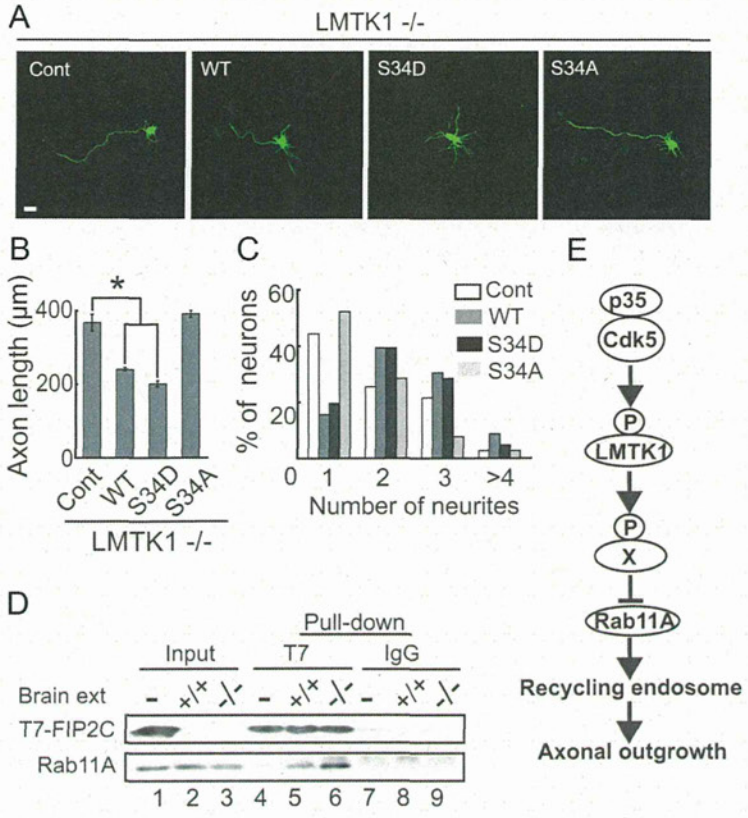

Figure 9. Expression of LMTK1 reverses the effect of *LMTK1* knock-out on axonal outgrowth. ***A***, Overexpression of LMTK1-WT or S34D reverses the *LMTK1* $^{-/-}$ phenotype. Empty vector (Cont), LMTK1-WT, LMTK1-S34D, and LMTK1-S34A were individually cotransfected with EGFP into *LMTK1* $^{-/-}$ neurons at DIV0, and axonal outgrowth was observed at DIV3. Scale bar, 20 μm. ***B***, Axon length in EGFP-expressing neurons (mean ± SEM of 90 neurons from three independent experiments; $^{*}p < 0.01$, Student's *t* test). ***C***, Number of neurites extending from EGFP-expressing neurons (90 neurons from three independent experiments). ***D***, Rab11A-GTP is increased in *LMTK1* $^{-/-}$ brains. T7-FIP2C was expressed in COS-7 cells (lane 1) and bound to protein G-Sepharose beads linked to anti-T7. T7-FIP2C-bound beads were incubated with brain extracts (ext) from *LMTK1* $^{+/+}$ (lane 2) and *LMTK1* $^{-/-}$ (lane 3) mice. Rab11A was pulled down and detected by immunoblotting with anti-Rab11A (bottom). T7-FIP2C was detected by immunoblotting with anti-T7 (top). Lane 4, Control T7-FIP2C beads; lane 5, Rab11A pulled down from *LMTK1* $^{+/+}$ mouse brain extract; lane 6, Rab11A from *LMTK1* $^{-/-}$ mouse brain extract. Control experiments using control IgG are shown in lanes 7–9. ***E***, Scheme representing the role of LMTK1 in axonal outgrowth. Cdk5 phosphorylates LMTK1 at Ser34. Phosphorylated LMTK1 may phosphorylate an unknown substrate (X), which would inhibit Rab11A activity, leading to reduced transport of recycling endosomes to the growing tip of the axon, resulting in suppression of its overelongation. The Cdk5-LMTK1-Rab11A pathway is a novel regulatory mechanism for axonal outgrowth of mammalian neurons.

high, LMTK1 would be phosphorylated and would suppress Rab11A activity to maintain the proper elongation rate of the axon.

Thus, Cdk5 plays an important role in the regulation of LMTK1 activity and then axonal outgrowth. According to the above scenario, Cdk5 would be a negative factor for axonal outgrowth. There is, however, disagreement on this point. Cdk5 phosphorylates the axonal cytoskeletal proteins CRMP-2 and MAP1B to reduce their interactions with microtubules (Brown et al., 2004; Uchida et al., 2005). Activation of Cdk5 also induces the shrinkage of growth cones (Sasaki et al., 2002). These results suggest that Cdk5 plays a negative role in axon formation. In contrast, previous studies have demonstrated a positive role for Cdk5 in neurite outgrowth: neurite length is reduced by dominant-negative Cdk5 and by the inhibition of Cdk5 by antisense oligonucleotides in cerebellar and cortical neurons (Nikolic et al., 1996; Paglini et al., 1998) and by the Cdk5 inhibitor roscovitine in retinal ganglion cells (Hahn et al., 2005). Overexpression of Mib1 ubiquitin ligase inhibits neurite outgrowth, and this inhibitory effect is blocked by p35 coexpression (Choe et al., 2007). These results indicate that Cdk5-p35 has a positive effect on axon formation, prompting us to examine the role of Cdk5 in axonal outgrowth using our experimental paradigm. When p35 was knocked down with shRNA, axon length was decreased slightly in cortical neurons (data not shown), consistent with the positive role of Cdk5-p35 in axonal outgrowth (Nikolic et al., 1996; Paglini et al., 1998) rather than our earlier suggestion to the contrary. However, when we conducted similar experiments with hippocampal neurons, p35 knockdown increased axonal outgrowth slightly (data not shown). Cdk5 is a multifunctional protein kinase that phosphorylates many cytoskeletal and membrane proteins. The knockdown effect of p35 would thus appear to be the overall outcome of various effects on the cytoskeleton, membrane trafficking and so on, instead of a simple output from the Cdk5–LMTK1 pathway. To understand the role of Cdk5 in axonal outgrowth, it would be necessary to investigate more axonal substrates for Cdk5 in detail and to determine how they are coordinated and integrated in the processes that lead to axonal outgrowth.

Axon length in *LMTK1*-null neurons was much longer than that in wild-type neurons. What is the mechanism? Neither axon formation nor polarity was affected by knockdown or knockout of LMTK1. Several molecules with axonal outgrowth activity determine polarity rather than regulate axon length itself (Polleux and Snider, 2010), but LMTK1 is not a polarity-establishing protein. Because total neurite length did not change after the overexpression of LMTK1-S34A or the knockdown of LMTK1, axons were lengthened at the expense of the elongation of other neurites. LMTK1 may modulate the direction of membrane sorting between the axon and dendrites. Alternatively, LMTK1 may activate the dynamics of Rab11A-dependent recycling endosomes only in the axon. LMTK1 may promote a specific subtype of recycling endosomes that contains neither neurotrophic factor receptors nor β-integrin adhesion protein. It would be interesting to determine what kind of recycling endosomes are conveyed under LMTK1 regulation. Depletion of the inhibitory LMTK1 activity should release the suppression of axonal outgrowth. In axons from *LMTK1* knock-out neurons, Rab11A-positive vesicles were abundant, dynamic, and rapidly transported anterogradely. These results suggest that membrane supply is a rate-limiting step in axonal elongation. The length of axons in *LMTK1* knock-out neurons was similar to that observed in LMTK1-S34A-overexpressing neurons, in which the membrane supply might have been excessive as a result of the activation of the Rab11A-dependent recycling endosome

pathway. The maximum elongation rate may also be determined by the ability of the cytoskeleton to lengthen the axon.

The axonal outgrowth activity of LMTK1-S34A was compromised by dominant-negative Rab11A-S25N, and LMTK1-S34D did not display negative activity in the presence of constitutively active Rab11A-Q70L, indicating that LMTK1 functions upstream of Rab11A. LMTK1 is the first and only reported factor upstream of Rab11A to act on axonal outgrowth. Regarding downstream effectors or Rab11-binding proteins, only two proteins, Rab coupling protein and protrudin, have been reported to play a role in neurite extension (Shirane and Nakayama, 2006; Eva et al., 2010). Because LMTK1 does not interact with Rab11A directly (Takano et al., 2010), there should be an unknown component that functions between LMTK1 and Rab11A (Fig. 9*E*, component X). Our previous results suggest that this component affects the exchange rate of guanine nucleotide bound to Rab11A (Takano et al., 2010). Because the kinase-negative mutant of LMTK1 lost axonal outgrowth inhibitory activity, as did LMTK1-S34A, it may be that LMTK1 functions by phosphorylating component X to suppress Rab11A activity (Fig. 9*E*). Although component X remains to be identified, we would like to propose that the Cdk5–LMTK1–Rab11A pathway is a novel signaling pathway that regulates axonal outgrowth during early neuronal development.

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Phosphorylation of p35 and p39 by Cdk5 determines the subcellular location of the holokinase in a phosphorylation-site-specific manner

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Summary

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family, which is activated by neuronal activators p35 or p39. Cdk5 regulates a variety of neuronal activities including migration, synaptic activity and neuronal death. p35 and p39 impart cytoplasmic membrane association of p35–Cdk5 and p39–Cdk5, respectively, through their myristoylation, but it is not clearly understood how the cellular localization is related to different functions. We investigated the role of Cdk5 activity in the subcellular localization of p35–Cdk5 and p39–Cdk5. Cdk5 activity affected the localization of p35–Cdk5 and p39–Cdk5 through phosphorylation of p35 or p39. Using unphosphorylated or phosphomimetic mutants of p35 and p39, we found that phosphorylation at Ser8, common to p35 and p39, by Cdk5 regulated the cytoplasmic localization and perinuclear accumulation of unphosphorylated S8A mutants, and whole cytoplasmic distribution of phosphomimetic S8E mutants. Cdk5 activity was necessary to retain Cdk5-activator complexes in the cytoplasm. Nevertheless, small but distinct amounts of p35 and p39 were detected in the nucleus. In particular, nuclear p35 and p39 were increased when the Cdk5 activity was inhibited. p39 had a greater propensity to accumulate in the nucleus than p35, and phosphorylation at Thr84, specific to p39, regulated the potential nuclear localization activity of the Lys cluster in p39. These results suggest that the subcellular localization of the Cdk5-activator complexes is determined by its kinase activity, and also implicate a role for p39–Cdk5 in the nucleus.

Key words: Cdk5, Subcellular localization, Membrane association, Nuclear translocation, Kinase activity, Neuron

Introduction

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family that is activated by the regulatory subunits p35 and p39. In contrast to the cell cycle-related Cdks in proliferating cells, Cdk5 plays a role in various activities unrelated to cell-cycle progression, including neuronal migration, synaptic activity and neuronal cell death in postmitotic neurons (Dhavan and Tsai, 2001; Shelton and Johnson, 2004; Cheung et al., 2006; Hisanaga and Endo, 2010). However, the molecular mechanisms by which Cdk5 regulates these activities are not fully understood. The subcellular localization of proteins is closely related to their function and, for active Cdk5, is determined by p35 and p39 (Nikolic et al., 1996; Humbert et al., 2000; Asada et al., 2008). p35–Cdk5 and p39–Cdk5 are predominantly cytoplasmic and membrane-associated due to myristoylation of p35 and p39 (Patrick et al., 1999; Asada et al., 2008). However, when Cdk5 is dysregulated by calpain-mediated cleavage of p35 to p25, the C-terminal fragment of p35, hyperactive p25–Cdk5 translocates to the nucleus, leading to cell death in neurodegenerative diseases (Patrick et al., 1999; Gong et al., 2003; Smith et al., 2006; Saito et al., 2007; Kim et al., 2008; Wen et al., 2008; Chang et al., 2011). In contrast, several reports also implicate p35 in nuclear Cdk5 activity, indicated by the presence of nuclear p35–Cdk5 complexes (Qu et al., 2002; Fu et al., 2004; Zhang et al., 2008). For a comprehensive understanding of Cdk5 function, it is important to determine how cytoplasmic and nuclear localization of active Cdk5 is regulated.

p39 is an isoform of p35 with 57% amino acid homology and a particularly high similarity (72%) in the C-terminal Cdk5 activation domain (Tang et al., 1995; Zheng et al., 1998). Gene deletion studies suggest that p39 has its own functions, though largely overlapping with p35. Mice lacking Cdk5 display perinatal lethality with extensive defects in neuronal organization of various brain regions (Ohshima et al., 1996). Mice lacking p35 show lamination defects in the cerebral cortex, but are viable and fertile (Chae et al., 1997; Hallows et al., 2003), whereas p39 deficiency does not show apparent abnormalities. However, double-null p35 and p39 mice display phenotypes identical to those of *Cdk5*$^{-/-}$ mice (Ko et al., 2001). Expression studies of p39 at transcriptional levels revealed that p39 is expressed in the more caudal brain at later stages of development than p35, which is expressed in rostral brain from the early stage of brain development (Wu et al., 2000; Takahashi et al., 2003). Because of the instability of the p39–Cdk5 complex and the lack of specific antibodies against p39, only a few biochemical and cell biological studies have been done on p39–Cdk5 (Humbert et al., 2000; Yamada et al., 2007; Asada et al., 2008). Although there are some reports describing the association of p39 with the actin cytoskeleton (Paglini et al., 1998; Humbert et al., 2000), further studies are required to identify the mechanisms of subcellular localization of p39–Cdk5 and p35–Cdk5.

There are two determinants in the N-terminal p10 region of p35 and p39 for the cytoplasmic membrane localization:

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myristoylation and the Lys cluster. Myristoylation, the N-terminal addition of a 14-hydrocarbon chain, is the primary determinant. Because myristoylation is not sufficient to anchor proteins to membranes, the Lys cluster is suggested to assist the binding to membranes by electrostatic interaction with phospholipids (McLaughlin and Aderem, 1995). Because myristoylated proteins are found in various intracellular membrane-bound compartments, it is unlikely that myristoylation determines the specific membrane compartments in which myristoylated proteins accumulate. Subcellular localization of proteins would be regulated by other factors, such as phosphorylation. p35 is phosphorylated at Ser8 and Thr138 by Cdk5 (Kamei et al., 2007; Hosokawa et al., 2010). Phosphorylation near myristoylation sites affects the interaction of myristoylated proteins to membranes (Taniguchi and Manenti, 1993; Hayashi and Titani, 2010), and Ser8 of p35 is located particularly close to the myristoylation site. Whether or not Cdk5 phosphorylation of p35 and p39 affects their subcellular localization has not previously been studied.

Another concern is nuclear Cdk5. Although the majority of p35–Cdk5 and p39–Cdk5 are cytoplasmic, the presence of nuclear Cdk5 has been suggested. The Lys cluster in both p35 and p39 conforms to the classical-type NLS (Dingwall and Laskey, 1991), and it is reported that importin β/5/7 binds to the Lys cluster of p35 to introduce p35 into the nucleus (Fu et al., 2006). Thus, the Lys clusters can serve a dual function for both nuclear and membrane localization (Maures et al., 2011). It is interesting to see in what cellular situations these Lys clusters act as the NLS in p35–Cdk5 and p39–Cdk5.

Here, we investigated the role of Cdk5 activity in the subcellular localization of p35–Cdk5 and p39–Cdk5. Phosphorylation at Ser8, common to p35 and p39, controls the cytoplasmic distribution of p35 and p39, and Thr84, specific to p39, suppressed the nuclear localization activity of the Lys cluster in p39. Further, Cdk5 activity was crucial to maintain the Cdk5-activator complexes in the cytoplasm. These results indicate that Cdk5 determines its cytoplasmic localization by its own kinase activity and suggest that p39 has a specific role in the nucleus.

Results

Cdk5-activity-dependent subcellular localization of p35 and p39

Cdk5 activators p35 and p39 determine the subcellular localization of Cdk5-activator complexes. We have reported that N-terminal myristoylation and the Lys cluster in the N-terminal p10 region of p35 and p39 are critical for their localization at the perinuclear region and at plasma membranes (Asada et al., 2008). Here, we studied the effect of the kinase activity of Cdk5 on p35 and p39 subcellular localization using an overexpression system in COS-7 cells. Since p35 and p39 are short-lived proteins, the amount of p35 and p39 expressed is lower than that of Cdk5 (Hosokawa et al., 2010). Further, active Cdk5 is always complexed with p35 or p39, indicating that the localization of the Cdk5 complexes should be examined with p35 or p39. When Cdk5 was coexpressed with p35 or p39 in COS-7 cells, both p35 and p39 displayed perinuclear and plasma membrane localization (Fig. 1A) (Asada et al., 2008). When Cdk5 activity is inhibited with roscovitine, a Cdk5 inhibitor, the perinuclear localization of both p35 and p39 was reduced and distributed more widely in the whole cytoplasm whereas their plasma membrane staining was still found (Fig. 1A, Cdk5+Ros).

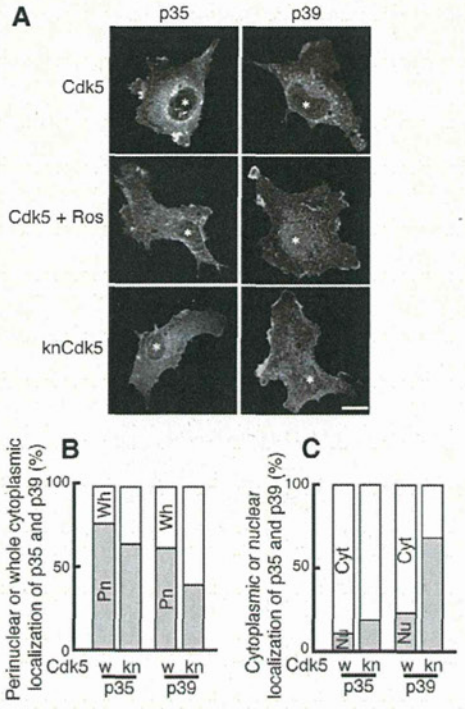

Fig. 1. Effect of Cdk5 activity on the intracellular localization of p35 and p39. (**A**) Subcellular distribution of p35 or p39 coexpressed with Cdk5. p35 or p39 was cotransfected with Cdk5 in the presence (+Ros) or absence of 20 μM roscovitine, or kinase negative (kn) Cdk5, into COS-7 cells. The subcellular localization of p35 and p39 is shown in left and right panels, respectively. The positions of the nucleus are indicated by asterisks. Scale bar: 10 μm. (**B**) Effect of Cdk5 activity on cytoplasmic distribution of p35 or p39. Perinuclear (Pn) and whole cytoplasmic (Wh) distribution of p35 or p39 coexpressed with Cdk5 (w) or knCdk5 (kn) were estimated as described in Materials and Methods, and their mean percentage ratios calculated (400 from three independent experiments). (**C**) Increased nuclear localization of p35 or p39 when coexpressed with knCdk5. The fluorescence of cytoplasmic or nuclear staining of p35 or p39 was measured as described in Materials and Methods and cells were grouped into two classes, higher cytoplasm staining (Cyt) and higher nuclear staining (Nu) and their mean percentage ratios calculated (400 from three independent experiments).

Similar results were obtained when the Cdk5 activity was suppressed by expression of kinase negative (kn) Cdk5 (Fig. 1A, knCdk5). When the perinuclear (Pn) and whole cytoplasmic (Wh) distribution were quantified as described in the Materials and Methods, coexpression with knCdk5 increased the ratio of cells with whole cytoplasmic distribution of p35 from 22.7% to 35% and of p39 from 37.5% to 60.2% (Fig. 2B). p39 showed diffuse distribution in the cytoplasm more so than p35, particularly when coexpressed with knCdk5.

In addition to these changes in the cytoplasmic distribution, there was increased staining in the nucleus for both p35 and p39 when the kinase activity was inhibited. While the nucleus was clearly less stained by p35 or p39 compared to the cytoplasm in the coexpression of wild-type Cdk5, the difference in the nuclear and cytoplasmic staining became obscured when Cdk5 activity was suppressed. In particular, the ratio of the nuclear staining became higher when p39 was coexpressed with knCdk5 (Fig. 1A, lower panels). It is important to determine if Cdk5 translocates to the nucleus or not, because nuclear Cdk5 is implicated in the

induction of neuronal cell death in many neurodegenerative diseases. To confirm higher nuclear staining, we measured fluorescence intensity in the cytoplasm and nucleus, and estimated the ratio of cells with higher cytoplasmic (Cyt) or nuclear (Nu) staining for both p35 and p39 (supplementary material Fig. S1; Fig. 1C). Coexpression with knCdk5 increased the number of cells with intensely p35 stained nuclei from 10.5% to 18.9% and with p39 from 23.1% to 68.3%. Further, when p35 and p39 were compared, p39 showed a stronger propensity to accumulate within the nucleus.

Phosphorylation of p35 by Cdk5 determines its cytoplasmic localization, and Cdk5 activity is involved in p35 nuclear localization

p35 is phosphorylated at Ser8 and Thr138 by Cdk5 (Kamei et al., 2007; Hosokawa et al., 2010). Therefore, there are two possibilities as to how Cdk5 activity affects subcellular localization; through phosphorylation of p35 or of other proteins. We examined these possibilities by using p35 mutants whose phosphorylation sites were replaced with Ala (unphosphorylated mutant 2A), or Glu (phosphomimetic mutant 2E; Fig. 2A). First, we expressed p35 WT, 2A and 2E with knCdk5 (Fig. 2B). In this experiment, we could evaluate the role of p35 phosphorylation independent of Cdk5 activity. p35 WT showed a similar staining pattern to p35 2A, which localized at the perinuclear and plasma membranes (Fig. 2Ba,b), but accumulation at the perinuclear region was less than p35 2A. In contrast, p35 2E displayed an even distribution throughout the whole cytoplasm (Fig. 2Bc). These results suggest that p35 phosphorylation at Ser8 and Thr138 affects its cytoplasmic distribution.

We can see the effect of Cdk5 activity on subcellular distribution by comparing the distribution of p35 2A coexpressed with Cdk5 or knCdk5. p35 WT, p35 2A or p35 2E coexpressed with Cdk5 is shown in Fig. 2Bd–f. Each p35 construct showed similar distribution patterns when coexpressed with knCdk5. Again, p35 2A showed a high concentration at perinuclear region and p35 2E showed weaker perinuclear staining and relatively diffuse cytoplasmic staining. A difference between Cdk5 and knCdk5 was nuclear staining of p35 2A (Fig. 2Bb,e). p35 2A showed a slightly increased nuclear staining when coexpressed with knCdk5 compared to expression with Cdk5. These results suggest that the cytoplasmic localization is controlled by phosphorylation of p35 by Cdk5 and nuclear translocation is regulated by Cdk5 activity in other ways.

Next, we examined the effect of the binding of Cdk5 to p35 on subcellular localization by expressing p35 WT, p35 2A and p35 2E alone. Since COS-7 cells express small amounts of Cdk5 endogenously, some of p35 expressed formed a complex with Cdk5 but the majority of p35 should be free from Cdk5 in this expression condition. This was confirmed by Phos-tag SDS-PAGE immunoblotting (supplementary material Fig. S2). While p35 bound to Cdk5 appeared as upward shifted bands by phosphorylation with Cdk5, p35 expressed alone moved to the unphosphorylated position as was observed with p35 coexpressed with knCdk5. Each p35 mutant showed almost identical staining patterns (Fig. 2C) to those expressed with knCdk5 as described above (Fig. 2B), indicating that the binding of Cdk5 did not affect the subcellular localization of p35.

We determined which of Ser8 or Thr138 is more critical for the cytoplasmic localization of p35 by using single Ala (A) and/or

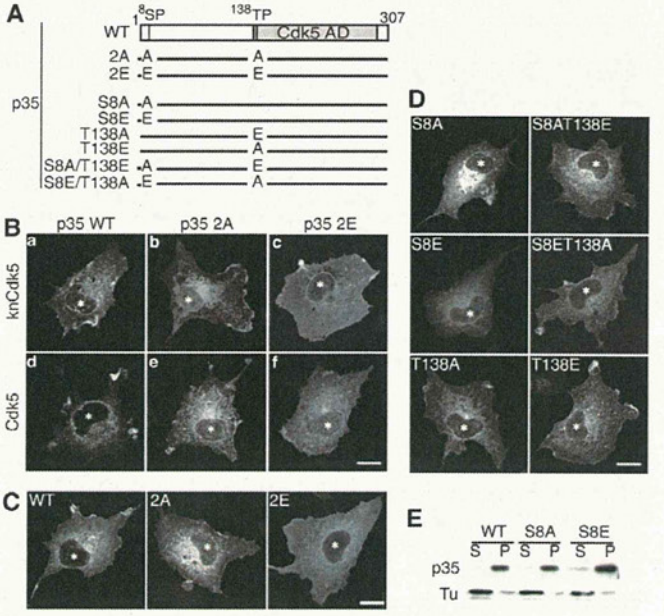

Fig. 2. Phosphorylation-site-specific effect on subcellular localization of p35. (**A**) Schematic representation of Ala or Glu mutants of p35 at Ser8 and Thr138 phosphorylation sites. The Cdk5 activation domain (Cdk5 AD) is present in the C-terminal half of p35, and Ser8 and Thr138 are major Cdk5-dependent phosphorylation sites (Kamei et al., 2007; Hosokawa et al., 2010). The Ala (A) or Glu (E) mutants at Ser8 and/or Thr138 are shown below. (**B**) The subcellular distribution of p35 WT (a,d), 2A (b,e) and 2E (c,f) complexed with knCdk5 (a–c) or Cdk5 (d–f). COS-7 cells were transfected with p35 WT, 2A or 2E in combination with Cdk5 or knCdk5, and the subcellular localization of p35 was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**C**) Subcellular localization of p35 WT, 2A and 2E. p35 WT, 2A or 2E was transfected into COS-7 cells, and their subcellular localization was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**D**) The subcellular localization of single Ala (A) or Glu (E) mutants of p35 in COS-7 cells. COS-7 cells were transfected with the indicated constructs of p35, and the subcellular localization was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**E**) Immunoblots indicating the association of p35 S8E with membranes. p35 WT, S8A or S8E was transfected into COS-7 cells and 24 h after transfection the post-nuclear extracts of COS-7 cells were centrifuged at 100,000 ***g*** for 1 h at 4℃ to separate the soluble (S) and membrane (P) fractions. Tubulin (Tu) was used as a marker of soluble proteins.

Glu (E) mutants. In this experiment, p35 mutants were expressed alone and their localization was observed (Fig. 2D). p35 S8A and p35 S8A/T138E showed greater localization at the perinuclear region and no plasma membrane staining (Fig. 2D, S8A, S8A/T138E). In contrast, p35 S8E and p35 S8E/T138A displayed a rather diffuse distribution in the cytoplasm by reducing perinuclear staining. Thr138 mutation to either Ala (p35 T138A) or Glu (p35 T138E) did not change the cytoplasmic distribution of p35. These results indicate that phosphorylation at Ser8 regulates the cytoplasmic distribution of p35, p35 accumulates at the perinuclear region when unphosphorylated, and evenly distributes throughout the whole cytoplasm when phosphorylated.

p35 associates with membranes via myristoylation at the N-terminal Gly residue (Patrick et al., 1999; Asada et al., 2008). Phosphorylation of Ser8 close to the myristoylation site may

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affect the interaction with membranes, and then induce the diffuse distribution of p35 2E or p35 S8E. We tested if phosphorylation may dissociate p35 from membranes by cell fractionation using the p35 S8E mutant (Fig. 2E). Postnuclear supernatants of COS-7 cells expressing p35 WT, p35 S8A and p35 S8E were separated into the soluble fraction in the supernatant (S) and membrane fraction in the pellet (P) by ultracentrifugation. Tubulin blotting (Tu) indicates the proper fractionation of soluble proteins. All p35 constructs were detected in the pellet fraction, indicating that p35 S8E still associates with membranes. These results suggest that Ser8 phosphorylation affects the distribution of p35 without causing dissociation from membranes.

Subcellular localization of p39–Cdk5 is also regulated by p39 phosphorylation and Cdk5 activity

We next investigated whether the subcellular localization of p39–Cdk5 is also controlled by both p39 phosphorylation and Cdk5 activity. Because p39 is a homologous isoform of p35, it is likely phosphorylated by Cdk5. However, it has not been shown that p39 is indeed phosphorylated by Cdk5 and if so, where Cdk5 phosphorylation sites reside. First, we examined whether p39 is phosphorylated by Cdk5 using Phos-tag SDS-PAGE (Kinoshita et al., 2006; Hosokawa et al., 2010). When p39 was expressed alone or with knCdk5 in HEK293 cells, p39 appeared as a single band at the faster migrating position (p39 in Fig. 3B). Coexpression with Cdk5 greatly shifted the electrophoretic mobility upward, indicating phosphorylation by Cdk5. There were two major and one minor p39 bands, suggesting three different phosphorylation states.

There are six SP and TP possible Cdk5 phosphorylation sites at Ser8, Thr84, Ser173, Ser205, Thr232 and Ser305 in p39 with the best consensus motif of (S/T)PX(K/R) at Ser173 (Fig. 3A). Sequence alignment with p35 using Genetics software (Genetics) indicated that Ser8 and Ser173 are sites corresponding to Ser8 and Thr138 of p35. We constructed Ala mutants of each phosphorylation site of p39, and used Phos-tag SDS-PAGE to confirm phosphorylation. Fig. 3C shows electrophoretic mobilities of each p39 Ala mutant together with p39 WT coexpressed with Cdk5 or knCdk5 for references. The change of the electrophoretic mobility is clear with Ser8 to Ala mutant (S8A) and Ser173 to Ala mutant (S173A). The Ala mutation at Ser205, Thr232 and Ser305 did not affect the mobility, indicating

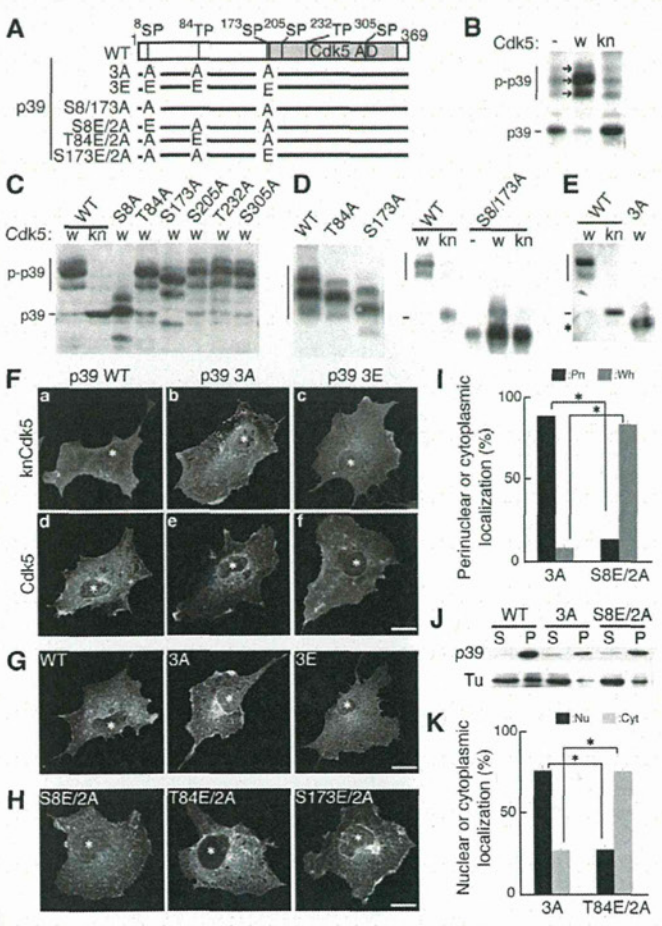

Fig. 3. Phosphorylation-site-specific effect on the subcellular localization of p39. (**A**) Schematic representation of p39 and its mutant constructs. Among six SP/TP possible phosphorylation sequences for Cdk5 (upper panel), Ser8, Thr84 and Ser173 were identified to be phosphorylation sites in following experiments (B–E). The Ala (A) or Glu (E) mutants at the phosphorylation sites are described below. (**B**) Immunoblot showing Cdk5-dependent phosphorylation of p39. p39 was expressed alone (–) or with Cdk5 (w) or knCdk5 (kn) in HEK293 cells. Phosphorylation of p39 was analyzed by immunoblotting following Phos-tag SDS-PAGE as described in Materials and Methods. (**C**) Phos-tag SDS-PAGE immunoblot of p39 or its Ala mutants at possible Cdk5 phosphorylation sites. p39 or its Ala mutants were expressed in HEK293 cells. Immunoblotting of p39 was performed after Phos-tag SDS-PAGE using 50 μM Phos-tag. (**D**) Left panel: Phos-tag SDS-PAGE immunoblot of p39 WT, T84A and S173A. To see the phosphorylation-dependent electrophoretic mobility shift more clearly, the concentration of Phos-tag was increased to 150 μM. Right panel: Phos-tag SDS-PAGE immunoblot of p39 S8/173A expressed alone (–) or coexpressed with Cdk5 (w) or knCdk5 (kn) in HEK293 cells. p39 (WT) coexpressed with Cdk5 (w) or knCdk5 (kn) is shown for reference. (**E**) Phos-tag SDS-PAGE immunoblot of p39 3A. p39 3A was expressed with Cdk5 in HEK293 cells and its phosphorylation state was examined by immunoblotting after Phos-tag (50 μM) SDS-PAGE. p39 coexpressed with Cdk5 (w) or knCdk5 (kn) is shown for reference. (**F**) The subcellular localization of p39 WT, 3A or 3E complexed with knCdk5 or Cdk5. p39 WT, 3A or 3E was transfected into COS-7 cells together with knCdk5 or Cdk5, and 24 h after transfection the subcellular localization of p39 or its mutants was examined. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**G**) Subcellular localization of p39 WT, 3A, or 3E. p39 WT, 3A, or 3E was transfected alone in COS-7 cells, and their subcellular localization was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**H**) Subcellular distribution of single site Glu (E) mutants of p39. p39 S8E/2A, T84E/2A or S173E/2A was expressed in COS-7 cells, and their subcellular localization was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**I**) Effect of a Glu (E) mutation at Ser8 on cytosolic distribution of p39. The perinuclear (Pn) and whole cytoplasmic (Wh) distribution of p39 3A or S8E/2A was quantitatively measured using ZEN software and is expressed as a ratio of the total in the cell (means ± s.e.m., n=500 from three independent experiments; *P<0.001, Student's t-test). (**J**) Immunoblots to determine the membrane association of p39 S8E/2A. p39 WT, 3A or S8E/2A was expressed in COS-7 cells and the cell homogenates were fractionated into the soluble (S) and membrane (P) proteins by centrifugation at 100,000 ***g*** for 1 h at 4℃ after brief centrifugation at 100 ***g*** for 5 min. Tubulin (Tu) was a marker of the soluble fraction. (**K**) Effect of a Glu (E) mutation at Thr84 in the nuclear translocation of p39. The cytoplasmic and nuclear staining of p39 3A and p39 T84E/2A was measured using Zen software, and the percentage ratios of the higher nuclear staining cells (Nu) and higher cytoplasmic staining cells (Cyt) were expressed as the means ± s.e.m. (n=500 from three independent experiments; *P<0.001, Student's t-test).

that they are not phosphorylation sites. Since Ala mutation at Thr84 (T84A) showed slightly faster but equivocal migration on SDS-PAGE using 50 μM Phos-tag, it was reexamined by increasing Phos-tag at 150 μM. As shown in the left panel of Fig. 3D, p39 T84A showed faster migration than p39. Further, to confirm Thr84 phosphorylation by Cdk5, we used an opposite type of construct, p39 double Ala mutant at Ser8 and Ser173 with intact Thr84 (S8/173A). A part of p39 S8/173A appeared as a slower migrating band when it was co-expressed with Cdk5 but not with knCdk5 (Fig. 3D, right panel), indicating Thr84 is also a Cdk5 phosphorylation site. Next, we examined whether these three sites encompass all of Cdk5-dependent phosphorylation sites in p39, using a triple Ala mutant (3A) at Ser8, Thr84 and Ser173. p39 3A exhibited faster migration (Fig. 3E, asterisk), than p39 coexpressed with knCdk5 indicating not only that these three sites cover all Cdk5 phosphorylation sites but also the possibility that one of these phosphorylation sites could induce additional phosphorylation by other kinases. These results indicate that p39 is phosphorylated by Cdk5 at Ser8, Thr84 and Ser173.

We also constructed triple Glu (3E) mutants at these sites of p39 and examined their subcellular distribution after coexpression with knCdk5 or Cdk5 (Fig. 3F). While p39 3A showed perinuclear and plasma membrane staining, p39 3E distributed more evenly in the whole cytoplasm as was observed with p35 2E. Comparison between p39 3A coexpressed with knCdk5 and Cdk5 revealed again stronger nuclear staining of p39 3A when coexpressed with knCdk5 (Fig. 3Fb,e). These results suggest that as well as p35, phosphorylation of p39 affects the cytoplasmic localization of p39–Cdk5 and the kinase activity of Cdk5 modulates its nuclear translocation.

Next, we examined the effect of Cdk5 binding on subcellular distribution of p39. Even when p39 WT, p39 3A, or p39 3E was expressed alone (Fig. 3G), each showed a distribution similar to those expressed with knCdk5 (Fig. 3F), indicating that the binding of Cdk5 does not affect the localization of p39.

To identify the critical phosphorylation site, we constructed single Glu (E) mutants with Ala at the other two sites, p39 S8E/2A, p39 T84E/2A, and p39 S173E/2A, and examined their subcellular localization (Fig. 3H). p39 S8E/2A distributed throughout cells (Fig. 3H, S8E/2A). By comparing the distribution pattern of p39 S8E/2A with p39 3A, it was known that phosphorylation at Ser8 reduces the perinuclear accumulation of p39. To confirm the role of Ser8 phosphorylation quantitatively, we counted the number of cells with perinuclear and whole cytoplasmic staining. The results shown in Fig. 3I clearly indicate that Ser8 mutation to Glu (E) makes the distribution of p39 uniform in the whole cytoplasm. The association of p39 S8E/2A with membranes is confirmed by cell fractionation. p39 S8E/2A expressed in COS-7 cells was recovered in the pellet fraction by ultracentrifugation as well as p39 WT or p39 3A (Fig. 3J). These results indicate that phosphorylation at Ser8 regulates the cytoplasmic localization of p39–Cdk5 and its unphosphorylated form accumulates at the perinuclear region, as was shown with p35.

p39 T84E/2A was found mainly at the perinuclear region with the loss of its nuclear staining (Fig. 3H). We compared quantitatively the number of cells with nuclear staining between p39 T84E/2A and p39 3A. As shown in Fig. 3K, while more than 70% of cells expressing p39 T84E/2A showed weaker nuclear staining, more than 70% of cells expressing p39 3A showed higher nuclear staining. These results suggest that phosphorylation at Thr84 plays a role in the nuclear localization of p39–Cdk5.

In contrast to S8E or T84E mutant, p39 S173E mutant did not display the difference from the distribution of p39 3A, indicating that phosphorylation at Ser173 does not influence the subcellular localization of p39. Together, these results suggest that in the case of p39, two different phosphorylation sites play a role in different aspects of p39 localization; Ser8 in the cytoplasmic distribution and Thr84 in nuclear localization.

The nuclear localization activity of the Lys cluster at amino acids 75–79 in p39

Phosphorylation at Thr84 in p39 decreased nuclear localization of p39 (Fig. 3H,K). p39 has a basic amino acid region at the N-terminal side of Thr84, seven Lys residues in amino acids 75–86 and four continuous Lys residues from amino acids 75–79 (Fig. 4A), conforming to the complete nuclear localization signal (NLS). The corresponding Lys cluster in p35 is shown to work as NLS (Fu et al., 2006). Further, there are many instances in which phosphorylation close to NLS plays a regulatory role against the NLS activity (Sekimoto et al., 2004; Mahalakshmi et al., 2007). Therefore, we examined whether the Lys cluster of p39 has the NLS activity or not. For this purpose, we mutated four Lys residues at amino acids 75–78 to Ala, p39 K75–78A, and expressed it in COS-7 cells. As we showed in Fig. 3, p39 localized to the perinuclear region when coexpressed with Cdk5

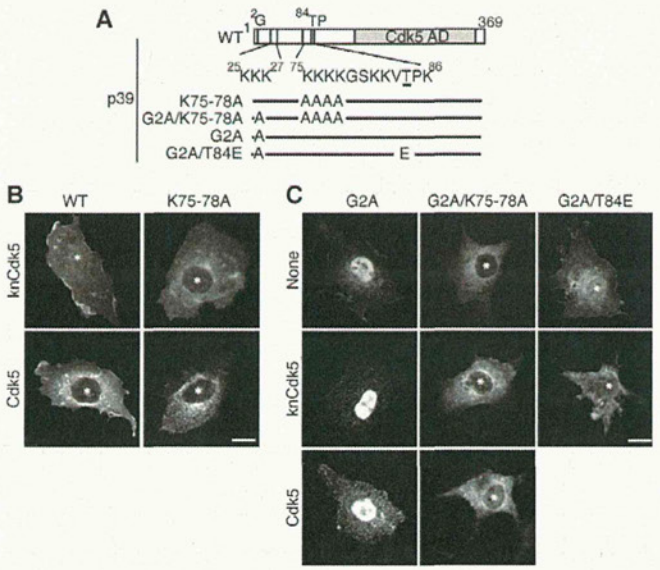

Fig. 4. The role of the Lys cluster in p39 on its nuclear localization and its regulation by Thr84 phosphorylation. (**A**) Schematic representation of p39 indicating the positions and amino acid sequences of the Lys clusters. The Thr84 phosphorylation site and the G2 myristoylation sites are also indicated. Ala (A) mutants of Gly2 or Lys residues at 75KKKK78, and a Glu (E) mutant at T84 are shown below. (**B**) Subcellular distribution of p39 WT and K75–78A. p39 WT or K75–78A was coexpressed with knCdk5 or Cdk5 in COS-7 cells and their subcellular localization was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**C**) Subcellular distribution of p39 G2A, G2A/K75–78A or G2A/T84E. p39 G2A, G2A/K75–78A or G2A/T84E was transfected alone (None) or with knCdk5 or Cdk5 in COS-7 cells and their subcellular distribution was examined 24 h after transfection. Nuclei are indicated by asterisks. Scale bar: 10 μm.

(Fig. 4B, lower left). A part of p39 was found in the nucleus when p39 was coexpressed with knCdk5 (Fig. 4B, upper left). This nuclear staining was greatly reduced with p39 K75–78A coexpressed with knCdk5 (Fig. 4B, upper right). Concomitantly, plasma membrane staining was lost with p39 K75–78A whether it was coexpressed with Cdk5 or knCdk5 (Fig. 4B, right panels), suggesting that K75–78 also participates in the plasma membrane binding of p39.

The cytoplasmic retention of p39 is conferred by myristoylation at Gly2. To increase the nuclear localization activity of p39, we used G2A mutant of p39, in which Gly2 is replaced with Ala to inhibit myristoylation (Fig. 4A). p39 G2A showed strong nuclear accumulation (Fig. 4C, upper left) as was reported previously (Asada et al., 2008). Coexpression with knCdk5 eliminated p39 completely from the cytoplasm (Fig. 4C, left panel of middle row), whereas coexpression with Cdk5 resulted in some p39 G2A remaining in the cytoplasm (Fig. 4C, left panel of bottom row). In contrast, p39 G2A with K75–78A mutation localized mainly in the cytoplasm either in the absence or presence of Cdk5 (Fig. 4C, middle panels), indicating that the basic amino-acid clusters in amino acids 75–86 in p39 have the NLS activity.

We suggested in Fig. 3 that phosphorylation at Thr84 regulates nuclear translocation of p39. To illustrate this more clearly, we introduced a Glu mutation in p39 G2A to mimic the phosphorylated state at Thr84 (Fig. 4A). T84E mutation greatly reduced nuclear accumulation of p39 G2A (Fig. 4C, right panel of upper row), indicating that Thr84 phosphorylation inhibits the nuclear localization activity of K75–78 in p39 G2A. This effect was not affected by binding to knCdk5 (Fig. 4C, right panel of middle row). These results show that the Lys cluster at amino acids 75–78 of p39 has the NLS activity and phosphorylation at Thr84 modulates the activity.

Nuclear Cdk5 has been suggested to induce cell death of neurons. p35 G2A or p39 G2A accumulating exclusively in the nucleus of COS-7 cells would provide the experimental paradigm to see the role of nuclear p35–Cdk5 or p39–Cdk5 in cultured cells. We examined cell death of COS-7 or Neuro-2a cells with p35 G2A–Cdk5 or p39 G2A–Cdk5 in the nucleus by LDH activity, nuclear condensation and TUNEL staining. However, we could not detect the increased cell death of those cells transfected with p35 G2A–Cdk5 or p39 G2A–Cdk5, compared with those expressing p35–knCdk5 or p39-knCdk5 or control mock transfected cells (data not shown).

The localization of p39–Cdk5 in primary neurons is not affected by p35 expression

In the above experiments, we expressed p35 and p39 separately in COS-7 cells to observe their respective cellular localization. However, p35 and p39 are co-expressed in neurons. To see if the localization of p39 is affected by p35, we examined the effect of p35 knockdown on the localization of p39 using primary cultured cortical neurons. Knockdown of p35 is indicated by immunoblotting (Fig. 5A) and immunostaining (arrowhead in KD of Fig. 5B). p35 showed dotted staining mainly at the perinuclear region of control neuron (Fig. 5B, upper left) as was reported previously (Asada et al., 2008). Because of the lack of anti-p39 antibody available for immunostaining, we cotransfected the plasmid encoding p39-myc into primary neurons with p35 knockdown or control scramble vector, where transfected neurons were indicated by GFP (Fig. 5C). p39

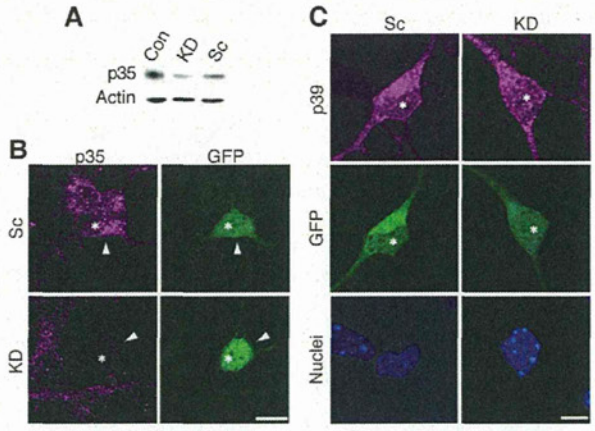

Fig. 5. Effect of p35 knockdown on the localization of p39 expressed in primary cortical neurons. (**A**) Immunoblot analysis of p35 in control neurons (Con) or in neurons transfected with p35 knockdown shRNA (KD) or scrambled shRNA (Sc) vector. (**B**) Immunostaining of p35 in cultured primary neurons to show the knockdown of p35. p35 knockdown or scrambled vector was transfected in primary cortical neurons, followed by immunostaining with anti-p35 antibody. GFP was used as a marker of transfection. Arrowheads indicate transfected neurons. Nuclei are indicated by asterisks. Scale bar: 10 μm. (**C**) Subcellular distribution of p39 in neurons. p35 knockdown or scramble vector was co-transfected with p39-myc in primary cultured neurons and the subcellular localization of p39 was examined with anti-myc antibody. GFP was used as the transfection marker. Nuclei are indicated by asterisks. Scale bar: 10 μm.

showed dotted distribution in the cytoplasm of cell soma and neurites with strongest staining at the perinuclear region when transfected alone or with control vector (Fig. 5C, left panel). The staining patterns were not changed by p35 knockdown (Fig. 5C, right panel). These results suggest that the cellular localization we observed in COS-7 cells can reflect those of p35 and p39 in neurons.

Discussion

Disruption of the subcellular localization system of Cdk5 causes neurodegenerative disease. However, it is not understood how the localization of Cdk5 is regulated. We investigated the effect of Cdk5 activity on the subcellular localization of the Cdk5 complexes, p35–Cdk5 and p39–Cdk5. Cdk5 activity affected the localization of p35 and p39, representative of the Cdk5 complexes, in two distinct manners. Phosphorylation at Ser8, the phosphorylation site common to p35 and p39, by Cdk5 affected the cytoplasmic localization of p35 and p39, perinuclear or whole cytoplasm. Moreover, Cdk5 activity excluded p35 and p39 from the nucleus. Nevertheless, p39 showed stronger nuclear localization than p35. This is because of a larger number of Lys in the N-terminal p10 region of p39. Phosphorylation at Thr84, which is a p39-specific phosphorylation site in the C-terminal side of the Lys cluster, countered the stronger nuclear localization activity of p39. These findings suggest that the cytoplasmic localization of the Cdk5-activator complex is determined by its kinase activity, and that the nuclear transported p39 would confer a specific function to p39–Cdk5 in the nucleus.

p35–Cdk5 and p39–Cdk5 are cytoplasmic protein kinases, which differ from other Cdks that play roles primarily in the nucleus of proliferating cells. Ectopic activation of cell cycle

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proteins in the nucleus of post-mitotic neurons induces neuron death (Herrup and Yang, 2007), which could be a cause of many neurodegenerative diseases. Cdk5 has the kinase activity very similar to Cdk1-cyclin B (Hisanaga et al., 1995). Therefore, it is very important to exclude Cdk5 activity from the nucleus for neurons to avoid cell death. We have previously shown that both p35 and p39 are myristoylated and that the myristoylation that anchors p35–Cdk5 and p39–Cdk5 to membranes is the primary mechanism keeping active Cdk5 in the cytoplasm. Here, we report Cdk5 activity as a second mechanism of p35–Cdk5 and p39–Cdk5 localization. We found that the inhibition of Cdk5 activity increased nuclear p35 and p39. In other words, the Cdk5 activity excludes p35–Cdk5 and p39–Cdk5 from nucleus. Since it is currently undetermined whether Cdk5 activity affects generally the nuclear import or export machinery, here we would like to discuss the issues related only to the Cdk5 complexes.

In spite of the predominant cytoplasmic localization of p35 and p39, a small but distinct number of cells showed nuclear staining for p35 and p39, even when they were coexpressed with Cdk5. Inhibition of kinase activity increased the ratio, particularly for p39, approximately 70% of p39 expressing cells showing stronger nuclear staining of p39. These results indicate that a certain amount of p35 and p39 can enter into the nucleus. Nuclear Cdk5 or nuclear free p35 is suggested to prevent re-entry of post-mitotic neurons into cell cycle (Zhang et al., 2008; Zhang et al., 2010). On the other hand, it is well documented that dysregulation of Cdk5 activity by cleavage of p35 to p25 by calpain induces neuronal death (Patrick et al., 1999; Gong et al., 2003; Smith et al., 2006; Saito et al., 2007; Kim et al., 2008; Wen et al., 2008; Chang et al., 2011). p25–Cdk5, which has lost p35 membrane binding sites, is shown to translocate into the nucleus to exert its cell death activity. A large amount of p25 is produced when calcium homeostasis is disrupted by strong stressors or death signals. However, neurons are excitable cells, in which a local cytoplasmic calcium increase occurs when excited. A subtle amount of p25–Cdk5 may be produced at the time of excitation. Cdk5 activity may be required to inhibit nuclear transport of the membrane-unbound Cdk5 complex, such as excitation-induced p25–Cdk5. This could be a reason for cells to prepare a second system to prevent the accumulation of active Cdk5 in the nucleus.

p39 displayed stronger nuclear staining than p35. This was always observed with any combination of p39 and Cdk5 constructs used here. p39 should have a greater propensity to enter into the nucleus than p35. This made us consider the distinct meanings of nuclear localization between p35 and p39. We initially suspected that cells expressing high nuclear p35 would undergo cell death, but there were no differences in cell death between cells, either COS-7 or Neuro-2a, expressing p35–Cdk5 and p39–Cdk5. Even though their nuclear accumulation was enhanced by using G2A mutants, the levels of the cell death did not increase more that those observed by mock or knCdk5 transfection. These results may suggest that the Cdk5 complexes have a physiological, but not apoptotic, role in the nucleus. Several nuclear functions have been proposed for p35 (Qu et al., 2002; Li et al., 2004; Tian et al., 2009), including phosphorylation of transcription factors such as STAT3 or MEF2 (Gong et al., 2003; Fu et al., 2004). Since p39–Cdk5 has similar kinase activity to p35–Cdk5 (Yamada et al., 2007), some of them, which have been assigned to p35–Cdk5, might be carried out by p39–Cdk5.

The higher nuclear localization of p39 can be explained by the amino acid sequence of the N-terminal p10 regions that contains a number of Lys residues including four consecutive Lys residues at amino acids 75–78 and the three Lys residues at positions 25–27 (Fig. 4A). The Lys cluster of amino acids 75–78 was indeed shown to have NLS activity using an Ala mutant. The NLS activity was more clearly shown with the G2A mutant of p39. The strong nuclear accumulation of p39 G2A was eliminated by Ala mutation of Lys75–78. It is reported that importin β/5/7 bind to the Lys cluster of p35 at amino acids 61-63 and transports p35 into the nucleus (Fu et al., 2006). The comparable amino acid sequence around the Lys cluster of p39 suggest that a similar importin species binds to p39 for translocation into the nucleus. Further, phosphorylation at Thr84, close to Lys75–78 inhibited nuclear accumulation of p39. As is known for importin-dependent nuclear transport of several proteins, nuclear transport of p39 is also regulated by phosphorylation.

While p35 and p39 WT were mainly present at the perinuclear region in the cytoplasm, p35 S8E and p39 S8E distributed relatively evenly in the whole cytoplasm. In contrast, p35 2A and p39 3A showed higher accumulation in a more compact area of the perinuclear region. Thus, phosphorylation at Ser8 appears to regulate the cytoplasmic localization of p35 and p39. However, there was disagreement between the results using knCdk5 (Fig. 1B) and S8E mutants of p35 and p39 (Fig. 2C, Fig. 3H,I); the inhibition of Cdk5 activity by knCdk5 induced the whole cytoplasmic distribution, as was observed with S8E phosphorylation mimic mutants. The results with knCdk5 may be explained in the following manner. Cdk5 activity is required for maintaining perinuclear localization and its inhibition by knCdk5 may allow the transport of the Cdk5 complexes into the nucleus from the perinuclear region, resulting in the decreased perinuclear staining and apparent whole cytoplasmic distribution. In fact, most cells with the nuclear staining displayed a low perinuclear accumulation (see Fig. 1A for example). Alternatively, dynamic phosphorylation/dephosphorylation reaction of Ser8 may be needed for perinuclear accumulation.

Considering that Ser8 is only six amino acids apart from the N-terminal myristoylation site within the completely identical first N-terminal nine amino acids of p35 or p39, phosphorylation at Ser8 may influence the interaction of p35 and p39 with membranes via myristoylation. MARCKS, a myristoylated protein, showed dissociation from the membranes by phosphorylation (Taniguchi and Manenti, 1993). However, the phosphorylation did not induce dissociation of p35 or p39 from membranes (Fig. 2E, Fig. 3J). It seems more likely that Ser8 phosphorylation modifies the interaction of p35 and p39 with membranes directly or through other membrane associated proteins. Introduction of a negative charge at Ser8 may reduce the interaction of p35 and p39 with phospholipids in membranes, and increases their mobility on membranes, leading to dispersion to the whole cytoplasm. Otherwise, phosphorylation may abolish the binding to a protein, which anchors p35 or p39 on the perinuclear membrane compartment. By these mechanisms, in any case, the phosphorylation may change the membranes with which p35 or p39 associates or the distribution of membranes to which p35 or p39 binds. p35 localizes to recycling endosomes or Golgi apparatus at the perinuclear region (Paglini et al., 2001; Tsutsumi et al., 2008; Takano et al., 2010). p39 is also present on recycling endosomes and partly in the Golgi apparatus (supplementary material Fig. S3) (Asada et al., 2008).