A Casein Kinase II–related Activity Is Involved in Phosphorylation of Microtubule-associated Protein MAP-1B during Neuroblastoma Cell Differentiation

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Abstract. A neuroblastoma protein related to the brain microtubule-associated protein, MAP-1B, as determined by immunoprecipitation and coassembly with brain microtubules, becomes phosphorylated when N2A mouse neuroblastoma cells are induced to generate microtubule-containing neurites. To characterize the protein kinases that may be involved in this in vivo phosphorylation of MAP-1B, we have studied its in vitro phosphorylation.

In brain microtubule protein, MAP-1B appears to be phosphorylated in vitro by an endogenous casein kinase II–like activity which also phosphorylates the related protein MAP-1A but scarcely phosphorylates MAP-2. A similar kinase activity has been detected in cell-free extracts of differentiating N2A cells.

Using brain MAP preparations devoid of endogenous kinase activities and different purified protein kinases, we have found that MAP-1B is barely phosphorylated by cAMP-dependent protein kinase, Ca/calmodulin-dependent protein kinase, or Ca/phospholipid-dependent protein kinase whereas MAP-1B is one of the preferred substrates, together with MAP-1A, for casein kinase II.

Brain MAP-1B phosphorylated in vitro by casein kinase II efficiently coassembles with microtubule proteins in the same way as in vivo phosphorylated MAP-1B from neuroblastoma cells. Furthermore, the phosphopeptide patterns of brain MAP-1B phosphorylated in vitro by either purified casein kinase II or an extract obtained from differentiating neuroblastoma cells are identical to each other and similar to that of in vivo phosphorylated neuroblastoma MAP-1B.

Thus, we suggest that the observed phosphorylation of a protein identified as MAP-1B during neurite outgrowth is mainly due to the activation of a casein kinase II–related activity in differentiating neuroblastoma cells. This kinase activity, previously implicated in β-tubulin phosphorylation (Serrano, L., J. Díaz-Nido, F. Wandosell, and J. Avila, 1987. *J. Cell Biol.* 105: 1731–1739), may consequently have an important role in posttranslational modifications of microtubule proteins required for neuronal differentiation.

NEUROBLASTOMA cell cultures constitute a good model system for the study of neuronal differentiation. Most neuroblastoma tumors arise in early childhood from neuroblast precursors of sympathetic neurons, and it has been suggested that these neoplasms may result from defective differentiation of neural crest derivatives that continue proliferating. A large number of cloned cell lines have been derived from neuroblastomas of mouse, rat, and human origin. Of these, the murine C1300 clones are the best characterized. Different treatments (hypertonic medium, serum deprivation, dibutyryl-cAMP, etc.) can induce differentiation in these neuroblastoma cell cultures (32, 34). Although the various clonal cell lines differ in their degree of maturation, most neuroblastoma cells become amitotic, lose their oncogenic capacity and express characteristics of mature neurons such as the extension of neurites, development of electrically excitable membranes, activation of neuron-specific enzymes involved in neurotransmitter metabolism, etc. (32, 34).

In differentiating neuroblastoma cells, it has been established that increased microtubule assembly occurs during process extension (2, 14, 30). Moreover, it has been shown that neurite outgrowth is abolished by drugs that inhibit microtubule assembly (9, 35, 49). Thus, neurite extension and maintenance seem to be dependent on the assembly and stabilization of internal microtubule arrays. It has been proposed that such an assembly and stabilization could be triggered by nerve growth factor or other neurotrophic factors by two distinct mechanisms (6, 18, 27). One involves the enhanced expression of genes coding for microtubule-associated proteins (MAPs),[1] such as tau and MAP-1 (14), and takes place in cells that have never been exposed to neuro-

1. *Abbreviation used in this paper*: MAP, microtubule-associated protein.

trophic factors, such as "naive" PC12 rat pheochromocytoma cells or neurons at very early ontogenetic stages. The second occurs in "primed" PC12 cells, sensory and sympathetic neurons, and most types of neuroblastoma cells, including the most undifferentiated "round-cell" neuroblastomas with no features of maturation, since these cell types have accumulated a critical amount of these MAPs (1, 27, 48). This mechanism does not require protein synthesis and seems to be mediated by posttranslational modifications, mainly phosphorylation, which could control the function of MAPs in promoting microtubule assembly and stabilizing assembled microtubules. Among the MAPs that are phosphorylated during neurite outgrowth, MAP-1 (a group of polypeptides that differ in their structure and cell localization [4, 23, 38, 44]) has been the most widely described in various cell lines, such as the PC12 rat pheochromocytoma and N115 mouse neuroblastoma cells (7, 17, 19, 20). At present, it is unknown which MAP-1 polypeptide is phosphorylated during neurite outgrowth and which kinase is involved in that process.

In this report, we have promoted neurite outgrowth in N2A mouse neuroblastoma cells by serum withdrawal and studied whether MAP-1 is phosphorylated. To identify the kinase involved in in vivo MAP-1 phosphorylation, we have analyzed the in vitro phosphorylation of MAP-1 polypeptides in microtubule protein preparations and used purified protein kinases and extracts of differentiating neuroblastoma cells. Finally, we have compared the characteristics of in vivo phosphorylated MAP-1 with those of the in vitro phosphorylated protein. A preliminary account of these results has been reported in abstract form (12).

Materials and Methods

Cell Culture

N2A mouse neuroblastoma cells (ATCC CCL 131, American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum, and induced to differentiate by transfer to DME without serum.

Preparation of Phosphorylated MAPs from Differentiating N2A Cells

100-mm culture dishes of differentiating N2A cells were rinsed with DME containing one-tenth the normal phosphate concentration and incubated overnight in the same medium to which 1 mCi/ml $^{32}PO_4$ (HCl- and carrier-free) (Amersham Corp., Arlington Heights, IL) had been added. Cells were then washed with 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) and gently scraped from the culture dish with a rubber policeman into 1 ml of cold PBS, centrifuged for 2 min at 1,000 *g*, and resuspended in 100 μl of buffer A (0.1 M 4-morpholinoethane sulfonic acid [MES], 0.5 mM $MgCl_2$, and 2.5 mM EGTA, pH 6.4) supplemented with 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 20 mM NaF, and 1 mM ATP. The cells were then homogenized with a teflon-glass homogenizer, the cell homogenates were centrifuged in a Beckman Instruments, Inc. (Fullerton, CA) airfuge (5 min at 100,000 *g*, 4°C), and the supernatants were collected. The labeled cell extracts were fractionated by chromatography on a phosphocellulose column under the conditions described by Weingarten et al. (47). The column was washed extensively with buffer A and the fractions containing neuroblastoma MAPs eluted with 0.5 M NaCl in buffer A, dialyzed against buffer A, and copolymerized with added brain microtubule protein in the presence of 1 mM GTP, 1 mM ATP, 20 mM NaF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM PMSF. After centrifugation in a Beckman airfuge (5 min at 100,000 *g*, 25°C), the pellet containing copolymerized phosphorylated N2A MAPs was subjected to SDS-PAGE.

Immunoprecipitation of In Vivo Phosphorylated MAP-1 from Labeled Cell Extracts

Immunoprecipitation of MAP-1 from labeled cell extracts was performed essentially as described by Gard and Kirschner (17), using the monoclonal antibody from clone 8D12 (IgM) (a generous gift of Dr. I. V. Sandoval) (5, 11).

Briefly, the labeled cell extract was brought to 100 μl with immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 2 mM EGTA, 150 mM NaCl, 20 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM ATP, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM PMSF) and preadsorbed for 5 min with 100 μl of 10% *Staphylococcus aureus* (Pansorbin) (Calbiochem-Behring Corp., San Digeo, CA). After centrifugation to remove *S. aureus*, 100 μl of 8D12 hybridoma-conditioned culture medium (in immunoprecipitation buffer) was added. The sample was incubated 1 h on ice, followed by addition of 4 μl of a rabbit anti-mouse immunoglobulin (IgA, IgG, and IgM, the kind gift of Dr. L. Kremer) and was reincubated for 1 h on ice. Subsequently, 100 μl of 10% *S. aureus* was added and the sample was incubated for 10 min on ice. The sample was then centrifuged and the *S. aureus* pellet was washed five times in immunoprecipitation buffer. The *S. aureus* pellet was finally resuspended in SDS sample buffer (1% [wt/vol] SDS, 2% 2-mercaptoethanol, 10% [vol/vol] glycerol), and subjected to SDS-PAGE.

Protein Preparations

Microtubule protein from pig or mouse brain was prepared by temperature-dependent cycles of assembly-disassembly according to Shelanski et al. (37) and stored in buffer A containing 50% glycerol at −70°C. MAPs were obtained from these preparations by chromatography on phosphocellulose columns as described by Weingarten et al. (47). In some cases, these tubulin-free MAP preparations were brought to 1 M NaCl and 10 mM dithiothreitol (DTT) in buffer A, incubated in a boiling water bath for 5 min, chilled in ice for 5 min, and centrifuged at 30,000 *g* for 30 min at 2°C, as described by Vallee (43). The resulting supernatant contained MAPs with no associated protein kinase activity and was used as a substrate for protein kinase assays. Alternatively, brain MAP preparations were obtained by phosphocellulose chromatography of microtubule protein pretreated with alkaline phosphatase (see below) and heat treatment was omitted.

Casein kinase II was purified from rat liver according to the method of Meggio et al. (29). The Ca^{2+}/calmodulin-dependent protein kinase from porcine brain was obtained by the procedure described by Wandosell et al. (46). The Ca^{2+}/phospholipid-dependent protein kinase from porcine brain was obtained by the method described by Walsh et al. (45). Calmodulin was purified from porcine brain homogenates by the procedure of Dedman et al. (10). The cAMP-dependent protein kinase was purchased from Sigma Chemical Co. (St. Louis, MO).

Phosphatase Treatment

Alkaline phosphatase from calf intestine (Boehringer Mannheim, Indianapolis, IN) was added to microtubule protein or MAP preparations (0.2 U/μg of substrate) in buffer A and incubated for 30 min at 37°C. For MAP preparations, phosphatase was inactivated by placing the sample in a boiling water bath for 2 min. For microtubule protein, phosphatase was removed by an additional cycle of polymerization as previously described (36).

Endogenous Phosphorylation of Brain Microtubule Protein

Purified brain microtubule protein (2 mg/ml), in some cases pretreated with alkaline phosphatase, was incubated for 15 min at 37°C in buffer A without EGTA and containing 5 mM $MgCl_2$ and 10 μM [γ-^{32}P]ATP (Amersham Corp.). These assays were also performed in the presence of 10 μM heparin alone; 1 mM $CaCl_2$ alone; 1 mM $CaCl_2$ and 1 mg/ml calmodulin; 1 mM $CaCl_2$ and 80 μg/ml phosphatidylserine; or 10 μM cAMP, as indicated in Results. Phosphorylation assays were stopped with SDS-sample buffer.

In Vitro Phosphorylation of Brain MAP Preparations with Purified Protein Kinases

The purified brain MAP preparations devoid of endogenous kinase activities were phosphorylated with purified casein kinase II, Ca^{2+}/calmodulin-dependent protein kinase II, Ca^{2+}/phospholipid-dependent protein kinase, or cAMP-dependent protein kinase.

The phosphorylation of MAPs (20 μg in buffer A) with purified casein kinase II (1 μg) was performed in the presence of 5 mM $MgCl_2$, 1 mM PMSF, and 10 μM [γ-^{32}P]ATP in a final volume of 100 μl. The Ca^{2+}/calmodulin-dependent phosphorylation of MAPs was done with 25 μg of the purified kinase and 2 μg of calmodulin in buffer A without EGTA, supplemented with 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM $CaCl_2$, 1 mM PMSF, and 10 μM [γ-^{32}P]ATP in a final volume of 100 μl. Ca/phospholipid-dependent phosphorylation of MAPs with purified kinase C was performed in buffer A without EGTA, and supplemented with 5 mM $MgCl_2$, 1 mM $CaCl_2$, 80 μg/ml phosphatidyl serine, 1 mM PMSF, 10 μM [γ-^{32}P]ATP. The phosphorylation of MAPs with purified cAMP-dependent protein kinase was performed in buffer A supplemented with 5 mM $MgCl_2$, 10 μM cAMP, 1 mM PMSF, and 10 μM [γ-^{32}P]ATP.

Phosphorylation reactions were stopped by addition of boiling SDS sample buffer.

In Vitro Phosphorylation of Brain MAP Preparations with Neuroblastoma Cell Extracts

Undifferentiated or differentiating neuroblastoma cells were washed with PBS and scraped from the culture dishes in 100 μl of buffer A supplemented with 20 mM NaF, 20 mM Na_3VO_4, 10 mM $MgCl_2$, and 10 mM PMSF. The cells were homogenized with a teflon-glass homogenizer and the resulting homogenate centrifuged in a Beckman airfuge (5 min at 100,000 *g*, 4°C). Aliquots of the supernatants containing the same amount of protein were mixed with purified brain MAPs (20 μg in buffer A), brought to a final volume of 100 μl with buffer A, and the phosphorylation assays performed in the presence of 5 mM $MgCl_2$, 1 mM PMSF, and 10 μM [γ-^{32}P]ATP. Phosphorylation reactions were stopped by addition of SDS sample buffer.

Polymerization Assays

Microtubule protein was assembled by incubation at 37°C for 30 min in buffer A in the presence of 1 mM GTP. The sample was layered onto a cushion of 40% (wt/vol) sucrose in buffer A and centrifuged in a Beckman airfuge (7 min at 100,000 *g*). The polymerized and unpolymerized proteins were boiled in the presence of 1% (wt/vol) SDS, 2% β-mercaptoethanol, and 10% (vol/vol) glycerol and subjected to electrophoresis.

Gel Electrophoresis

SDS-PAGE was performed according to the procedure of Laemmli (26), and gels were stained with Coomassie Blue as indicated by Fairbanks et al. (16). Phospholabeled proteins were detected by autoradiography of dried gels exposed to Kodak-X-Omat films. The radioactivity associated with phosphorylated MAP-1 was determined by measuring the Cerenkov radiation of the excised phospholabeled band in a scintillation spectrophotometer (Beckman Instruments, Inc.).

Phosphopeptide Mapping

Limited proteolysis of phosphorylated MAP-1 with *S. aureus* V8 protease was performed according to the method of Cleveland et al. (8), as described previously for MAP-2 (22). Essentially, each phospholabeled protein band was cut out from the gel, mixed with 100 μg of bovine serum albumin (BSA), and placed in a 4% polyacrylamide stacking gel. The protein mixture was cleaved in the stacking gel with V8 protease (1 μg) for 30 min, and the resulting phosphopeptides were separated on a 15% polyacrylamide gel.

Extensive trypsin digestions of the phospholabeled MAP-1 bands were also performed as described by Herrmann et al. (23). The resulting phosphopeptides, in 0.1% (vol/vol) trifluoroacetic acid, were applied to a reverse-phase HPLC column (NOVAPACK C-18; Waters Millipore Corp., Bedford, MA) and fractionated using a 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid with a flow rate of 0.5 ml/min for 260 min. Fractions of 0.5 ml were collected and the associated radioactivity was determined by measuring Cerenkov radiation.

Phosphoamino Acid Analysis

MAP-1 phosphopeptides were eluted from gel slices with *S. aureus* V8 protease as described above and were hydrolyzed in 6 N HCl for 2 h at 100°C. Samples were spotted on cellulose thin-layer chromatography plates (Eastman Kodak Co., Rochester, NY) and electrophoresed in acetic acid/pyridine/water (50:5:945) for 60 min at 900 V. Phosphoamino acids were detected by autoradiography and unlabeled standards were located by staining with 1% ninhydrin.

Immunofluorescence Microscopy

N2A cells were grown on glass coverslips and induced to differentiate by serum withdrawal. After 1 d of serum deprivation, they were fixed for 10 min with cold methanol. The cells were then washed with PBS, followed by a 1-h incubation with 8D12 hybridoma-conditioned culture medium. They were then washed with PBS, incubated 30 min with fluorescein-conjugated goat anti-mouse IgM (Cappel Laboratories, West Chester, PA), and washed again with PBS and the coverslips mounted on glass slides using glycerol (90% in PBS).

Results

Phosphorylation of a Neuroblastoma Protein Related to Brain MAP-1B during Neurite Outgrowth

N2A mouse neuroblastoma cells develop neurites upon serum withdrawal. Within 1 h, most cells extend short processes; long neurites, which are stained with the monoclonal antibody 8D12 (5), are found after 1 d of serum deprivation (Fig. 1 *A*).

Phosphorylation of a MAP related to MAP-1 coupled to neurite outgrowth has been described for other neuroblastoma cell lines (17); we have therefore studied whether a similar modification occurs in N2A cells, to identify which component of MAP-1 is involved. Fig. 1 shows that a high-molecular-weight MAP becomes phosphorylated in differentiating N2A cells. Such a phosphoprotein has been identified as a MAP by coassembly with pig brain microtubules (Fig. 1 *B*). We suggest this protein represents the neuroblastoma counterpart to brain MAP-1B, since both proteins have the same electrophoretic mobility and are recognized by the monoclonal antibody 8D12 (Fig. 1 *B*) previously reported as specific for MAP-1B (12). Moreover, both proteins have similar peptide maps (see below).

To quantitate the level of phosphorylated MAP-1B during neuroblastoma cell differentiation, N2A cells were labeled overnight with $^{32}PO_4$ after 0–48 h of serum deprivation. The amount of phosphorylated MAP-1B was determined by immunoprecipitation from aliquots of labeled cell extracts containing equal amounts of TCA-precipitable $^{32}PO_4$ counts with the monoclonal antibody 8D12 as indicated in Materials and Methods. Fig. 2 shows a four- to fivefold increase in phosphorylated MAP-1B, the major portion of which occurs during the first 12 h after serum withdrawal. In this time, neurite extension is clearly seen. Inclusion of colcemid, which abolishes neurite outgrowth, during the labeling of N2A cells does not affect MAP-1B phosphorylation (Fig. 1 *C*). This indicates that MAP-1B phosphorylation is not coupled to microtubule assembly. On the other hand, no differences in the level of MAP-1B are observed after [^{35}S]methionine labeling (data not shown). Thus, the increase in phosphorylated MAP-1B is not due to protein synthesis but to enhanced phosphorylation. This must be the consequence of an increased MAP-1B kinase activity by activation of the kinase itself or inhibition of a MAP-1 phosphatase activity.

In Vitro Phosphorylation of MAP-1B in Brain Microtubule Preparations

Since some pioneering works reported an association of protein kinase activity with microtubules assembled in vitro (15, 31, 33, 39), several reports have dealt with the characterization of a cAMP-dependent protein kinase (42), a Ca/cal-

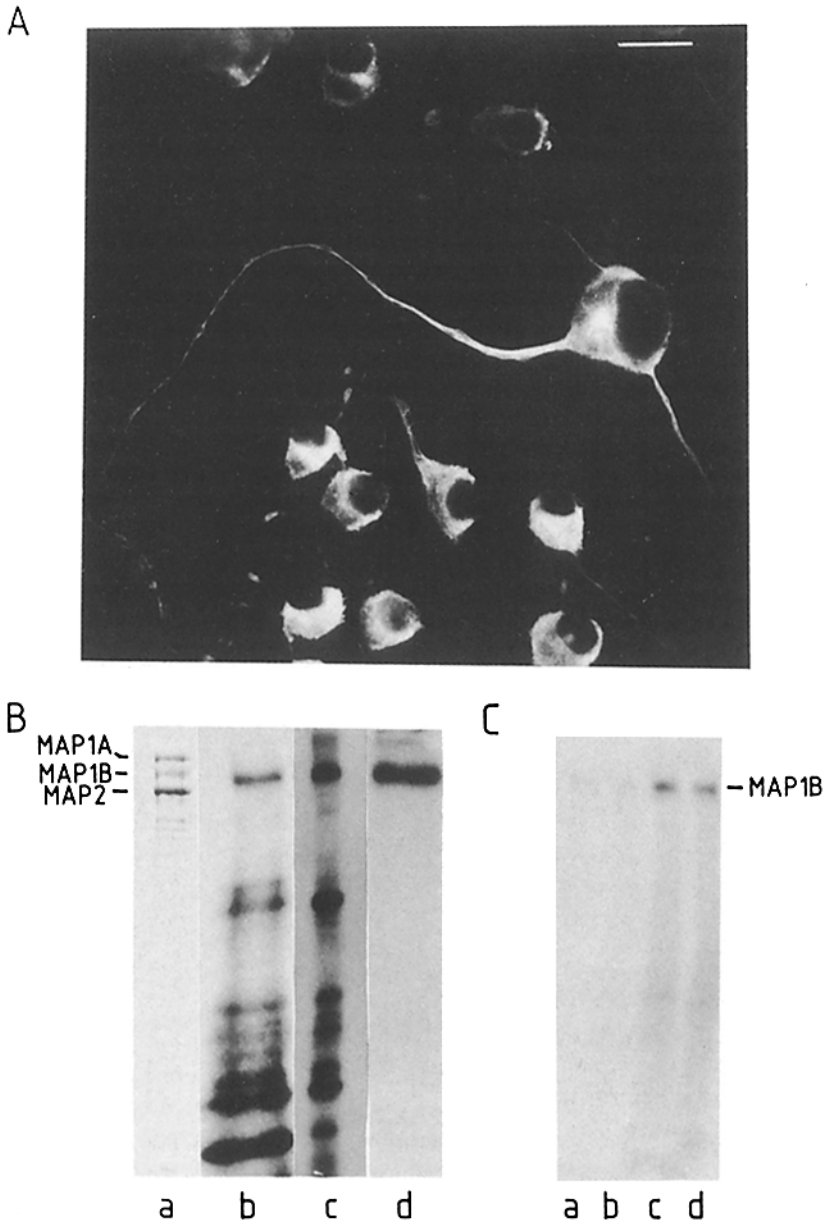

Figure 1. Phosphorylation of microtubule-associated protein MAP-1B in differentiating N2A neuroblastoma cells. (*A*) Immunofluorescent image of N2A neuroblastoma cells by 8D12 antibody after 1 d of serum deprivation. Bar, 25 μm. (*B*) Phosphorylated MAPs from differentiating N2A cells were prepared as described in Materials and Methods and subjected to SDS-PAGE. Lane *a* corresponds to the Coomassie Blue-stained pattern of mouse brain MAPs; the positions of MAP-1A, MAP-1B, and MAP-2 are indicated. Lanes *b* and *c* correspond to the autoradiographies of the supernatant (lane *b*) and the pellet (lane *c*) of ^{32}P-labeled neuroblastoma proteins copolymerized with brain microtubule protein. Lane *d* shows that the 325-kD phosphoprotein identified as a MAP comigrating with MAP-1B is immunoprecipitated from labeled cell extracts with the monoclonal antibody 8D12. (*C*) Extracts from N2A cells labeled in the presence (lanes *b* and *d*) or in the absence (lanes *a* and *c*) of 2 μM colcemid after 0 (lanes *a* and *b*) or 24 h (lanes *c* and *d*) of serum deprivation were immunoprecipitated with the monoclonal antibody 8D12 showing that colcemid does not affect MAP-1B phosphorylation.

Table I. Quantitation of Radioactivity Associated with High-Molecular-Weight MAPs upon Addition of [γ-^{32}P]ATP to Brain Microtubule Protein

	No addition	Heparin	Protein inhibitor	Calcium and calmodulin	Calcium and phosphatidylserine	cAMP
Untreated						
MAP-1A	1 (3,932 cpm)	0.32	1.06	1.05	1.01	1.03
MAP-1B	1 (3,234 cpm)	0.41	1.05	1.01	1.01	1.02
MAP-2	1 (25,265 cpm)	0.87	0.61	1.41	1.37	1.74
Treated with alkaline phosphatase						
MAP-1A	1 (5,048 cpm)	0.20	0.87	0.97	1.01	1.27
MAP-1B	1 (6,731 cpm)	0.18	0.83	0.87	0.98	1.23
MAP-2	1 (36,580 cpm)	0.78	0.41	1.51	0.69	2.12

Brain microtubule protein was phosphorylated using the endogenous kinase activities as indicated in Materials and Methods and subjected to SDS-PAGE. The radioactivity associated with each phospholabeled band was measured. The ratio of counts incorporated is indicated in the presence of heparin, the protein inhibitor of cAMP-dependent protein kinase, calcium and calmodulin, calcium and phosphatidylserine (PS), or cAMP, relative to those incorporated in the absence of these molecules. Microtubule proteins were untreated or previously treated with alkaline phosphatase. Standard deviations were in all cases under 0.05.

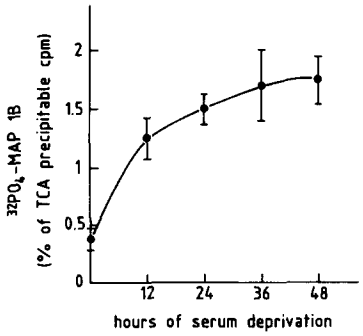

Figure 2. Increase in the level of phosphorylated MAP-1B during neuroblastoma cell differentiation determined by immunoprecipitation from aliquots of cell extracts labeled overnight with $^{32}PO_4$ after 0–48 h of serum withdrawal as indicated in the text. Error bars represent standard deviations (n = 5).

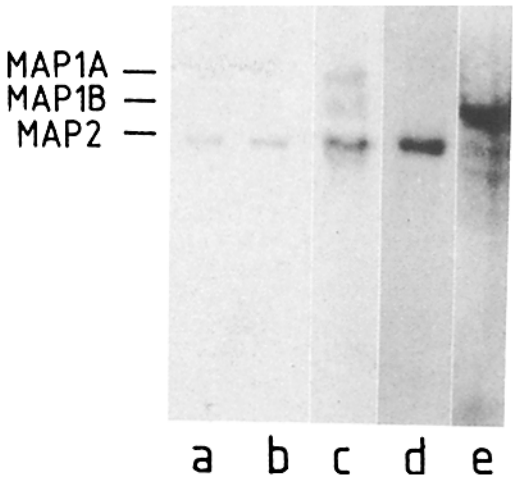

Figure 3. Phosphorylation of brain MAPs by cell-free lysates obtained from N2A neuroblastoma cells. Brain MAPs devoid of endogenous kinase activities were incubated in the presence (lanes *b* and *d*) or in the absence (lanes *a* and *c*) of 10 μM heparin with cell extracts obtained from proliferating (lanes *a* and *b*) or differentiating N2A cells (lanes *c* and *d*). An aliquot of the sample shown in lane *b* was immunoprecipitated with the monoclonal antibody 8D12 to better visualize phosphorylated MAP-1B (lane *e*).

modulin-dependent protein kinase (41), and a casein kinase II–like activity (36) in these preparations. Different specific inhibitors and activators let us identify the preferred substrates for these protein kinases in microtubule protein. Table I shows that the in vitro phosphorylation of brain MAP-1B is inhibited by heparin, a known inhibitor of casein kinase II (21), and is barely affected by modulators of other kinase activities. The same is observed for MAP-1A whereas the in vitro phosphorylation of MAP-2 is strongly affected by modulators of cAMP-dependent protein kinase, Ca/calmodulin-dependent protein kinase, and Ca/phospholipid-dependent protein kinase and scarcely inhibited by heparin. Thus, a casein kinase II–like activity is the only one that preferentially phosphorylates MAP-1B, together with MAP-1A, in microtubule protein. This appears even more evident when microtubule protein is pretreated with phosphatase. Interestingly, the fact that the level of phosphorylated MAP-1B increases upon phosphatase treatment (see Table I) indicates that MAP-1B contains bound phosphate when purified from brain. Moreover, the above-mentioned casein kinase II–like activity can replace phosphate residues released previously by the phosphatase treatment. With respect to other protein kinases, it appears that cAMP-dependent protein kinase could be responsible for only a very minor fraction of in vitro MAP-1B phosphorylation in microtubule protein, differing from MAP-2 (see Table I).

In Vitro Phosphorylation of Brain MAPs by N2A Cell-free Extracts

It thus appears that brain MAP-1 is mainly phosphorylated by a microtubule-associated casein kinase II–like activity. To test whether differentiating N2A neuroblastoma cells contain a similar protein kinase, we have studied the in vitro phosphorylation by neuroblastoma cell-free extracts of brain MAP preparations devoid of endogenous kinase activities (obtained as described in Materials and Methods).

Fig. 3 shows that cell extracts from differentiating N2A cells can phosphorylate brain MAPs including MAP-1B (Fig. 3, lane *c*). Brain MAP-1B phosphorylation appears to be due to a casein kinase II–related activity, since it is inhibited by heparin (Fig. 3, lane *d*) and is not affected by cAMP, Ca^{2+}/calmodulin, or Ca^{2+}/phosphatidyl serine (data not shown). The same results are observed from brain MAP-1A, whereas the in vitro phosphorylation of MAP-2 by cell-free extracts is barely affected by heparin. Part of the material comigrating with MAP-2 corresponds to MAP-1B degradation products, as shown by immunoprecipitation (Fig. 3, lane *e*).

In cell-free lysates obtained from proliferating neuroblastoma cells (Fig. 3, lanes *a* and *b*), the casein kinase II–related activity is significantly reduced, suggesting that it is activated upon serum deprivation. This points strongly to an involvement of such a kinase in neuroblastoma MAP-1B phosphorylation during neurite outgrowth.

On the other hand, MAP-1B phosphorylated in vitro with cell-free extracts obtained from differentiating N2A cells can also efficiently coassemble with microtubule protein (data not shown) in the same way as it will be described for MAP-1B phosphorylated in vitro by purified casein kinase II (see below).

In Vitro Phosphorylation of Brain MAPs with Purified Protein Kinases

In microtubule preparations and neuroblastoma cell extracts, brain MAP-1B is mainly phosphorylated by a casein kinase II–related activity as indicated above. To further check these results, brain MAP preparations, which contain MAP-1A, MAP-1B, MAP-2, and other MAPs, but are devoid of endogenous kinase activities, were pretreated with phosphatase and incubated with several purified protein kinases (see Materials and Methods). Among them, Ca/calmodulin-dependent protein kinase II and Ca/phospholipid-dependent protein kinase C does not seem to phosphorylate MAP-1B at all (data not shown). Whereas cAMP-dependent protein kinase phosphorylates MAP-1B and MAP-1A to a much lower

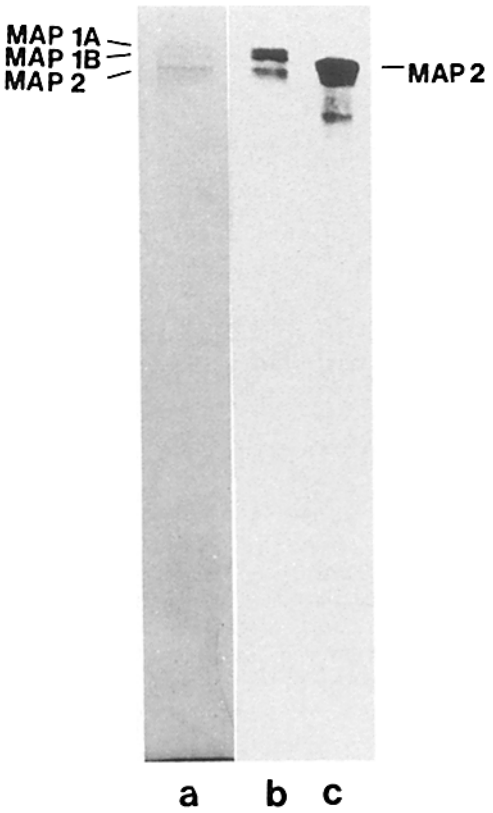

Figure 4. Phosphorylation of brain MAPs by casein kinase II and cAMP-dependent protein kinase. Brain MAPs devoid of kinase activities were incubated with purified protein kinases and [γ-^{32}P]-ATP as indicated in Materials and Methods and subjected to SDS-PAGE. Lane *a* shows the Coomassie Blue-stained pattern of the MAP preparation. Lanes *b* and *c* correspond to the autoradiographies of MAPs phosphorylated with casein kinase II (*b*) or cAMP-dependent protein kinase (*c*).

extent than it phosphorylates MAP-2 (Fig. 4), casein kinase II phosphorylates MAP-1B and MAP-1A almost exclusively (Fig. 4). MAP-2 seems to be slightly phosphorylated by casein kinase II, but most phospholabeled material comigrating with MAP-2 (Fig. 4) corresponds to a degradation product of MAP-1B, as indicated above. This degradation product has been previously reported (5, 17).

Coassembly Properties of Brain MAP-1B Phosphorylated In Vitro by Casein Kinase II

Phosphorylation of MAP-1B by purified casein kinase II does not inhibit its coassembly with microtubule protein. Moreover, phosphorylated MAP-1B is enriched twofold in twice cycled microtubule protein as compared with unphosphorylated MAP-1B (data not shown). The same result is observed for MAP-1B phosphorylated in vitro by a cell-free extract of serum-deprived neuroblastoma cells. This is consistent with the fact that in vivo phosphorylated N2A MAP-1 can also coassemble with microtubules.

Interestingly, in vitro phosphorylated MAP-1B is enriched almost fourfold in the cold-resistant pellet when a cycle of assembly–disassembly is performed. In the absence of microtubule protein, phosphorylated MAP-1B is not found in the pellet, suggesting that there is no aggregation of MAP-1B as a consequence of phosphorylation (data not shown). Thus, a possible explanation for the presence of [^{32}P]MAP-1B in the cold-resistant fraction is their association with cold-stable microtubules.

It also appears that in vitro phosphorylated MAP-1B is found enriched 1.3-fold in the polymers assembled early during incubation (data not shown). Since under these conditions, the polymers present are not microtubules (13), it is suggested that phosphorylated MAP-1B preferentially binds to large oligomers, which may be involved in microtubule nucleation and are especially enriched in high-molecular-weight MAPs.

On the other hand, MAP-1B phosphorylation by casein kinase II occurs regardless of whether MAP-1B is bound to taxol-polymerized microtubules or not (data not shown), thus differing from the results for β-tubulin (36).

Comparison of In Vivo Phosphorylated MAP-1B in N2A Neuroblastoma Cells and Mouse Brain MAP-1B Polypeptides Phosphorylated In Vitro

S. aureus V8 phosphopeptide maps of in vivo phosphorylated neuroblastoma MAP-1B and brain MAP-1B polypeptides phosphorylated in vitro with purified casein kinase II and cAMP-dependent protein kinase were performed as indicated in Materials and Methods. V8 phosphopeptide maps of in vitro phosphorylated brain MAP-1A were also performed for comparison. Fig. 5 shows that the V8 phosphopeptide patterns of MAP-1A and MAP-1B phosphorylated in vitro are similar to each other. However, the V8 phosphopeptide patterns of both MAP-1B and MAP-1A phosphorylated in vitro

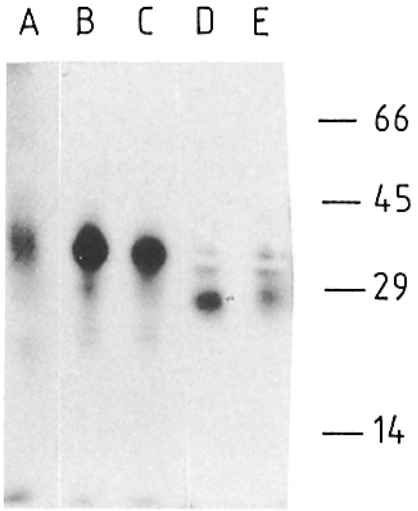

Figure 5. V8 phosphopeptide mapping of in vitro and in vivo phosphorylated MAP-1 polypeptides. The autoradiograph shown corresponds to neuroblastoma MAP-1B phosphorylated in vivo (*A*), brain MAP-1B phosphorylated in vitro by purified casein kinase II (*B*), brain MAP-1A phosphorylated in vitro by casein kinase II (*C*), brain MAP-1B phosphorylated in vitro by cAMP-dependent protein kinase (*D*), and brain MAP-1A phosphorylated in vitro by cAMP-dependent protein kinase (*E*). Bovine serum albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000), and lysozyme (14,000) were used as molecular weight markers. The numbers to the right refer to the molecular masses (in kilodaltons) of markers.

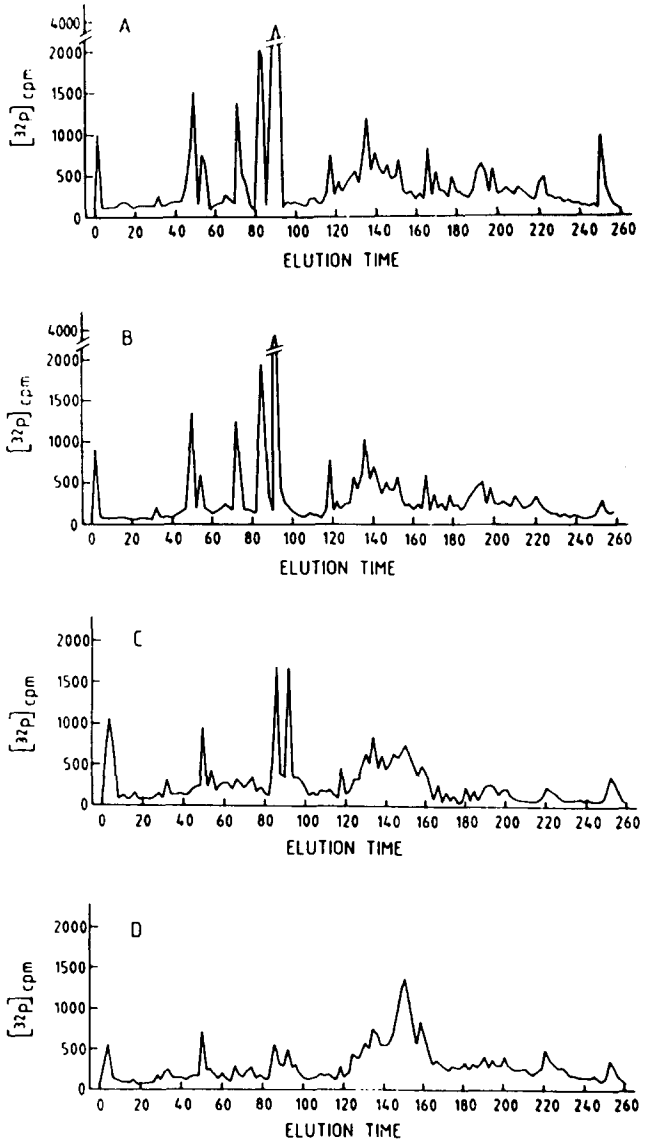

Figure 6. HPLC analyses of phosphopeptides from in vivo phosphorylated MAP-1B and MAP-1B phosphorylated in vitro by casein kinase II. (*A*) HPLC phosphopeptide pattern of heated MAP-1B phosphorylated in vitro with a cell-free lysate obtained from differentiating N2A cells. (*B*) HPLC phosphopeptide pattern of heated MAP-1B phosphorylated in vitro with purified casein kinase II. (*C*) HPLC phosphopeptide pattern of unheated MAP-1B phosphorylated in vitro with purified casein kinase II. (*D*) HPLC phosphopeptide pattern of neuroblastoma MAP-1B phosphorylated in vivo in differentiating N2A cells.

with cAMP-dependent protein kinase are clearly different from those of both MAP-1B and MAP-1A phosphorylated in vitro with casein kinase II, which is consistent with the different substrate specificity of these protein kinases (40).

Fig. 5 also shows that the phosphopeptide pattern of in vivo phosphorylated neuroblastoma MAP-1B is very similar to that of brain MAP-1B phosphorylated in vitro with casein kinase II. In both cases the phosphorylated residues were serine and threonine (data not shown). This clearly suggests that a casein kinase II–related enzyme is involved in neuroblastoma MAP-1B phosphorylation.

For a further comparison, these phosphoproteins were subjected to exhaustive trypsin digestion, and the resulting phosphopeptides were separated by HPLC. Fig. 6 shows that the HPLC phosphopeptide pattern of brain MAP-1B phosphorylated in vitro with purified casein kinase II is essentially identical to that of brain MAP-1B phosphorylated in vitro with cell-free extracts obtained from differentiating neuroblastoma cells. These neuroblastoma cell extracts therefore contain a casein kinase II–related activity (as indicated above) which phosphorylates brain MAP-1B at the same sites that are phosphorylated by purified casein kinase II.

The HPLC phosphopeptide pattern corresponding to in vivo phosphorylated neuroblastoma MAP-1B is similar to that of in vitro phosphorylated mouse brain MAP-1B. However, despite the fact that the same peaks are observed in both HPLC phosphopeptide patterns, the amounts of radioactivity associated with each peak are different. These differences may be due to the fact that brain MAP preparations are boiled to inactivate phosphatase before performing the phosphorylation reactions. To test this possibility, brain MAP preparations were obtained by an alternative procedure omitting heat treatment (see Materials and Methods). These nonheated brain MAP preparations were phosphorylated in vitro by either purified casein kinase II or a cell extract from differentiating neuroblastoma cells and the resulting phosphorylated MAP-1B bands subjected to trypsin digestion and HPLC phosphopeptide mapping. The HPLC phosphopeptide patterns of unheated brain MAP-1B phosphorylated by either purified casein kinase II or a neuroblastoma cell extract are essentially identical (not shown). Interestingly, unheated brain MAP-1B phosphorylated in vitro has a HPLC phosphopeptide pattern notably more similar to that of in vivo phosphorylated neuroblastoma MAP-1B than that of in vitro phosphorylated brain MAP-1B which has previously been heated (Fig. 6). The main points are the reduction in the amount of radioactivity associated with the two peaks eluting betwen 80 and 100 min and the increased proportion of radioactivity associated with the peak eluting around 145 min in the HPLC phosphopeptide pattern of unheated brain MAP-1B when compared to that of brain MAP-1B which has previously been heated. These differences may therefore be due to heat-induced conformational changes which could expose new phosphorylation sites.

Discussion

It has been suggested that posttranslational modifications of microtubule proteins are involved in the formation of microtubule arrays which are necessary for neurite extension and maintenance in differentiating neuroblastoma cells. In neuronal cells induced to differentiate, the phosphorylation of β-tubulin and MAP-1, among other MAPs, has been described (7, 17, 19, 20, 36). We have previously reported that a casein kinase II–related activity should be considered responsible for β-tubulin phosphorylation in differentiating N2A neuroblastoma cells (36). Here we suggest that such a kinase can also be involved in neuroblastoma MAP-1B phosphorylation.

We have found that casein kinase II is the only kinase that primarily phosphorylates brain MAP-1B, together with MAP-1A, rather than MAP-2, which is the preferred substrate for other protein kinases in vitro. Thus, it appears that

brain MAP-1B and MAP-2 may be regulated by different phosphorylation mechanisms. In this context, it has been described that phosphorylation of MAP-2 results in a decreased interaction with microtubules (24), whereas phosphorylated MAP-1B shows a similar or even higher affinity for microtubules than unphosphorylated MAP-1B.

In addition to its increased binding to microtubules, phosphorylated MAP-1B is enriched in the cold-resistant fraction, which might represent an association with cold-stable microtubules, and is also enriched in polymers found early in the assembly process. These polymers may serve as "seeds" of microtubule assembly in vitro and could constitute the in vitro counterpart of the stable microtubule fragments found in axons after depolymerization (3, 25). Thus, MAP-1B phosphorylation may favor microtubule nucleation and stabilization which is consistent with the fact that neuroblastoma MAP-1B becomes phosphorylated in vivo during neurite outgrowth.

Interestingly, a casein kinase II–related activity that can phosphorylate brain MAP-1B in vitro in the same way as purified casein kinase II appears to be activated in neuroblastoma cells after serum deprivation.

However, both purified casein kinase II and the casein kinase II–related activity detected in neuroblastoma cell-free extracts phosphorylate both brain MAP-1B and MAP-1A in vitro, whereas only MAP-1B is phosphorylated in vivo in N2A neuroblastoma cells. With respect to this point, it should be stressed that brain MAP-1A and MAP-1B are distinct but related proteins (4, 23, 44). Here we show that their phosphopeptide patterns are very similar, despite the differences in their silver-stained peptide patterns (4). We are now studying whether the phosphorylation sites are scattered on the molecules or clustered in defined regions. If the latter is the case, as suggested by V8 phosphopeptide mapping, it will imply that MAP-1A and MAP-1B share some homologous regions which contain the phosphorylation sites. In this context, it has been described that a monoclonal antibody recognizing a phosphorylated epitope reacts with both brain MAP-1A and MAP-1B (28). The fact that differentiated neuroblastoma cells only contain phosphorylated MAP-1B probably reflects the different distribution of MAP-1 polypeptides in neurons. Whereas MAP-1B is a major component of axonal cytoskeleton (4), MAP-1A appears to be localized to dendrites together with MAP-2 (38). In this way, serum deprivation would make neuroblastoma cells generate axonlike processes in which MAP-1B is a major component. Since brain MAP-1A is also a phosphoprotein, its phosphorylation may be connected with later events in neuronal differentiations that are not observed in neuroblastoma cell cultures.

On the other hand, the fact that the phosphopeptide patterns of brain MAP-1B phosphorylated in vitro by purified casein kinase II or cell extract are identical to each other clearly indicates that the cell extract contains a casein kinase II–related enzyme which may be involved in neuroblastoma MAP-1B phosphorylation. The quantitative differences observed in the otherwise similar phosphopeptide patterns of brain MAP-1B phosphorylated in vitro by casein kinase II and neuroblastoma MAP-1B phosphorylated in vivo might be due to the conditions required to purify MAPs and perform phosphorylation assays.

In view of these results, we suggest that the neuroblastoma protein identified as MAP-1B is phosphorylated by a protein kinase similar to casein kinase II which is activated in N2A neuroblastoma cells after serum withdrawal.

As indicated above, this kinase has previously been involved in β-tubulin phosphorylation (36). However, there are some differences between β-tubulin and MAP-1B phosphorylation. Casein kinase II preferentially phosphorylates tubulin in the polymerized form, whereas MAP-1B is phosphorylated by casein kinase II regardless of its binding to tubulin. Furthermore, colcemid (and other microtubule-depolymerizing drugs) prevents β-tubulin phosphorylation in differentiating neuroblastoma cells (17, 36) whereas MAP-1B phosphorylation is not affected. Thus, MAP-1B phosphorylation may be a prior step to β-tubulin phosphorylation in differentiating neuroblastoma cells. In this way, the sequential action on MAP-1B and β-tubulin of a casein kinase II–related activity, whose mechanism of activation remains to be established, could play an essential role during neuronal differentiation, since it might initiate microtubule assembly and stabilize the resulting microtubules inside developing axons.

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