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Microtubule-Stimulated Phosphorylation of tau at Ser202 and Thr205 by cdk5 Decreases Its Microtubule Nucleation Activity[1]

Yasutaka Wada,*[,2] Koichi Ishiguro,† Tomohiko J. Itoh,‡ Tsuneko Uchida,† Hirokazu Hotani,‡ Taro Saito,†[,3] Takeo Kishimoto,† and Shin-ichi Hisanaga*[,3,4]

*Laboratory of Cell and Developmental Biology, Faculty of Biosciences, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501; †Mitsubishi Kasei Institute of Life Sciences, Minamiooya, Machida, Tokyo 194-8511; and ‡Division of Biological Science, Graduate School of Science, Nagoya University, Furocho, Chikusa-ku, Nagoya 464-8602

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Phosphorylation of tau, a heat-stable neuron-specific microtubule-associated protein, by cdk5 was stimulated in the presence of microtubules (MTs). This stimulation was due to an increased phosphorylation rate and there was no increase in total amount of phosphorylation. Two-dimensional phosphopeptide map analysis showed that MTs stimulated phosphorylation of a specific peptide. Using Western blotting with antibodies that the recognized phosphorylation-dependent epitopes within tau, the phosphorylation sites stimulated by the presence of MTs were found to be Ser202 and Thr205 (numbered according to the human tau isoform containing 441 residues). MT-dependent phosphorylation at Thr205 was observed *in situ* in rat cerebrum primary cultured neurons. Stimulated phosphorylation at Ser202 and Thr205 decreased the MT-nucleation activity of tau, which is in contrast to MT-independent phosphorylation at Ser235 and Ser404.

Key words: cdk, microtubule-associated proteins, neurons, phosphorylation, tau.

Tau is a heat-stable microtubule-associated protein (MAP) predominantly expressed in neurons and localized mainly in the axons (*1-4*). Tau has the capacity to polymerize and stabilize microtubules (MTs) and is thought to play a role in forming and sustaining neurites. The MT-stabilizing ability of tau is regulated by phosphorylation (*5-7*). Phosphorylation of tau by second messenger-dependent protein kinases including cAMP-dependent protein kinase, Ca-calmodulin-dependent protein kinase, and protein kinase C has been reported to decrease its polymerization ability and its affinity for MTs (*8-11*). However, recent studies revealed that phosphorylation of tau *in vivo* occurs mainly at Ser and Thr residues followed by a proline residue (S-P and T-P sites), that is, at sites not phosphorylated by the above kinases (*12, 13*). Instead, three types of so-called proline-directed kinases [mitogen-activated protein kinase (MAP kinase), glycogen synthase kinase 3β (GSK3β), and cyclin-dependent kinase 5 (cdk5)] have been suggested as being responsible for phosphorylation at tau's S-P and T-P sites (*14-16*). Using butyrolactone I, a specific inhibitor of cdk5 and cdc2 kinase, we have shown that cdk5 was a major tau phosphorylating kinase in porcine brain extract (*17*). Phosphorylation of tau by cdk5 has been fully analyzed *in vitro*, and the phosphorylation sites identified are Ser202, Thr205, Ser235, and Ser404, with minor sites at Ser195, Thr231, and Ser396 (*16, 18*). However, it is still not known yet how phosphorylation at these sites is regulated and what role it plays.

Tau is a potent promoter of tubulin polymerization. An increase in tau protein concentration correlates with an increase in the levels of polymerized and stable MTs in PC12 cells and developing cerebellar nerve cells (*19, 20*). Tau induced ectopically into cells that do not normally express tau proteins enhances MT stability and, in some cases, induces MT bundling (*21, 22*). Most tau molecules appear to associate with MTs in these cells. The report that the phosphorylation of tau by cdk5 was stimulated in the presence of tubulin (*15*) raised the possibility that tau might be differently phosphorylated when it is associated with MTs. However, in most previous experiments (*8-11, 14-17, 23*), tau protein in the absence of MTs was used as a substrate for phosphorylation. Thus, we studied the phosphorylation of tau in the presence and absence of MTs. In this report, we show that phosphorylation of tau by cdk5 at Ser202 and Thr205 [numbered according to a human tau isoform containing 441 residues (*24*)] was stimulated in the presence of MTs, and that phosphorylation at these

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Present addresses: [2]Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, Hongo, Tokyo 113-0033; [3] Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, 1-1 Minamiosawa, Hachioji, Tokyo 192-0397.

[4] To whom correspondence should be addressed to the present address. Tel: +81-426-77-2577, Fax: +81-426-77-2559, E-mail: hisanaga-shinichi@c.metro-u.ac.jp

Abbreviations: cdk5, cyclin-dependent kinase 5; dP-tau, dephosphorylated tau; GSK3β, glycogen synthase kinase 3β; G-tau, tau proteins prepared from microtubule fraction polymerized with GTP; MAPs, microtubule-associated proteins; MAP kinase, mitogen-activated protein kinase; MT, microtubule; PC-tubulin, tubulin purified from microtubule proteins by phosphocellulose column chromatography.

sites reduced the MT-binding activity and MT-nucleating capacity of tau.

EXPERIMENTAL PROCEDURES

Purification of Tubulin, tau, and cdk5—MT proteins were prepared from porcine brains by two or three cycles of temperature-dependent polymerization/depolymerization (*25*). Tubulin was purified from MTs by either phosphocellulose or DEAE-cellulose column chromatography (*26, 27*). Tubulin purified by use of a phosphocellulose column was called PC-tubulin. Tau was purified from MT pellets by method 3 of Grundke-Iqbal *et al.* (*28*). Tau prepared from MT fractions polymerized with GTP and tau dephosphorylated by *Escherichia coli* alkaline phosphatase were designated as G-tau and dP-tau, respectively (*17*). cdk5/p26 was purified from porcine brains using dephosphorylated NF proteins as substrates as described (*29*).

Phosphorylation of tau and Preparation of Phosphorylated tau—Tau proteins (0.72 μM for dP-tau and 0.85 μM for G-tau) were phosphorylated by cdk5 (43 nM) at 35°C for 30 min in a reaction mixture consisting of 20 mM MOPS, pH 6.8, 1 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]ATP, with or without MTs (5 μM tubulin). For measurement of the initial rate of tau phosphorylation, the reaction was carried out at 15°C. The reaction was stopped by boiling samples in the presence of 0.75 M NaCl. The extent of phosphorylation was determined by Cerenkov counting of the amount of radioisotope incorporated into tau proteins after SDS-PAGE. Phosphorylated tau in the heat-stable fraction was concentrated with a Centricon-30 device (Amicon, Beverly, MA) and dialyzed against PEM buffer (0.1 M Pipes, pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$) containing 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin for examination of the MT-binding and polymerizing ability.

Two-Dimensional Phosphopeptide Map Analysis—Phosphorylated tau was separated on SDS-PAGE, and eluted from polyacrylamide gels by overnight (17 h) digestion with trypsin (50 μg/ml) at 37°C. The digested peptides were subjected to 2D phosphopeptide mapping using silica gel thin layer plates (Merck, Darmstadt, Germany). Electrophoresis was performed with acetic acid/formic acid/water (150:50:800). Ascending chromatography was performed in *n*-butanol/pyridine/acetic acid/water (325: 250: 50:200) (*30*).

Examination of MT-Binding and MT-Polymerization Activity of tau—Tau (1.2 μM) and tubulin (5 μM) were incubated in PEM containing 20 μM taxol and various concentrations of NaCl at 37°C for 30 min. After centrifugation at 100,000 × *g* for 30 min, the precipitates were suspended in PEM containing 1 M NaCl, then boiled for 5 min. The supernatants of 100,000 × *g* centrifugation were subjected to SDS-PAGE together with four known concentrations of tau as standards. The amount of tau was estimated by densitometric scanning (ACI-Japan, Tokyo).

MT polymerization was measured by monitoring light scattering at 450 nm using a F-300 Fluorescent Spectrophotometer (Hitachi, Tokyo) equipped with a water circulation system. Tubulin (26 μM) was mixed with tau (1.2 μM) in various phosphorylation states at 0°C. Polymerization was initiated by raising the temperature in the spectrophotometer to 37°C.

MT polymerization was also observed under dark field illumination at room temperature (29°C) and recorded on videotape as described previously (*31*). Compared to MAP2- or MAP4-decorated MTs, tau-decorated MTs showed less adhesiveness to glass surface and tended to flow away from the microscopic field during observation. To increase the number of MTs remaining stably in the observation field, the coverslips were washed with fuming nitric acid. Furthermore, bovine serum albumin (BSA), which was usually added at 2 mg/ml to prevent loss of tubulin by adsorption to the glass surface, was omitted from the incubation mixture. To overcome these unfavorable polymerization conditions, initial protein concentrations of tubulin and tau were increased to 40 and 2.0 μM, respectively, in this experiment.

Cell Culture and Metabolic Phosphorylation of tau—Cerebral cortical neurons were prepared from embryonic day-17 rat brains as described previously (*32*). Ten days after plating into culture dishes, cells were treated with 100 ng/ml nocodazole (Sigma, St. Louis, MO) for 0, 1, 3, 6, and 24 h. Heat-stable supernatants of the cell lysates were used for Western blotting with anti-phosphopeptide antibodies.

For *in vivo* phosphorylation of tau, 3 h after nocodazole addition, cultured neurons were incubated with approximately 9 MBq/ml [^{32}P]orthophosphoric acid in phosphate-free DME culture medium containing 5% dialyzed fetal calf serum and 5% dialyzed horse serum for 3 h.

Miscellaneous—SDS-PAGE was carried out according to the method of Laemmli with 9% polyacrylamide (*33*). Western blots were performed with anti-phosphopeptide antiserum (PS202, PT205, human or rat tau PS235, and PS404) at dilutions ranging from 1:30 to 1:200. Phosphopeptides were produced as described (*34*) and, after conjugation with keyhole limpet hemocyanin, were used to immunize rabbits (*35*). Detailed properties of these antibodies were described elsewhere (*36*). Protein concentrations were measured with the bicinchoninic acid protein assay reagent (Pierce Chemical, IL) or Coomassie Brilliant Blue (*37*) using BSA as a standard. The molar concentration of proteins was calculated by assuming that the molecular masses of the tubulin dimer and tau were respectively 100 and 41 kDa, and the purity of cdk5/p26 complex was 70%.

RESULTS

Phosphorylation of tau by cdk5 Is Stimulated in the Presence of MTs—We used dP-tau (tau dephosphorylated with alkaline phosphatase) and G-tau [tau prepared from the MT fraction polymerized with GTP (*17*)], both of which were in relatively low phosphorylation states (*17*), as substrates for phosphorylation with cdk5. Phosphorylation of both dP-tau and G-tau was stimulated by the addition of tubulin to the reaction mixture (Fig. 1), confirming the previous result of Ishiguro *et al.* (*15*). A plateau was obtained at concentrations greater than 1 mg/ml (10 μM) tubulin, at which a 1.5 to 2-fold stimulation was always observed. This stimulation appeared to be specific to tubulin, since it was not observed when bovine serum albumin was used instead of tubulin (Fig. 1, square). It has been suggested that PC-tubulin contains some protein kinase(s). However, the stimulated phosphorylation observed here was not due to contaminating kinases in PC-

tubulin fractions, because when cdk5 was omitted from the reaction mixture, no phosphorylation of tau was detected (data not shown). Furthermore, the possibility that cdk5 is activated by tubulin itself or contaminating proteins was excluded by using a synthetic peptide as substrate. No stimulation was observed in the presence of tubulin when the synthetic peptide containing NF-H phosphorylation site (*38*) was phosphorylated by cdk5 (data not shown). We used 0.5 mg/ml (5 μM) tubulin for further studies because higher concentrations of tubulin might otherwise disturb the electrophoretic migration of tau, or hinder the accurate quantification of tau phosphorylation.

To determine if MT formation is required for this stimulation, we added nocodazole to the reaction mixture but could not completely suppress polymerization of MTs by dephosphorylated tau probably because of tau's strong MT-polymerizing activity. We tried to find phosphorylation conditions where MTs did not polymerize even in the presence of unphosphorylated tau, but we could not. Therefore, we were unable to determine whether the unpolymerized form of tubulin is sufficient for stimulation of tau phosphorylation. On the other hand, MTs were usually formed in tau:tubulin mixtures used for the phosphorylation reaction, as was confirmed by negative staining electron microscopy and cosedimentation of tau with MTs. Since the addition of taxol to tau:tubulin mixtures did not change the extent of stimulation (data not shown), most tau in the reaction mixtures appeared to be bound to tubulin and thus promoted MT polymerization.

Identification of MT-Stimulated Phosphorylation Sites— We tested the possibility that MT-dependent stimulation of tau phosphorylation results from generation of new phosphorylation sites on tau. This experiment was performed by lengthening the incubation time for phosphorylation, and measuring the final amount of phosphate incorporated into tau. The final phosphate content was similar in the absence and presence of MTs (Fig. 2A), suggesting that the stimulation of phosphorylation is due to an increased rate of phosphorylation of tau when it is bound to MTs, rather than an increase in the final extent of phosphorylation. This was confirmed by measuring the initial rate of tau phosphorylation by cdk5. As shown in Fig. 2B, the presence of MTs increased the rate of tau phosphorylation about twofold.

We next examined whether MTs stimulate phosphorylation at all sites evenly or at a specific subset of sites by performing two-dimensional phosphopeptide mapping. Figure 3 clearly shows a marked increase in phosphorylation of spot 1, in contrast to the three other major spots (*2-4*) in which the phosphate incorporation was unchanged. The same result was obtained with either G-tau (A and B) or dP-tau (C and D). It was also confirmed that the longer incubation (3 h) with cdk5 in the absence of MTs resulted in the appearance of spot 1 (data not shown). Thus, binding of tau to MTs resulted in the stimulation of phosphorylation primarily at a single spot.

To identify the phosphorylation sites stimulated by the presence of MTs, we employed Western blotting with antibodies specifically reactive with each cdk5 phosphorylation site. The major sites at which cdk5 phosphorylates tau are Ser202, Thr205, Ser235, and Ser404 (*18*), although phosphorylation at other sites has also been reported (*16*). dP-tau did not react with antibodies to any of the above sites, indicating that dephosphorylation, at least at these sites, was complete (Fig. 4, dP). In this experiment, tubulin was removed by boiling and centrifugation after the

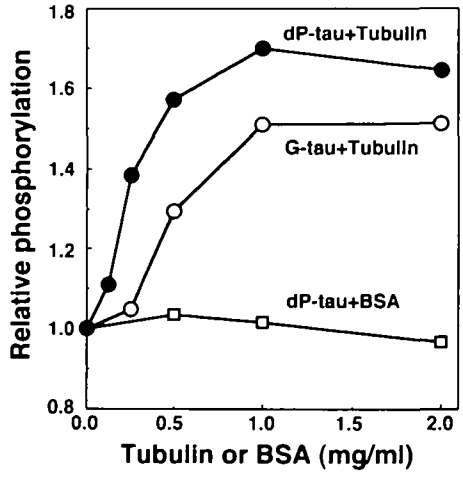

Fig. 1. **Stimulation of phosphorylation of tau in the presence of tubulin.** dP-tau (0.73 μM, ●) or G-tau (0.85 μM, ○) was phosphorylated by cdk5 (43 nM) for 30 min at 35°C. BSA was replaced with tubulin to demonstrate the specificity of tubulin (□). The ordinate shows phosphorylation of tau relative to that in the absence of tubulin.

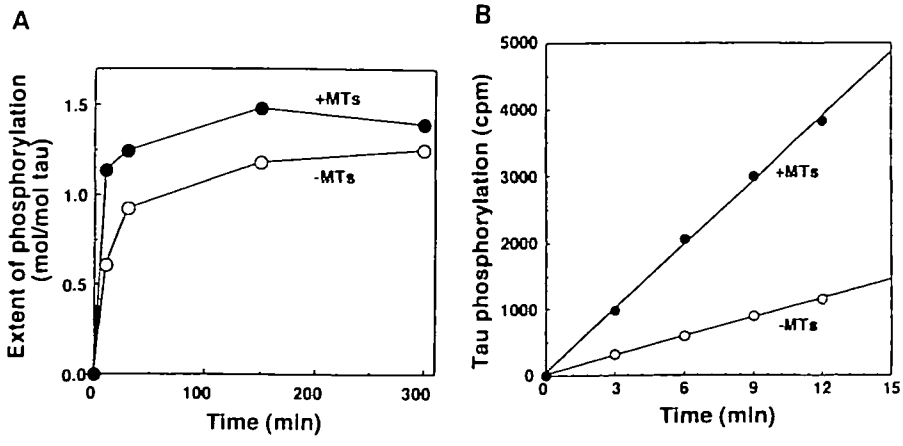

Fig. 2. **Time course of MT-stimulated tau phosphorylation by cdk5.** (A) Long-time incubation to determine the total amount of phosphorylation. dP-tau (0.73 μM) was phosphorylated by cdk5 (43 nM) in the presence (●) or absence (○) of MTs (4.5 μM) at 35°C. (B) Short-time incubation to measure the initial rate of phosphorylation. dP-tau (0.73 μM) was phosphorylated by cdk5 (43 nM) in the presence (●) or absence (○) of MTs (5 μM) at 15°C.

phosphorylation reaction, in order to avoid tubulin's effect on the electrophoretic mobility of tau. Dephosphorylated porcine tau was composed of two major and two minor bands. This band pattern did not change after rephosphorylation with cdk5, although the electrophoretic mobility decreased (CBB in Fig. 4). Phosphorylation in the presence of MTs increased the shift further, although only slightly. A blot that reacted with a phosphorylation-independent antibody is shown in the upper row (Tau in Fig. 4).

Reaction with anti-PS202 and anti-PT205 antibodies was greatly enhanced after phosphorylation by cdk5 in the presence of tubulin (Fig. 4, +), whereas the reaction with anti-human tau PS235 and anti-PS404 antibodies was not, although these antibodies reacted strongly with tau phosphorylated in the absence of MTs (Fig. 4, −). Because Ser202 and Thr205 are contained in the same tryptic

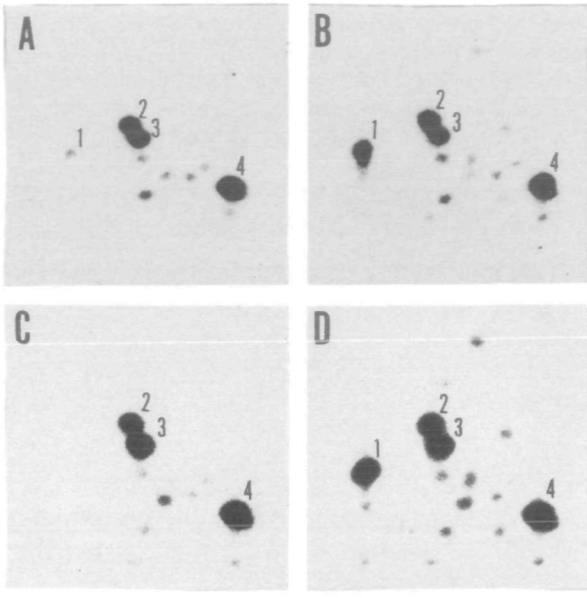

Fig. 3. **Phosphotryptic peptide maps of tau phosphorylated by cdk5 in the absence or presence of MTs.** G-tau (0.85 μM, A and B) or dP-tau (0.73 μM, C and D) was phosphorylated by cdk5 in the absence (A and C) or presence (B and D) of MTs (5 μM) for 30 min at 35°C. Tryptic digests of tau separated by 9% SDS-PAGE were analyzed by electrophoresis at pH 1.9 in the horizontal dimension, followed by ascending chromatography as described in "EXPERIMENTAL PROCEDURES."

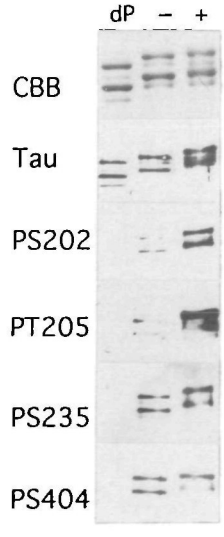

Fig. 4. **Immunoblot analysis of the phosphorylation sites in tau with anti-phosphorylation-dependent antibodies.** dP-tau (dP) phosphorylated by cdk5 in the presence (+) or absence (−) of MTs for 30 min at 35°C was run on 9% SDS-PAGE (CBB, protein staining), then transferred onto a PVDF membrane. Membranes were blotted with an anti-tau antibody (Tau, the phosphorylation-independent antibody) and anti-phosphorylation-dependent antibodies that recognize the phosphorylated form of Ser202 (PS202, ×200 dilution), Thr205 (PT205, ×100 dilution), Ser235 (human tau PS235, ×30 dilution), and Ser404 (PS404, ×200 dilution).

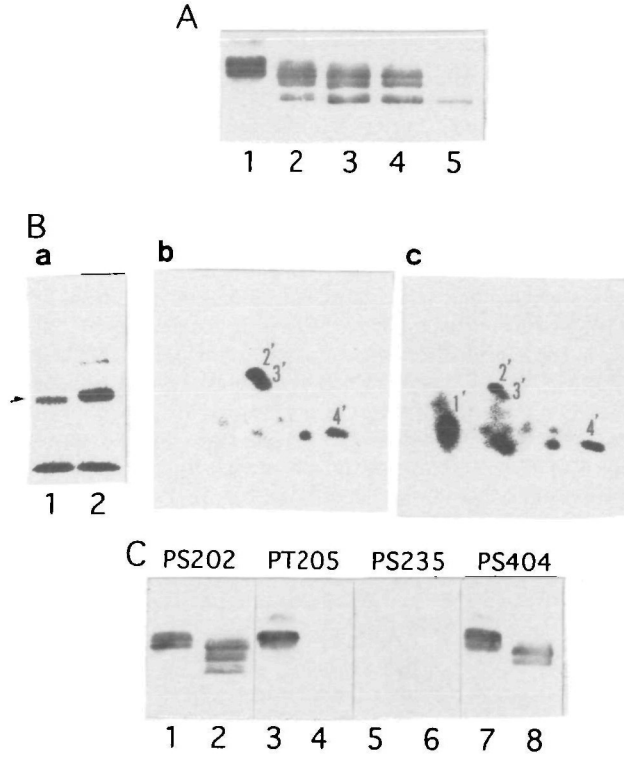

Fig. 5. **Effects of nocodazole on *in situ* phosphorylation of tau in rat primary cultured neurons.** Cerebral cortical neurons were prepared from 17-day-old embryonic rat brains. On day 10 after plating, cultured neurons were incubated with [^{32}P]orthophosphoric acid for 3 h in the presence or absence of 100 ng/ml nocodazole. The heat stable extracts of cultured neurons were also used for analysis of the phosphorylation states of tau by blotting with anti-phosphorylation-dependent antibodies. (A) Blotting of the extracts of control neurons (lane 1), neurons treated with nocodazole for 1 h (lane 2), for 3 h (lane 3), for 6 h (lane 4), and for 24 h (lane 5) with anti-human tau antibody (phosphorylation-independent) to show the effect of nocodazole on the electrophoretic mobility of tau. (B) (a) An autoradiograph of the heat stable supernatant of cell extracts prepared from primary cultured neurons metabolically labeled for 3 h in the presence (lane 1) or absence (lane 2) of nocodazole. Arrowhead indicates tau protein. (b) and (c), 2D phosphopeptide map of tau shown in lanes 1 and 2 of (a), respectively. Spot 1′ in (c), which may correspond to spot 1 of the *in vitro* phosphorylation by cdk5 (Fig. 3), was shown to be MT-dependent. Spots whose migration were same as those of rat tau phosphorylated by cdk5 *in vitro* are labeled as 2′ to 4′. (C) Blotting of the control extract (lanes 1, 3, 5, and 7) and the nocodazole-treated extract for 6 h (lanes 2, 4, 6, and 8) with anti-phosphorylation dependent antibodies: lanes 1 and 2, PS202; lanes 3 and 4, PT205; lanes 5 and 6, rat tau PS235; lanes 7 and 8, PS404.

peptide, the increased signal in spot 1 shown in Fig. 3 probably corresponds to the peptide containing these two phosphorylation sites.

MT-Dependent Phosphorylation of tau at Thr205 in Primary Cultured Neurons—To determine whether phosphorylation of Ser202 and Thr205 is also stimulated in the presence of MTs *in vivo*, we used cortical primary cultured neurons prepared from rat embryonic brains. We treated the cultured neurons with nocodazole to depolymerize MTs and compared the phosphorylation state of tau with that in control cultures. Tau in embryonic cultured neurons was a juvenile type composed of two bands with different phosphorylation states (Fig. 5A, lane 1, see also Ref. *34*). Nocodazole treatment increased the electrophoretic mobility of tau, which was probed with an anti-human tau antibody (phosphorylation-independent antibody) (Fig. 5A, lanes 1-4). This SDS-PAGE pattern continued at least for 6 h, while the overnight treatment resulted in degradation of tau (Fig. 5A, lane 5).

We examined tau phosphorylation 3 to 6 h after nocodazole treatment by incubating primary cultured neurons with [^{32}P]orthophosphoric acid, when a new equilibrium in phosphorylation was maintained. The heat-stable supernatant of the cell extract was subjected to autoradiography after SDS-PAGE (Fig. 5B a). A major labeled band indicated by arrowhead was identified as tau by immunoblotting and cosedimentation with MTs (data not shown). Nocodazole treatment decreased the extent of phosphorylation of tau (Fig. 5B a, lane 1). Both tau proteins were analyzed by 2D phosphopeptide mapping. The overall phosphorylation pattern resembled that of porcine tau phosphorylated by cdk5 *in vitro* (compare Fig. 5B b with Fig. 3, A or C). The major phosphorylation spots of 2′-4′ in Fig. 5B, b and c, comigrated with corresponding spots of rat tau phosphorylated by cdk5 *in vitro*, suggesting that cdk5 is one of the major tau kinases in rat cortical cultured neurons. Differences in the exact migration from those of Fig. 3 may be due to differences in species used, *i.e.*, porcine and rat. Further, the appearance of additional spots suggested that additional kinases may phosphorylate tau *in vivo*. Spot 1′ (Fig. 5B c), which seemed to correspond to spot 1 of *in vitro* phosphorylation, disappeared on treatment with nocodazole. Figure 5C shows a blotting of nocodazole-treated tau with phos-

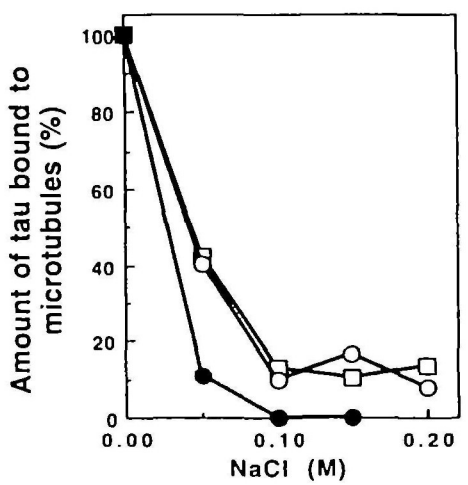

Fig. 6. **MT-binding assay of tau phosphorylated in the absence or presence of MTs.** Tau (1.2 μM) in different phosphorylation states [dephosphorylated tau (○), tau phosphorylated in the absence of MTs (□), and tau phosphorylated in the presence of MTs (●)] was incubated with MTs polymerized with 20 μM taxol in various concentrations of NaCl at 37°C for 30 min. After centrifugation, the pellets were suspended with PEM containing 1 M NaCl, then boiled for 5 min. Denatured tubulin was removed by centrifugation, and the supernatants were analyzed by SDS-PAGE. The amount of tau bound to MTs in the absence of NaCl was assigned as 100%. Without NaCl, virtually all tau bound to MTs.

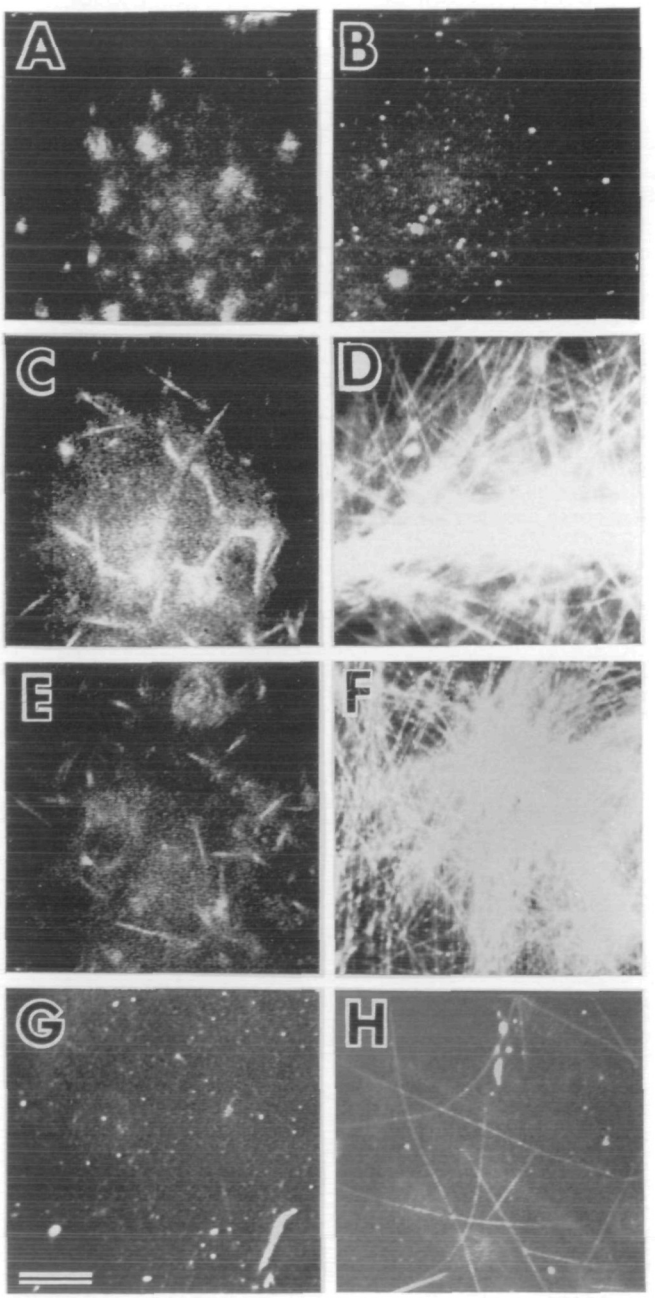

Fig. 7. **Video images of dark field microscopy demonstrating polymerization of MTs.** Mixtures of tubulin (40 μM) and tau (1.95 μM) were incubated on coverslips at 29°C for 2.5 min (A, C, E, and G) or 12 min (B, D, F, and H). A and B, tubulin alone; C and D, tubulin and dephosphorylated tau; E and F, tubulin and tau phosphorylated in the absence of MTs; G and H, tubulin and tau phosphorylated in the presence of MTs. Bar represents 10 μm.

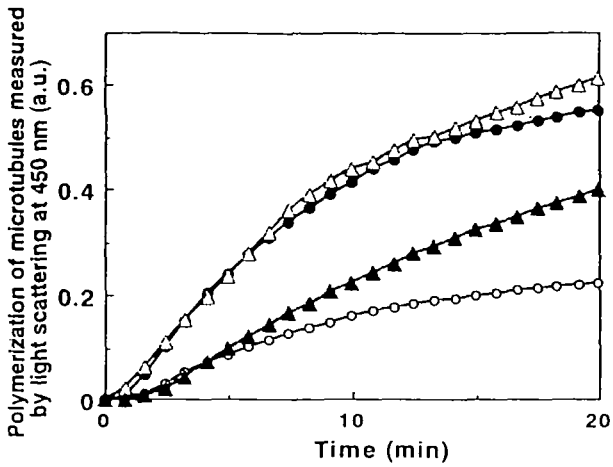

Fig. 8. **MT-polymerization monitored by light scattering.** Tubulin (26 μM) was incubated with tau (1.2 μM) at 37°C. MT-polymerization was measured by light scattering at 450 nm in a fluorescence spectrophotometer (Hitachi, Tokyo). The ordinate is expressed in an arbitrary unit. ●, Dephosphorylated tau; △, tau phosphorylated in the absence of MTs; ▲, tau phosphorylated in the presence of MTs; ○, tubulin alone.

phorylation-dependent antibodies used in Fig. 4. Ser202, Thr205, and Ser404, but not Ser235, were phosphorylated *in vivo* in the control cultured neurons (Fig. 5C, lanes 1, 3, 5, and 7), which is consistent with previous reports (*32, 35*). Among these three phosphorylation sites, Thr205 was unphosphorylated in cultured neurons treated with nocodazole (Fig. 5C, lanes 2, 4, 6, and 8). These results indicate that phosphorylation of tau at Thr205 is MT-dependent also in cultured neurons.

In contrast to *in vitro* phosphorylation by cdk5, phosphorylation at Ser202 was not MT-dependent in brain cortical neurons. We do not know the reason, but one possible explanation is that Ser202, which was phosphorylated in tau molecules bound to MTs before the nocodazole treatment, maintained its phosphorylation state during the 6 h treatment with nocodazole. While this study was in progress, a similar result was reported by Merrick *et al.* (*39*), who showed selective dephosphorylation of Ser202 and Thr205 by protein phosphatase 2A when NT2N cultured neurons were treated with nocodazole. Therefore, the difference in the phosphorylation at tau's Ser202 residue may be caused by the difference in neurons used.

Phosphorylation on Ser202 and Thr205 Decreased MT-Nucleation Activity of tau—Next we examined the effects of phosphorylation at these sites on tau's ability to bind to MTs. Each tau was almost completely cosedimented with MTs in PEM solution. Increasing the concentration of NaCl gradually decreased the ratio of tau bound to MTs, as shown in Fig. 6. While tau phosphorylated by cdk5 in the absence of MTs showed a MT-binding profile similar to dephosphorylated tau, tau phosphorylated in the presence of MTs was more readily released from MTs than dephosphorylated tau.

The effects of tau on polymerization of MTs were examined using dark field microscopic observation. Compared to MAP2- or MAP4-decorated MTs, tau-decorated MTs were less adhesive to the glass coverslips. To obtain clear images, glass coverslips were cleaned elaborately and the incubation mixture was altered, as described in "EXPERIMENTAL PROCEDURES." Under these conditions, tubulin alone did not polymerize even at 40 μM at 29°C (Fig. 7, A and B). Addition of dephosphorylated tau induced formation of short MTs in 2.5 min and many long MTs in 12 min (Fig. 7, C and D). An apparently similar extent of MT polymerization was observed on addition of tau phosphorylated in the absence of MTs (Fig. 7, E and F), while the number of MTs formed was greatly reduced on addition of tau phosphorylated in the presence of MTs (Fig. 7, G and H).

To confirm the above results quantitatively, MT polymerization was monitored by light scattering at 450 nm (Fig. 8). Tubulin (26 μM) alone showed little increase in light scattering (Fig. 8, ○); and dephosphorylated tau (1.5 μM) enhanced MTs polymerization (Fig. 8, ●), as expected. While the tau phosphorylated in the absence of MTs showed a similar MT-polymerization activity to that of dephosphorylated tau (Fig. 8, △), phosphorylation in the presence of MTs resulted in a prolonged lag phase and a slower rate of MT formation (Fig. 8, ▲), suggesting decreased nucleation activity.

DISCUSSION

In this study, we have demonstrated that (a) MT-stimulated phosphorylation sites of tau by cdk5 were Ser202 and Thr205, and (b) phosphorylation at these sites decreased the MT-nucleation activity of tau. Special features of the current study are the use of cdk5 as a kinase and the comparison of phosphorylation of tau bound to MTs with that of free tau. Considering that (i) cdk5, a kinase likely to phosphorylate tau *in vivo*, binds to MTs (*15, 29*), (ii) most tau proteins in neurons bind to MTs, and (iii) the MT-dependent phosphorylation is observed in cultured neurons (*39*, this study), this reaction is likely to occur on MTs *in vivo*.

Spot 1 (Fig. 3) was the only spot on the 2D phosphopeptide map whose appearance was dependent on MTs. The appearance of immunoreactivity to anti-PS202 and PT205, but not Ser235 and Ser404, was also MT-dependent. We also confirmed that the other S/T-P sequences of Ser199, Ser231, Ser396, Ser413, and Ser422 were not phosphorylated by cdk5 by Western blotting with a panel of phosphorylation-dependent antibodies (data not shown, *36*). Considering that these sites include all cdk5-phosphorylation sites reported so far, spot 1 should correspond to the peptide containing Ser202 and Thr205. This is supported by the data of Trinczek *et al.* (*40*), who showed an identical phosphopeptide map of tau phosphorylated by cdk5 to ours. By determining the amino acid sequence of each peptide, they suggested that the spot, corresponding to our spot 1, contains doubly phosphorylated peptides, Ser202 in combination with either Ser198, Ser199, or Thr205. Our results showed Thr205 to be another phosphorylation site. Phosphorylation of Ser199 was not detected by blotting with an anti-phosphorylation-dependent antibody against Ser199 as described above. Ser198, whose phosphorylation by cdk5 has not yet been reported, is not a consensus phosphorylation site for cdk5 (*16, 18*).

The MT-stimulated phosphorylation sites, Ser202 and Thr205, are located at the N-terminal of tau's MT-binding region, 54 and 51 amino acid residues, respectively, distant

from the imperfect MT-binding repeats in the C-terminal domain (*24, 41*). In contrast, phosphorylation of Ser235 and Ser404, which are closer to the MT-binding repeats, was not affected by the presence of MTs. How might phosphorylation of Ser202 and Thr205 sites be selectively stimulated in the presence of MTs? In other words, could these phosphorylation sites be exposed in tau bound to MTs and masked in unbound tau? There are two possible explanations: intramolecular folding (*10*) and dimerization. The occurrence of either or both is suggested from the observation of tau molecules by low angle rotary shadowing (*42*). The overall length of a single tau molecule observed by this technique was about 25-33 nm, which corresponds to the length of the region bearing MT-binding repeats, and suggests a hairpin-like folding in a molecule. Anti-parallel dimer formation, resulting from interactions between the repeat domains, was also shown by Wille *et al.* (*42*). The intramolecular interaction between the proline-rich and repeat regions was supported by a recent study by Goode *et al.* (*43*) using amino-terminal deletion mutants of tau. The region containing Ser202 and Thr205 may be a site for folding or hidden by dimerization. The interaction of tau with MTs would be expected to extend a tau molecule or break tau dimers, resulting in exposure of Ser202 and Thr205 for phosphorylation. If this is so, phosphorylation of other sites close to Ser202 and Thr205 might also be stimulated by the presence of MTs. In fact, tau phosphorylation by GSK3β, one of whose preferred phosphorylation sites is Ser199, was also shown to be stimulated by tubulin (*15*). Furthermore, phosphorylation at Ser214 (corresponding to Ser156 of the fetal-specific human tau isoform containing 352 residues) with protein kinase A is stimulated by heparin, and this stimulated phosphorylation reduced tau's activity to nucleate MTs much more than to promote MT growth (*44*). Heparin, an acidic polysaccharide, might act on tau to change its conformation, in the same way that the acidic C-terminal MAP-binding domain of tubulin molecules does.

Although phosphorylation of tau is known to decrease its MT-polymerizing activity (*8-11, 23, 45, 46*), there has been no detailed examination of which phosphorylation site plays a role in which step of polymerization, except for Ser262 and Ser396. Their phosphorylation has been reported to reduce binding of tau to MTs (*5, 6*). In this study, tau in three different known phosphorylation states was used to examine its binding to MTs and MT-polymerization ability *in vitro*. Dephosphorylated tau showed a strong capacity to induce polymerization of MTs, while tau phosphorylated at Ser235 and Ser404 showed a similar polymerizing ability to dephosphorylated tau. However, further phosphorylation at Ser202 and Thr205 decreased the polymerization activity with little decrease in the MT-binding activity. Seubert *et al.* (*47*) reported that phosphorylation at Ser396/Ser404 or Ser202/Thr205 did not eliminate binding of tau to MTs, and Biernat *et al.* (*6*) reported that phosphorylation at Ser202, Ser235, Ser396, and Ser404 was less important than that of Ser262 for altering tau's interaction with MTs. Our result that phosphorylation at Ser202, Thr205, Ser235, and Ser404 did not change the binding to MTs in the absence of salt are consistent with these previous results (*6, 47*). However, addition of salt to the binding solution demonstrated a difference in desorption from MTs between tau phosphorylated at two (Ser235/Ser404) sites and at four (Ser202/Thr205/Ser235/Ser404) sites. On the other hand, MT-polymerization activity of MAP4 has been shown to be more sensitive to phosphorylation than its binding to taxol-stabilized MTs (*48*). Whether phosphorylation at both Ser202 and Thr205 contributes equally to the decrease in tau's ability for MT polymerization is not known at present. Trinczek *et al.* (*40*) showed that phosphorylation of tau mainly at Ser202, Ser235, and Ser404 by cdk5 decreased the MT-stabilizing ability moderately by increasing the frequency of catastrophe about fourfold. As shown here, while phosphorylation at Ser235 and Ser404 did not affect the MT-polymerization activity of tau, additional phosphorylation at Ser202 and Thr205 in the presence of MTs decreased it markedly. Although it is difficult to draw a conclusion from these different types of experiments, each phosphorylation site may act additively to decrease tau's MT-stabilizing ability. To determine concretely the role of each phosphorylation site, it will also be necessary to use proteins whose possible phosphorylation sites are changed to unphosphorylatable amino acid residues.

Undoubtedly, *in vivo* phosphorylation states and regulation are likely to be very complex. In addition to cdk5-dependent phosphorylation sites, there are several more sites that are phosphorylated by other kinases in the flanking regions of the MT-binding repeat sequence (*12, 13*). Their function remains to be addressed. Furthermore, *in vivo* phosphorylation is regulated by protein phosphatases as well as protein kinases (*32*). Tau is reported to be dephosphorylated by several protein phosphatases in a site-specific manner (*49*). In any case, some degree of phosphorylation of tau may be required in order to maintain proper MT dynamics in neurons. If all tau were in the dephosphorylated state in axons, for example, MTs would become very stable, rather than possessing appropriate plasticity. On the other hand, excess phosphorylation would be expected to decrease MT number. In particular, Ser202 and Thr205 sites in unbound tau molecules might be kept in an unphosphorylated state to allow new MT assembly. Decreased accessibility of cdk5, and probably increased accessibility of protein phosphatase 2A (*40, 45*), to Ser202 and Thr205 sites in tau free of MTs are a plausible mechanism to protect these sites from phosphorylation.

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