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p39 activates cdk5 in neurons, and is associated with the actin cytoskeleton

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SUMMARY

Cyclin-dependent kinase 5 (cdk5) is a small serine/threonine kinase that displays close sequence homology to the mitotically active cyclin-dependent kinases. Cdk5 has been shown to play an essential role in the development of the nervous system, including neuronal migration and neurite outgrowth. Cdk5 activation requires the presence of a regulatory activator such as p35. cdk5 −/− mice have much more extensive defects in the development of the nervous system than p35 −/− mice, leading to the speculation that other regulatory activators of cdk5 exist. Indeed, p39 is a p35 related protein isolated by sequence homology to p35. We show here that p39 associates with cdk5 in brain lysates, and that this complex is active in phosphorylation of histone H1. By extensive characterization of p39 subcellular localization in different cell types, we demonstrate the presence of p39 in lamellipodial and fillopodial structures of cells and in growth cones of neurons. We show that p39 colocalizes with actin, and cofractionates with the detergent insoluble cytoskeleton from brain. Further, p39 coimmunoprecipitates with actin in brain lysates. Finally, disruption of the actin cytoskeleton alters p39 subcellular localization as well as kinase activity of the p39/cdk5 complex. Therefore, our results reveal the existence of the p39/cdk5 complex in vivo and suggest that it might play a role in regulating actin cytoskeletal dynamics in cells.

Key words: cdk5, p39, p35, Kinase, Neuron, Actin cytoskeleton

INTRODUCTION

Cyclin-dependent kinase 5 (cdk5) is a member of the cdk family of proteins that requires association with a regulatory subunit for activation. Unlike the other cdks, cdk5 and its associated kinase activity is most abundant in the nervous system (Tsai et al., 1993). p35 was identified as the first regulatory subunit of cdk5 through its physical interaction with cdk5 in brain lysates and by its ability to activate cdk5 upon direct binding (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994).

p35 is a very unstable protein (Patrick et al., 1998), whose expression is restricted to post-mitotic neurons of the central nervous system (CNS; Delalle et al., 1997). Immunocytochemistry and subcellular fractionation experiments indicate that p35 is membrane associated (Nikolic et al., 1998). In developing neurons, both cdk5 and p35 are enriched in the growing tips of neurites. Furthermore, in primary cortical cultures, inactivation of cdk5 by expression of a dominant negative mutant of cdk5 or antisense p35 results in inhibition of neurite outgrowth (Nikolic et al., 1996). Similar observations have been made in immortalized hippocampal cells (Xiong et al., 1997) and in cultured cerebellar macroneurons (Pigino et al., 1997; Paglini et al., 1998). Thus, the p35/cdk5 kinase plays a role in the morphological maturation of neurons.

The localization of p35 at the tip of growth cones and its association with lamellar and filopodial structures (Nikolic et al., 1998) indicate that the p35/cdk5 kinase might regulate actin cytoskeletal dynamics. We recently found that p35/cdk5 interacts with the small GTPase Rac when it is in the active GTP-bound configuration (Nikolic et al., 1998). This interaction allows Rac and the Cdc42 effector, Pak1, to be hyperphosphorylated by cdk5, resulting in down-regulation of Pak1 kinase activity. Therefore, the p35/cdk5 kinase is involved in signal transduction pathways mediated by the small GTPases, most likely to regulate actin cytoskeleton reorganization. Furthermore, the phosphorylation of neuron-specific cytoskeletal proteins such as tau and neurofilaments (Ishiguro et al., 1992; Lew et al., 1992; Baumann et al., 1993; Hisanaga et al., 1993; Paudel et al., 1993) suggests that the p35/cdk5 kinase indeed plays a broader role in neuronal cytoskeletal organization.

Several homologs of p35 have been identified. In *Xenopus*, two different p35 homologs were isolated, Xp35.1 and Xp35.2 (Philpott et al., 1997, 1999). p35-like sequences also exist in the nematode *Caenorhabditis elegans*. In mammals, the only homologue identified so far, by virtue of its sequence homology to p35, is p39 (Tang et al., 1995). p39 and p35 share 57% identity at the protein level. The p39 coding sequence contains several putative cdk5 phosphorylation sites, a myristoylation site and a long proline rich region. Furthermore, p39 has a 32 amino acid insert at the C terminus which is not present in p35. The ability of p39 to activate cdk5 histone H1

kinase activity, to a similar extent as p35, was shown in vitro using a truncated version of p39 (Tang et al., 1995). Expression of p39 during rat embryonic development was investigated by in situ hybridization. p39 expression, as that of p35, is specific to post-mitotic neurons of the nervous system (Cai et al., 1997; Zheng et al., 1998). However, p39 temporal and spatial expression patterns are different from and complementary to that of p35. In particular, p39 is expressed later in the CNS during embryonic development.

The role of p39 in neurite outgrowth has been investigated (Xiong et al., 1997; Paglini et al., 1998). In immortalized hippocampal cells, p39 expression stimulated neurite outgrowth and a dominant-negative mutant of cdk5 blocked p39-induced neurite extension (Xiong et al., 1997). In addition, in this cell line, antisense p39 prevented bFGF-induced neurite outgrowth. However, Paglini and collaborators (1998) showed that p39 has no role in neurite outgrowth and axonal extension in cerebellar macroneurons. These contrasting results suggest that a role for p39 in neurite outgrowth is cell type specific and may also depend on environmental cues.

The functional importance of cdk5 regulatory subunits other than p35 is suggested by more severe defects of the CNS observed in cdk5 null mice than those observed in the p35 knockout animals. Mice lacking p35 are viable but display a disrupted cortical lamination pattern due to an inverted layering of neurons during corticogenesis (Chae et al., 1997; Kwon and Tsai, 1998) In addition, p35 –/– animals have increased susceptibility to seizures (Chae et al., 1997). In contrast, Cdk5 null mice show late embryonic or perinatal lethality and disordered lamination of the cerebral cortex, abnormal hippocampal formation and disrupted cerebellar development (Ohshima et al., 1996).

Despite the above studies, association of endogenous p39 and cdk5 has not been shown. It is also not clear if an endogenous p39/cdk5 complex is active since p39 was not previously purified as a component of cdk5 associated histone H1 kinase. Using a p39 specific antibody, we show here that indeed p39 and cdk5 form a functional kinase complex in vivo. Our study emphasizes that p39 is located in growth cones of neurons and in lamellipodial and filopodial structures. Furthermore, we present evidence that p39 is associated with the actin cytoskeleton. Indeed, we show that p39 colocalizes with actin and cofractionates with the detergent insoluble cytoskeleton in brain. A p39 specific antibody coimmunoprecipitates p39 and actin from brain extracts. Finally, cytochalasin D, an actin disorganizing agent, modifies p39 subcellular localization and p39/cdk5 activity. Therefore, the p39/cdk5 complex may play a role in regulating actin cytoskeletal dynamics in cells.

MATERIALS AND METHODS

Antibodies

The p39 antibody was raised by immunization of rabbits with an N-terminal truncation of human p39 (amino acids 111 to 367) fused to a 6-histidine tag. The antibody was affinity purified on a GST-p39 column. The cdk5 mouse monoclonal antibodies DC17 and DC39 (Kinetek Biology Corporation) were used for western blots and immunostaining, respectively. Polyclonal anti-p35 C19 (Santa Cruz) and monoclonal anti-β-actin AC15 (Sigma) antibodies were used for western blots. Anti-HA antibody 12CA5 was used as a control (Boehringer Mannheim). Anti-src antibody (mAb 327) is a gift from J. Brugge (Harvard Medical School, Boston) and anti-MAP2 antibody (5F9) of K. Kosik (Harvard Medical School, Boston).

Plasmids and constructs

CMV-p35, CMV-cdk5 and dominant negative cdk5 (dncdk5) were previously described (Nikolic et al., 1996). A p39 mammalian expression construct was obtained by PCR amplification of the mouse p39 cDNA and cloning into the *Bam*HI site of pcDNA3 (Invitrogen). The bacterial p39-GST construct was generated by cloning a *Bam*HI fragment containing the full length p39 mouse cDNA into pGEX-4T-2 (Pharmacia). The 6-histidine tagged p39 was made by cloning a *Bam*HI fragment containing the C-terminal portion of the p39 human protein (amino acids 111 to 367) into pET19b (Novagen).

Kinase assays

Transfected COS7 or whole brains from P10 mice were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA pH 8, 0.1% Nonidet P-40, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin. Western blot analysis was performed on 5 μg of transfected cell lysate or 25 μg of brain lysate. To assay for p39 associated kinase activity, 500 μg of protein from each lysate was immunoprecipitated with affinity purified rabbit polyclonal antibody to p39. Histone H1 was used as a substrate for in vitro kinase assays which were performed as previously described (Nikolic et al., 1996). In Fig. 1D, histone H1 was not added in order to assay p39 phosphorylation by cdk5.

Preparation of growth cones

Lysed growth cones were prepared from brains of fetal (E17-E19) Sprague-Dawley rats as described elsewhere (Moss, 1983; Pfenninger et al., 1983). Briefly, brains were homogenized in 8 volumes (wet weight:volume) of 0.32 M sucrose containing 1 mM $MgCl_2$, 1 mM TES-NaOH, pH 7.3, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF. The homogenate was spun at 1660 ***g*** for 15 minutes and the low speed supernatant (H) was layered over a 0.75 M sucrose solution. After centrifugation at 242,000 ***g*** for 40 minutes, the interface material containing the growth cones (GC) was collected and lysed by dilution. The lysed growth cones were pelleted at 160,000 ***g*** for 30 minutes (GCP). Fractions were analysed by SDS-PAGE and western blotting.

Isolation and subfractionation of brain cytoskeleton

Cytoskeleton was isolated from adult mouse brain and subfractionated by the method already described (Moss, 1883; Arai and Cohen, 1994) with minor modifications. Briefly, mouse brains were homogenized in 2% Triton X-100 in Tris/$MgCl_2$ buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 1 mM PMSF). The homogenate (H) was centrifuged at 100,000 ***g*** for 30 minutes. The detergent soluble supernatant (Det. Sol.) was recovered. The detergent insoluble pellet (Det. Insol.) was homogenized in 10% sucrose in Tris/$MgCl_2$ buffer, and overlayed on a discontinous sucrose gradient consisting of 50% and 30% sucrose layers in Tris/$MgCl_2$ buffer. After centrifugation at 100,000 ***g*** for 3 hours in a Beckman SW41 rotor, fractions were collected from the 10/30% sucrose interface, the 30/50% sucrose interface and the 50% sucrose fraction. Aliquots containing 50 μg protein were analysed by SDS-PAGE and western blotting.

Cell cultures and transfection

E19 pregnant rats (Long Evans strain) were purchased from Harland Sprague-Dawley. For cortical cultures, embryos were surgically removed, and their cortices were dissected and cultured as previously described (Nikolic et al., 1996). Dissociated cultures of cerebellar neurons were established from P8 rat pups (Galli et al., 1995). Hippocampal cultures were obtained as described elsewhere (Finkbeiner, 1992) except that: the cortices were obtained from E19 rats; cells were plated at high density (150000 cells/cm^2) and cytosine-β-D-arabinofuranoside (Ara-C) was added at a concentration of 5 μM.

DRG neurons from adult rats were cultured as described (Smith and Sken, 1997). COS7 cells were maintained in DMEM supplemented with 10% fetal calf serum and transiently transfected with various plasmid constructs using the calcium phosphate precipitation method (Nikolic et al., 1996). Cells were harvested 24 hours post-transfection. To study the relationship between p39 and cytoskeletal elements, p39 transfected COS7 cells were treated with 2 μM cytochalasin D or 5 μg/ml nocodazole (Calbiochem) before fixation.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in 1× PBS for 10 minutes at room temperature followed by permeabilization with 0.3% Triton X-100 in 1× PBS. Immunostaining was performed as previously described (Nikolic et al., 1996) using anti-p39, anti-cdk5, anti-acetylated tubulin (Sigma), anti-glial fibrillary acidic protein (GFAP; Sigma) antibodies followed by Texas Red- or FITC-conjugated anti-rabbit, and/or FITC-conjugated anti-mouse antibodies. For visualization of F-actin, rhodamine phalloidin or Oregon Green 488 phalloidin (Molecular Probes) was added to the coverslips (20 minutes at room temperature) before washing once in 1× PBS and mounting (Prolong Antifade Kit, Molecular Probes). Images were made using a Nikon inverted microscope or a Leica confocal microscope.

Coimmunoprecipitation of p39 and actin

P10 brains were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 140 mM NaCl, 0.1% SDS, 0.1% deoxycholic acid, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml leupeptin) and centrifuged at 12000 ***g*** for 15 minutes at 4°C. The supernatants were incubated with either no antibody, the p39 polyclonal antibody or anti-HA antibody followed by protein A/agarose precipitation. Agarose beads were washed 3 times in RIPA buffer, twice in 1× PBS and separated on a 10% SDS-polyacrylamide gel.

RESULTS

p39 activates cdk5 in vivo

A polyclonal antibody specific to p39 was prepared by immunization of rabbits with a recombinant human partial p39 fused to a 6×histidine tag. After purification on a GST-p39 column, this antibody recognized a 41 kDa protein, synthesized by in vitro transcription and translation (IVT) of p39 cDNA (Fig. 1A, lane 1 left panel) or in brain extracts (Fig. 1A, lane 2 left panel). The antibody did not cross react with endogenous or IVT p35 (Fig. 1A, lanes 2 and 3 left panel).

An N-terminally truncated version of p39 fused to GST has been shown to activate cdk5 histone H1 kinase activity in vitro (Tang et al., 1995) but this truncated p39 is not present in vivo (Fig. 1A; data not shown). To determine whether full length p39 can activate cdk5, in vitro H1 kinase assays (Fig. 1B) were performed using lysates of COS7 cells transfected with p39, cdk5 or both. Equal amounts of total protein were immunoprecipitated with anti-p39 antibody, and either tested for their histone H1 kinase activity or analyzed by western blot using anti-cdk5 antibody. Robust p39 associated H1 kinase activity could be detected only when p39 and cdk5 were cotransfected, but not when either one was expressed alone (Fig. 1B). This activity was due to the association of these two proteins, as cdk5 was present in the p39 immunoprecipitation (Fig. 1B). The p39/cdk5 complexes, as well as p39 associated histone H1 kinase activity was also detected from postnatal day 10 (P10) mouse brain extracts using the p39 specific antibody (Fig. 1C). These results show that p39 binds to cdk5 in neurons, and support the notion that p39 is a regulatory activator of cdk5. In addition, when we immunoprecipitated p39 from p39/cdk5 co-transfected COS7 cells using anti-p39 antibody, p39 was phosphorylated in an in vitro kinase assay (Fig. 1D). This is likely to be the consequence of phosphorylation by cdk5 as no p39 phosphorylation was seen when it was coexpressed with a dominant negative mutant of cdk5

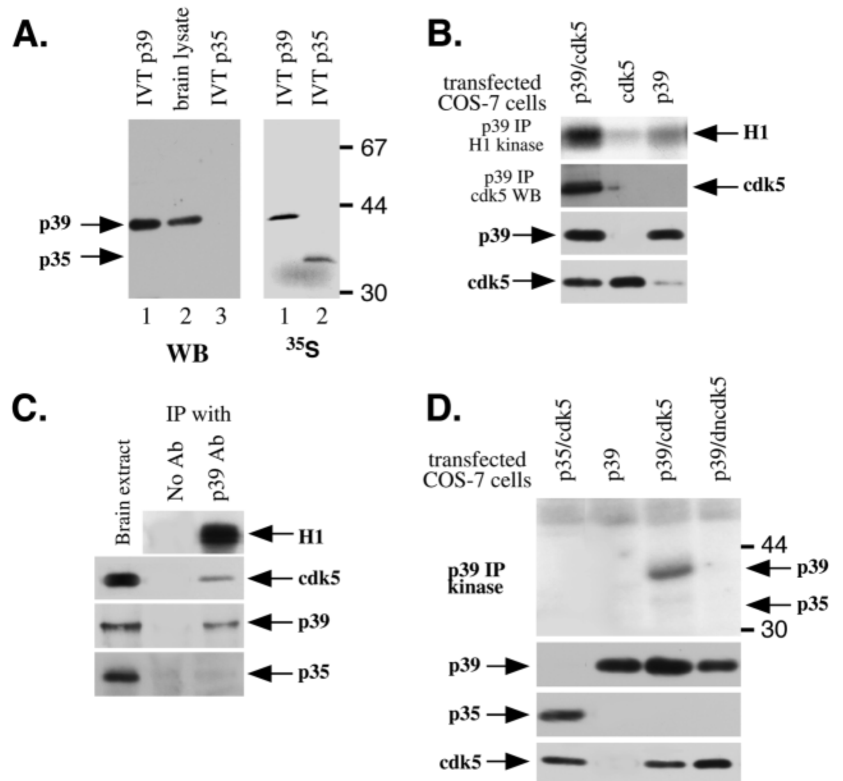

Fig. 1. p39 is a cdk5 activator in transfected COS7 cells and in brain extracts. (A) Specifity of the p39 affinity purified rabbit polyclonal antibody is revealed by western blot (left panel) analysis of either in vitro translated (IVT) p39 (lane 1), total P10 mouse brain extract (lane 2) or IVT p35 (lane 3). The antibody labels a single immunoreactive protein species in brain with an apparent molecular mass of 41 kDa. ^{35}S-labeled IVT p39 (lane 1) or p35 (lane 2) are shown in right panel. (B) COS7 cells were transfected with p39, cdk5 or both. Lysates were immunoprecipitated with anti-p39 antibody and tested for histone H1 kinase activity (p39 IP H1 kinase) or analyzed by western blot using anti-cdk5 antibody (p39 IP cdk5 WB). Lower panels show p39 and cdk5 protein levels in lysates. (C) P10 mouse brain extracts were immunoprecipitated with no antibody (No Ab) or p39 rabbit polyclonal antibody (p39 Ab) and tested for histone H1 kinase activity or analyzed by cdk5, p39 or p35 western blot. (D) Lysates from p35/cdk5, p39, p39/cdk5 and p39/dncdk5 transfected COS7 cells were immunoprecipitated with p39 antibody and kinase reactions without histone H1 were performed. Lower panels show p39, p35 or cdk5 western blot of the lysates.

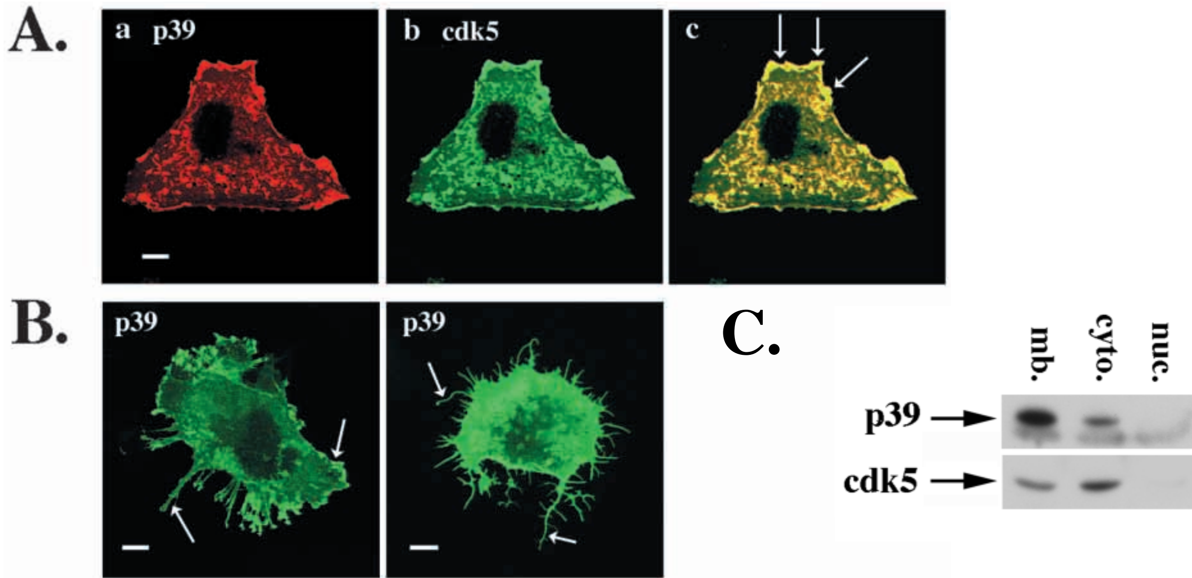

Fig. 2. p39 and cdk5 colocalize in COS7 cells and p39 is enriched at the membrane. (A) p39 and cdk5 colocalize in COS7 cells. COS7 cells were transfected with p39 and cdk5 and double immunostained using anti-p39 and anti-cdk5 antibodies. p39 was detected using Texas Red (a) and cdk5 using FITC-conjugated (b) secondary antibodies. Arrows indicate strong colocalization of p39 and cdk5. (B) COS7 cells transfected with p39 were immunostained with anti-p39 antibodies and FITC-conjugated secondary antibodies. Arrows indicate cells protrusions where p39 stains. Bars, 10 µm. Images were obtained using a Leica confocal microscope. (C) E18 rat brain lysates were fractionated into membrane (mb.), cytoplasmic (cyto.) and nuclear (nuc.) pools and 20 µg of the fractions were analyzed by p39 and cdk5 western blot.

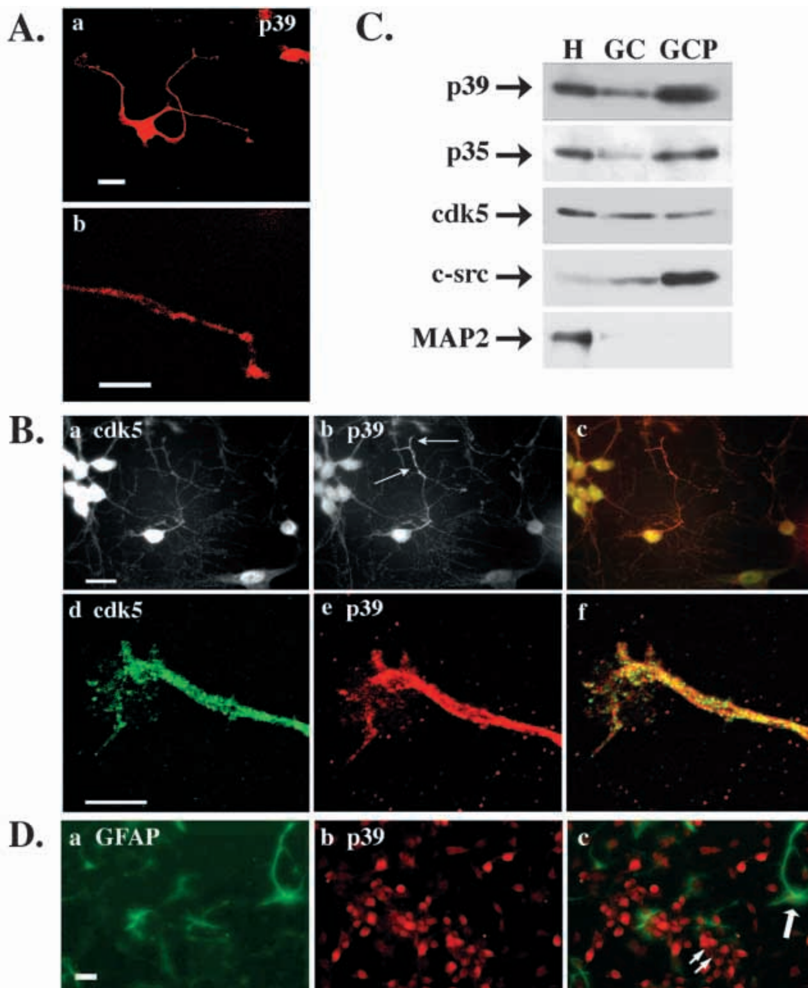

Fig. 3. p39 is present in growth cones of neurons and colocalizes with cdk5. (A) 1 day-old cultures of adult rat DRG neurons were immunostained with anti-p39 antibody and Texas Red-conjugated secondary antibody. (B) Double immunostaining of P8 rat cerebellar neurons was performed with an anti-cdk5 polyclonal antibody (a,d) and an anti-p39 polyclonal antibody (b,e) followed by FITC or Texas Red-conjugated secondary antibodies. The arrows point to the neurites strongly stained for p39. Images were obtained using a Nikon inverted microscope (B, a,b,c) or a Leica confocal microscope (A; B, d,e,f). Bars: 5 µm (A, b); 10 µm (A, a, B, d); 15 µm (B,D). (C) E18 rat brain homogenates (H) were loaded on a discontinous sucrose gradient to obtain the growth cone enriched interface (GC). This fraction was lysed and pelleted to obtain growth cone particles (GCP). 50 µg of H and 20 µg of the GC and GCP fractions were analysed by western blot for presence of p39, p35, cdk5, c-src and MAP2. (D) Rat cortical neurons were immunostained with anti-GFAP (D,a) and anti-p39 antibodies (D,b) followed by FITC or Texas Red-conjugated secondary antibodies. The arrows point to the neurons stained for p39 (double arrows) and to a glial cell stained for GFAP (single arrow).

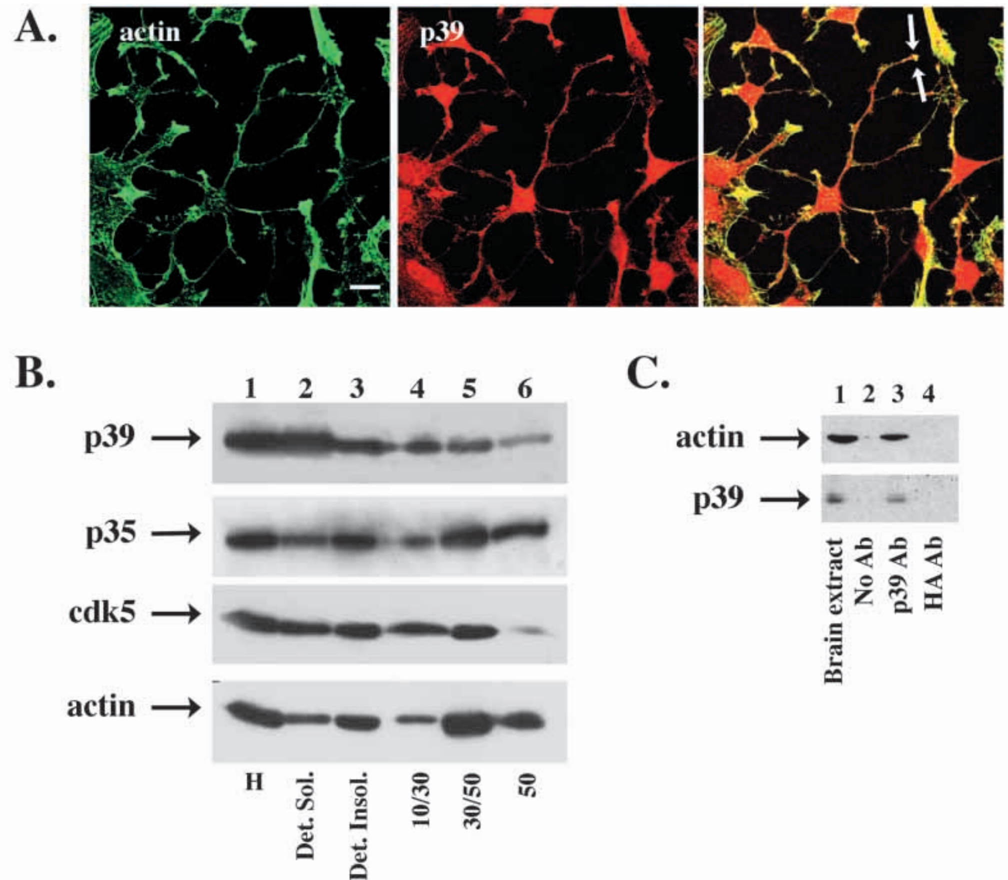

Fig. 4. p39 colocalizes and coimmunoprecipitates with actin. (A) 2-week-old hippocampal cultures were stained with Oregon Green-phalloidin and anti-p39 antibody followed by Texas Red-conjugated secondary antibody. Colocalization of actin and p39 is seen in the neurites and in the growth cones (white arrows). Bar, 10 μm. (B) Adult rat brain homogenate (H) was extracted with 2% Triton X-100 to produce the detergent soluble (Det. Sol.) and detergent insoluble (Det. Insol.) lysates. The detergent insoluble lysate was fractionated by discontinous sucrose gradient centrifugation. 50 μg protein of the 10/30% sucrose interface (membrane associated cytoskeleton), 30/50% interface (mixture of membrane and cytoplasmic cytoskeleton) and the 50% sucrose fractions (cytoplasmic cytoskeleton) were analyzed by SDS-PAGE and western blotting for the presence of p39, p35, cdk5 and actin. (C) 1 mg of P10 brains extracts was incubated with either no antibody (No Ab), the p39 polyclonal antibody (p39 Ab) or anti-HA antibody (HA Ab) followed by protein A/agarose precipitation. The precipitates were analysed by western blotting for the presence of p39 and actin. Brain extract: 1 μg of extract was loaded.

(dncdk5). As shown in Fig. 1C,D, the p39 antibody immunoprecipitates p39 specifically and not p35.

These results demonstrate the existence of a functional p39/cdk5 complex in brain and suggest that p39 becomes autophosphorylated by activated cdk5.

p39 colocalizes with cdk5 and is present at the membrane of cells and growth cones of neurons

To further characterize the p39/cdk5 kinase, we exogenously expressed p39 and cdk5 in COS7 cells (Fig. 2) and looked at their subcellular distribution. Complete colocalization of p39 and cdk5 was observed (Fig. 2A, a-c). In particular, p39 and cdk5 are enriched in the cell periphery and membrane ruffles (white arrows). Immunostaining of p39 transfected COS7 cells further reveals that p39 is strikingly present at the membrane and at the filopodial and lamellipodial structures of the cell (Fig. 2B, white arrows). To confirm the membrane association of p39, lysates obtained from mouse cortices at embryonic day 18 (E18) were fractionated into membrane, cytoplasmic and nuclear pools and were analysed by western blotting with anti-p39 and anti-cdk5 antibodies. The majority of p39 was associated with the membranes (Fig. 2C) whereas, as previously described (Nikolic et al., 1998), most cdk5 was present in the cytoplasm with a smaller proportion at the membranes. Fractionation into membrane, cytoplasm and nuclear pools of p39/cdk5 transfected COS7 cells gave similar distribution of p39 and cdk5 (data not shown).

We next examined the subcellular distribution of endogenous p39 in primary cultures of rat dorsal root ganglia (DRG; Fig. 3A) by immunocytochemistry. p39 is present throughout the entire neuron including cell bodies, axons and dendrites (Fig. 3A, a). Staining was seen along entire neurites including the growth cones (Fig. 3A, b). Colocalization of p39 and cdk5 was then assessed in rat cerebellar granule cells. As in DRG neurons, p39 antibody stained cell soma, axons and dendrites (Fig. 3B, b). The subcellular localization of cdk5, previously described in cortical neurons (Nikolic et al., 1996), was similar to that of p39 (Fig. 3B, a). Interestingly, though p39 and cdk5 largely colocalized in neurons, cdk5 staining was stronger in the soma and p39 staining was often stronger in the neurites (white arrows) while both cdk5 and p39 were evident in the axonal growth cones (Fig. 3B, d-f). The presence of both proteins in growth cones was further assessed by western blot analysis of growth cone particles isolated by subcellular fractionation (Moss, 1983; Pfenninger et al., 1983). Fig. 3C shows that p39 was enriched in the growth cone pellet (GCP). Cdk5 is also present in the GCP, although it is present at high levels in the GC fraction, indicating a broader distribution than p39. p35 western blot analysis was used as a control, as p35 was reported to be abundant in the GCP (Paglini et al., 1998). To test for the purity of the fractions, we analysed the distribution of c-src which has been shown to be present in the GC and the GCP fractions at significant levels (Helmke and Pfenninger, 1996). By contrast, as previously decribed (Lohse et al., 1996), the GCP fraction does not contain detectable amounts of high molecular mass MAP2. GFAP and p39

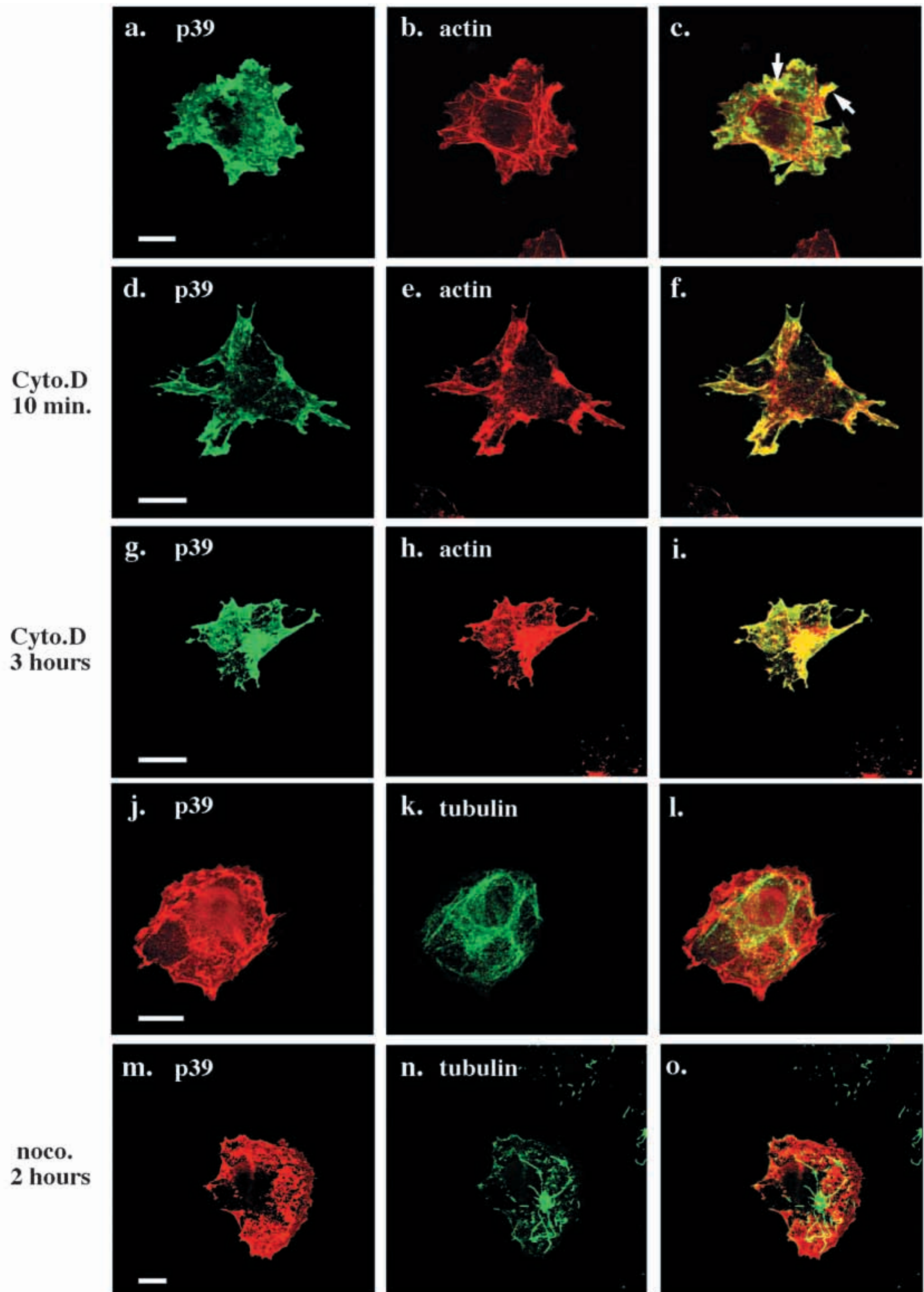

Fig. 5. Cytochalasin D modifies p39 distribution in cells. To visualize F-actin and p39, p39 transfected COS7 cells were stained with anti-p39 antibody followed by FITC-conjugated secondary antibody and rhodamine phalloidin (a-i). (a-c) p39 colocalizes with actin in the cortical areas (white arrow) while less colocalization is observed in the stress fibers (white arrowhead). (d-i) To determine if p39 distribution depends on the actin network, cells were treated 10 minutes (cyto. D 10 min., d-f) or 3 hours (cyto D 3 hours, g-i) with 2 μM of cytochalasin D. Cytochalasin D treatment resulted in the formation of large F-actin clusters where p39 localizes. (j-o) COS7 cells transiently transfected with p39 were double immunostained using anti-p39 and anti-tubulin antibodies (revealed, respectively, by Texas Red and FITC-conjugated secondary antibodies). After 5 μg/ml nocodazole treatment for 2 hours (noco. 2 hours; m-o), tubulin staining changed whereas p39 localization remained unchanged. Bars, 10 μm.

costaining of cortical cultures was done to show that p39 is expressed exclusively in neurons (Fig. 3D). There is no significant overlap of these two proteins in our cultures.

In conclusion, p39 and cdk5 colocalize in both transfected COS7 cells and neurons. In addition, the analysis of the subcellular distribution of p39 clearly demonstrates p39 localization in the growth cones and its enrichment at the membrane.

p39 colocalizes and coimmunoprecipitates with actin

Because p39 is enriched at the cell periphery where actin filaments are highly enriched, we examined whether p39 is found in similar subcellular compartments as actin by immunostaining of 2-week-old primary cultures of hippocampal neurons (Fig. 4A). As in DRG and cerebellar neurons, p39 was found in cell bodies, neurites and growth cones of hippocampal neurons (Fig. 4A). Strong colocalization of p39 and actin was observed in the neurites and the growth cones (white arrows). However, in the cell body where p39 is abundant, much less actin staining is observed.

We next isolated the cytoskeleton by detergent extraction of adult rat brain lysates and fractionated it by discontinuous sucrose gradient centrifugation (Fig. 4B). Using this procedure, membrane-associated cytoskeleton is enriched in the 10/30% sucrose interface fraction (Moss, 1983). The 50% sucrose step

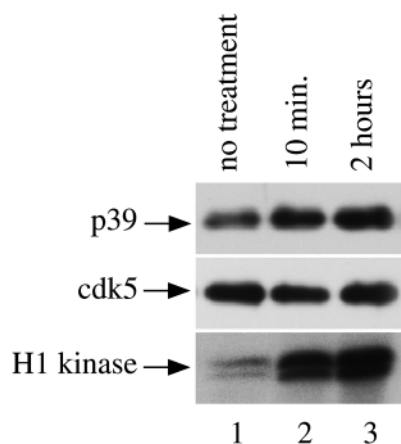

Fig. 6. Cytochalasin D induces p39/cdk5 kinase. p39/cdk5 transfected COS7 cells were treated with 4 μM of cytochalasin D for 10 minutes or 2 hours. The amount of p39 and cdk5 proteins was determined by western blot of 20 μg of the lysates of the untreated (lane 1) or treated cells (lanes 2-3) by anti-p39 and anti-cdk5 antibodies. The lower panel shows the associated kinase activity of the same lysates immunoprecipitated with p39 antibody.

contains cytoplasmic cytoskeleton and the 30/50% sucrose interface contains a mixture of the two previous fractions. The different fractions were tested for the presence of p39, p35, cdk5 and actin by western blotting. As expected, actin was found in all three cytoskeletal fractions (Arai and Cohen, 1994). p39 and cdk5 were predominantly found in the membrane associated component of the cytoskeleton (Fig. 4B, lane 4) and less in the 50% sucrose step (Fig. 4B, lane 6). Interestingly, p35 distribution is different from that of p39. While p35 immunoreactivity was enriched in the cytoplasmic cytoskeleton, less p35 was found in the membrane associated cytoskeleton 10/30% fraction (Fig. 4B, lanes 6 and 4). This fractionation experiment is consistent with our immunostaining data (Fig. 2) and further emphasizes the enrichment of p39 at the membrane. It also reveals that a significant proportion of p39 and cdk5 in brain is associated with the cytoskeleton, predominantly with the membrane rather than the cytoplasmic cytoskeleton.

As p39 codistributes with actin, we decided to further investigate the interaction of p39 and actin (Fig. 4C). P10 mouse brain lysates were prepared in stringent conditions (RIPA buffer, see Materials and Methods) and incubated with either no antibody (lane 2), purified p39 polyclonal antibody (lane 3) or anti-HA antibody (lane 4). The presence of p39 and actin was then analysed by western blot. As shown, the p39 antibody immunoprecipitates specifically p39 and actin whereas no actin was immunoprecipitated when Protein A-Sepharose beads alone (lane 2) or anti-HA antibody (lane 4) were used.

Altogether these experiments indicate that p39 is associated with actin. Therefore the p39/cdk5 kinase could play a role in the dynamics of the actin-based cytoskeleton.

Disruption of the actin based cytoskeleton modifies p39 distribution in cells and kinase activity of the p39/cdk5 complex

We then determined if distribution of p39 depends on the actin network (Fig. 5). The distribution of p39 with respect to F-actin was investigated in p39 expressing COS7 cells. In these cells, the F-actin containing network could be readily observed after labeling with rhodamine-conjugated phalloidin (Fig. 5, b). Colocalization of p39 and F-actin was evident in the cortical areas and in membrane extensions (Fig. 5, c, white arrows) and less colocalization was seen in the stress fibers (white arrowheads). This distribution of p39 was further examined in cytochalasin D treated cells where the actin cytoskeleton is disrupted. Cytochalasin D treatment resulted in a progressive loss of stress fibers and formation of large F-actin clusters (Fig. 5, e and h). The treatment also led to a major reorganization of p39 protein causing most of the p39 to accumulate in clusters containing F-actin (Fig. 5, d-i). After 10 minutes of treatment, p39 begins to colocalize to F-actin clusters (Fig. 5, d-f). By 3 hours, p39 is localized to the cell center corresponding to F-actin framework retraction (Fig. 5, g-i). As a control we stained for tubulin in COS7 cells transfected with p39. Limited colocalization was observed between p39 and tubulin (Fig. 5, j-l). However, disruption of microtubules by nocodazole treatment did not appear to cause detectable change in the subcellular distribution of p39 (Fig. 5, m-o).

To explore a possible effect of cytochalasin D on the p39/cdk5 kinase, we assayed the level of p39/cdk5 H1 associated kinase activity after treating COS7 cells transfected with p39 and cdk5 with 2 μM cytochalasin D. As shown in Fig. 6, 10 minutes after cytochalasin D treatment, p39 levels in the soluble fraction increased to reach a maximum after 2 hours of treatment (compare lanes 1-3, upper panel) while the amount of cdk5 in the soluble fraction remained constant (lanes 1-3, middle panel). The p39/cdk5 histone H1 associated kinase activity increased in levels corresponding to the increase in p39 levels (lanes 1-3, lower panel). In summary, these experiments show that p39 localization and associated kinase activity are modified after disruption of the actin cytoskeleton. This suggests that the p39/cdk5 kinase could be regulated by the configuration of the actin cytoskeleton and/or play a role in regulating the actin-based cytoskeleton.

DISCUSSION

p39 is a homolog of the neuron specific activator of cdk5, p35. In this communication we establish the in vivo association of p39 and cdk5 and demonstrate that the p39/cdk5 kinase is active in brain lysates. This constitutes the first study describing p39 at the protein level and in particular the first extensive characterization of its subcellular localization in different cell types. We show that p39 interacts with actin, and that p39 subcellular distribution, and kinase activity of the p39/cdk5 complex are affected by changes in the actin cytoskeleton.

p39 is a regulatory subunit of cdk5

Primary sequence conservation between p39 and p35 suggests that p39 can serve as a regulatory activator for cdk5. Also, N-terminally truncated p39 fragment was shown to activate cdk5 kinase using histone H1 as substrate in an in vitro kinase assay (Tang et al., 1995). We present evidence here that p39 binds to cdk5 both in COS7 cells and in brain and that p39 can activate cdk5 kinase activity in vivo. Indeed, when co-transfected in

COS7 cells, association between p39 and cdk5 was readily detectable. Similarly, cdk5 was present in anti-p39 immunoprecipitates from lysates of P10 mouse cortex. Using histone H1 as an in vitro substrate, activation of cdk5 by p39 was evident in both transfected COS7 cells and P10 mouse brain lysates. Colocalization of p39 and cdk5 in COS7 cells and in cerebellar neurons also supports the role of p39 as a regulatory partner of cdk5. The fact that p39 becomes autophosphorylated suggests that phosphorylation of p39 by cdk5 might play a role in the regulation of p39/cdk5 kinase, as is the case for the p35/cdk5 kinase (Patrick et al., 1998).

The functional implications for the in vivo presence of two cdk5 activators, p39 and p35, still remain to be determined. However, expression studies have revealed that p39 and p35 have complementary expression patterns (Cai et al., 1997; Paglini et al., 1998; Zheng et al., 1998; S. Humbert and L.-H. Tsai unpublished data). The peak of p39 expression in the CNS occurs postnatally, much later during development than that of p35 (Paglini et al., 1998; S. Humbert and L.-H. Tsai, unpublished observations). In addition, their spatial expression patterns are different. In particular, whereas levels of p39 are high in the postnatal cerebellum (S. Humbert and L.-H. Tsai, unpublished data), p35 peaks embryonically in the forming cortical plate (Delalle et al., 1997). This suggests that p39 and p35 would activate cdk5 in a stage and region specific manner in the nervous system and for this reason they may have different functional roles in distinct brain regions. The two kinases might also display differences in their substrate specificity which we are currently investigating. Finally, the more severe phenotype of the cdk5 –/– mice compared to that of the p35 null mice emphasizes the importance of p39 as a cdk5 regulator. The p39 and the double p39/35 knockout mice models should provide insights on the role of the p39/cdk5 kinase during development.

p39 is a membrane associated protein present at the growth cone

Interestingly, p39 accumulates in filopodial and lamellipodial protrusions in transfected COS7 cells and is enriched at the membrane. The association of p39 with membranes is further demonstrated by fractionation of p39 transfected COS7 cells (data not shown) or E18 rat brain lysates into membrane, nucleus and cytoplasmic pools. The localization of p39 at membranes is suggestive of a role for p39 in the regulation of cell shape changes.

Conflicting results have been published on the role of p39 in axonal growth. In immortalized hippocampal cells, cdk5 mediates the formation of neurites specifically through p39 (Xiong et al., 1997). In this cell line, p39 is required for differentiation. However, in cerebellar macroneurons, suppression of p39 by antisense oligonucleotide treatment has no effect on axonal elongation (Paglini et al., 1998). In this communication, we clearly assess by immunostaining experiments that p39 is present at the growth cone of 3 different types of neurons, namely DRG, hippocampal and cerebellar neurons. p39 is also present in growth cone containing fractions obtained from subcellular fractionation of brains extracts. In the growth cone fractions, the p39/cdk5 kinase is active in an in vitro H1 kinase assay (data not shown). Altogether, these data suggest that the p39/cdk5 kinase is associated with growth cone membranes and may regulate growth cone mobility and axonal growth. Moreover, this function could be differently regulated depending on cell type and time of development as suggested by the cell-type specific effect of p39 on axonal elongation.

p39 is associated with the actin cytoskeleton

Our results show that a significant portion of p39 is present in cytoskeleton subcellular fractions of detergent extracted adult rat brain lysates. We also observe that many p39 enriched areas contained F-actin as demonstrated by phalloidin staining. In particular, p39 and actin colocalize in regions of active actin dynamics namely at growth cones of hippocampal neurons and at membranes of p39 transfected COS7 cells. To further investigate the interaction of p39 and the actin cytoskeleton, we performed immunoprecipitation experiments that shows that p39 binds to actin in brain extracts. Whether this interaction is direct or not remains to be established. p39 has been shown to play a role in neurite outgrowth in immortalized hippocampal cells (Xiong et al., 1997). p39 binding to actin cytoskeleton suggests that the p39/cdk5 kinase may regulate the dynamics of actin microfilaments for example by phosphorylation of actin associated proteins and therefore may induce neurite outgrowth.

Treatment of these COS7 cells with the actin inhibitor cytochalasin D caused a drastic reorganization of the actin cytoskeleton and also caused in a rapid redistribution of p39 protein to F-actin clusters. This observation suggests that intact actin cytoskeleton is required for the normal subcellular localization of p39. Finally, we treated p39 transfected COS7 cells with cytochalasin D and analyzed p39/cdk5 associated histone HI activity in the soluble fraction of these cell lysates. After only 10 minutes of treatment, p39 levels in the soluble fraction and the H1 associated p39/cdk5 kinase activity increased, whereas cdk5 levels remained constant. This suggests that availability of p39 is the limiting factor in the formation of the p39/cdk5 kinase. The rapid increase in p39 levels and the p39/cdk5 associated kinase activity are unlikely to be due to de novo protein synthesis of p39. It is possible that a pool of p39 is associated with an inhibitory complex that requires an intact actin cytoskeleton. Disruption of the actin framework with cytochalasin D could destabilize this complex, causing a change in p39 subcellular localization as well as making more p39 available for association with cdk5 to form an active kinase complex. Lee et al. (1996) have presented evidence for the presence of p35/cdk5 in a macromolecular complex that contains a kinase inhibitory factor. p39 could similarly be associated with an inhibitor complex that is stabilized by an intact cytoskeleton, and its dissociation upon cytochalasin treatment could explain the increase in p39/cdk5 kinase activity. Alternatively, activation of the p39/cdk5 kinase may be the consequence of a yet to be identified cytochalasin signalling cascade.

We have shown previously that p35 binds to Rac and Pak1 (Nikolic et al., 1998), both of them involved in regulation of the actin cytoskeleton and cell shape (for reviews see Lim et al., 1996; Eby et al., 1998; Sells et al., 1999). The interaction of p35/cdk5 with the small GTPase Rac and its effector Pak1 is likely to regulate the dynamics of actin cytoskeleton. We have no evidence to show an interaction between p39, Rac and Pak1 nor modification of Pak1 by the p39/cdk5 kinase (S. Humbert and L.-H. Tsai, unpublished observation). Therefore,

it is of great interest to elucidate the mechanism by which p39 is associated with actin and to determine if p39/cdk5 plays a role in the regulation of the actin cytoskeleton.

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