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Biochemical and Biophysical Research Communications 331 (2005) 50–55
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MAP1B phosphorylation is differentially regulated by Cdk5/p35, Cdk5/p25, and JNK

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Received 15 March 2005
Available online 30 March 2005

Abstract

Mode I phosphorylated MAP1B is observed in developing and pathogenic brains. Although Cdk5 has been believed to phosphorylate MAP1B in the developing cerebral cortex, we show that a Cdk5 inhibitor does not suppress mode I phosphorylation of MAP1B in primary and slice cultures, while a JNK inhibitor does. Coincidently, an increase in phosphorylated MAP1B was not observed in COS7 cells when Cdk5 was cotransfected with p35, but this did occur with p25 which is specifically produced in pathogenic brains. Our primary culture studies showed an involvement of Cdk5 in regulating microtubule dynamics without affecting MAP1B phosphorylation status. The importance of regulating microtubule dynamics in neuronal migration was also demonstrated by in utero electroporation experiments. These findings suggest that mode I phosphorylation of MAP1B is facilitated by JNK but not Cdk5/p35 in the developing cerebral cortex and by Cdk5/p25 in pathogenic brains, contributing to various biological events.
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Keywords: Microtubule-associated protein; MAP1B; Cdk5; p35; p25; Neuronal migration; Neurodegenerative disease; Alzheimer's disease; Roscovitine; SP600125

Microtubules play a crucial organizing role in neurons, conferring appropriate morphology on leading processes or neurites. The stability of microtubules is dynamically regulated, contributing to dynamic cellular events such as neuronal migration and neurite extension. At the tips of neuronal processes, which are in constant motion while searching for appropriate targets, dynamic rather than stable microtubules are principally observed [1]. Microtubule dynamics are thought to be regulated by microtubule-associated proteins (MAPs) in various cell types including neurons. One of the MAPs, MAP1B, is a microtubule-stabilizing phosphoprotein that is expressed in the nervous system including radial migrating neurons in the developing cerebral cortex [2,3]. There are two phosphorylated forms of MAP1B; mode I phosphorylation which is detected only in the developing nervous system, and mode II phosphorylation which is observed throughout developmental stages and adulthood. It has been reported that MAP1B is phosphorylated at mode I sites by glycogen synthase kinase 3β (GSK3β) in cultured dorsal root ganglion cells, and that this results in a loss of the microtubule-stabilizing ability [4,5]. Mode I phosphorylated MAP1B is also observed in the neurofibrillary tangles (NFT) and dystrophic neurites (DN) in Alzheimer's diseased brains [6,7]. Furthermore, overexpression of MAP1B promotes not only neurite growth but also neuronal apoptosis [8]. These facts suggest that MAP1B and its phosphorylation are

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.132

involved in both the development and pathogenesis of the nervous system.

It has been suggested that proline-directed protein kinases (PDPKs) may phosphorylate MAP1B at mode I sites. Previously we reported that c-Jun N-terminal kinase (JNK), one of the PDPKs, is involved in mode I phosphorylation of MAP1B and neuronal migration in the developing cerebral cortex [9]. Another PDPK, cyclin-dependent kinase 5 (Cdk5), has also been implicated for involvement in mode I phosphorylation; antisense oligonucleotides for Cdk5 in cultured macroneurons from the cerebellum were shown to decrease mode I phosphorylation of MAP1B [10,11]. However, direct evidence for Cdk5 involvement in MAP1B phosphorylation in the cerebral cortex is still lacking.

It has been known that Cdk5 requires its activator, p35, to exhibit kinase activity [12]. In pathological circumstances such as neurodegenerative disorders, p35 is processed to a more stable form, p25, which confers a stronger kinase activity upon Cdk5. Furthermore, it has been suggested that the substrate specificity and subcellular localization of p25 are distinct [13].

We show that Cdk5 is not involved in the phosphorylation of MAP1B at mode I sites in the developing cerebral cortex where p35, but not p25, is expressed. We also show that Cdk5 phosphorylates MAP1B at mode I sites only as a complex with p25, but not p35, in cultured cells. Furthermore, we show the importance of MAP1B regulation of microtubule stability in cortical neuronal migration through in utero electroporation experiments. These findings suggest that mode I phosphorylation of MAP1B is differentially regulated by Cdk5/p35, Cdk5/p25, and JNK in developing and pathogenic brains, contributing to a variety of physiological and pathologic events.

Experimental procedures

Plasmids. Plasmids were prepared using Endo Free plasmid purification kits (Qiagen). Oligonucleotides containing multicloning sites were inserted into pcCAG [9] to generate pCAG-MCS2. MAP1B [8], Cdk5 [14], p35 or p25 [15] cDNA was inserted into the pCAG-MCS2 vector. JNK expression vector was a generous gift from Dr. S. Tamura [16].

Antibodies and chemical regents. Primary antibodies used in this study were SMI31 (Sternberger Monoclonals), anti-MAP1B (Santa Cruz N-19), anti-phospho-Ser732 FAK (Sigma), anti-FAK (Santa Cruz C-903), anti-activated JNK1/2 (Promega), anti-JNK1/2 (BD Biosciences), and anti-p35 (Santa Cruz C-19) antibodies. Roscovitine and SP600125 were purchased from Sigma and BIOMOL Research Laboratories, respectively.

Cell culture and transfection. COS7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum and antibiotics. Transfections were performed as described previously [17]. One molar D-sorbitol (Sigma) was added into the culture media for 30 min to activate JNK (Fig. 2).

In utero electroporation, primary culture, slice culture, and Western analyses. In utero electroporation, primary culture of E15 mouse embryonic cortical neurons, slice culture of E16 mouse embryonic cortex, and Western analyses were performed as described previously [9,18].

Results and discussion

E15 cerebral cortices were dissociated and after 1 day in culture, inhibitors or solvent was added for another 24 h. Cells were harvested and subjected to immunoblot analysis with SMI31 antibody which recognizes mode I phosphorylated MAP1B [19]. As previously reported [9,20], treatment with a JNK inhibitor, SP600125, resulted in decreased mode I phosphorylation of MAP1B (Fig. 1A, SP25 and SP50). In contrast, treatment with a Cdk5 inhibitor, roscovitine, had no effect (Fig. 1A, R100), although this inhibitor decreased the phosphorylation of FAK at Ser732 (Fig. 1A, R100), which was reported to be phosphorylated by Cdk5 [21]. Interestingly, administration of a higher concentration of roscovitine (200 μM) to the primary cortical culture increased the mode I phosphorylation of MAP1B (Fig. 1A, R200) and resulted in a nearly 6-fold increase of phosphorylated JNK1 at Thr and Tyr (Fig. 1B), corresponding to an activated JNK1 [22]. These results suggest that Cdk5 suppresses the activity of JNK1 as well as phosphorylation of MAP1B in cultured cortical neurons. Although it is possible that higher concentrations of roscovitine may affect the activity of molecules other than Cdk5, the report that Cdk5 inhibits JNK3 through the phosphorylation of Thr131 [23] suggests that the suppression of JNK1 activity may occur through similar molecular mechanisms.

To confirm these results in vivo, we used slice cultures of mouse E16 cerebral cortices in which many radially migrating neurons are observed [9]. To determine whether treatment with 100 μM roscovitine can inhibit neuronal migration, we used enhanced GFP (EGFP) fluorescence to allow visualization of migrating neurons. Embryos were electroporated with EGFP expression vectors at E14 and coronal slices were taken at E16 and cultured for 20 h with or without 100 μM roscovitine to observe the migration of GFP-positive cells. While GFP-positive cells migrated toward the pial surface in the control slices, roscovitine treatment inhibited cell migration, mimicking the cerebral cortex phenotype of Cdk5-deficient mice as well as that of dominant negative Cdk5-electroporated neurons (Fig. 1C) [9,24,25]. Consistent with this result, treatment with 100 μM roscovitine to slice cultures resulted in suppression of FAK phosphorylation at Ser732 (Fig. 1D), suggesting that 100 μM roscovitine inhibits Cdk5 kinase activity and Cdk5-dependent neuronal migration. However, treatment with 100 μM roscovitine did not inhibit the mode I phosphorylation of MAP1B, while administration of 50 μM SP600125 decreased this phosphorylation

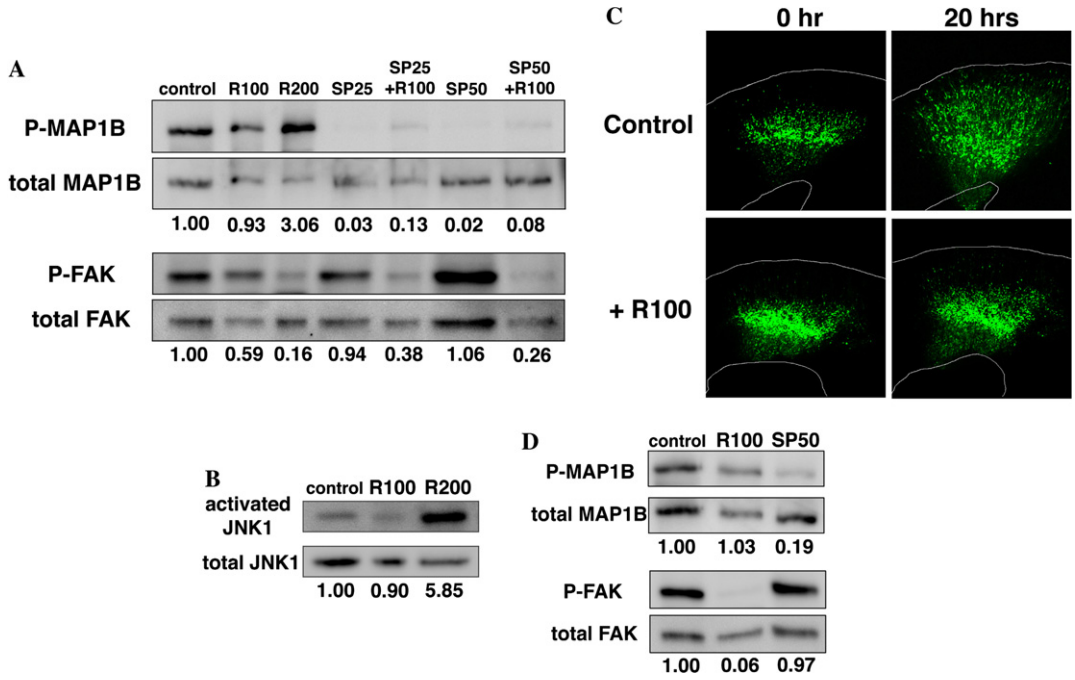

Fig. 1. Influence of roscovitine and SP600125 on the mode I phosphorylation of MAP1B and activation of JNK. (A) Primary cultures of E15 cerebral cortices (2DIV) were treated with 100 or 200 μM roscovitine (R100 or R200), 25 or 50 μM SP600125 (SP25 or SP50) or both (SP25 + R100 or SP50 + R100) for 24 h, and subjected to immunoblot analyses with SMI31 which recognizes mode I phosphorylated MAP1B (P-MAP1B), anti-MAP1B antibody (total MAP1B), anti-phospho-Ser732 FAK antibody (P-FAK) or anti-FAK antibody (total FAK). The numbers indicate the ratio of P-MAP1B/total-MAP1B and P-FAK/total-FAK, which were determined by chemiluminescence (FUJIFILM). While treatment with SP600125 resulted in decrease of the P-MAP1B/total-MAP1B ratio (SP25 and SP50), treatment with 100 μM roscovitine had no effect (R100). Interestingly, the higher concentration of roscovitine (200 μM) increased the ratio (R200). Addition of roscovitine to SP600125-treated cells caused a slight increase of P-MAP1B/total MAP1B ratio (SP25 + R100 and SP50 + R100). Similar results were obtained from five independent experiments. (B) Primary cultures of E15 cerebral cortices (2DIV) were treated with 100 or 200 μM roscovitine (R100 or R200) or solvent (DMSO; control) for 24 h, and subjected to immunoblot analyses with anti-activated JNK1/2 or anti-JNK1/2 antibodies. JNK1 (46 kDa) signals are shown. In contrast to JNK1 signals, JNK2 (54 kDa) signals were very weak (data not shown). Experiments were performed in triplicate. (C) E14 brains were electroporated with EGFP expression vector and at E16, 300 μm coronal sections were cultured on membrane inserts for 20 h with or without 100 μM roscovitine (R100). Addition of 100 μM roscovitine resulted in inhibition of neuronal migration. Similar results were obtained from five independent experiments. (D) Three hundred micrometers E16 coronal brain sections were cultured on membrane inserts for 24 h with 100 μM roscovitine (R100), 50 μM SP600125 (SP50) or solvent (DMSO; control). The numbers indicate the ratios of P-MAP1B/total-MAP1B and P-FAK/total-FAK, which were determined by chemiluminescence. Similar results were obtained from five independent experiments.

efficiently, suggesting that the phosphorylation of MAP1B mainly depends on JNK, but not Cdk5, in the developing cerebral cortex (Fig. 1D). These results suggest that Cdk5 regulates cortical neuronal migration through the phosphorylation of substrates other than MAP1B. Actually, Xie et al. reported that Cdk5 phosphorylates FAK, contributing to cortical neuronal migration [21,25]. Accordingly, in our experiments, 100 μM roscovitine suppressed phosphorylation of FAK at Ser732 and consequently, neuronal migration. Although a high concentration of roscovitine (200 μM) increased JNK activity (Fig. 1B), it also resulted in suppression of neuronal migration in this slice culture system (data not shown), exhibiting an effect indistinguishable from that of 100 μM roscovitine shown in Fig. 1C. However, this result is not paradoxical, as it has previously been reported that increased activation of JNK by overexpression of MUK (MAPK-upstream protein kinase) perturbs cortical neuronal migration [26]. Furthermore, overexpression of JNK by means of in utero electroporation also suppressed neuronal migration in vivo (our unpublished data). These facts may suggest that proper neuronal migration requires the appropriate activities of Cdk5 and JNK.

To further elucidate the ability of Cdk5 to phosphorylate MAP1B, we performed in vitro experiments using COS7 cells, in which Cdk5 activators, p35 and p25, were not detected by immunoblot analysis (data not shown). Because p35 was expressed in the embryonic cerebral cortex (Fig. 2A), COS7 cells were cotransfected with Cdk5 and p35 expression vectors, and subsequently subjected to immunoblotting with SMI31. A complex of Cdk5 with p35 was detected by immunoprecipitation from the transfected cells, suggesting that they can associate in COS7 cells (data not shown). However, expression of Cdk5/p35 resulted in little increase of phosphorylated MAP1B in the COS7 cells compared to the control cells transfected only with Cdk5, although

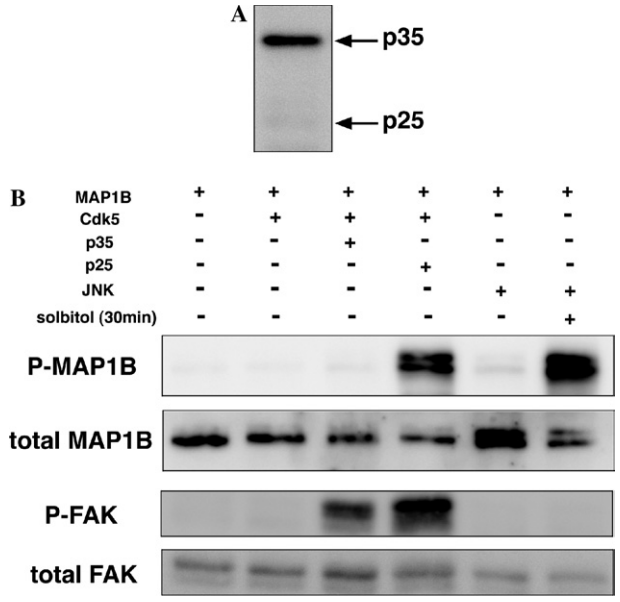

Fig. 2. Cdk5/p25 but not Cdk5/p35 phosphorylates MAP1B. (A) Immunoblot analysis of extract from E15 mouse embryonic cerebral cortex using an anti-p35 antibody (C-19, Santa Cruz), which recognizes both p35 and p25. This antibody recognizes both p35 and p25 in lysates from p35- or p25-transfected COS7 cells as well as brains with Alzheimer's disease (data not shown). (B) COS7 cells were cotransfected with the indicated plasmids and subsequently subjected to immunoblot analyses with SMI31 (P-MAP1B), anti-MAP1B (total MAP1B), anti-phospho-Ser732 FAK (P-FAK) or anti-FAK (total FAK) antibodies. Experiments were performed in quadruplicate.

Ser732 on FAK was strongly phosphorylated (Fig. 2B). This suggests that Cdk5 does not phosphorylate MAP1B when it associates with p35, consistent with the results in Fig. 1. In pathological circumstances such as neurodegenerative disorders, p35 is processed to a more stable form, p25 [13]. Only p35, not p25, is observed in the normal embryonic cerebral cortex (Fig. 2A). Because it has been suggested that Cdk5/p25 exhibits a substrate specificity distinct from Cdk5/p35 [13] and that mode I phosphorylated MAP1B and p25 are detected in brains with neurodegenerative disorders [6], we tested whether Cdk5/p25 phosphorylates MAP1B. Interestingly, when Cdk5 was cotransfected with p25, strong phosphorylation of MAP1B at mode I sites as well as FAK at Ser732 was detected (Fig. 2B). These facts suggest that Cdk5 phosphorylates MAP1B only upon association with p25, not with p35. Together with the result that JNK phosphorylates MAP1B in the embryonic cerebral cortex (Fig. 1), these findings suggest that mode I phosphorylation of MAP1B is facilitated by JNK but not Cdk5 in developing cerebral cortex, and further imply that Cdk5 may be involved in phosphorylation of MAP1B in p25-producing pathogenic brains.

Mode I phosphorylation of MAP1B is known to affect its ability to regulate microtubule dynamics [4]. However, whether proper regulation of microtubule dynamics is actually required for neuronal migration is still unclear. Transfection of MAP1B into the primary cultured neurons of E15 cerebral cortex has been shown to significantly increase microtubule stability at the tips of the neuronal processes, confirming the positive effect of MAP1B on the microtubule stability in the transfected neurons (data not shown). We introduced MAP1B expression plasmids into ventricular zone cells of the mouse cerebral cortex at E14 using in utero electroporation. In control brains of P0 animals (5 days after electroporation), GFP-labeled transfected cells reached the superficial layer of the cortical plate as reported previously (Fig. 3A) [9]. However, in MAP1B-overexpressing brains, some of the transfected cells were found to stall in the intermediate zone, probably due to perturbed neuronal migration, although a portion of the transfected cells reached the superficial layer of the cortical plate (Fig. 3B). To estimate the extent of cell migration statistically, the fluorescence intensities in distinct layers of the electroporated cortex were recorded. $34.0 \pm 5.3\%$ of the EGFP fluorescence was detected in the cortical plate of cortices overexpressing MAP1B, compared to $78.1 \pm 4.9\%$ in the control cortices (Figs. 3C and D). This strongly suggests that proper regulation of microtubule dynamics is required for normal cortical neuronal migration.

While Cdk5 does not phosphorylate MAP1B in the developing cerebral cortex, it is known to phosphorylate several other microtubule-regulatory proteins [11,25].

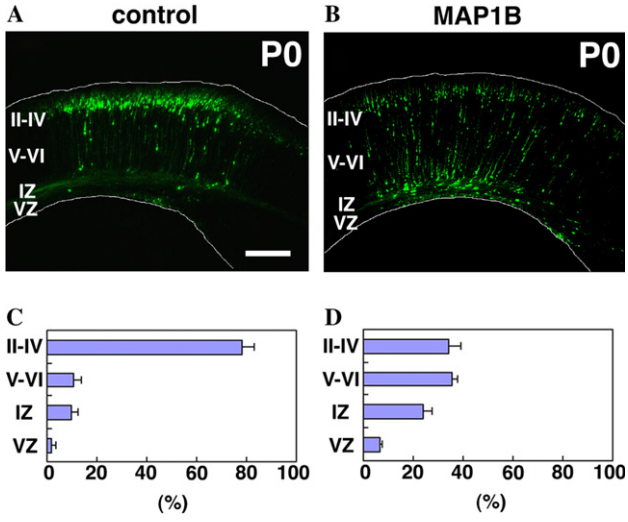

Fig. 3. Overexpression of MAP1B in the developing cerebral cortex. Control (A,C) or MAP1B expressing (B,D) vectors were transfected with pEGFP into E14 mouse embryonic brains by in utero electroporation. (A,B) At P0, 5 days after electroporation, frozen brain sections were examined for EGFP fluorescence. White lines represent pial and ventricular surfaces. (C,D) Estimation of cell migration by recording fluorescence intensities of EGFP in distinct regions of the cerebral cortex [9]. Each bar represents the mean percentage of relative intensity ± SE $n = 6$. II–IV, layers II–IV of the cortical plate; V–VI, layers V–VI of the cortical plate; IZ, intermediate zone; VZ, ventricular zone. Scale bar: 200 μm.

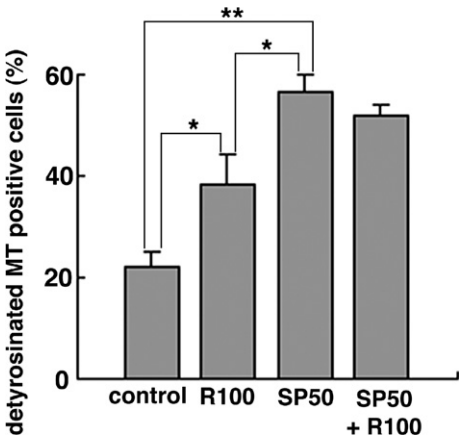

Fig. 4. E15 cerebral cortices were dissociated and cultured for 20 h, and subjected to an additional 8 h incubation with 100 μM roscovitine (R100), 50 μM SP600125 (SP50) or solvent (DMSO; control). Cells were stained with anti-α-tubulin and anti-detyrosinated tubulin antibodies, and the ratios of the cells containing detyrosinated microtubules at their process tips were determined. When the distance between the distal ends of detyrosinated microtubules and entire microtubules at the tip of the longest neurite was <5 μm, that cell was counted as a 'detyrosinated MT-positive cell' [9]. Scores represent mean percentage ± SE; $n = 5$ brains; *$p < 0.05$, **$p < 0.02$, t test.

Because both the Cdk5 kinase activity and proper regulation of microtubule dynamics were required for normal neuronal migration (Figs. 1C and 3), we suspected that Cdk5 may influence microtubule stability to regulate neuronal migration. To test this, we investigated the effect of roscovitine treatment on microtubules in cultured cortical neurons. Primary cultured neurons were coimmunostained with anti-detyrosinated α-tubulin (a marker for stable microtubules) and anti-α-tubulin antibodies. Treatment with roscovitine significantly decreased detyrosinated microtubules at the tips of neuronal processes, indicating that Cdk5 can regulate microtubule dynamics without affecting the MAP1B phosphorylation status (Fig. 4). Cdk5 is known to phosphorylate PAK1 at Thr212 and this alters microtubule dynamics without affecting the kinase activity of PAK1 [27]. Therefore, Cdk5 may regulate microtubule dynamics at the tips of neuronal processes through phosphorylation of PAK1, although how the PAK1 activity controls the microtubule dynamics remains unclear.

Although Cdk5 is believed to phosphorylate MAP1B at mode I sites in the developing cerebral cortex [11,25], our results showed that this phosphorylation is dependent on JNK, not Cdk5. However, we also demonstrated that both Cdk5 and JNK are required for regulating microtubule dynamics and neuronal migration. These results suggest that these proline-directed protein kinases regulate different downstream pathways to control similar cellular events. On the other hand, the activities of both kinases can be regulated by the same upstream molecule, Rac1 [9,28]. These facts imply that microtubule dynamics are complexly but coordinatedly regulated in the cerebral cortex during development.

Our experiments also suggest that Cdk5 suppresses mode I phosphorylation of MAP1B in developing cortical neurons, possibly through the inhibition of JNK activity. Furthermore, it was demonstrated that Cdk5 can phosphorylate MAP1B at mode I sites only when it associates with p25. These results suggest that conversion of p35 to p25 transforms Cdk5 from a negative regulator to a strong positive regulator for MAP1B phosphorylation. This conversion might be important in better understanding the pathology of neurodegenerative disorders, since both mode I phosphorylated MAP1B and p25 are frequently and abundantly observed in the neurofibrillary tangles of Alzheimer's disease [6,7,13,29].

Thus, mode I phosphorylation of MAP1B seems to be differentially regulated by Cdk5/p35, Cdk5/p25, and JNK in the developing as well as pathogenic brains. Further studies are required to understand the implications of MAP1B phosphorylation in the nervous system development and in neurodegenerative disorders.

Acknowledgments

We thank A. Takashima, S. Tamura, L.H. Tsai, and Y. Uchida for providing plasmids. We also thank R. Yu and M. Sone for helpful comments and technical help. This work was supported by the center of excellence (COE) grant and Grant-in-Aid for Scientific Research on Priority Areas, ''Elucidation of glia–neuron network mediated information processing systems (#16047220),'' and ''Membrane Traffic (#16044225)'' from the Ministry of Education, Culture, Sports and Science and Technology, Japan, and by grants from Japan Brain Foundation and Takeda Science Foundation.

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