Phosphorylation is a major post-translational modification widely used in the regulation of many cellular processes. Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase activated by activation subunit p35. Cdk5-p35 regulates various neuronal activities such as neuronal migration, spine formation, synaptic activity, and cell death. The kinase activity of Cdk5 is regulated by proteolysis of p35: proteosomal degradation causes down-regulation of Cdk5, whereas cleavage of p35 by calpain causes overactivation of Cdk5. Phosphorylation of p35 determines the proteolytic pathway. We have previously identified Ser8 and Thr138 as major phosphorylation sites using metabolic labeling of cultured cells followed by two-dimensional phosphopeptide mapping and phosphospecific antibodies. However, these approaches cannot determine the extent of p35 phosphorylation in vivo. Here we report the use of Phos-tag SDS-PAGE to reveal the phosphorylation states of p35 in neuronal culture and brain. Using Phos-tag acrylamide, the electrophoretic mobility of phosphorylated p35 was delayed because it is trapped at Phos-tag sites. We found a novel phosphorylation site at Ser51, which was phosphorylated by Ca2+-calmodulin-dependent protein kinase II in vitro. We constructed phosphorylation-dependent banding profiles of p35 and Ala substitution mutants at phosphorylation sites co-expressed with Cdk5 in COS-7 cells. Using the standard banding profiles, we assigned respective bands of endogenous p35 with combinations of phosphospecific antibodies. These are the first quantitative and site-specific measurements of phosphorylation of p35, demonstrating the usefulness of Phos-tag SDS-PAGE for analysis of phosphorylation states of in vivo proteins.

Molecular & Cellular Proteomics 9:1133–1143, 2010.
postmitotic neurons and regulates various neuronal events such as neuronal migration, spine formation, synaptic activity, and cell death (22–24). Cdk5 is activated by binding to activation subunit p35 and inactivated by proteosomal degradation of p35 (25). In addition, Cdk5 activity is deregulated by cleavage of p35 to p25 with calpain, resulting in abnormal activation and ultimately causing neuronal cell death (26–29). Proteolysis of p35, either by proteosomal degradation or cleavage by calpain, is regulated by phosphorylation of p35 by Cdk5 (30–33). Therefore, phosphorylation of p35 is essential for proper regulation of Cdk5 activity and function. We previously identified Ser9 and Thr138 as major p35 phosphorylation sites (33). We also showed that phosphorylation of p35 decreased during brain development and proposed its relationship to age-dependent vulnerability of neurons to stress stimuli (32). Thus, to understand the in vivo regulation of Cdk5 activity, it is critical to analyze the phosphorylation states of p35 in brain. However, there is no convenient method to analyze the precise in vivo phosphorylation status of the endogenous proteins.

In this study, we applied the Phos-tag SDS-PAGE method to analyze the phosphorylation states of p35 in vivo and in cultured neurons. We constructed standard band profiles of phosphorylated p35 by Phos-tag SDS-PAGE using Ala mutants at Ser and/or Thr as major p35 phosphorylation sites (33). From these experiments, we observed an unidentified in vivo phosphorylation site at Ser91. We quantified the phosphorylation at each site in cultured neurons and brain, providing the first quantitative estimate of the in vivo phosphorylation states of p35. We discuss the usefulness of Phos-tag SDS-PAGE to analyze the in vivo phosphorylation states of proteins.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Antibodies, and Expression Plasmids—** Phos-tag acrylamide was purchased from NARD Institute Ltd. (Amagasaki, Japan). Microcystin LR and bacterial alkaline phosphatase (BAP) were obtained from Wako (Osaka, Japan). N-Methyl-d-aspartate (NMDA) was obtained from Sigma. Pefabloc SC was obtained from Merck. Polyfect transfection reagent was purchased from Qiagen (Hilden, Germany). The site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). [γ-32P]ATP was obtained from PerkinElmer Life Sciences. Anti-p35 (C19) and anti-Cdk5 (DC17) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Thr[32P] was obtained from Millipore (Billerica, MA). Anti-Ser was obtained from Millipore (Billerica, MA).

**Phosphorylation of p35 Analyzed by Phos-tag SDS-PAGE**

Phosphorylation of p35 was analyzed by Phos-tag SDS-PAGE when [32P]ATP was added to cell extracts. The phosphorylated peptides were then detected by autoradiography. The Phos-tag acrylamide gels were washed with transfer buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM DTT) containing protease inhibitors (0.4 mM Pefabloc SC and 10 μg/ml leupeptin) and phosphatase inhibitors (10 mM β-glycerophosphate, 5 mM NaF, 1 mM Na3VO4, and 1 μM Microcystin LR). After centrifugation at 10,000 × g for 15 min, the supernatant was collected as COS-7 cell extracts. For dephosphorylation experiments, cells were homogenized in 100 mM Tris-HCl, pH 8.0, containing protease inhibitors, and the cell extracts were incubated with 2 units of BAP at 37 °C for 2 h.

Mice (ICR) or rats (Wistar S/T) were obtained from Sankyo Labo (Tokyo, Japan). Brain cortex of rats at postnatal day 1 (P1) was homogenized in homogenization buffer (25 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM DTT) with a Teflon pestle homogenizer. After centrifugation at 10,000 × g for 15 min, the supernatant was collected as the rat brain extract. Brain cortices of embryonic day 17 (E17) or adult (10-week-old) mice were homogenized in RIPA buffer containing protease inhibitors and phosphatase inhibitors with a Teflon pestle homogenizer. After centrifugation at 10,000 × g for 15 min, the supernatant was collected as fetal or adult mouse brain extract. Rat and mouse brain extracts were boiled in Laemmli sample buffer and subjected to SDS-PAGE or Phos-tag SDS-PAGE.

**Primary Neuronal Culture and Glutamate or NMDA Treatment—** Cerebral cortical neurons were prepared from E17–E18 rat brains as described (31). Neurons were treated with 100 μM glutamate or NMDA for 1 or 5 min at 10 days in vitro (DIV 10) and were immediately lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors.

**Phosphorylation of p35 by CaMKII—** p35 kinase-negative Cdk5 D144N (knCdk5) complex was purified from S9H cells as described (35). p35 was phosphorylated by 0.5 μg/ml CaMKII in the presence of 50 μg/ml calmodulin and 0.5 mM CaCl2 in MOPS buffer (20 mM MOPS, pH 7.2, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM MgCl2, 1 mM ATP) at 37 °C for 60 min. Phosphorylation was detected by electrophoretic mobility shift following SDS-PAGE or by autoradiography after SDS-PAGE when [γ-32P]ATP was used for phosphorylation.

**Laemmli SDS-PAGE, Phos-tag SDS-PAGE, and Immunoblotting—** Laemmli SDS-PAGE was carried out with 10% polyacrylamide gels. Proteins were transferred to PVDF (Millipore, Bedford, MA) membranes using a semidyblotting apparatus. Phos-tag SDS-PAGE was performed with 7.5% polyacrylamide gels containing 50–100 μM Phos-tag acrylamide and 100–200 μM MnCl2. After electrophoresis, Phos-tag acrylamide gels were washed with transfer buffer (50 mM Tris, 384 mM glycine, 0.1% SDS, 20% methanol) containing 1 mM EDTA for 10 min with gentle shaking and then with transfer buffer without EDTA for 10 min according to the manufacturer’s protocol. Proteins were transferred to PVDF membranes using a submarine blotting apparatus. Membranes were probed with anti-p35, anti-Ser, anti-Thr, or anti-Cdk5. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Immunodetection was carried out with an ECL system (GE Healthcare) or Millipore Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore).

**Quantitative Measurement and Statistical Analysis—** Immunoreaction was captured as digital images (eight-bit gray scale) using a Chemidoc XRS apparatus (Bio-Rad) or by scanning χ-ray film, and band intensities were measured by Image J software. The digital

1134 Molecular & Cellular Proteomics 9.6
Phosphorylation of p35 Analyzed by Phos-tag SDS-PAGE

Fig. 1. Comparison of electrophoretic mobility shift of p35 using Laemmli and Phos-tag SDS-PAGE. Brain extracts prepared from rat at P1 were incubated at 37 °C for 60 min in the presence or absence of 1 mM ATP and/or 10 μM Microcystin LR (MC) as indicated. The extracts were subjected to Laemmli SDS-PAGE (upper panel) or Phos-tag SDS-PAGE (lower panel), and p35 was detected by immunoblotting with anti-p35. Lane 1 is the brain extract before incubation.

We first examined whether Phos-tag SDS-PAGE can be used to analyze the phosphorylation states of p35. p35 is phosphorylated at Ser^8 by Cdk5 in adult rat brain (33). p35 in P1 rat brain extract was compared between Laemmli and Phos-tag SDS-PAGE after incubation with ATP and/or the phosphatase inhibitor Microcystin LR. The dephosphorylated and phosphorylated forms of p35 were then detected by immunoblotting using anti-p35 antibody. After incubation, this analysis showed a slight mobility shift on Laemmli SDS-PAGE (Fig. 1, upper panel). In contrast, using Phos-tag SDS-PAGE (Fig. 1, lower panel), rat brain p35 was separated into several bands with large mobility differences. Incubation in the absence of ATP and Microcystin LR shifted the bands downward to produce a new band (Fig. 1, lane 2), probably due to dephosphorylation. Incubation with Microcystin LR (Fig. 1, lane 3) or ATP (Fig. 1, lane 4) inhibited this downward shift. Thus, phosphorylation states before incubation might be retained under these conditions. Incubation of rat brain with both ATP and Microcystin LR produced considerably upward shifted smear bands (Fig. 1, lane 5), probably due to further phosphorylation. These results indicate that Phos-tag SDS-PAGE can be used to analyze phosphorylation states of p35.

To see the mobility shift by phosphorylation with Cdk5, p35 was co-expressed in COS-7 cells with Cdk5 or kinase-negative Cdk5 D144N (knCdk5). When p35 was expressed alone (Fig. 2A, lane 1) or with knCdk5 (Fig. 2A, lane 3), p35 appeared as two close but distinct bands at about 37 kDa, approximately the same mobility as that of p35 in Laemmli SDS-PAGE. However, co-expression with Cdk5 shifted the p35 mobility remarkably upward (Fig. 2A, lane 2). To confirm that these mobility shifts are indeed caused by phosphorylation, we treated the COS-7 cell extract with BAP. Only a single band was detected after BAP treatment (Fig. 2B, lanes 2 and 4). Dephosphorylation with BAP shifted the upper bands to the lowest position, suggesting that the lowest band corresponds to unphosphorylated p35.

Next, we evaluated the phosphorylation state of each band using p35 mutants with Ala substitutions at known phosphorylation sites (Fig. 2C): Ser^8 (mutant S8A), Thr^138 (mutant T138A), and both Ser^8 and Thr^138 (mutant 2A). These mutants were co-transfected with either Cdk5 or knCdk5 in COS-7 cells, and the cell extracts were subjected to Phos-tag SDS-PAGE and immunoblotting with anti-p35 (Fig. 2D, top panel). When co-expressed with knCdk5, 2A and T138A mutants exhibited a single band with the fastest mobility (Fig. 2D, lanes 2 and 4), whereas wild-type (WT) p35 and the S8A mutant displayed two closely migrating bands with a slightly stronger signal in the upper band (Fig. 2D, lanes 1 and 3), indicating that the upper band was phosphorylated at Thr^138. Thus, Thr^138 can be phosphorylated by another kinase. When p35 was co-transfected with Cdk5, p35 apparently shifted to a single band with a much slower mobility than that of p35 co-expressed with knCdk5 (Fig. 2D, lane 5). This band pattern is different from that of p35 in lane 3 of Fig. 2B, presumably due to the amount (i.e., kinase activity) of the expressed Cdk5. Upon increasing the expression of Cdk5 in COS-7 cells, the faster migrating bands of p35 shifted to the upper band (supplemental Fig. S1). In contrast, the 2A mutant migrated as two bands at a position close to the unphosphorylated band (Fig. 2D, lane 6), confirming that Ser^8 and Thr^138 are the major phosphorylation sites on p35 in COS-7 cells. The S8A mutant shifted p35 slightly upward (Fig. 2D, lane 7), and the T138A mutant shifted the band substantially upward (Fig. 2D, lane 8). The mobility change of WT p35 from the position of 2A mutant was almost equal to the combined changes of S8A and T138A mutants. We confirmed phosphorylation at Ser^8 and Thr^138 using phosphospecific antibodies (Fig. 2D, second and third panels). Ser(P)^8 was detected in the major band of WT p35 and T138A, and Thr(P)^138 was detected in the major band of WT p35 and S8A, indicating that the mobility shift of p35 is caused by phosphorylation at Ser^8 and Thr^138 by Cdk5 in COS-7 cells. We labeled the bands for WT p35 and T138A, both with phosphorylation at Ser^8, as M1 and M2, respectively (Fig. 2D, top panel).

We observed two other bands that were reproducibly detected throughout these experiments, although they showed some variability based on the method of Phos-tag SDS-PAGE. The upper band associated with the 2A mutant appeared when 2A was co-expressed with Cdk5 (Fig. 2D, lane 6). The phosphorylation site producing this band might cor-
respond to Ser\textsuperscript{170} or Thr\textsuperscript{197} in (S/T)P Cdk5 consensus sequences of p35 because the phosphorylation occurred by co-expression with Cdk5. We tentatively identified this phosphorylation site as “X1.” This upward shift was similar to but slightly smaller than that of Thr\textsuperscript{138} phosphorylation (Fig. 2D, lane 4). Therefore, three slightly separated bands were detected just above the unphosphorylated band. These four bands, including the unphosphorylated p35, were named L1 to L4 from the upper to the lower band, respectively (Fig. 2D, top panel).

Minor phosphorylation bands were detected by exposing blots for a longer time (Fig. 2D, bottom panel). A band with even slower migration was found in WT p35 (lane 5), and corresponding bands with similar upward displacement were seen in the S8A, T138A, and 2A mutants (lanes 6–8), suggesting phosphorylation at a site other than Ser\textsuperscript{8} and Thr\textsuperscript{138}. We hypothesized that these upper bands are phosphorylated at one site, and we tentatively termed the unidentified phosphorylation site as “X2.” The most highly shifted bands, observed with wild-type p35 and T138A, were designated as H1 and H2, respectively. The corresponding bands observed with S8A and 2A were named H1’ and H2’, respectively. These results enabled us to identify phosphorylation sites in each band of p35. The deduced phosphorylation site(s) at each band are indicated at the right side of the bottom panel of Fig. 2D.

**Phosphorylation States of p35 in Cultured Neurons and Mouse Brain**—We previously reported that Ser\textsuperscript{8} was a major phosphorylation site in cultured rat cortical neurons and that Thr\textsuperscript{138} became phosphorylated in the presence of the protein phosphatase inhibitor Microcystin LR (33). We therefore applied Phos-tag SDS-PAGE to p35 in extracts from rat brain primary neuronal cultures (Fig. 3A and supplemental Fig. S2A). p35 in cultured cortical neurons at DIV 10 was compared with standard phosphorylation-dependent banding profiles of p35 by immunoblotting with anti-p35 (Fig. 3B). The p35 banding patterns were similar in neurons cultured...
from 5 to 16 days, although the H2 band appeared slightly stronger in neurons cultured over a longer period. These results indicate that p35 in cultured neurons is phosphorylated mainly at Ser8, moderately at Thr138, and partially at the unidentified X2 site.

Next we examined the phosphorylation states of p35 in mouse brain. Adult mouse brain extract was immunoblotted with anti-p35 after Phos-tag SDS-PAGE and compared with mouse brain. Adult mouse brain extract was immunoblotted with anti-p35 after Phos-tag SDS-PAGE and compared with mouse brain p35, despite the similar banding profile. The H2 band was stronger in fetal brain than in adult. There was no unphosphorylated p35 in fetal brain, whereas some p35 remained unphosphorylated in adult brain. These results confirm our previous findings that p35 is more highly phosphorylated in fetal brain than in adult brain (32).

**Phosphorylation of p35 by CaMKII**—Because the unknown X2 site was phosphorylated in vivo, particularly in fetal brain, we turned our attention to characterizing the X2 site. Because the intensity of band H increased shortly after treatment of primary neurons with NMDA or glutamate (Fig. 4A), we hypothesized that CaMKII is a protein kinase that phosphorylates p35 at the X2 site. First, we phosphorylated p35 bound to knCdk5 in vitro by purified CaMKII in the presence of calcium and calmodulin (Ca2+-CaM). We used p35-knCdk5 in this experiment instead of p35-Cdk5 to avoid phosphorylation of p35 by Cdk5. We found that p35 was shifted substantially upward even in Laemmli SDS-PAGE by incubation with CaMKII in the presence of Ca2+-CaM (Fig. 4B). Autophosphorylation of CaMKII is shown to represent the activation of CaMKII (Fig. 4B, upper panel). The upward shift of p35 was also detected in lysates of HEK293 cells expressing p35 (supplemental Fig. S3A) and in p35 immunoprecipitates of primary neurons (supplemental Fig. S3B) when they were incubated with CaMKII in the presence of Ca2+-CaM (Fig. 4B). When p25 was expressed in HEK293 cells and incubated in vitro with CaMKII, phosphorylation of p25 was not seen, whether assayed by electrophoretic mobility shift or by 32P incorporation (Fig. 4D). These results suggested that the phosphorylation site should be present in the N-terminal p10 region.

The phosphorylation consensus site for CaMKII is Ser or Thr in (R/K)XX(S/T) (36). There are four such sites at Thr15,
Ser45, Ser65, and Ser91 in the p10 region (Fig. 5A). To determine which site is phosphorylated by CaMKII, we constructed an Ala mutant of each site in p35, expressed the mutants in HEK293 cells, and phosphorylated them by CaMKII after immunoprecipitation (Fig. 5B). The S91A mutant was the only one that failed to be shifted upward by incubation with CaMKII, suggesting that Ser91 is a CaMKII phosphorylation site. To see whether Ser91 phosphorylation shifts p35 upward to the position of band H1 on Phos-tag SDS-PAGE, each Ala mutant was expressed with Cdk5 in COS-7 cells, and the cell extracts were subjected to Phos-tag SDS-PAGE followed by immunoblotting with anti-p35. C, Phos-tag SDS-PAGE analysis of p35 Ala mutants phosphorylated by CaMKII. Ala mutants of p35 at CaMKII consensus sites were co-expressed with Cdk5 and subjected to Phos-tag SDS-PAGE followed by immunoblotting with anti-p35.

Phosphorylation of p35 at Ser91 in Primary Neurons and Brains —To confirm the phosphorylation of Ser91 in endogenous p35, we generated an anti-phospho-Ser91-specific antibody (anti-Ser(P)91) as described under “Experimental Procedures.” Anti-Ser(P)91 reacted specifically with p35 phosphorylated by CaMKII (Fig. 6A, middle panel). Preabsorption with the antigenic peptide eliminated the reaction (Fig. 6A, right panel). Anti-Ser(P)91 also recognized WT p35 (Fig. 6B, lane 1) but not the S91A mutant (Fig. 6B, lane 2) expressed in COS-7 cells. Furthermore, anti-Ser(P)91 recognized T138A and T138A/S8A mutants but not in combination with the mutation at Ser91 (Fig. 6C). Preabsorption of anti-Ser(P)91 with the phospho-Ser91 antigenic peptide eliminated the immunologic reaction with T138A and T138A/S8A mutants. Band H’ was detected when p35-knCdk5 purified from Sf9 cells was phosphorylated in vitro by CaMKII. Anti-Ser(P)91 reacted specifically with band H’ on Phos-tag SDS-PAGE, although anti-Ser(P)91 preabsorbed with the phospho-Ser91 antigenic peptide did not react, indicating that bands H1 and H2’, and probably bands H1 and H2, contain phospho-Ser91 (Fig. 6D). Taken together, these data support the specific reaction of anti-Ser(P)91 to phosphorylated Ser91.

Using this antibody, we analyzed the phosphorylation of endogenous p35 in mouse brain. Because the antibody could not detect phosphorylated Ser91 in brain extracts, immunoprecipitated p35 was examined in this experiment. Anti-Ser(P)91 reacted more strongly to p35 in immunoprecipitates...
preparing from fetal brain than those prepared from adult brain (Fig. 6E). This indicates that Ser91 is phosphorylated in fetal brains more than in adult brains, consistent with the result of Fig. 3D, if the X2 site is indeed Ser91.

Mutating Ser91 to Ala in T138A or S8A/T138A mutants, we further examined whether band H2 of primary cultured neurons (Fig. 7A) or adult mouse brain (Fig. 7B) includes phosphorylated Ser91. T138A mutants were used in this experiment because they showed a similar p35 banding patterns in brain and primary cultured neurons (Fig. 3, A and C). The S91A mutation abolished band H2 from the T138A mutant (Fig. 7, A and B, lane 3) and band H2' from the T138A/S8A mutant (Fig. 7, A and B, lane 5), indicating that band H2 is phosphorylated at least at Ser8 and Ser91 and that band H2' is phosphorylated at Ser91. All phosphorylated bands at the H and M regions disappeared with triple mutations at Thr138, Ser8, and Ser91. In the L region, L3 was still detected in the triple mutant S8A/S91A/T138A due to phosphorylation at the X1 site in COS-7 cells. These results indicate that p35 is primarily phosphorylated at Ser8 and partially at Ser91 in cultured cortical neurons and mouse brain.

Quantitative Analysis of Phosphorylated p35 in Cultured Neurons and Mouse Brain—Because one phosphorylation-independent antibody (C19) is sufficient for detection of different phosphorylation states of p35 by immunoblotting after Phos-tag SDS-PAGE, we can estimate the phosphorylation states of p35 quantitatively by densitometric measurement of the respective p35 bands. Tables I and II show the results of p35 in primary cultured neurons at DIV 10–12 and mouse brain at E17 and 10 weeks as well as p35 in COS-7 cells in which high Cdk5 activity was expressed. Table I shows the percent ratio of each band, and Table II shows the percent ratio of phosphorylation at each site. Fig. 8 shows a graphic representation of these calculations (left panel) and schematic representations of p35 with corresponding phosphorylation states and their responsible kinases (right panel). Phosphorylation states differed between COS-7 cells and cultured neurons. Although Ser8 was phosphorylated strongly in both types of cells, Thr138 was more highly phosphorylated in COS-7 cells than in cultured neurons, and Ser91 was more highly phosphorylated in cultured neurons than in COS-7 cells.

Phosphorylation of p35 also changed in mouse brain based on age. In fetal brain, almost all p35 molecules were phosphorylated at Ser8, 59% of p35 molecules were phosphorylated at Ser91, and ~12% of p35 molecules were phosphorylated at Thr138. Phosphorylation at all three sites decreased in adult brains, down to 69% for Ser8, down to 8% for Ser91, and to undetectable levels for Thr138. As a result of decreased phosphorylation, the unphosphorylated form of p35 increased to 31% in adult brains from an undetectable amount in fetal brain.

DISCUSSION

In this study, we analyzed the in vivo phosphorylation states of a p35 Cdk5 activator quantitatively using Phos-tag SDS-PAGE followed by immunoblotting with a phosphorylation-independent antibody to p35. p35 displayed phosphorylation site-dependent upward shifts in electrophoretic mobility on

| Table I |
| Percent ratio of p35 in respective phosphorylation states |

p35 prepared from COS-7 cells, primary neurons, or mouse brains was separated into three to five bands, H1, H2, M1, M2, L2, or L4, depending on their phosphorylation states in Phos-tag SDS-PAGE. The amount of each band was estimated by densitometric scanning, and their ratios were expressed as percentage of the total amount of p35. Data are represented as mean ± S.E. from four independent preparations of COS-7 cells transfected with Cdk5-p35, 10 preparations of primary cultured cortical neurons at DIV 10–12, six preparations of fetal mouse brains, and four preparations of adult mouse brains.

|            | COS-7 Cdk5-p35 (n = 4) | Primary (n = 10) | Fetal (n = 6) | Adult (n = 4) |
|------------|-------------------------|-----------------|--------------|--------------|
| H1 (Ser8/Ser91/Thr138) | 9.06 ± 2.9          | 5.30 ± 1.4      | 11.7 ± 2.6   | N.D.*       |
| H2 (Ser8/Ser91)         | N.D.                 | 16.0 ± 3.0      | 47.3 ± 1.7   | 8.40 ± 0.4  |
| M1 (Ser9/Ser91/Thr138)  | 70.6 ± 10.1         | 35.2 ± 2.3      | N.D.         | N.D.        |
| M2 (Ser9)               | N.D.                 | 37.4 ± 3.0      | 41.0 ± 3.3   | 60.3 ± 0.5  |
| L2 (Thr138)             | 11.5 ± 5.6          | N.D.            | N.D.         | N.D.        |
| L4 (unphosphorylated)   | 8.94 ± 3.7          | 6.15 ± 0.9      | N.D.         | 31.3 ± 0.5  |

* N.D., not detected.
Phosphorylation of particular proteins. A quantitative application of Phos-tag SDS-PAGE was reported with myosin light chain, but site-specific quantification was not addressed (15). We report here that the upward mobility shifts differed among the phosphorylation sites in p35 where the largest upward shift of phosphorylation was at Ser\(^{91}\) followed by Ser\(^{8}\) and finally Thr\(^{138}\), which was a small but distinct shift. Although the chemical basis for phosphorylation site-specific mobility variation is not known, we were able to use this method successfully to identify site-specific phosphorylation-dependent shifts.

Using two-dimensional phosphopeptide mapping and anti-phosphoantibody analysis, we previously reported that p35 is phosphorylated at Ser\(^{8}\) and Thr\(^{138}\) in COS-7 cells and at Ser\(^{8}\) in cultured neurons (33). Furthermore, we showed that phosphorylation decreases in developing rat brain (32). However, we did not have a method to address quantitative phosphorylation of endogenous p35 in cultured neurons or brain at that time. The quantitative measurement obtained here supports the previous qualitative results and further demonstrates that there are differences in the extent of phosphorylation between transfected cells, primary cultured neurons, and brain cells in vivo. Ser\(^{8}\) was phosphorylated in most p35 molecules whether they were exogenously expressed in transfected COS-7 cells or endogenously expressed in primary cultured neurons and embryonic brain. The phosphorylation of Thr\(^{138}\) was detected most extensively in COS-7 cells (91\% of p35 molecules) followed by primary neurons (40\%) and embryonic brain (<12\%). In contrast, the phosphorylation of Ser\(^{91}\) was least extensive in COS-7 cells (9\%) followed by primary neurons (21\%) and embryonic brain (59\%). These results suggest that phosphorylation of ectopically expressed proteins is not always identical to that of proteins in endogenous tissues.

The phosphorylation of Ser\(^{8}\) was found in p35 co-expressed with Cdk5 but not with knCdk5. Thus, Ser\(^{8}\) was phosphorylated by Cdk5. In fetal brain, Ser\(^{8}\) was phosphory-
lated in all p35 molecules. This result was somewhat surprising even though Ser8 is autophosphorylated. This high phosphorylation level is not due to its inability to dephosphorylate after it has been once phosphorylated. Ser8 was dephosphorylated by incubation of brain extract in the absence of phosphatase inhibitors. Ser8 was strongly phosphorylated over a short time during metabolic labeling experiments of cultured neurons (33). These results suggest that phosphorylation of Ser8 is maintained by continuous phosphorylation even after it is dephosphorylated. Continuous phosphorylation may be accomplished by intramolecular phosphorylation in the active Cdk5-p35 complex. In contrast, phosphorylation of Thr138 was not the case. We hypothesized that Thr138 could be the phosphorylation site for Cdk5 because Thr138 is in the TPKR sequence, the most preferred consensus sequence for Cdk5 (37). This possibility is supported by the observation that co-expression of p35 with Cdk5 enhanced phosphorylation of Thr138. However, this site was also phosphorylated when p35 was co-expressed with knCdk5 in COS-7 cells. Cycling Cdks such as Cdk1 and Cdk2, whose substrate specificity is similar to Cdk5 (38), are potential candidates for phosphorylating Thr138 in COS-7 cells. These results indicate that Thr138 can be phosphorylated by other proline-directed kinases and that, as opposed to Ser8, Thr138 phosphorylation would be intermolecular even when the site is phosphorylated by Cdk5.

In addition to Ser8 and Thr138, several unidentified minor phosphorylation sites were detected in p35 expressed in COS-7 cells using Phos-tag SDS-PAGE analysis. For the site designated X1, phosphorylation shifted the 2A mutant to the L3 position by Phos-tag SDS-PAGE when co-expressed with Cdk5. Because this phosphorylation was observed in the 2A mutant by co-expression with Cdk5 but not with knCdk5, this site could be one of the other p35 (S/T)P sites, Ser91 and Thr197 (Fig. 2C). This is consistent with the recent report by He et al. (39) that the 2A mutant co-expressed with Cdk5 in HEK293 cells is still phosphorylated to some extent. However, this phosphorylation appeared only to a small extent, if at all, in neurons of mouse brain because the bands that could possibly include phosphorylated X1 were not clearly detectable in p35 prepared from brain. Therefore, in this study, we did not attempt to determine whether Ser170 or Thr197 corresponds to the X1 site. Another novel phosphorylation site named X2 became obvious when the immunoblot using antibody C19 (anti-p35) was overexposed. This band was detected even in the 2A mutant co-expressed with knCdk5, indicating that the site was phosphorylated at an amino acid other than the (S/T)P sites by a different kinase. The finding that phosphorylation at this site was detected in endogenous p35 in neurons prompted us to identify the X2 site (see below). These two phosphorylation sites were not evident in our previous analysis by two-dimensional phosphopeptide mapping following metabolic labeling of neurons or COS-7 cells with [32P]phosphate (33). Lower levels of phosphorylation and/or a slower turnover rate may explain why the X1 and X2 sites were not identified in earlier studies. In either case, Phos-tag SDS-PAGE can be applied to detect minor phosphorylation sites as well as those with a slow turnover rate.

Because phosphorylation of X2 increased upon NMDA treatment, which activates CaMKII (40), we hypothesized that the X2 site could be a CaMKII phosphorylation site. Using Ala mutants at CaMKII consensus (R/K)XX(S/T) phosphorylation sites, the X2 site was identified as Ser91. We confirmed the phosphorylation of Ser91 in fetal and adult brains using phosphospecific antibody (Ser(P)91) generated in this study. Mass spectrometric analysis may be an alternative way to confirm this phosphorylation, although this might not be easy for p35 because only a small percentage of p35, that is a small amount of unstable protein, was phosphorylated at Ser91 in adult brains. In general, however, combinatorial use of mass spectroscopy to map phosphorylation sites after identifying phosphorylation using Phos-tag SDS-PAGE will be a powerful biochemical analytic tool.

Although we cannot conclude that Ser91 is phosphorylated exclusively by CaMKII in vivo, based on published results that a population of p35 localizes in the postsynaptic region (41, 42) and associates with CaMKII (43), it is likely that CaMKII phosphorylates p35 at Ser91 in neurons. Indeed, the phosphorylation of Ser91 was lower in COS-7 cells than in primary neurons and fetal brain, reflecting expression levels of CaMKII, which are abundant in neuronal cells compared with rat kidney cells (44). Ser91 phosphorylation was reduced considerably with development, dropping from 59% in embryonic brain to 8% in adult brain. This result may simply reflect the decreased activation frequency of CaMKII in adult mouse brain. In fact, it is reported that active CaMKII decreases during brain development (45, 46). On the other hand, we reported previously that Cdk5 activity suppresses CaMKII activation (41). Thus, the interaction between Cdk5 and CaMKII is bidirectional. CaMKII is a well known mediator of long term potentiation (47, 48), and Cdk5 has also been shown to be involved in memory formation (42, 49–52). The cross-talk between these molecules would be an important regulatory mechanism in synaptic plasticity.

In summary, we used Phos-tag SDS-PAGE for the first time to estimate site-specific in vivo phosphorylation of p35. Quantification of site-specific phosphorylation can be investigated by other means, for example by stable isotope mass spectrometry using multiple reaction monitoring (53–55). However, the number of studies using this method has been limited. We successfully used the Phos-tag SDS-PAGE method to determine populations of p35 with different combinations of phosphorylation sites. The successful use of the method is due to our previous determination of two major phosphorylation sites in p35 and the limited number of phosphorylation sites. We note that this method has advantages over other methods that have been used to analyze phosphorylation of proteins. This method does not require special equipment other than that used for typical electrophoresis and blotting, nor does it...
require radiolabeling, purification of the proteins, or a catalogue of site-specific phosphorylation-dependent antibodies. Lysates of tissues or cells can be directly subjected to this method followed by immunoblotting with a phosphorylation-independent antibody, although prior identification of phosphorylation sites of target proteins would be preferable to maximally utilize this method. We also have to note the negative aspects of this method. This method takes more time than mass spectroscopy and requires reliable antibody for quantification. Nevertheless, we believe that this method is a simple and convenient way to quantify the in vivo phosphorylation states of proteins.

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REFERENCES

1. Hunter, T. (2000) Signaling—2000 and beyond. Cell 100, 113–127

2. Cohen, P. (2002) Protein kinases—the major drug targets of the twenty-first century? Nat. Rev. Drug. Discov. 1, 309–315

3. Thingholm, T. E., Jensen, O. N., and Larsen, M. R. (2009) Analytical strategies for phosphoproteomics. Proteomics 9, 1451–1468

4. Kalume, D. E., Molina, H., and Pandey, A. (2003) Tackling the phosphoproteome: tools and strategies. Curr. Opin. Chem. Biol. 7, 64–69

5. Inagaki, M., Inagaki, N., Takahashi, T., and Takai, Y. (1997) Phosphorylation-dependent control of structures of intermediate filaments: a novel approach using site- and phosphorylation state-specific antibodies. J. Biochem. 121, 407–414

6. Kaufmann, H., Bailey, J. E., and Fusenegger, M. (2001) Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. Proteomics 1, 194–199

7. Wegener, A. D., and Jones, L. R. (1984) Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. Evidence for a protein structure consisting of multiple identical phosphorylatable subunits. J. Biol. Chem. 259, 1834–1841

8. Baudier, J., and Cole, R. D. (1987) Phosphorylation of tau proteins to a state like that in Alzheimer’s brain is catalyzed by a calcium/calmodulin-dependent kinase and modulated by phosphorylisis. J. Biol. Chem. 262, 17571–17583

9. Morishima, Y., and Ihara, Y. (1994) Posttranslational modifications of tau in Alzheimer’s disease: evidence for a paired helical filament. Dementia 5, 282–288

10. Hisanaga, S., Kusubata, M., Okumura, E., and Kishimoto, T. (1991) Phosphorylation of neurofilament H subunit at the tail domain by CDC2 kinase and modulated by phospholipids. J. Biol. Chem. 266, 21798–21803

11. Kinoshita, E., Kinoshita-Kikuta, E., Takayama, K., and Koike, T. (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol. Cell. Proteomics 5, 749–757

12. Kinoshita-Kikuta, E., Aoki, Y., Kinoshita, E., and Koike, T. (2007) Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. Mol. Cell. Proteomics 6, 356–366

13. Miyata, Y., and Nishida, E. (2007) Analysis of the CK2-dependent phosphorylation of serine 13 in Cdc37 using a phospho-specific antibody and phospho-affinity gel electrophoresis. FEBS J. 274, 5690–5703

14. Oh, H., and Irvine, K. D. (2008) In vivo regulation of Yorkie phosphorylation and localization. Development 135, 1081–1088

15. Takeya, K., Loutzenhiser, K., Shiraishi, M., Loutzenhiser, R., and Walsh, M. P. (2008) A highly sensitive technique to measure myosin regulatory light chain phosphorylation: the first quantification in renal arterioles. Am. J. Physiol. Renal Physiol. 294, F1487–F1492

16. Tatatematsu, K., Yoshimoto, N., Okajima, T., Tanizawa, K., and Kuroda, S. (2008) Identification of ubiquitin ligase activity of RBCK1 and its inhibition by splice variant RBCK2 and protein kinase Cbeta. J. Biol. Chem. 283, 11575–11585

17. Igarashi, J., Murase, M., Iizuka, A., Pichierry, F., Martinova, M., and Shimizu, T. (2008) Elucidation of the heme binding site of heme-regulated eukaryotic initiation factor 2alpha kinase and the role of the regulatory motif in heme sensing by spectroscopic and catalytic studies of mutant proteins. J. Biol. Chem. 283, 18782–18791

18. Sumara, G., Formentini, I., Collins, S., Sumara, I., Windak, R., Bodenmiller, B., Ramacheya, R., Caille, D., Jiang, H., Platt, K. A., Meda, P., Aebersold, R., Rorsman, P., and Ricci, R. (2009) Regulation of PKD by the MAPK p38delta in insulin secretion and glucose homeostasis. Cell 136, 235–248

19. Kinoshita, E., Kinoshita-Kikuta, E., Matsubara, M., Aoki, Y., Ohie, S., Mouri, Y., and Koike, T. (2009) Two-dimensional phosphate-affinity gel electrophoresis for the analysis of phosphoprotein isotypes. Electrophoresis 30, 550–559

20. Grob, A., Roussel, P., Wright, J. E., McStay, B., Hernandez-Verduin, D., and Sini, V. (2009) Involvement of SIRT7 in resumption of rDNA transcription at the exit from mitosis. J. Cell Sci. 122, 489–498

21. Oyama, S., Yamakawa, H., Sasagawa, N., Hosoi, Y., Futai, E., and Ishiura, S. (2009) Dysbindin-1, a schizophrenia-related protein, functionally interacts with the DNA-dependent protein kinase complex in an isoform-dependent manner. PLoS One 4, e14199

22. Dhavan, R., and Tsai, L. H. (2001) A decade of CDK5. Nat. Rev. Mol. Cell Biol. 2, 749–759

23. Chung, Z. H., and Ip, N. Y. (2007) The roles of cyclin-dependent kinase 5 in dendrite and synapse development. Biotechnol. J. 2, 949–957

24. Lim, A. C., and Qi, R. Z. (2003) Cyclin-dependent kinases in neural development and degeneration. J. Alzheimers Dis. 5, 329–335

25. Hisanaga, S., and Saito, T. (2003) The regulation of cyclin-dependent kinase 5 activity through the metabolism of p35 or p39 Cdk5 activator. Neurosignals 12, 221–229

26. Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L. H. (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402, 615–622

27. Kusakawa, G., Saito, T., Onuki, R., Ishiguro, K., Kishimoto, T., and Hisanaga, S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J. Biol. Chem. 275, 17166–17172

28. Patrick, G. N., Zhou, P., Kwon, Y. T., Howley, P. M., and Tsai, L. H. (1998) p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5), is degraded by the ubiquitin-proteasome pathway. J. Biol. Chem. 273, 7499–7506

29. Kamei, H., Saito, T., Ozawa, M., Fujita, Y., Asada, A., Bibb, J. A., Saido, T. C., Sorimachi, H., and Hisanaga, S. (2007) Suppression of calpain-dependent cleavage of the CDK5 activator p35 to p25 by site-specific
phosphorylation. J. Biol. Chem. 282, 1687–1694
34. Liu, J., Fukunaga, K., Yamamoto, H., Nishi, K., and Miyamoto, E. (1999) Differential roles of Ca2+/calmodulin-dependent protein kinase II and mitogen-activated protein kinase activation in hippocampal long-term potentiation. J. Neurosci. 19, 8292–8299
35. Yamada, M., Saito, T., Sato, Y., Kawai, Y., Sekigawa, A., Hamazumi, Y., Asada, A., Wada, M., Doi, H., and Hisanaga, S. (2007) Cdk5-p39 is a labile complex with the similar substrate specificity to Cdk5-p35. J. Neurochem. 102, 1477–1487
36. Means, A. R. (2000) Regulatory cascades involving calmodulin-dependent protein kinases. Mol. Endocrinol. 14, 4–13
37. Kesavapany, S., Li, B. S., Amin, N., Zheng, Y. L., Grant, P., and Pant, H. C. (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proc. Natl. Acad. Sci. U.S.A. 104, 1012–1019
38. Mayya, V., Rezual, K., Wu, L., Fong, M. B., and Han, D. K. (2006) Absolute quantification of multisite phosphorylation by selective reaction monitoring mass spectrometry: determination of inhibitory phosphorylation status of cyclin-dependent kinases. Mol. Cell. Proteomics 5, 1146–1157
39. Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A., and White, F. M. (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proc. Natl. Acad. Sci. U.S.A. 104, 5860–5865
40. Ciccimaro, E., Hanks, S. K., Yu, K. H., and Blair, I. A. (2009) Absolute quantification of phosphorylation on the kinase activation loop of cellular focal adhesion kinase by stable isotope dilution liquid chromatography/mass spectrometry. Anal. Chem. 81, 3304–3313
41. Lai, K. O., and Ip, N. Y. (2009) Recent advances in understanding the roles of Ca2+/calmodulin-dependent protein kinase II (Cdk5) activity by glutamatergic regulation of p35 stability. J. Neurochem. 93, 502–512
42. Sananbenesi, F., Fischer, A., Wang, X., Schrick, C., Neve, R., Radulovic, J., and Tsai, L. H. (2007) A hippocampal Cdk5 pathway regulates extinction of contextual fear. Nat. Neurosci. 10, 880–886
43. Thiel, G., Czerneck, A. J., Gorelick, F., Naim, A. C., and Greengard, P. (1988) Calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the alpha subunit associated with the generation of Ca2+-independent activity. Proc. Natl. Acad. Sci. U.S.A. 85, 6337–6341
44. Tobimatsu, T., and Fujisawa, H. (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol. Chem. 264, 17907–17912
45. Molloy, S. S., and Kennedy, M. B. (1991) Autophosphorylation of type II Ca2+/calmodulin-dependent protein kinase in cultures of postnatal rat hippocampal slices. Proc. Natl. Acad. Sci. U.S.A. 88, 4756–4760
46. Yamamoto, H., Hiragami, Y., Murayama, M., Ishizuka, K., Kawahara, M., and Takashima, A. (2005) Phosphorylation of tau at serine 416 by Ca2+/calmodulin-dependent protein kinase II in neuronal soma in brain. J. Neurochem. 94, 1438–1447
47. Thiel, G., Czerneck, A. J., Gorelick, F., Naim, A. C., and Greengard, P. (1988) Calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the alpha subunit associated with the generation of Ca2+-independent activity. Proc. Natl. Acad. Sci. U.S.A. 85, 6337–6341
48. Fukunaga, K., Stoppini, L., Miyamoto, E., and Muller, D. (1993) Long-term potentiation is associated with an increased activity of Ca2+/calmodulin-dependent protein kinase II. J. Biol. Chem. 268, 7863–7867
49. Wei, F. Y., Tomizawa, K., Ohshima, T., Asada, A., Saito, T., Nguyen, C., Bibb, J. A., Ishiguro, K., Kulkarni, A. B., Pant, H. C., Mikoshiba, K., Matsui, H., and Hisanaga, S. (2005) Control of cyclin-dependent kinase 5 (Cdk5) activity by glutamatergic regulation of p35 stability. J. Neurochem. 93, 502–512
50. Ohshima, T., Ogura, H., Tomizawa, K., Hayashi, K., Suzuki, H., Saito, T., Kamei, H., Nishi, A., Bibb, J. A., Hisanaga, S., Matsui, H., and Mikoshiba, K. (2005) Impairment of hippocampal long-term depression and defective spatial learning and memory in p35 mice. J. Neurochem. 94, 917–925
51. Hosokawa, T., Saito, T., Sato, Y., Asada, A., Ohshima, T., Asada, A., Wada, M., Doi, H., and Hisanaga, S. (2007) Cdk5-p39 is a labile complex with the similar substrate specificity to Cdk5-p35. J. Neurosci. Res. 84, 747–754
52. Lai, K. O., and Ip, N. Y. (2000) Recent advances in understanding the roles of Cdk5 in synaptotoxic plasticity. Biochim. Biophys. Acta 1792, 741–745
53. Hisanaga, S., Uchiyama, M., Hosoi, T., Yamada, K., Honma, N., Ishiguro, K., Uchida, T., Dahl, D., Ohsumi, K., and Kishimoto, T. (1995) Porcine brain neurofilament-H tail domain kinase: its identification as cdk5/p26 complex and comparison with cdc2/cyclin B kinase. Cell. Motil. Cytoskeleton 31, 283–297
54. Bliss, T. V., and Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39
55. Ciccimaro, E., Hanks, S. K., Yu, K. H., and Blair, I. A. (2009) Absolute quantification of cellular signaling networks. Proc. Natl. Acad. Sci. U.S.A. 106, 1012–1019