin (Frödin et al., 1994), and phorbol ester (Heasley and Johnson., 1994). Immunoblot analysis of cell lysates obtained from cells stimulated with each reagent showed that elevated MPM2
reactivity of MAP1B was only detected in NGF and FGF treated cells (Figure 2, lanes 1 and 2), while virtually no reactivity was detected in EGF or forskolin treated cells (Figure 2, lanes 3 and 4). Treatment of PC12 cells with FGF also induced elaborate neurite outgrowth, similar to that obtained with NGF (Figure 3). Forskolin treated cells, on the other hand, only developed short neuritic processes. These processes were not stable and usually retracted back. Extensive neurite networks did not develop following forskolin treatment. EGF treatment did not induce differentiation and neurite outgrowth (Figure 3), but rather stimulated proliferation of PC12 cells. These results confirm that the up-regulation of the MPM2 phosphoepitope on MAP1B is only associated with stimulation that induces the growth of neurites in PC12 cells.

**Spatial distribution of MPM2 antigens in differentiated PC12 cells**

Having established that the MPM2 antibody specifically recognizes a phosphorylated form of MAP1B in differentiated PC12 cells, we next examined the cellular distribution of the MPM2-reactive MAP1B by indirect immunofluorescence (Figure 4). Differentiated PC12 cells were stained either with MPM2 or tubulin antibodies, and the specific localization was examined using FITC-conjugated secondary antibodies. The MPM2 antibody did not label the cell body of NGF-treated cells (Figure 4, panel A, arrowhead), whereas tubulin antibody clearly labeled the individual microtubule fibers in the periphery of the cell (Figure 4, panel B). MPM2 staining started to increase at the hillock region (Figure 4, panel A,
arrow), where filamentous staining was seen to coalesce into the neurite. The increase in MPM2 staining intensity along the neurite shaft was dramatic. In contrast, tubulin antibody more uniformly labeled all the microtubules regardless of cellular compartments. Common to both antibodies, no staining of individual filamentous structures could be visualized in the neurite shaft due to the density and tight packing of the microtubules. Fiber staining was once again present in the distal portion of the neurite near the growth cone in both MPM2 (Figure 4, panel C) and tubulin antibody (Figure 4, panel D) staining. The MPM2 antibody labeled microtubules in the growth cone in a punctate pattern, whereas the tubulin labeling was continuous. Therefore, the immunolocalization showed that MPM2-reactive MAP1B was concentrated in the neurite and growth cone, but was not associated with microtubules in the cell body.

To further demonstrate whether the MPM2 staining is associated with microtubules, we examined the localization of MPM2 staining in differentiated PC12 cells at the ultrastructural level using immunoelectron microscopy. To visualize the MPM2 staining, secondary antibodies conjugated with 5 nm gold particles were used. As shown in Figure 5A, clusters of gold particles were associated exclusively with neurite microtubules. While some portions of microtubules were studded with gold particles, others were free of labeling. This pattern of gold labeling correlates well with the punctate staining of microtubules in the growth cone observed by immunofluorescence staining. As a control, cells were also labeled with tubulin
antibodies (Figure 5B). Neurite microtubules were heavily and evenly labeled with gold particles. In fact, the labeling was so dense, microtubules appeared thicker than those present in panel A of Figure 5 at the same magnification. The labeling with control tubulin antibodies indicated that the labeling pattern observed with the MPM2 antibody could not derived from artifact, since samples were lysed and fixed in the same way for staining with both antibodies. The microtubule and MPM2 labeling patterns were further examined in the cell body (Figure 6) of differentiated PC12 cells. No gold particles were seen on microtubules in the cell body incubated with the MPM2 antibody, whereas the tubulin antibody again labeled microtubules in the cell body (Figure 6, panel B). Microtubules were again uniformly decorated with gold particles. It should also be pointed out that microtubules in the neurite shaft were organized in parallel bundled group, compared to the randomly orientated individual microtubules in the cell body. The immunogold labeling experiment confirms that the MPM2 staining seen in the neurite by immunofluorescence staining is indeed associated with microtubules. These results indicated that the phosphorylated MAP1B recognized by MPM2 antibody was closely associated with the bundled microtubules present in the neurite, and further suggests that MPM2 phosphorylation of MAP1B may be functionally important for the bundling of neurite microtubules.
DISCUSSION

In this study we have shown that MAP1B in developing neurons is highly phosphorylated on an epitope recognized by the MPM2 antibody. In NGF differentiated PC12 cells, MPM2-reactive phosphorylated MAP1B is associated with microtubules in neurites, but not in cell bodies where microtubules do not form tight bundles. Phosphorylation of the MPM-2 site coincides with the development of the neurite, and levels of MPM2-reactive MAP1B accumulate as neurites elongate. MAP1B was also heavily phosphorylated at an MPM2 site following FGF stimulation of neurite outgrowth in PC12 cells. Stimulation of cells with forskolin or EGF does not induce neurite outgrowth or an increase in MPM2 reactivity above control levels. Therefore, phosphorylation of MAP1B at the MPM2 epitope correlates with neurite outgrowth in PC12 cells.

Immunofluorescence studies showed that MPM2 staining was specifically localized to the neurite shaft and growth cone. Since MAP1B is virtually the only MAP in differentiated PC12 cells and neurite MTs are more dynamic than these in the cell body, this high concentration of phosphorylated MAP1B may play a role in the plasticity of MTs. Thus, the concentration of phosphorylated MAP1B in neurites seems to be important for continued neurite outgrowth. Immunogold labeling showed that phosphorylated MAP1B is associated with MT bundles in the neurite
The formation of MT bundles is also thought to be an essential step of neurite outgrowth and elongation. Previous studies showed that purified MAP1B formed cross-bridges between microtubules *in vitro* (Sato-Yoshitake et al., 1989), which provides the molecular base for the formation of microtubule bundles. The specific localization of the phosphorylated MAP1B on these tightly bundled MTs provides *in vivo* evidence in supporting its role in the formation of MT bundles.

In bundling microtubules, MAP1B may use different mechanisms from other neuronal MAPs such as MAP2 and tau. Transfection of either MAP2 or tau cDNA into non-neuronal cells such as CHO, COS, or 3T3 cells, which normally do not express these neuronal MAPs, causes the formation of microtubule bundles (Takemura et al., 1992; Barlow et al., 1994). In contrast, exogeneous MAP1B does not cause microtubule bundling in transfected COS cells. However, microtubules are stabilized against microtubule-depolymerizing drugs, and acetylated or mature α-tubulins are accumulated in MAP1B-transfected CHO cells (Takemura et al., 1992). The PC12 cells we used express extremely low levels of MAP2 and tau, and MAP1B is the major MAP. Since bundles of microtubules were observed in the neurite shaft of differentiated PC12 cells, it is likely that MAP1B has the ability to crosslink microtubules and induce the formation of microtubule bundles in these cells. The microtubule-binding domain of MAP1B is composed of 21 copies of the basic amino acid motif KKEE(I/V) on its amino terminal. In addition, the microtubule-binding domain of MAP1B only occupies a small portion of the
molecule. The functional properties of the other domains on MAP1B have not been determined. It is likely that the projection domain of MAP1B may be involved in forming crossbridges between microtubules. Stabilization and bundling of microtubules by MAP1B may be regulated by the phosphorylation state of certain portions of the protein such as on the MPM2 epitope. The phosphorylation of this MPM2 site may be catalyzed by a kinase only activated in the neuronal system, but not in non-neuronal cells.

The phosphorylation of MAPs has been considered to be an important mechanism to regulate the binding affinity of MAPs to MTs. For MAP2, MAP4, and tau, their affinity to MTs is reduced following phosphorylation (Lindwall and Cole, 1984; Drechsel et al., 1992; Faruki et al., 1992; Ookata et al., 1995). In the neurofibrillary tangles (NFT) of Alzheimer’s disease, hyperphosphorylated tau proteins lose the ability to bind to microtubules, and therefore dissociate from MTs and then self-associate forming paired-helical filaments (PHF). Kinases capable of phosphorylating tau include glycogen synthase kinase 3β (GSK3β) (Ishiguro et al., 1993), cdk2, cdk5 (Baumann et al., 1993), and p110\textsuperscript{mark} (Drewes et al., 1995). They all have the ability to transform the normal tau into Alzheimer’s disease-like tau in vitro. Except for cdk2, the other kinases GSK3β, cdk5 and p110\textsuperscript{mark} were all found in normal brain tissues. Among these three kinases, cdk5 is especially interesting. First, it has been reported that cdk5 was essential for neurite outgrowth of cortical neurons. The expression of a dominant-negative mutant of cdk5 abolished neurite...
outgrowth, which was then rescued by co-expression of the wild-type cdk5 (Nikolic et al., 1996). Second, cdk5 is a member of the cyclin-dependent kinase family, which are the major kinases controlling the eukaryotic cell cycle. While other cdk family members control cell proliferation, their neuronal counterpart, cdk5, may regulate the differentiation of neuronal cells. Most MPM2 antigens are present in mitotic cells and could have regulatory activity in cell cycle progression. Here we have demonstrated that MAP1B, which mainly exists in the nervous system, is also MPM2 reactive. Therefore, the differential functions of the cdk family of kinases fit the association of MPM2 substrates with mitosis and neuronal differentiation.

The MAPK pathway is activated in PC12 cells by treatment with NGF, FGF, EGF, and forskolin through the activation of MAPKK (Qiu and Green, 1991; Traverse et al., 1992; Frodin et al., 1994). Yet the elevation of MPM2 reactivity was only detected in NGF and FGF treated cells, which developed elaborate neurite networks. Differentiation of PC12 cells was not induced by EGF or forskolin. EGF induces transient activity of MAPK and no nuclear translocation of MAPK occurs in EGF treated cells (Traverse et al., 1992). Forskolin-induced MAPK activity reaches the maximal within 5-25 min and drops quickly (Frodin et al., 1994). Our observation is consistent with previous reports showing that sustained activity of MAPK and nuclear translocation of MAPK induced by NGF and FGF is required for the neurite outgrowth of PC12 cells. No significant increase of MPM2 reactivity in PC12 cells was detected until about 3-5 days following the addition of NGF,
whereas the MAPK activity stimulated by NGF or FGF reaches a plateau after only 3-5 min (Traverse et al., 1992). Thus, while MAPK itself is not likely to be the neuronal MPM2 kinase, our result also indicates that the activation of the MAPK by EGF or forskolin is not sufficient to activate the MPM2 kinase in PC12 cells. It will be interesting to see if the activation of the MPM2 kinase in PC12 cells is associated with prolonged activity of MAPK. In addition to the duration of the kinase activity, the magnitude of kinase stimulation may also be a factor in inducing the differentiation of PC12 cells. In a separate study, it was found that a kinase activity phosphorylating MAP1 was increased by 15-20-fold with NGF and FGF treatment, while EGF only induced a 4-fold activation, and the stimulation by forskolin was even less (Tsao et al., 1990). Different from results obtained with PC12 cells, MAPK purified from unfertilized *Xenopus* eggs has been reported to be able to phosphorylate and convert MPM2-negative interphase oocyte extracts into MPM2-reactive proteins (Kuang and Ashom, 1993). While MAPK did phosphorylate a 150 kD protein in *Xenopus* oocytes, it is possibly not the major MPM2 kinase in this system, because another kinase activity, designated ME kinase-H, was able to phosphorylate more MPM2 antigens. The different systems and substrates used in these studies may be responsible for the different effects of MAPK observed.

Hyperphosphorylated MAP1B was also present in the NFT of Alzheimer's disease, where there is an attempt at massive neurite regeneration (Hasegawa et al., 1990). Whether MAP1B is phosphorylated by cdk5 is not known, although it has
been shown that in mitotic CHO cells MAP1B was phosphorylated by a spindle-associated kinase, which may be related to cdc2 (Tombes et al., 1991). The spindle-associated kinase activity was cell-cycle dependent. It conferred the MPM-2 reactivity to MAP1B very effectively in metaphase, only weakly in anaphase, and almost none in telophase. However, for NGF stimulated differentiation of PC12 cells down-regulation of cdk2 activity is a crucial event (Dobashi et al., 1995), while cdk5 activity is required for neurogenesis (Nikolic et al., 1996). Therefore, it will be interesting to see if cdk5 or any other kinases are responsible for phosphorylating the MPM-2 epitope on MAP1B in the nervous system. It is speculated that phosphorylation of MAP1B on the MPM2 epitope by cdc2 kinase in the spindle pole may enhance its functions in nucleating and stabilizing spindle microtubules, whereas the same modification by neuron-specific kinase activity may allow MAP1B to maintain the proper plasticity of neurite microtubules by balancing the stability and dynamics of microtubules. Identification of this putative neuronal MPM2-kinase will help us to understand further the regulation of neurogenesis, and possibly the relationship of neuronal differentiation to the proliferation of somatic cells.
Figure 1. Time course study of MPM2 reactivity with MAP1B in differentiated PC12 cells.

Equal amount of protein lysates from PC12 cells treated with NGF were separated by 4% urea/SDS-PAGE, and transferred to nitrocellulose membrane. The cell lysates in each lane were:

Lane 1: control cells without NGF treatment.
Lane 2: synchronized mitotic PC12 cells.
Lane 3: 1 day treatment with NGF.
Lane 4: 5 day treatment with NGF.
Lane 5: 8 day treatment with NGF.

Immunoblots were probed with MPM2 antibody (A) or a MAP1B antibody, MAP1B-4 (B). Although MPM2 antibody recognized multiple bands in mitotic PC12 cells, it only recognized MAP1B (shown by the arrow) in differentiated cells.
Figure 2. MPM2 reactivity of MAP1B in PC12 cells treated with different growth factors.

Equal amounts of protein from PC12 cells treated with NGF (Lane 1), FGF (Lane 2), EGF (Lane 3), and forskolin (Lane 4) for 5 days were resolved on a 4% urea/SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot analysis against MPM2 antibody was performed. The MAP1B band recognized by MPM2 antibody was designated with the arrow. While both NGF and FGF treated cells had strong MPM2 reactivity, EGF or forskolin treated cells showed no MPM2 reactivity.
Figure 3. Morphology of PC12 cells treated with different growth factors.

Micrographs of PC12 cells treated with NGF (a), FGF (b), EGF (c), and forskolin (d) were taken from cultures on the 5th day of each treatment. NGF and FGF-treated cells grew elaborate neurites, whereas EGF or forskolin-treated cells did not develop mature neurites. Although forskolin-treated cells had some short neuritic processes, these processes never matured into neurites.
Figure 4. Localization of MPM2-reactive MAP1B in differentiated PC12 cells by indirect immunofluorescence.

Fully differentiated PC12 cells grown on matrigel-coated coverslips were fixed and permeabilized as described in Materials and Methods. Cells were incubated with MPM2 (A and C) or tubulin (B and D) antibodies. MPM2 antibody did not label the cytoplasm (arrow head in A), and the staining became bright at the hillock region (arrow in A), where filamentous structures were seen. Tubulin antibodies labeled individual microtubule fibers in the cell body. Neurites were labeled by both antibodies, although individual fibers in the neurite could not be visualized. In the growth cone (C and D), both MPM2 and tubulin antibodies stained microtubules. While MPM2 antibody staining had a punctate pattern (C), tubulin labeling was continuous (D). The fluorescence in the center of the cell body in (A) was due to autofluorescence from glutaraldehyde used to fix cells.
Figure 4
Figure 5. Immunogold labeling of the neurite shaft in differentiated PC12 cells with MPM2 and tubulin antibodies.

Differentiated PC12 cells were processed for immunogold labeling as described in Materials and Methods. Localization of MPM2 (A) or tubulin (B) antibodies were shown by labeling with 5 nm-gold particles. MPM2 labeling in the neurite shaft was associated with clusters of gold particles that were associated with microtubules, while tubulin labeling was uniform along the whole length of microtubules. Also note that microtubules in the neurite shaft run parallel to each other. Two panels were at the same magnification.
Figure 6. Immunogold labeling of the cell body in differentiated PC12 cells with MPM2 and tubulin antibodies.

PC12 cells were processed as in Figure 5. While MPM2 antibody (A) did not label microtubules in the cell body, tubulin antibody (B) uniformly labeled microtubules. Microtubules in the cell body were randomly orientated and did not form bundles.
CHAPTER 2

Identification of An MPM2 Epitope on Microtubule-associated Protein 1B (MAP1B)

Introduction

The MPM2 monoclonal antibody was raised against the protein extract of mitotic HeLa cells and selected by preferential staining of mitotic versus interphase cells by indirect immunofluorescence labeling (Davis et al., 1983). MPM2 antibody stains components of the mitotic apparatus such as spindle poles, kinetochores, kinetochore fibers, chromosome axis and midbodies (Vandré et al., 1984; Vandré and Burry, 1992). On immunoblots, MPM2 antibody recognizes a series of proteins with molecular mass ranging from 40 to 200 kD in mitotic cells regardless of the species (Davis et al., 1983). Known MPM2 antigens identified to date include MAP1 (Vandré et al., 1986; Tombes et al, 1991), MAP4 (Vandré et al., 1991), DNA topoisomerase IIα and β (Taagepera et al., 1993b), Cdc25 protein phosphatase (Kuang et al., 1994), p42^mapk (Taagepera et al., 1994), NIMA protein kinase in Aspergillus nidulans (Ye et al., 1995), wee1 kinase (Mueller et al., 1995a), Myt1
kinase (Mueller et al., 1995b), and ten previously undescribed proteins (Matsumoto-Taniura et al., 1996). A recent report (Stukenberg et al., 1997) adds eleven new members, with some of them related to translation and transcription factors. The importance of the MPM2 antibody is evidenced not only by the fact that it recognizes a number of cell cycle regulatory proteins, but also by the microinjection of MPM2 antibody, which blocks the completion of M phase in HeLa cells and Xenopus eggs (Davis et al., 1989; Kuang et al., 1989).

Because MPM2 is a monoclonal antibody, it is likely that proteins reacting to this antibody share the same antigenic site or epitope. MPM2 antigens are synthesized in interphase, however, most of them are not MPM2-reactive until G2/M transition, when they are phosphorylated directly or indirectly by the activity of the M phase promoting factor. The antigenicity to MPM2 antibody is lost when the phosphate is removed from proteins by alkaline phosphatase (Vandré et al., 1991). The phosphorylation of threonine/serine residues, but not tyrosine, was shown to be an essential part of the MPM2 epitope, with phosphorylated threonine being a better site than phosphoserine (Zhao et al., 1989). Amino acids flanking the phosphothreonine are also thought to be required in order to bind the MPM2 antibody. Using an expression cloning method, peptides containing the sequences LTPLK and FTPLQ were found to be able to bind MPM2 antibody after they were phosphorylated by the M-phase kinase activity from mitotic extracts (Westendorf et al., 1994). Two possible M-phase phosphoproteins, termed MPP1 and MPP2,
containing these sequences were identified *in vitro*. Later MPP1 and MPP2 were indeed shown to be MPM2-reactive in mitotic HeLa cells. Therefore, these authors suggested that the phosphorylation of a proline directed site (T/SP) was a part of the MPM2 epitope (Matsumoto-Taniura et al., 1996). However, it was also reported that mitogen-activated protein kinase (MAPK), p42\textsuperscript{mapk}, contains an MPM2 reactive epitope, and the important amino acids for MPM2 antibody binding were FLTEY (Taagepera et al., 1994). Therefore, MPM2 recognition of a phosphorylated epitope did not require a proline adjacent to the phosphorylated amino acid. Research from our laboratory has also demonstrated that the synthetic peptide containing the MPM2 epitope sequence WLTNF, which is present in DNA topoisomerase IIα and β, binds the MPM2 antibody. Substitution of selected amino acids in the reactive peptide showed that aromatic amino acids at either -2 (N-terminus) or +2 (C-terminus) are important for MPM2 antibody binding (Ding et al., 1997) (Table 2). Despite the recent progress on defining the MPM2 epitope, the complete characteristics of this epitope are still not fully understood. Identification of the epitope on more MPM2 antigens will be helpful in understanding the nature of the epitope, and thereby, the nature of the kinase or kinases capable of modifying the epitope.

Microtubule associated proteins (MAPs) are a group of heterogeneous proteins that co-purify with tubulins, and promote both the polymerization and stability of microtubules (Wiche, 1989; Pryer et al., 1992). MAP1B has an important
role in neurogenesis and maintenance of neuronal plasticity (Matus, 1988). We found that in differentiated PC12 cells and fetal and newborn brain tissues MAP1B is very strongly recognized by the MPM2 antibody. In Chapter 1, it is shown that the phosphorylation level of the MPM2 epitope on MAP1B increases as PC12 cells grow neurites. We have also reported that MAP1B in mitotic CHO cells is also MPM2-reactive (Vandré et al., 1986; Tombes et al., 1991). The elevation of the MPM2 reactivity in developing neurons and dividing cells implies that there might be a common pathway activating the kinase(s) phosphorylating MPM2 antigens during neuronal development and cell proliferation. While the role of MPM2 antigens in the cell division has been partially established, it remains unclear how the phosphorylation of the MPM2 epitope on MAP1B affects the differentiation of postmitotic neuronal cells. To achieve this goal, we first attempted to localize the MPM2 epitope on MAP1B. In this study, we cleaved the MAP1B molecule with proteases and distinguished the proteolytic fragments with antibodies to identify those that were MPM2-reactive. The proteolytic digestion results together with our previous knowledge of the characteristics of the MPM-2 epitope, allowed us to predict the identity of a potential MPM2 site on MAP1B. The 11-amino acid synthetic peptide containing the predicted site was highly reactive with the MPM2 antibody, and furthermore, the peptide efficiently competed for the MPM2 antibody binding with its parental protein, MAP1B, as well as other MPM2-reactive phosphoproteins in mitotic cells.
Materials and Methods

Materials

Endoproteinase Asp-N and keyhole limpet hemocyanin (KLH) were purchased from Calbiochem (Lo Jolla, CA). Peptide TP-MAP1B (LSEFTpEYLSES) and M-MAP1B (LSEFTEYLSES) were synthesized by Genosys (The Woodlands, TX). Glutaraldehyde was purchased from Polysciences (Warrington, PA). MPM2 antibody was a generous gift from Dr. Potu N. Rao (Department of Chemotherapy Research, The University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston TX). Antibody MAP1B-3 was kindly provided by Dr. Richard B. Vallee (Worcester Foundation for Experimental Biology, Shrewsbury, MA). Polyclonal antibody PIN was prepared against a recombinant protein including amino acid 63-455 of the mouse MAP1B in our laboratory. Peroxidase conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Protease chymotrypsin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO)

Isolation of MAPs From Bovine Brain

MAP fractions were prepared according to Pedrotti and Islam (Pedrotti and Islam, 1994) with slight modifications. Briefly, twice-cycled microtubule proteins were prepared in MES (0.1 M MES, 2.5 mM EGTA, 0.5 mM MgCl₂, 0.1 mM EDTA, and 1 mM DTT, pH 6.4, with KOH) buffer from bovine brain tissues, and MAPs were dissociated with PIPES (0.1 M PIPES, 2.5 mM EGTA, 0.5 mM MgCl₂, 0.1 mM
EDTA, and 1 mM DTT, pH 6.9, with KOH) buffer from the microtubule proteins. The supernatant was recovered after taxol-induced polymerization in PIPES. This preparation was highly enriched with MAP1B and used as the substrate for the proteolytic cleavage.

**Proteolytic Digestion**

The MAP preparation in PIPES buffer was mixed with chymotrypsin in TRIS buffer (50 mM Tris-HCl, pH 8.0, 10 mM CaCl₂). Endoproteinase Asp-N digestion was performed in 50 mM sodium phosphate buffer, pH 7.6. The substrate to protease ratio was 200:1 (wt:wt) for both enzymes. The digestion was carried out at 37°C for various time and the reaction was terminated by the addition of PMSF and 5×SDS sample buffer. The mixture was then heated at 95°C for 5 min, and resolved on the SDS-PAGE for immunoblot analysis.

**Peptide Conjugation**

The carrier protein KLH (10 μg) was dialyzed against 4 liters of PBS (140 mM NaCl, 1.5 mM KH₂PO₄, 2.7 mM KCl, 6.5 mM Na₂HPO₄, pH 7.2) at 4°C overnight followed by dialysis against a second buffer change. The KLH solution was then mixed with the peptide dissolved in PBS with gentle agitation. Freshly prepared 0.3% glutaraldehyde was slowly added to the peptide/KLH mixture to a final concentration of 0.1% while stirring at room temperature. The reaction was allowed to proceed for 2 h, by which time the final solution turned yellow. To block
unreacted glutaraldehyde, 0.25 ml of 1 M glycine was added and allowed to react for 30 min. The peptide/KLH conjugate was collected and dialyzed against two changes of distilled water and then lyophilized.

**Dot Immunoblot**

A nitrocellulose membrane was presoaked in TBS (154 mM NaCl, 10 mM Tris-Base, pH 7.4) and assembled in the Bio-Dot™ apparatus (BioRad Laboratory, Hercules, CA). The lyophilized peptide/KLH conjugate was dissolved in TBS. The solution was applied to wells (50 µl/well) and allowed to bind to the membrane for 5 min before the vacuum was turned on. Wells were then washed three times with excess TBS. The NC membrane was then taken out of the apparatus, rinsed in TBS, and blocked in 10 % horse serum for 1 h. It was then incubated with MPM2 antibody (1:1000) for 1 h, rinsed, and incubated with peroxidase-conjugated donkey anti-mouse secondary antibody for 1 h prior to development. The TP-MAP1B/KLH and M-MAP1B/KLH conjugates were each blotted at various amounts: 0.01, 0.033, 0.1, 0.33, 1.0, 3.3 and 10 µg/well. KLH alone was also tested as a negative control. The bovine brain MAP preparation was used as a positive control.

MPM2 antibody titration: TP-MAP1B/KLH was blotted on the NC membrane at 0.1 µg/dot. Each dot was probed with MPM2 antibody at following dilutions: 1:1,000; 1:10,000; 1:20,000; 1:100,000; 1:1,000,000. The rest of the steps were the same as in the dot blot protocol described above.
Competition assay of MAP1B peptide by immunofluorescence on LLC-PK cells and differentiated PC12 cells

Pig kidney LLC-PK cells were grown on glass coverslips at 2,500 cells/cm² for 48 h before the experiment. Rat pheochromocytoma PC12 cells were cultured on matrigel-coated glass coverslips at 10,000 cells/cm² and differentiated with 50 ng/ml nerve growth factor (NGF, from Boehringer Mannheim, Indianapolis, IN) for 5 days. The immunofluorescence staining was performed as described by Vandré et al. (1984). Before being applied to the coverslips, MPM2 antibody (1:8,000) was preincubated with peptide TP-MAP1B or M-MAP1B for 1 h at room temperature using the following concentrations of peptide: 0.1, 1, 10 and 100 µg/ml.

Competition assay of MAP1B peptide by immunoblot

HeLa cells were synchronized in mitosis by incubation with nocodazole (40 ng/ml) for 16~24 hrs. Mitotic cells were collected by shake off and lysed in PBS containing 1% SDS. NGF differentiated PC12 cells were collected in RIPA lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 0.1 M NaH₂PO₄, pH 7.2, 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 0.1 mM sodium vanadate) supplemented with a protease inhibitor cocktail (aprotinin 100kU, PMSF 1 mM, leupeptin 1 µg/ml, pepstatin 1 µg/ml). The protein concentration of the cell lysates was determined by the bicinchoninic acid (BCA) method (Smith et al., 1985). The cell lysates were resolved on SDS-PAGE prior to immunoblotting. MPM2 antibody solution (1:8,000) was preincubated with peptide TP-MAP1B or M-MAP1B for 1h at
room temperature using the following concentrations of peptide: 0.1, 1, 10 and 100 µg/ml. The remaining steps were the same as in the regular immunoblotting experiments.
Results

Proteolytic digestion of MAP1B and identification of MPM2 reactive fragments

MAP1B is a large molecule consisting of 2,464 amino acids. In order to identify and characterize the MPM-2 epitope in detail, we first determined the approximate position of the epitope on MAP1B. The MAP1B molecule was cleaved with proteases, and the digestion products were resolved on SDS-PAGE prior to immunoblot analysis. Proteolytic fragments generated by each enzyme were probed with the MPM2 antibody and MAP1B antibodies, specific to known regions of the MAP1B molecule. The monoclonal antibody MAP1B-3 binds to MAP1B near the N-terminal portion of MAP1B, including part of its microtubule-binding domain (amino acids 517-848). Polyclonal antibody P1N was raised against a recombinant MAP1B fragment including amino acid 63-455, and therefore is specific to the N-terminal portion of MAP1B. MAP1B from bovine brain was first subjected to chymotrypsin digestion, which cleaves peptide bonds on the C-terminus of aromatic amino acids. Chymotrypsin digestion (0.5 to 2 min) generated a relatively large stable fragment of 200 kD, which was recognized by both MPM-2 and MAP1B-3 antibodies (Figure 7). This was the smallest fragment generated after chymotrypsin digestion that still remained MPM2-reactive. Smaller MPM2-reactive fragments were not detected, possibly because chymotrypsin destroys the epitope. Since the epitope appeared to be sensitive to chymotrypsin, we examined endoproteinase Asp-N digestion, which cleaves peptide bonds on the N-terminal side of aspartic
acid. Unlike chymotrypsin, Asp-N digestion of MAP1B generated a series of small fragments ranging from 70~40 kD. The smallest MPM2-reactive fragment from Asp-N digestion (Figure 8) was about 40 kD. This fragment was recognized by both MPM2 and the PIN antibody, which placed the MPM2 epitope toward the N-terminus of MAP1B.

The MPM2-reactive 40 kD Asp-N fragment was also detected on coomassie blue stained gels, which allowed us to collect the band following transfer to PVDF membrane for peptide microsequencing (data not presented). However, during microsequencing, the 40 kD fragment did not yield clear products of Edman degradation, indicating that the N-terminus of this 40 kD fragment is blocked and may therefore be derived from the N-terminus of the protein (see also discussion). This correlates with the result obtained from the immunoblot assay indicating that the 40 kD fragment was reactive with antibodies specific for the N-terminus. Our working model of the MPM2 epitope (Ding et al., 1997) includes four criteria: (1) a phosphothreonine or phosphoserine residue is required for the MPM2 antibody binding, with preference for phosphothreonine; (2) a proline residue immediately following the phosphorylated residue is not necessary; (3) an aromatic amino acid within 3 amino acids of the phosphorylation site on the N-terminal side greatly enhances the antibody binding; and (4) an aromatic amino acid spaced two or three residues from the phosphorylation site to the C-terminus also may enhance MPM2 recognition, but may not be required. According to this working model, we searched
the mouse MAP1B sequence, and found that there were four sites fitting the model: T-230, T-384, S-1168 and S-1793 (Diagram 4). Among the four potential sites, only the first two are localized in the region included by the PIN antibody. While there are several aspartic acid residues at positions 344, 353 and 367, cleavage on the N-terminal side of either one of these positions could generate a fragment of about 40 kD in size from the N-terminus of MAP1B. In order to generate an Asp-N fragment that included T-384, the next available aspartic acid residue in the sequence is 448, at which position Asp-N will cleave the N-terminal part of the MAP1B molecule into a fragment larger than 40 kD. Therefore, the potential MPM2 epitope is likely to contain T-230. Aromatic amino acids flank both sides of threonine-230 and are within 2 amino acids (229FTEYL233). This site fits the working model stringently. Our efforts were, therefore, focused on this site.

The proposed MAP1B synthetic peptide is MPM2-reactive

According to the above analysis, two MAP1B peptides with sequence LSEFTEYLSES_{236} (M-MAP1B) and its phosphorylated form LSEFTpEYLSES (TP-MAP1B, Tp=phosphothreonine) (Table 3) were synthesized to test their MPM2 reactivities. The peptides were first conjugated to a carrier protein, keyhole limpet hemocyanin (KLH). The peptide/KLH conjugate was then spotted onto a nitrocellulose membrane for immunoblot analysis (Figure 9). The phosphopeptide TP-MAP1B/KLH was highly reactive with MPM2 even at the low amount of 0.01 μg/dot. The intensity of MPM2 staining increased when 0.033, 0.1 and 0.33 μg of
peptide/KLH conjugate were added to each spot. The dephosphopeptide M-MAP1B/KLH, however, did not react with the MPM2 antibody even at amounts as high as 10 μg. The carrier protein KLH itself also did not react with the MPM2 antibody. The bovine brain MAP preparation was included as a positive control. These results showed that the phosphopeptide containing the sequence FTPpEYL has the ability to react with the MPM2 antibody with a high efficiency. The negative reaction of the dephosphopeptide M-MAP1B/KLH conjugate and KLH alone show that phosphorylation on threonine is required for MPM2 antibody binding, and that it is the phosphopeptide that conferred the TP-MAP1B/KLH conjugate its MPM2 reactivity.

To examine the affinity of the TP-MAP1B/KLH conjugate for the MPM2 antibody, a titration of the antibody was performed against 0.1 μg/dot of TP-MAP1B/KLH on the nitrocellulose membrane (Figure 10). MPM2 antibody diluted to 1:1,000,000 still exhibited a significant level of reaction with TP-MAP1B/KLH, whereas 0.1 μg of the bovine brain MAP preparation had the comparable intensity at a higher concentration (1:1,000) of MPM2 antibody. The titration experiment demonstrated that the phosphopeptide binds to MPM2 antibody with a very high affinity, confirming that the sequence we have proposed is a potent MPM2 epitope.

The MAP1B synthetic phosphopeptide competes for MPM2 antibody binding

To determine whether the phosphopeptide TP-MAP1B shares characteristics
of native MPM2-reactive epitopes, we examined its ability to compete for MPM2 binding using PC12 cells first. MAP1B is the major MAP in differentiated PC12 cells. As PC12 cells grow neurites, MAP1B is heavily phosphorylated on the MPM2 epitope, and immunoblots demonstrate that phosphorylated MAP1B is the dominant band recognized by the MPM2 antibody in these cells (see Chapter 1). Thus, the unconjugated free phosphopeptide TP-MAP1B should compete for MPM2 binding with a native antigen, in this case the phosphorylated forms of MAP1B in PC12 cells. In the first assay (Figure 11), differentiated PC12 cells were stained with MPM2 antibody (1:8,000) preincubated with various amounts of unconjugated peptide TP-MAP1B or M-MAP1B. Normally, MPM2 antibody labels the neurites of PC12 cells at the dilution of 1:8,000 used in this assay. In the growth cones, a pattern of bright and punctate (or filamentous) staining is observed. When MPM2 antibody was preincubated with the peptide M-MAP1B (0.1~100 μg/ml) or 0.1 μg/ml of TP-MAP1B, the staining patterns obtained were identical to the control sample (Figure 11, panels a-e). However, in the samples of which MPM2 antibody had been preincubated with 1.0 μg/ml of TP-MAP1B, the labeling in neurites was significantly reduced (Figure 11, panel f), and in the growth cone (Figure 12), no punctate staining was observed. Higher concentrations of the phosphopeptide TP-MAP1B (10 and 100 μg/ml) further decreased the intensity of the total MPM2 staining.
In mitotic cells, MPM2 antibody recognizes a family of proteins with the molecular weight ranging from 40 to >200 kD (Davis et al., 1983). Presumably, all the mitotic MPM2 antigens share a consensus site required for MPM2 antibody binding. Here we carried out competition assays with mitotic cells to test if the phosphopeptide derived from MAP1B could compete for the MPM2 antibody binding to phosphoproteins other than MAP1B.

LLC-PK cells grown on coverslips were stained with MPM2 antibody preincubated with the MAP1B peptides. The intensity of MPM2 staining varies depending on the stage of cell cycle (Figure 13). To compare the staining intensity in different samples, metaphase cells were chosen. Normally, MPM-2 antibody labels spindle poles, kinetochores, kinetochore fibers and cytoplasm in mitotic cells (Figure 13, C). Again, preincubation of MPM2 antibody with the phosphopeptide TP-MAP1B decreased the staining intensity in mitotic cells in a concentration-dependent fashion (Figure 14), whereas preincubation with the dephosphopeptide M-MAP1B did not change the staining intensity (Figure 15).

Competition assay was also performed using immunoblots (Figure 16). Cell lysates (30 μg/lane) of mitotic HeLa cells (Figure 16, A) and differentiated PC12 cells (Figure 16, B) were separated on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with MPM2 (1:8,000) preincubated with various amounts of TP-MAP1B or M-MAP1B. On the
immunoblot, 1 μg/ml of TP-MAP1B greatly inhibited the reaction between MPM2 antibody and mitotic phosphoproteins from HeLa cells and MAP1B from PC12 cells. Preincubation with 10 μg/ml of the phosphopeptide almost abolished the reaction. Virtually no band was detected in either sample when MPM2 antibody was preincubated with 100 μg/ml TP-MAP1B. The dephosphopeptide M-MAP1B did not interfere with the reaction regardless the amount included during the preincubation. Therefore, this result is consistent with that of the phosphopeptide competition assay observed by immunofluorescence in PC12 cells and mitotic LLC-PK cells. Together, these results indicate that the phosphopeptide TP-MAP1B has the ability to compete with phosphoproteins in mitotic cells, as well as phosphorylated MAP1B in PC12 cells, for MPM2 antibody binding.
Discussion

MPM2 antibody, a widely-accepted marker of mitosis-associated phosphoepitopes, recognizes a number of phosphoproteins in mitotic cells regardless of the species of origin. Only a few of the MPM2 reactive proteins have been identified, and even fewer of the epitopes recognized by the MPM2 antibody have been defined on these proteins. Here we have identified the MPM2 epitope on MAP1B. The authenticity of the epitope is supported by: (1) a synthetic phosphopeptide containing the proposed sequence FTEYL is highly reactive with MPM2 antibody; (2) the phosphopeptide competes for MPM2 antibody binding with native MAP1B, as well as other phosphoproteins in mitotic cells.

When MAP1B was cleaved with chymotrypsin, which cleaves peptide bonds at the C-terminus of aromatic amino acid residues, only large fragments remained MPM2 reactive. The MPM2 epitope site we have identified on MAP1B contains both phenylalanine and tyrosine residues, which explains the extreme sensitivity of the epitope to chymotrypsin. This is consistent with the fact that there are aromatic amino acid residues flanking the phosphothreonine in the MPM2 epitope of topoisomerase II (WLTNF) and p42

\[^{\text{mapk}}\] (FLTEY). In addition, Ding et al. (1997) have shown that substitution of either W or F with alanine significantly reduces the MPM2 antibody recognition of the topoisomerase II epitope. The 40 kD fragment generated from Asp-N digestion did not yield any N-terminal sequence information.
Therefore, the 40 kD fragment appeared to contain a modified and blocked N-terminus. Previously, Hammarback et al. (1991) reported that a 120 kD fragment derived from the N-terminal portion of MAP1B failed to yield N-terminal sequence information. Further digestion and mapping located the fragment to the N-terminus of MAP1B, suggesting that MAP1B is indeed N-terminally blocked. Together with the information obtained from MAP1B antibody recognition, the potential MPM2 epitope on MAP1B can be narrowed down to its N-terminus 40 kD. In addition, another high-molecular weight MAP, MAP1A, sharing sequences with MAP1B starting from amino acid 223, is also an MPM2 antigen. However, on MAP1A the amino acid corresponding to threonine-230 of MAP1B is a serine. This is consistent with the fact that MAP1A is a much weaker MPM2 antigen than MAP1B (our observation). Furthermore, a molecule in chicken nervous systems, named claustrin, is highly homologous to the N-terminus of mouse MAP1B. Clastrin also has FTEYL sequence in the corresponding region, and more importantly, it is a strong MPM2 antigen (data not published).

The synthetic phosphopeptide containing the proposed sequence FTEYL bound MPM2 antibody with a high affinity, when it was conjugated with KLH. The unconjugated free peptide TP-MAP1B also competed for MPM2 antibody binding with MAP1B both on immunoblot and by immunofluorescence staining of fixed cells. Competition of the peptide with MPM2 recognition of phosphoproteins in mitotic cells further supports that the TP-MAP1B phosphopeptide has the antigenic
properties of native MPM2 antigens. It should be pointed out that the epitope we proposed does not have a proline residue following the phosphothreonine. However, Westendorf and co-workers stated that peptides containing sequences FTPLQ and LTPLK were able to bind MPM2 antibody when they are phosphorylated by an M phase cell extract (Westendorf et al., 1994). They also cloned two proteins, MPP1 and MPP2, that contained the above sequences as well as other S/TP sites. In a more recent report, using similar expression cloning techniques, they isolated cDNAs for 10 unknown M phase proteins (MPPs), including MPP1 and MPP2, and 3 previously known proteins (Matsumoto-Taniura et al., 1996). All ten MPPs are present in mitotic HeLa cells and recognized by the MPM2 antibody. Again, nine of the ten newly isolated MPPs have S/TP sites similar to the sequence FTPLQ. However, among the three known proteins, two of them do not have any S/TP sites in the region cloned that was MPM2 reactive (amino acid 222-295 of laminin-binding protein and amino acid 121-175 of ribosomal protein L18a). Examining the sequences of these two proteins, we found that they both have sites similar to the model we have proposed for the MPM2 epitope and related to that identified in MAP1B lacking a proline residue adjacent to the phosphothreonine. The expression cloning technique may select for an MPM2 reactive protein only if it could serve as a substrate for the MPM2 kinase activity in the M phase cell extract used. Therefore, the sequence FTPLQ and other S/TP sites may represent only a subgroup of the total MPM2 epitopes. The site we have identified on MAP1B supports this possibility.
To date, several kinases have been described that are able to generate MPM2 reactive proteins. They are MAPK (Kuang and Ashorn, 1993), p34\(^{\text{cdc2}}\) (Westendorf et al., 1994), MAPKK or MEK (Taagepera et al., 1994) and plx1 (Kumagai and Dunphy, 1996). All of these kinases are important for regulating the cell cycle and transducing signals. For example, MEK can phosphorylate p42\(^{\text{mapk}}\), thereby, generating its MPM2 phosphoepitope that includes threonine-183. Phosphorylation on this site is mandatory for the activity of p42\(^{\text{mapk}}\). In *Xenopus* eggs, plx1, a protein homologous to the polo kinase family in *Drosophila*, has been identified as another MPM2 kinase. Its serine/threonine kinase activity phosphorylates cdc25 and converts it into an MPM2 antigen. Phosphorylation of the MPM2 epitope modulated the dual phosphatase activity of cdc25, which is a key regulator of the cdc2-cyclin B complex. Among the kinases identified, each has a different substrate specificity. It appears likely that there may be a family of MPM2 epitope kinases with different substrate specificities rather than a single MPM2 kinase. Some of the substrates for each kinase may have a phosphorylation site recognized by the individual kinase that has also shared the properties of the MPM2 epitope. The MPM2 kinase activities described to date are all from dividing cells. Thus, the MAP1B-MPM2 kinase may be unique in that it is associated with a process of differentiation. The dephosphopeptide M-MAP1B should be able to serve as a substrate for the putative MPM2 kinase. Because MAP1B is such a large molecule with many potential phosphorylation sites, it is difficult to define
which site is phosphorylated by a specific kinase. The advantage of using an artificial peptide substrate is obvious. Therefore, identification of the epitope on MAP1B should aid in searching for the MPM2 kinase activity in neuronal systems.

Recently, there have been reports showing that the phosphoepitope identified by MPM2 antibody is associated with the pathological lesion of Alzheimer's disease (AD) (Vincent et al., 1996; Kondratick and Vandré, 1996). The MPM2 staining co-localizes with the staining of AT-8 antibody, a paired-helical filament (PHF)-tau specific antibody. The tau protein isolated from the PHF of AD reacts with MPM2, whereas neither the normal adult nor the fetal tau is recognized by MPM2 antibody (Kondratick and Vandré, 1996). The reappearance of the mitosis-specific phosphoepitope in terminally differentiated neurons implies that there may be an attempt of regeneration in these cells in AD brain. The understanding of the kinase responsible for the modification of the epitope will shed a new light on the etiology of AD. The MPM2 epitope on tau is not defined so far. The identity of the epitope on MAP1B should be informative in mapping the epitope on tau, and other MPM2 antigens, and in turn finding the kinase.

The functional significance of MAP1B phosphorylation is not well understood, possibly because modification on different sites may have different effects. Having defined the MPM2 phosphoepitope on MAP1B, we can further analyze its functional role. MAP1B fragments carrying mutations on the MPM2 epitope sites can be
expressed, and treated with isolated kinases or lysates from differentiated PC12 cells. Fragments can then be co-polymerized with microtubules, and their ability to promote microtubule polymerization and affinity for microtubules can be analyzed *in vitro*. Furthermore, MAP1B cDNA with variations on the MPM2 epitope site can be transfected into non-neuronal cells such as COS and 3T3 cells, to observe the possible different effects on changes in the microtubule organization of these cells.

In summary, we have identified an MPM2 epitope on MAP1B. The synthetic phosphopeptide containing the sequence we proposed is a strong MPM2 antigen, and it binds MPM2 antibody with a high affinity. The peptide can compete for MPM2 antibody binding with MAP1B and other phosphoproteins found in mitotic cells. Currently, we are generating an antibody against the epitope to analyze the functional significance of the epitope in neuronal cells.
Peptide sequence  | MPM2 reactivity
-----------------|------------------
RKEWLTPNFMEDRR  | +++              
RKEWLTNFMEDRR  | -                
RKEWLTPAFMEDRR  | +++              
RKEALTPNFMEDRR  | +/-              
RKEWLTPNAMEEDRR | ++                

*Tp=phosphothreonine
Letter underlined stands for the substituted amino acid.

Table 2.
Characteristics of the MPM2 Epitope in DNA Topoisomerase II
Table 3. Synthetic Peptides Derived From MAP1B

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphopeptide TP-MAP1B</td>
<td>NH$<em>2$$^{226}$LSEFTpEYLSES$</em>{236}$-COOH</td>
</tr>
<tr>
<td>Peptide M-MAP1B</td>
<td>NH$<em>2$$^{226}$LSEFTEYLSES$</em>{236}$-COOH</td>
</tr>
</tbody>
</table>

*Tp=phosphothreonine

Numbers indicate the amino acid positions on the mouse MAP1B.
Diagram 4
Potential MPM2 Sites in MAP1B
Figure 7. Proteolytic digestion of MAP1B with chymotrypsin.

Bovine brain MAPs were digested with chymotrypsin for 0, 0.5, 1.0, and 2.0 min at 37°C, and the digestion products resolved by a 7% SDS-PAGE. Immunoblot analysis with MPM2 (A) or MAP1B-3 (B) antibodies was conducted. A stable MAP1B fragment with the size of 200 kD (shown by the arrow) was recognized by both MPM2 and anti-MAP1B-3 antibodies. No smaller MPM2-reactive fragments were detected.
Figure 7
Figure 8. Proteolytic digestion of MAP1B with endoproteinase Asp-N.

Bovine brain MAPs were digested with Asp-N for 4 min at 37°C. Immunoblot analysis of the digestion products were carried out with MPM2 (Lane 1) or P1N (Lane 2) antibodies. Asp-N digestion generated a series of small MPM2-reactive fragments with molecular weights of 70, 60, and 40 kD. The smallest MPM2-reactive 40 kD band (designated by the arrow) was also recognized by the P1N antibody and detected on the coomassie blue stained gel (Lane 3). Lane M is molecular weight standards in kD.
Figure 8
Figure 9. Dot immunoblot analysis of MAP1B synthetic peptides against MPM2 antibody.

Synthetic MAP1B peptides were coupled to the carrier protein KLH and then spotted on nitrocellulose membrane as described in Materials and Methods. Dot immunoblot analysis was conducted using MPM2 antibody. Carrier protein KLH alone (row a) was also tested. Bovine brain MAP preparation (row d) was included as a positive control. For each protein preparation or peptide/KLH conjugate, different amounts were spotted to the membrane: column 1, 0.01; 2, 0.033; 3, 0.1, and 4, 0.33 µg/dot. The phosphopeptide TP-MAP1B/KLH conjugate (row b) was highly reactive with MPM2 antibody at the amount of 0.01 µg, whereas the dephosphopeptide M-MAP1B/KLH conjugate (row c) did not react. KLH also was not reactive. The bovine brain MAPs showed MPM2 reactivity.
Figure 10. Titration of MPM2 antibody on dot blots containing MAP1B phosphopeptide.

Dot immunoblot analysis was conducted as in Figure 9. In row a, 0.1 µg of TP-MAP1B/KLH conjugate was spotted on the nitrocellulose membrane. In row b and c, 0.1 and 1.0 µg of bovine brain MAP preparation were included, respectively. Blots were probed with MPM2 antibody at the following dilutions:

1. 1:1,000,000
2. 1: 100,000
3. 1: 20,000
4. 1: 10,000
5. 1: 1,000

The phosphopeptide TP-MAP1B/KLH conjugate reacted with MPM2 antibody at an MPM2 dilution of 1:1,000,000, while the same amount of brain MAPs did not. It required 10 times the amount of brain MAPs to reach a similar level of MPM2 reactivity at each dilution.
Immunofluorescence was conducted as usual except that MPM2 antibody (1: 8,000) was preincubated with various amounts of free peptides at room temperature for 1 h, before it being applied to PC12 cells fixed on the coverslip. Dephosphopeptide M-MAP1B (a, b, c, and d) or phosphopeptide TP-MAP1B (e, f, g, and h) was preincubated with MPM2 antibody solution at the following concentrations: 0.1 (a and e), 1.0 (b and f), 10 (c and g), and 100 µg/ml (d and h). Dephosphopeptide M-MAP1B did not affect the intensity of the MPM2 antibody staining, regardless of the amount of peptide included during preincubation. Phosphopeptide TP-MAP1B dramatically decreased the staining intensity in neurites at 1.0 µg/ml. Higher concentration of the phosphopeptide further eliminated the staining.
Figure 11
Figure 12. Competition assay with MAP1B peptides for MPM2 staining of growth cone in PC12 cells.

Competition assays were performed as in Figure 11. Micrographs were taken from samples labeled with MPM2 antibody preincubated with 1.0 μg/ml of dephosphopeptide (A and a) or phosphopeptide (B and b). Panel a and b are corresponding phase contrast micrograph. Filamentous staining in the growth cone was shown in A, while in B phosphopeptide eliminated the MPM2 staining.
Figure 13. MPM2 immunofluorescence staining of LLC-PK cells through the cell cycle.

LLC-PK cells grown on coverslips were incubated with MPM2 antibody and labeled with FITC-conjugated secondary antibodies. Fluorescence micrographs were taken of cells at interphase (A and a), prophase (B and b), metaphase (C and c), early anaphase (D and d) and late anaphase (E and e). Cells were also treated with DAPI to visualize DNA. Panels a, b, c, d, and e are corresponding DAPI staining of cells in A, B, C, D, and E, respectively. MPM2 antibody stained some foci in the interphase nucleus (A), but no staining was detected in the interphase cytoplasm. Prophase nucleus (B) was more heavily labeled, and more importantly, the centrosome was stained. In the metaphase cell (C), MPM2 antibody distinctly labeled spindle poles. Kinetochores and kinetochore fibers were also labeled. The staining started to decrease gradually at the early anaphase (D). In the late anaphase cell (E), the staining in spindle poles decreased further.
Figure 13
Figure 14. Competition assay with MAP1B phosphopeptide for MPM2 staining of mitotic LLC-PK cells.

LLC-PK cells were stained with MPM2 antibody (1: 8,000) preincubated with 0.1 (A and a), 10 (B and b), and 100 (C and c) μg/ml of phosphopeptide TP-MAP1B. Panels a, b, and c were DNA staining of corresponding cells in panels A, B, and C, respectively. The MPM2 staining was greatly decreased by preincubation with 10 μg/ml (B) of phosphopeptide TP-MAP1B. Higher concentration of the phosphopeptide almost abolished the staining (C).
Figure 15. Competition assay with MAP1B dephosphopeptide for MPM2 staining of mitotic LLC-PK cells.

Immunofluorescence was performed as in Figure 14 except that dephosphopeptide M-MAP1B was included during the preincubation at concentrations of 0.1 (A and a), 10 (B and b), and 100 (C and c) μg/ml. Preincubation with various amounts of dephosphopeptide did not change the staining intensity.
Figure 16. Competition assay with MAP1B peptides for MPM2 staining on immunoblots.

Lysates of synchronized mitotic HeLa cells (A) and differentiated PC12 cells (B) (25 μg/lane) were resolved on 7% SDS-PAGE (A) or 4% urea/SDS-PAGE (B). Immunoblot analysis was performed using MPM2 antibody (1: 8,000) preincubated with dephosphopeptide M-MAP1B (Lanes 1~4) or phosphopeptide TP-MAP1B (Lanes 5~8) at following concentrations: 0.1 (Lane 1 and 5), 1.0 (Lanes 2 and 6), 10 (Lanes 3 and 7), and 100 μg/ml (Lanes 4 and 8). Preincubation of MPM2 antibody with M-MAP1B did not affect the level of reactivity regardless of the peptide amount, while TP-MAP1B decreased the intensity of reaction at a concentration-dependent manner.
Figure 16

A

1 2 3 4

5 6 7 8

B

1 2 3 4

5 6 7 8

100
CHAPTER 3

Generation and Characterization of A Phosphorylation State- and Site-specific MAP1B Antibody

Introduction

The nervous system is responsible for transmitting electric impulses from one part of the body to another, thereby ensuring coordination of various organs and parts. The functional units of the nervous system, neurons, have a unique organization of their cytoskeletal filaments. The cytoskeletal components of the neuron, in particular, the microtubules, are involved in neuronal differentiation, morphogenesis, axonal transport, and synaptic transmission (Hirokawa, 1993). When cultured PC12 cells are treated with nerve growth factor, they develop elaborate neuritic processes, which are termed neurites. The organization of microtubules also undergoes dramatic alteration, changing from the radial pattern present in cell bodies of non-differentiated cells into the parallel microtubule arrays found in neurites of differentiated cells. Similar changes may also occur when neuronal precursor cells differentiate into mature neurons. The presence of parallel
microtubules allows the formation of microtubule bundles, which is an essential step for neurite formation and elongation (Gordon-Weeks, 1993). The mechanism underlying this dramatic change in microtubule organization is not known.

A large body of data suggests that microtubule-associated proteins (MAPs) are the main regulators of this process (Matus, 1988). According to their distribution in tissues, MAPs can be classified into two categories. MAP1A, MAP1B, MAP2, and tau are mainly found in neuronal tissues, therefore, they are usually called neuronal MAPs. MAP4 is mainly found in non-neuronal tissues, and it is the only non-neuronal MAP identified so far (Wiche, 1989). Non-neuronal cells, such as COS, 3T3 and CHO cells normally do not express the neuronal MAPs tau and MAP2, and only small amounts of MAP1. Microtubules in interphase non-neuronal cells typically radiate from the centrosome or microtubule organization center (MTOC) as individual fibers to the periphery of the cytoplasm (Barlow et al., 1994). Microtubules in these cells usually do not form bundles. However, when these cells are transfected with neuronal MAPs the expression of exogenous MAPs causes the formation of microtubule bundles in these non-neuronal cells (Takemura et al., 1992; Weisshaar and Matus, 1993; Barlow et al., 1994). Neuritic processes were also formed in some transfected cells. In the case of tau expression, the degree of microtubule bundling seems to correlate with its expression levels. Transfection of MAP1B into COS cells has been reported, however, the effect is different from that of tau or MAP2 (Takemura et al., 1992). Exogenous MAP1B expression in COS
cells does not cause reorganization of microtubules such as bundle formation. Microtubules in transfected cells are substantially stabilized against microtubule-depolymerizing reagents, however. Therefore, MAP1B may alter the behavior of neuronal microtubules in a different way than either MAP2 or tau.

The expression of MAP1B has been shown to be required for the differentiation of neuronal cells. Transfection of MAP1B antisense oligonucleotides into PC12 cells completely inhibits the expression of endogenous MAP1B. Meanwhile, MAP1B antisense-treated PC12 cells lose their ability to develop neurites in response to NGF. This strongly suggests that MAP1B plays a pivotal role during neurite outgrowth in PC12 cells. Recently, defects in the nervous system development have been observed in the MAP1B mutant mouse, which only expressed N-terminal 517 amino acids of MAP1B (Edelmann et al., 1996). The mutant homozygotes could not survive embryonic stages, and embryos die prior to 8.5-days of gestation, and the mutant heterozygotes display a number of abnormalities in the nervous system. A new study, which allowed even shorter truncated form (N-terminal 11 amino acids) of MAP1B expressed, showed less severe defects. Heterozygotes in this study did not exhibit obvious defects, while homozygotes had delayed nervous system development (Takei et al., 1997).

Previous reports have shown that functions of MAPs are regulated by their post-translational modifications. Both glycosylation (Ding and Vandré, 1996; Arnold et al., 1996) and phosphorylation of MAPs (Wiche, 1989; Vandré et al., 1991;
Phosphorylation of MAP1B has been reported in a number of neuronal tissues (Riederer, 1992; Mansfield et al., 1994) and cell lines (Greene et al., 1983; Aletta et al., 1988; Diaz-Nido et al., 1988). MAP1B isolated from brain tissue is phosphorylated to various extents, depending on the developmental stage of the animal. In fetal or neonatal brain, MAP1B is heavily phosphorylated, while in adult brain it is largely dephosphorylated (Diaz-Nido et al., 1990; Ulloa et al., 1993b). This change indicates that phosphorylation of MAP1B may be required during neurogenesis in early stages of development. In individual neurons, phosphorylated MAP1B is concentrated in the developing axon, especially the distal part of axons (Sato-Yoshitake et al., 1989; Nothias et al., 1996). Phosphorylated MAP1B has also been detected in neurofibrillary tangles in Alzheimer's disease brain (Hasegawa et al., 1990). Therefore, phosphorylation of MAP1B appears to correlate with the neurite formation and regeneration.

Unlike MAP2, MAP4, and tau, whose phosphorylation has been shown to reduce their affinity for microtubules (Lindwall and Cole, 1984; Drechsel et al., 1992; Faruki et al., 1992; Ookata et al., 1995), the effect of phosphorylation on the function of MAP1B remains to be determined. MAP1B phosphorylated in vitro by a casein kinase II-like activity has a higher affinity for microtubules than dephosphorylated MAP1B (Diaz-Nido et al., 1988). However, a recent paper using relatively pure MAP1B showed a different effect (Pedrotti et al., 1996). In this study, MAP1B purified from the bovine brain (1B-P), which has already been
phosphorylated, was used to co-sediment with taxol-stabilized microtubules. 1B-P was found to bind less tightly to microtubules than alkaline phosphatase-treated MAP1B (1B-AP). The effect of phosphorylation on the function of MAPs may also depend on the specific sites that are phosphorylated.

The importance of MAP1B in neuronal development has been established by a number of reports (Brugg et al., 1993; Edelmann et al., 1996), the exact role of MAP1B phosphorylation during neuronal differentiation, however, has not been well defined. To study the significance of MAP1B phosphorylation, specific antibody probes to selected phosphorylation sites would prove valuable. In Chapter 2, a phosphoepitope on MAP1B specified by the MPM2 antibody was identified. Although MPM2 antibody only recognizes MAP1B in differentiated PC12 cells as shown in Chapter 1, it recognizes a number of phosphoproteins in mitotic cells. Here we generated a MAP1B phosphospecific antibody by using the synthetic peptide containing the identified MPM2 epitope sequence as the immunogen.
Materials and Methods

Materials Complete and incomplete Freund's adjuvants were purchased from DIFCO LABORATORIES (Detroit, MI). HiTrap Protein G column and CNBr-activated Sepharose 4B beads were purchased from Pharmacia Biotech (Sweden). Alkaline phosphatase was purchased from Boehringer Mannheim (Germany). Peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Kirkegaard and Perry Laboratories (Gaithersburg MD). MAP1B synthetic peptide TP-MAP1B and M-MAP1B (see Table 3 for sequences) were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX). All other chemicals were purchased from Sigma (St. Louis, MO).

Immunization of Rabbit

The phosphopeptide derived from MAP1B, TP-MAP1B, was conjugated to a carrier protein KLH as described in chapter 2. The peptide/KLH conjugate (0.8 mg) was dissolved in 0.75 ml PBS. An aliquot of peptide conjugate (0.25 ml) was added to 0.75 ml of complete Freund's adjuvant and mixed between two glass syringes coupled by a connector. The remaining peptide was added and mixed in two additional aliquots of 0.25 ml to form a thick emulsion. Preimmune blood was collected from the central ear artery, and serum prepared prior to immunization. The
emulsion was injected subcutaneously along the side of the rabbit at multiple sites, with approximately 0.1 ml at each site. After 20 days, the rabbit was boosted with 0.8 mg peptide/KLH conjugate emulsified in incomplete Freunds adjuvant as described above. The animal was injected at a single subcutaneous site on the nape of the neck. Immune serum was obtained by bleeding the rabbit 10 days after boosting. To keep the antibody titer high, a second boost was carried out after a month, and the rabbit was bled once every two weeks thereafter.

**Affinity Purification of Antibody**

Total IgG was purified from the immune serum using a protein G column. A prepacked HiTrap Protein G column was washed and equilibrated with 3 column volumes of the starting buffer (20 mM NaH$_2$PO$_4$, pH 7.0). 2.5 ml of the immune serum was loaded onto the column with a syringe. Unbound proteins were washed off the column with the starting buffer until the $A_{280}$ of the effluent was zero. IgG bound to the column was eluted with the elution buffer (0.1 M glycine-HCl, pH 2.7) and 1.2 ml fractions were collected. Neutralization buffer (1 M Tris-HCl, pH 9.0) (80 µl) was added to each microfuge tube to neutralize the pH of eluates. Fractions at the peak were pooled and aliquoted into small volumes for storage(-70°C). The protein concentration was determined by the BCA method (Pierce).

The phosphopeptide TP-MAP1B (6.4 mg at the purity of 77%) was coupled to CNBr-activated sepharose 4B beads according to the manufacturer's instructions.
Briefly, 0.285 g of dried sepharose beads (forming 1 ml gel volume) were suspended in 1 mM HCl for 15 min. Beads were then washed in the coupling buffer (0.1 M NaHCO₃, pH 8.5, 0.5 M NaCl) 3 times. The free peptide to be coupled was dissolved in 0.5 ml of the coupling buffer and mixed with bead slurry. The total volume was adjusted to 2 ml before the mixture was rotated end-over-end at 4°C overnight. After mixing, the supernatant was saved to test the efficiency of coupling. The bead slurry was washed with 5 ml of coupling buffer three times. Remaining active groups on the beads were blocked by treatment with 0.1 M Tris-HCl, pH 8.0 for 2 h at room temperature. To remove ionically bound peptides, bead slurry was washed with 4 cycles of alternate pH. Each cycle consists of 5 ml of 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl, followed by a wash with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl.

The bead slurry was degassed, and packed into a 3 ml-syringe. The column was equilibrated with the same starting buffer used for the HiTrap Protein G column. 2 ml of the pooled peak fractions from the HiTrap Protein G column were loaded to the affinity column. The rest of the steps were the same as in the HiTrap Protein G protocol. Eluate fractions at the A₂₈₀ peak were collected.

**Preparation of Bovine Brain MAPs and PC12 Cell Lysates**

Brain MAPs were isolated from twice cycled microtubule proteins as shown in chapter 2. PC12 cells were differentiated as in chapter 1 and cell lysates were made in RIPA buffer.
**SDS-PAGE and Immunoblots**

SDS-polyacrylamide gel analysis and immunoblots of protein samples were performed as described in chapter I. To separate high molecular weight MAPs, bovine brain MAP preparations and lysates from differentiated PC12 cells were loaded on 4% urea/SDS-PAGE. The immune serum was used at a dilution of 1:100 for immunoblots, and affinity-purified antibody was used at 1:10.

**Alkaline Phosphatase Treatment**

After PC12 cell lysates were resolved on urea/SDS-PAGE and transferred, half of the nitrocellulose membrane was treated with alkaline phosphatase (1 U/10 μg protein) in buffer (50 mM Tris-HCl, pH 8.0, 2 mM MnCl₂, 1 mM PMSF) at 37°C overnight. Immunoblot analysis was carried out as described previously.

**Isolation of Spindles From CHO Cells**

Spindles were isolated from mitotic CHO cells according to Tombes et al. (1991) with modifications. CHO cells were cultured in 10 T-150 flasks and synchronized in G1/S and S phase with a thymidine (5 mM) block. After 13~16 h, cells were released from the block and cultured in growth medium Ham’s F-10 with 10% FBS for 2.5~3.5 h. Nocodazole (40 ng/ml) was added to the culture to synchronize cells in metaphase. After 3.5~4.5 h, mitotic cells were collected and released from nocodazole block. Cell pellets were collected in fresh F-10 medium with FBS in a glass conical centrifuge tube. Twelve minutes after release from
nocodazole block, 10 µg of taxol was added to each ml of cell suspension, followed by incubation for 2 min. Cell suspensions were then washed with warm distilled water twice, and lysed in isolation buffer (2 mM PIPES, pH 6.9, 0.5% Triton X-100, 5 mM 5,5'-dithiobis(2-bitrobenzoic acid), 100 nM Microcystin LR, 1 mM sodium orthovanadate). Spindles were liberated from cells, and were free of chromosomes and intermediate filaments as a result of the low ionic strength. The spindle pellet was quick frozen in liquid nitrogen for storage at -70°C.

For immunoblot analysis, the spindle pellet was dissolved in an equal volume of hot 1% SDS/PBS and sonicated to disrupt stable microtubules in the pellet.

**Immunofluorescence**

Immunofluorescence staining was performed as described in chapter 1. PC12 cells were fixed with 0.7% glutaraldehyde in PHEM buffer for 15 min, and then permeabilized with 0.5% Triton X-100 in PHEM for 15 min. For LLC-PK cells, extraction was conducted first with 0.1% Triton X-100 in PHEM, followed by fixation with 0.7% glutaraldehyde in PHEM. Residual glutaraldehyde was reduced with two changes of freshly made sodium borohydride (2 mg/ml) in TBS.
Results

The immune serum contains IgG specific to MAP1B.

The synthetic phosphopeptide TP-MAP1B/KLH conjugate was used to immunize rabbits, and the immune serum obtained after boosting was designated PMB1 antiserum (Phosphospecific MAP1B antibody). The phosphopeptide TP-MAP1B sequence is nearly conserved between MAP1B and MAP1A. The peptide TP-MAP1B derived from MAP1B has the sequence LSEFTpEYLSES, while in the corresponding region of MAP1A, the sequence is VAEFSEYVSET. Therefore, the immune serum was first tested to show whether it is sequence specific to MAP1B or not.

To test the specificity of the immune serum, a bovine brain MAP preparation, which contains mainly MAP1A, MAP1B, and MAP2, was used for immunoblot analysis with PMB1 antiserum, MPM2, and anti-MAP1B-4 antibodies (Figure 17). MPM2 antibody recognized both MAP1A and MAP1B, with MAP1B being the dominant band, verified by its reaction to anti-MAP1B-4 antibody. Immunoblot against PMB1 immune serum showed that only the MAP1B band was recognized by the antibody. MAP1A or other proteins in the brain MAP preparation were not reactive with PMB1, proving that PMB1 immune serum is specific to MAP1B. Next,
whole cell lysates made from differentiated PC12 cells were used for immunoblot against PMB1 (Figure 18). Among all the proteins on the blot, PMB1 antibody only recognized the MAP1B band, which was also reactive to MPM2 and anti-MAP1B-4 antibodies.

**Affinity purified PMB1 antibody is specific to the phosphorylated form of MAP1B.** Several phosphorylation state specific MAP1B antibodies have been reported such as anti-MAP1B-3 (Hammarback et al., 1991;), mAb150 and mAb125 (Ulloa et al., 1993a; Ulloa et al., 1993b). However, the specific epitope sites recognized by these antibodies have not been defined. The PMB1 immune serum raised against the synthetic phosphopeptide has the advantage that it recognizes a clearly known epitope site on MAP1B. The specificity of PMB1 for the phosphorylated form of MAP1B was tested on immunoblots.

As shown in Figure 19, lysates from differentiated PC12 cells were resolved on urea/SDS-PAGE, and transferred to nitrocellulose membrane. In panel B, proteins on the nitrocellulose membrane were extensively treated with alkaline phosphatase. Alkaline phosphatase removed phosphates from MAP1B, shown by elimination of the reaction of MAP1B with MPM2 antibody (Figure 19). The crude PMB1 immune serum, however, recognized both dephosphorylated and phosphorylated forms of MAP1B. The PMB1 immune serum was affinity purified in an attempt to obtain an antibody preparation specific for the phosphorylated form of MAP1B. The whole immune serum was first passed through a HiTrap Protein G
column, and total IgG was collected and subsequently loaded onto an affinity column coupled with the synthetic phosphopeptide TP-MAP1B. By immunoblot analysis, the affinity purified PMB1 only recognized the native phosphorylated MAP1B band, and did not recognize the dephosphorylated form of MAP1B (Figure 19, lane 3). This indicated that the affinity purified PMB1 antibody is a phosphospecific antibody recognizing the MPM2 epitope site on MAP1B. In all of the following experiments affinity purified PMB1 antibody was used unless otherwise stated.

To compare the specificity of PMB1 antibody with that of the MPM2 antibody, cell lysates from differentiated and non-differentiated PC12 cells were resolved by SDS-PAGE and prepared for immunoblots. Figure 20, panel A shows that PMB1 antibody did not recognize any band significantly in non-differentiated PC12 cells, just as MPM2 antibody did not recognize any band in non-differentiated cells as described in chapter 1 (see Figure 1, panel A, lane 1). In differentiated PC12 cells, increased reactions with both PMB1 (Figure 20, panel A, lane 2) and MPM2 antibody (Figure 1, panel A, lane 4) were observed. The preimmune PMB1 serum did not react with any band in either differentiated or non-differentiated cells (Figure 20, panel B).

**PMB1 antigen is localized to neurites in differentiated PC12 cells.**

As shown in chapter 1 (see Figure 4), the MPM2 antibody stained neurites of PC12 cells differentiated with NGF, indicating that MPM2-reactive MAP1B is
concentrated in growing neurites. To observe the distribution of PMB1 reactive-MAP1B, indirect immunofluorescence staining was also carried out using differentiated PC12 cells (Figure 21). Panel A shows that PMB1 antibody labeled neurites heavily. In a relatively flat cell (panel B), PMB1 antibody staining showed a weak background reaction in the cell body, similar to the low intensity non-specific staining of MPM2 antibody in cell bodies. The labeling increased at the axonal hillock region, and maximal staining was present in the neurite shaft and growth cone. This staining pattern was almost identical to that of MPM2 antibody (see Figure 4 in chapter 1). The labeling in cell bodies is likely resulting from autofluorescence, as similar intensity of fluorescence was also observed in cells stained with preimmune serum (Figure 21, panel C). Neurite staining was also not detected in cells stained with the preimmune serum.

Together with the immunoblot analysis, these results support the specificity of affinity purified PMB1 to the phosphorylated form of MAP1B.

Analysis of PMB1 antigens in mitotic cells.

The presence of PMB1 antigens in mitotic cells was also examined using immunobLOTS containing lysates prepared from interphase and mitotic CHO cells. Figure 22 shows that PMB1 antibody mainly detected 4 polypeptide species of ≈245, 240, 110 and 55 kD. The 245 and 240 kD bands specifically appearing in mitotic cells were also recognized by MPM2 antibody, indicating that these two
phosphoproteins are also MPM2 antigens. The 110 kD band was present in the interphase cell lysate, suggesting that the protein was phosphorylated during interphase. Interestingly, the 55kD PMB1-reactive band was not recognized by MPM2 antibody. No band at the position around 320 kD, at which MAP1B runs on SDS-PAGE, was detected. This may be explained by the extremely low expression level of MAP1B in non-neuronal CHO cells.

In order to test whether MAP1B in CHO cells is recognized by PMB1, spindles were isolated from synchronized metaphase CHO cells. MAP1B is significantly enriched in mitotic CHO spindles. Immunoblot analysis against anti-MAP1B-4 antibody (Figure 23, lane 1) showed a high molecular weight band at ~320 kD. There were two bands at a lower molecular weight also detected by anti-MAP1B-4 antibody. This may be due to proteolytic degradation. Importantly, the top MAP1B band, designated by the arrow in Figure 23, was also reactive to both PMB1 and MPM2 antibodies. Therefore, like MPM2 antibody, PMB1 recognizes the phosphorylated form of MAP1B as well as some other phosphoproteins in mitotic cells.

The localization of PMB1 antigens in mitotic cells was analyzed by indirect immunofluorescence staining (Figure 24) and compared to the staining pattern obtained with MPM2 antibody. Kidney epithelial LLC-PK cells were labeled with
PMB1 antibody. To distinguish the cell cycle stages, DNA was labeled with DAPI. In interphase cells, PMB1 did not specifically label any cellular structures in the cytoplasm, but the nucleolus appeared to be labeled. This suggests that the 110 kD band detected in PMB1 immunoblot may be a nuclear phosphoprotein. In prophase cells, the centrosome was distinctively labeled, while condensed chromosomes were not. Importantly, spindle poles were heavily stained in metaphase cells, as well as the cytoplasm. However, unlike MPM2 antibody, PMB1 did not label kinetochores or kinetochore fibers, suggesting that PMB1 is more selective than the MPM2 antibody. In anaphase cells, the intensity of PMB1 labeling in spindle poles decreased (Figure 24), similar to MPM2 antibody staining pattern (see Figure 12 in chapter 2). The staining in spindle poles by the PMB1 antibody is specific, because control cells stained with preimmune serum did not show any labeling of this structure. To further confirm the specificity of the antibody, LLC-PK cells were stained with PMB1 antibody that had been preincubated with the cognitive peptide TP-MAP1B and the dephosphopeptide M-MAP1B. As shown in Figure 25, (panel A), preincubation of the PMB1 antibody with the dephosphopeptide M-MAP1B did not affect the labeling of spindle pole. Preincubation of PMB1 with the phosphopeptide TP-MAP1B eliminated the staining (Figure 25, panel B). This indicates that PMB1 recognizes phosphoprotein components associated with the spindle pole in mitotic LLC-PK cells, and it is likely that at least one of these phosphoproteins is MAP1B.
Discussion

We have generated a phosphorylation state and site-specific MAP1B antibody by using a synthetic phosphopeptide derived from the MAP1B sequence as the immunogen. To our knowledge, this is the first MAP1B antibody of this kind. The synthetic peptide TP-MAP1B contains the MPM2 epitope sequence that has been identified in the study described in chapter 2. The specificity of PMB1 antibody against the phosphorylated form of MAP1B is demonstrated by the following experiments: 1) PMB1 reacted only to MAP1B in the brain MAP preparation and PC12 cell lysates, but not other proteins in these preparations; 2) the reaction with the phosphorylated form of MAP1B by the PMB1 antibody can be eliminated by the treatment of MAP1B with alkaline phosphatase prior to immunoblot analysis; 3) PMB1 antibody recognized the MPM2-reactive MAP1B band in CHO cell spindle preparations, and 4) PMB1 antibody specifically labeled spindle poles by indirect immunofluorescence. Preincubation of the antibody with the cognitive phosphopeptide TP-MAP1B abolished this specific staining.

The specificity of PMB1 antibody to MAP1B but not MAP1A is somewhat unexpected, because the peptide used for generating the polyclonal antibody
contains sequence that is highly homologous (72%) to the corresponding sequence in MAP1A. While MPM2 antibody recognizes both MAP1A and MAP1B, PMB1 is specific to MAP1B in the brain MAP preparation. The selectivity of PMB1 antibody should allow for a more accurate biochemical and immunological analysis of MAP1B phosphorylated on the MPM2 epitope. In addition, PMB1 may provide a promising reagent for the separation of MAP1B from MAP1A. Because of the similar size and biochemical behaviors shared by MAP1A and MAP1B, ideal separation of MAP1B from MAP1A has been difficult. This has also been one of the major difficulties in the purification of MAP1B. PMB1, therefore, may aid in the isolation of MAP1B from a crude mixture of brain MAPs.

The crude immune serum contains IgG recognizing MAP1B both in the phosphorylated and dephosphorylated forms in PC12 cell lysates, since alkaline phosphatase treatment only slightly reduced the intensity of the staining. Therefore, PMB1 antibody required further purification using an affinity column coupled with the MAP1B phosphopeptide TP-MAP1B. The affinity purified PMB1 only recognized the phosphorylated form of MAP1B, but not MAP1B dephosphorylated by alkaline phosphatase. Therefore, the portion of antibody eluted from the affinity column represents a pool of IgG specific to MAP1B phosphorylated on the MPM2 epitope. This also indicates that the MPM2 epitope site on MAP1B is highly likely to be phosphorylated in vivo. MAP1B phosphorylation on the MPM2 epitope site can be studied with both MPM2 and PMB1 antibodies. Since PMB1 is more selective than
MPM2 antibody, we can utilize the PMB1 antibody for the functional analysis of MAP1B phosphorylation at the MPM2 epitope during neuronal differentiation. PMB1 antibody can be introduced into PC12 cells at various time points following NGF stimulation, and the potential function of MAP1B phosphorylation on neurite outgrowth could be studied.

It is possible that the PMB1 antibody recognizes MAP1B on sites other than the MPM2 site. While this possibility seems unlikely, proof will require a complete proteolytic digestion of MAP1B, and microsequence analysis of the small PMB1-reactive fragment. If PMB1 indeed recognizes the MPM2 epitope sequence, it is clear that the immunolocalization of the two antibodies should show the same staining pattern in neuronal cells. Indirect immunofluorescence in differentiated PC12 cells with PMB1 has demonstrated an almost identical staining pattern to that obtained using the MPM2 antibody as described in chapter 1.

Because MPM2 antibody recognizes a series of mitotic phosphoproteins with molecular mass ranging from 200-40 kD on immunoblots (Davies et al., 1983), the antibody has been considered to be a marker for mitosis-specific phosphoepitopes. Recently, the appearance and up-regulation of the MPM2 epitope has been described in postmitotic neuronal cells (Kondratick and Vandré, 1996; results in Chapter 1). Because the PMB1 antibody was raised against a peptide containing the MPM2 epitope sequence, it was possible that this antibody also recognized
some mitotic phosphoproteins. Immunoblot analysis using mitotic CHO cell lysates showed that PMB1 antibody recognized 4 protein bands, among which two of these bands were also reactive to MPM2. However, PMB1 antibody also recognized a 55 kD band that was not identified by the MPM2 antibody. Thus, the epitope bound by PMB1 antibody represents a subset of epitopes required for MPM2 antibody binding. It has been reported that MPM2 epitope sites identified to date vary in their amino acid sequences, with some common conserved properties (Ding et al., 1997). The site on MAP1B specified by PMB1 antibody, appears to be related to some other MPM2 epitope sites. Like other MPM2 epitope sites, this particular site may also be conserved in proteins other than MAP1B, such as in the two proteins identified by both PMB1 and MPM2 antibodies. The 55 kD band, on the other hand, may have variations in its epitope site, which still allows the binding of PMB1 antibody, but not MPM2 antibody. Taken together, it can be concluded that PMB1 is a phosphospecific antibody, recognizing a subset of MPM2 antigens in mitotic cells. PMB1 antibody shows similar, but not identical, specificity to phosphoproteins in mitotic cells. Similar properties have also been observed with an antibody raised against the phosphopeptide containing the MPM2 epitope sequence in DNA topoisomerase II. The phosphorylated topoisomerase II epitope 1 (PTE1) antibody also recognizes phosphoproteins other than topoisomerase II in mitotic cell lysates. Several of these bands are shared by the MPM2 antibody, while some are not (Ph.D. Dissertation by Min Ding, 1996).
The immunolocalization of PMB1 antigens in mitotic cells also supports the idea that PMB1 antibody is MPM2-like but not identical, with PMB1 being more selective. First, in the interphase cells, PMB1 stained nuclear foci in a different pattern from that stained by the MPM2 antibody. Neither antibody stains the interphase cytoplasm. Their similarity and differences are better displayed in immunofluorescence staining of metaphase cells. Both antibodies distinctly label spindle poles as well as the mitotic cytoplasm. While MPM2 antibody clearly labels kinetochores and spindle fibers, PMB1 did not label any of these structures. The protein(s) recognized by PMB1 antibody appear to be associated with the spindle pole only, not other components of mitotic machinery.

Phosphorylated MAP1B associated with spindle poles has been reported before, and the phosphorylation appeared to depend on the cell cycle (Tombes et al., 1993). However, the nature of its role is not clear. We suspect that MAP1B may promote the nucleation of spindle microtubules during the mitosis. Microtubules in mitotic cells have a shorter half-life time than those in interphase cells. The quick turnover rate requires the MTOC to nucleate new microtubules rapidly to ensure proper spindle morphology and segregation of sister chromosomes. PMB1 antibody specifically labeled spindle poles in mitotic cells, but not the interphase centrosome, suggesting a potentially important role of the phosphorylated MAP1B in mitosis. Phosphorylation of MAP1B may enhance its ability to nucleate and stabilize new spindle microtubules. As shown in Figure 23,
even in isolated spindle samples, MAP1B is still a minor component represented on
the MPM2 immunoblot, whereas PMB1 selectively recognized MAP1B among many
other proteins. Thus, PMB1 antibody is more specific, and may be utilized to
analyze the role of MAP1B and its phosphorylation in maintaining proper functions
of the spindle pole. Proteins interacting with MAP1B can also be
immunoprecipitated from isolated spindles, and this may provide new piece of
evidence to understand how the dynamic function of the spindle pole is regulated.

Several kinases capable of modifying proteins and conferring on them MPM2
reactivity have been reported. p34\textsuperscript{cdc2} kinase from mitotic extracts can
phosphorylate artificial peptide substrates and render them reactive with MPM2
antibody (Westendorf et al., 1994). Kuang and Ashorn (1993) reported that p42\textsuperscript{mapk}
purified from unfertilized \textit{Xenopus} eggs generated the MPM2 phosphoepitope on
other proteins in interphase \textit{Xenopus} oocytes. Later, it was found that MAPK itself
is also an MPM2 antigen in meiotic \textit{Xenopus} eggs, and it gains the MPM2 reactivity
through the phosphorylation on threonine-183 by MAPKK (or MEK).
Phosphorylation on threonine-183 has been shown to be mandatory for the kinase
activity. Binding of MPM2 antibody to active p42\textsuperscript{mapk} inhibits its kinase activity
(Taagepera et al., 1994). Recently, a polo-like kinase, plx1, has been isolated from
\textit{Xenopus} eggs and shown to phosphorylate Cdc25 on the MPM2 epitope. Cdc25
is the key regulator of the p34\textsuperscript{cdc2}/cyclin B complex (Kumagai and Dunphy, 1996).
These MPM2 kinases exist mainly in mitotic and meiotic systems, and their
functions are important for the proper regulation of the cell division and oocyte maturation. To date no MPM2 kinase has been identified in the postmitotic neuronal system. In the case of MAP1B, several kinases have been shown to phosphorylate MAP1b \textit{in vitro}, however, the \textit{in vivo} kinase responsible for regulating MAP1B remains to be determined. In particular, the kinase that is responsible for the modification on the MPM2 epitope of MAP1B is still an open question. Our preliminary studies suggest that it may be a kinase requiring a cofactor for its full activity. In the experiment to isolate MAP1B-MPM2-kinase from the brain tissue, the kinase activity was restored by combining two fractions after the extensive fractionation, whereas a single fraction showed only a minimal activity. In this context, cdk5 kinase seems to be a good candidate. The expression of cdk5 is specific to the neuronal system (Ino et al., 1994) and its activity is essential for the differentiation of neuronal cells (Nikolic et al., 1996). Cdk5 activity requires an activator, p25^{nck5a}, rather than a cyclin subunit (Lew et al., 1994). The cdk kinase family is known to phosphorylate substrates on serine/threonine sites followed by a proline residue. Our study in chapter 2 shows that the MPM2 epitope site on MAP1B (FTEY) does not have an S/TP site. It is speculated that the unique regulatory subunit of cdk5, different from cyclin or other cdk family members, may give it a slightly different substrate specificity. Further studies should be able to clarify this. Alternatively, MAP1B-MPM2-kinase could be a neuronal counterpart of known kinases such as MEK, or a novel kinase. Identification of the kinase responsible for this modification will provide important clues in understanding the
pathway leading to neuronal differentiation, and common points conserved between cell division and differentiation.
Figure 17. Analysis of the polyclonal antibody PMB1 using a bovine brain MAP preparation.

The MAP1B phosphopeptide TP-MAP1B/KLH was injected into rabbits and the immune serum PMB1 (lane 4) was used for immunoblot analysis of samples containing bovine brain MAPs (12 μg/lane) resolved on a 4% urea/SDS-PAGE. Immunoblot analysis was also performed using MPM2, 1:3,000 (lane 1); anti-MAP1A, 1:500 (lane 2); anti-MAP1B-4, 1:1,000 (lane 3) antibodies, and preimmune serum of the rabbit, 1:50 (lane 5). MPM2-reactive bands were mainly MAP1A and MAP1B with MAP1B being the major band (shown by the arrow). The PMB1 antibody only recognized MAP1B, but not MAP1A. The preimmune serum did not react with any protein.
Figure 17

1  2  3  4  5

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Figure 18. Analysis of the polyclonal antibody PMB1 using PC12 cell lysates.

Lysates of differentiated PC12 cells were separated by a 4% urea/SDS-PAGE (25 μg/lane). Immunoblot analysis was conducted using MPM2, 1:2000 (lane 1); anti-MAP1B-4, 1: 1,000 (lane 2), and PMB1, 1: 50 (lane 3) antibodies. PMB1 antibody solution recognized the MAP1B band in differentiated PC12 cells.
Figure 19. Analysis of the specificity of PMB1 for phosphorylated MAP1B in PC12 cells.

Lysates of differentiated PC12 cells (25 μg/lane) were loaded and separated on a 4% urea/SDS-PAGE. Control samples (Panel A) or nitrocellulose membrane treated with alkaline phosphatase (Panel B) were examined. Alkaline phosphatase eliminated the MPM2 reactivity of MAP1B (compare Lanes 1 in panel A and B). PMB1 antibodies in the crude serum recognized both the phosphorylated and dephosphorylated forms of MAP1B (Lanes 2 in panel A and B). In lanes 3 of panel A and B, PMB1 antibodies purified from the affinity column coupled with phosphopeptide TP-MAP1B were used as the probe. While the purified PMB1 antibody recognized the phosphorylated form of MAP1B (Panel A, lane 3), it did not react with the dephosphorylated MAP1B (Panel B, lane 3).
Figure 19
Figure 20. Immunoreactivity of PMB1 antibody with differentiated and non-differentiated PC12 cells.

Equal amounts of protein lysates from NGF-differentiated PC12 cells (Lanes 2 in A and B) or non-differentiated cells (Lanes 1 in A and B) were loaded on a 4% urea/SDS-PAGE, and immunoblot analysis was performed against the purified PMB1 antibody (A) or preimmune serum (B). The PMB1 antibody did not react significantly with any proteins in control non-differentiated cells, whereas it recognized MAP1B in differentiated cells. The preimmune serum did not show any reactivity in either sample.
Figure 20
Figure 21. Immunofluorescence staining of PC12 cells with PMB1 antibody.

Fully differentiated PC12 cells were fixed and permeabilized as described in Materials and Methods. Cells were then incubated with purified PMB1 antibody (A and B) or preimmune serum (C). PMB1 antibody labeled neurites in the differentiated PC12 cells. In a relatively flat cell shown in B, PMB1 did not significantly stain the cytoplasm, while the staining in neurites was very intense. Preimmune serum did not stain neurites. Staining in the cell body was due to background since it was also present in cells incubated with the preimmune serum.
Figure 22. Comparison of immunoreactivity of MPM2 and PMB1 antibodies using CHO cell lysates.

Lysates of interphase (Lanes I) or synchronized mitotic (Lanes M) CHO cells were resolved by 7% SDS-PAGE (25 μg/lane). Blots were probed against MPM2 (A) or PMB1 (B) antibodies. PMB1 antibody recognized 4 polypeptides in mitotic cells: 245, 240, 110, and 55 kD. Among these four bands, the two high molecular weight bands were also reactive with the MPM2 antibody. The 55 kD band was not recognized by MPM2 antibody. The 110 kD band was also detected by PMB1 in the interphase sample.
Figure 22
Figure 23. Immunoblot analysis of isolated CHO cell spindles with MPM2 and MAP1B antibodies.

Equal amounts of spindle proteins isolated from metaphase CHO cells were separated by 7% SDS-PAGE. Blots were probed using MAP1B-4 (Lane 1), PMB1 (Lane 2), and MPM2 (Lane 3) antibodies. The top band on the MAP1B-4 blot was also recognized by PMB1 antibody. At the same position on the MPM2 blot there is also a corresponding band (shown by the arrow). Bands at lower positions in MAP1B-4 and PMB1 blots may be due to proteolytic degradation.
Figure 23
Figure 24. Immunofluorescence localization of PMB1 antigens in LLC-PK cells.

LLC-PK cells grown on glass coverslips were processed as described in Materials and Methods, and incubated with purified PMB1 antibody (1:50) (Left panels). Cells were also labeled with DAPI to visualize DNA (Right panels). Micrographs were taken from cells at different cell cycle stages. The PMB1 antibody did not stain interphase cytoplasm (A), but it appeared to label the nucleolus. The prophase centrosome (B) was stained by PMB1 antibody. In an early anaphase cell (C), spindle poles were heavily labeled with PMB1 antibody, while no labeling was detected in kinetochores or kinetochore fibers. The staining generally became weak in late anaphase cells (D). Panel E showed no staining with the preimmune serum.
Figure 25. Competition assay of PMB1 staining in mitotic LLC-PK cells using MAP1B peptides.

LLC-PK cells were processed as in Figure 24 using PMB1 antibody preincubated with 10 μg/ml of dephosphopeptide M-MAP1B (A) or phosphopeptide TP-MAP1B (B). The specific staining in mitotic LLC-PK cells was eliminated by the preincubation with TP-MAP1B but not M-MAP1B.
SUMMARY

Neurons are polarized cells characterized by long axonal and dendritic processes. Neuronal cytoskeletal proteins are responsible for the architectural integrity of the neurite and maintaining organelle transportation within neuritic processes. Among the neuronal cytoskeletal elements, microtubules and microtubule-associated proteins are of major interest because of their involvement in many functional activities of the neuronal cytoskeleton. Microtubules are polymerized from heterodimers composed of α and β tubulin subunits. The stability of microtubules is modulated by MAPs. In neurites, the organization of microtubules is unique in that neurite microtubules run parallel to each other and form tight bundles. This differs from non-neuronal cells where cytoplasmic microtubules radiate from the centrosome and project toward the periphery of the cell. The polarity of neurite microtubules is arranged in such a way that axonal microtubules all are oriented with their plus end toward the distal tip of axons, while in dendrites microtubules are randomly oriented. The polarity of microtubules has important functional roles in organelle transport from the cell body to the distal region of the axon. In neurites, the stability and dynamic properties of microtubules are
balanced to ensure the proper plasticity of growing processes. MAPs are believed to be the key regulator in controlling the dynamics and organization of microtubules within the cell.

MAPs are a group of heterogeneous proteins copurifying with tubulin through temperature-dependent assembly and disassembly cycles. According to their distribution, the conventional MAPs or structural MAPs can be divided into two categories, neuronal MAPs and non-neuronal MAPs. Neuronal MAPs include MAP1A, MAP1B, MAP2, and tau proteins. MAP4 is the only MAP mainly found in non-neuronal tissues. Both MAP1A and MAP1B have their microtubule-binding domain on their N-termini, and they share sequence homology in a large part of the N-terminal domain. However, they are expressed at different stages of development in neuronal systems. MAP1A is considered to be a mature or adult MAP, whereas MAP1B is mainly expressed in nervous tissues in late embryonic stages of development and in selected regions of the adult brain that retain plasticity. In developing neurons, MAP1B is concentrated in areas of axonal growth, while in mature neurons, its expression is low and non-specifically distributed in all cellular compartments. Therefore, the developmental expression of MAP1B in nervous tissues, and its localization in individual neurons, suggest an important role for MAP1B in neurogenesis.
Previous studies have shown that the expression of MAP1B in PC12 cells could be inhibited by antisense oligodeoxynucleotide transfection. The antisense transfected cells lose the ability to develop neurites following stimulation with NGF. Recently, MAP1B gene disruption studies have shown that MAP1B is essential for normal development of the murine nervous system. To date, among all of the MAPs, MAP1B is the only one whose function has been demonstrated to be indispensable.

The function of MAP1B is believed to be modulated by phosphorylation. MAP1B isolated from brain tissues is phosphorylated to various extent depending on the developmental stage of the animal. In neuronal cells such as differentiated PC12 cells and neuroblastoma cells, MAP1B is also heavily phosphorylated. It has been reported that the phosphorylation state of MAP1B may affect the affinity of MAP1B for microtubules. It is believed that the functional effect of phosphorylation is closely related to the site on MAP1B that is modified. In this study, a monoclonal antibody MPM2, specifying a conserved phosphoepitope in mitotic cells, is used to investigate the role of MAP1B in neuronal differentiation.

In Chapter 1, I show that MAP1B becomes phosphorylated and MPM2-reactive as PC12 cells develop neurites upon the stimulation of NGF and FGF. Treatment of cells with EGF or forskolin does not induce complete differentiation of
PC12 cells, nor does it increase the MPM2 reactivity. Therefore, the increase in MPM2 reactive phosphoproteins is only associated with signals that induce differentiation. Immunofluorescence and immunogold labeling experiments show that MPM2-reactive MAP1B is localized in neurites but not in cell bodies. Phosphorylated MAP1B is exclusively associated with bundled microtubules in the neurite. While MPM2 antibody recognizes a number of phosphoproteins in mitotic PC12 cells, it only recognizes MAP1B in differentiated PC12 cells. MPM2 antibody has been widely accepted as a marker specifying mitotic phosphoepitopes. The appearance and up-regulation of this epitope in differentiated PC12 cells suggest that there may exist a kinase activity that is conserved between pathways leading to cell division and differentiation. These results imply that phosphorylation of MAP1B on the MPM2 epitope may play an important role in neurite outgrowth.

Chapter 2 focuses on the identification of the MPM2 epitope on MAP1B molecule. Through proteolytic cleavage, the MPM2 epitope was narrowed down to the N-terminal 40 kD of MAP1B. Based upon a model of the MPM2 epitope constructed from other MPM2 antigens, a threonine site in the 40 kD N-terminal domain of MAP1B was selected as a potential MPM2 epitope site. Peptides including the selected sequence were synthesized in both phosphorylated and dephosphorylated forms. It was shown by immunoblot assay that the phosphopeptide/KLH conjugate reacts with the MPM2 antibody very, whereas the dephosphopeptide/KLH conjugate did not. The ability of free peptides to bind the
MPM2 antibody was also tested using competition assays. The free phosphopeptide was shown to compete for MPM2 antibody binding not only with native MAP1B protein but also other MPM2-reactive phosphoproteins found in mitotic cells. The dephosphopeptide did not have affinity for MPM2 antibody. Therefore, I conclude that the MAP1B synthetic phosphopeptide has all required elements for MPM2 antibody recognition, indicating that the proposed sequence is the MPM2 epitope on MAP1B.

Chapter 3 describes the characterization of a rabbit polyclonal antibody that was generated against the MAP1B phosphopeptide containing the MPM2 epitope sequence. The resultant antiserum, PMB1, was found to recognize MAP1B in a mixture of brain MAPs. Affinity purified PMB1 antibody specifically reacted with the phosphorylated form of MAP1B, but not the dephosphorylated form. This indicates that the epitope identified in chapter 2 is likely to be phosphorylated \textit{in vivo}. The PMB1 antibody labels neurites in differentiated PC12 cells similar to the staining observed with MPM2. By immunoblot, PMB1 recognizes a subset of MPM2 antigens present in mitotic cells, however, it also recognizes a protein not recognized by MPM2 antibody. In isolated CHO spindle samples, PMB1 specifically recognized phosphorylated MAP1B. Immunofluorescence staining of cultured cells showed that PMB1 labeled spindle poles, however, it did not stain kinetochore or kinetochore fibers in mitotic cells. Therefore, PMB1 antibody is an MPM2 like
antibody. The antibodies are not identical, however, as the PMB1 serum appears to have a more selective epitope recognition.

Like developing neurons, cultured PC12 cells also display a series of sequential steps during neurite extension, i.e., initiation, elongation and stabilization (Luckenbill-Edds, 1979; Nunez, 1986; Matus, 1988). After 24 to 48 h of NGF treatment, short processes are initiated and begin to extend from the cell body. MAP1B expression starts to increase from the basal pre-NGF level during this initiation phase. While some processes remain short or retract back to the cell body, selective processes elongate rapidly following 2-5 days of NGF induced differentiation. A dramatic increase in MAP1B protein level and a concomitant increase in MPM2 reactivity coincide with the elongation of neurites. Approximately 5 to 7 days after NGF treatment, neurites undergo a transition from the elongation phase to a stabilization phase. During this last phase of development, MAP1B may be replaced by so-called mature MAPs such as MAP1A. We observed an increase in the MPM2 reactivity of MAP1B during the late stages of the initiation phase and throughout the elongation phase. MAP1B promotes polymerization of MTs more efficiently than classic MAPs, i.e., MAP2, yet MAP1B-MTs are not as stable as MAP2-MTs (Pedroitti et al., 1996). Initiation and elongation of neurites call for the rapid polymerization of a large amount of MTs. These newly polymerized MTs must also be very dynamic to ensure the plasticity of neurites. Thus, MAP1B-MTs meet the requirement for a quick supply of dynamic MTs. Phosphorylation of MAP1B on
the MPM2 epitope(s) may enhance the efficiency of MAP1B to promote polymerization of neurite MTs. It may also improve the ability of MAP1B to form crosslinks between MTs and thereby the formation of tightly bundled MTs in neurite. Phosphorylation may also be involved in the transportation of MAP1B to neurites from the cell body.

In summary, this dissertation describes efforts to identify and characterize a phosphoepitope on MAP1B. The epitope identified by MPM2 antibody was previously found in mitotic system, and now also appears and is up-regulated in differentiated postmitotic neuronal cells. The epitope site is identified by the combination of proteolytic cleavage and analysis of synthetic peptide. The synthetic phosphopeptide is used to generate a phosphorylation and site-specific antibody PMB1. The specificity of PMB1 antibody is compared to that of MPM2. The synthetic peptides and PMB1 antibody provide tools for functional analysis of this epitope site. It is anticipated that understanding of functional properties of the MAP1B-MPM2 epitope-kinase will contribute to knowledge on the regulation of normal neuronal development, and mechanisms of neurodegenerative diseases.
REFERENCES


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