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Actin interaction and regulation of cyclin-dependent kinase 5/p35 complex activity

Jiqing Xu[1], **Koji Tsutsumi**[2], **Kiyotaka Tokuraku**[1], **Katherine A. Estes**[1], **Shin-ichi Hisanaga**[2], and **Tsuneya Ikezu**[1,3,*]

[1] Center for Neurodegenerative Disorders, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA

[2] Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Tokyo, Japan 192-0397

[3] Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA 02118, USA

Abstract

Cyclin-dependent kinase 5 (Cdk5) plays a critical role during neurodevelopment, synaptic plasticity, and neurodegeneration. Cdk5 activity depends on association with neuronal proteins p35 and p25, a proteolytic product of p35. Cdk5 regulates the actin cytoskeletal dynamics that are essential for neuronal migration, neuritic growth, and synaptogenesis. However, little is known about the interaction of actin and Cdk5 and its effect on neuronal Cdk5 activity. In a previous study, we observed that Cdk5/p35 activity is negatively correlated with co-immunoprecipitated F-actin (filamentous actin) amounts in the mouse brain, and suggested that F-actin inhibits the formation of the Cdk5/p35 complex (J Neurosci 28:14511, 2008). The experiments reported here were undertaken to elucidate the relationship between actin and the formation of the Cdk5/p35 complex and its activity. Instead of an F-actin-mediated inhibition, we propose that G-actin (globular actin) in the F-actin preparations is responsible for inhibiting Cdk5/p35 and Cdk5/p25 kinase activity. We found that F-actin binds to p35 but not p25 or Cdk5. We have shown that G-actin binds directly to Cdk5 without disrupting the formation of the Cdk5/p35 or Cdk5/p25 complexes. G-actin potently suppressed Cdk5/p35 and Cdk5/p25 activity when either histone H1 or purified human tau protein were used as substrates, indicating a substrate-independent inhibitory effect of G-actin on Cdk5 activity. Finally, G-actin suppressed the activity of Cdk5 immunoprecipitated from wild type and p35-deficient mouse brain, suggesting that G-actin suppresses endogenous Cdk5 activity in a p35-independent manner. Together, these results suggest a novel mechanism of actin cytoskeletal regulation of Cdk5/p35 activity.

Keywords

Cyclin-dependent kinase 5; actin; p25; p35; Alzheimer's disease

Introduction

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase, is a unique member of the Cdk family; its activity depends on association with two largely neuronal proteins p35, p39, and with Cyclin I (Dhavan & Tsai 2001, Brinkkoetter *et al.* 2009). In the developing brain, Cdk5 plays a critical role in neuronal migration, neurite growth and

*To whom correspondence should be addressed: Tel: 617-414-2658; tikezu@bu.edu.

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synaptogenesis, whereas in the adult brain Cdk5 may modulate synaptic plasticity (Lai & Ip 2009). This suggests that Cdk5 activity is normally under tight regulation. Indeed, deregulation of Cdk5 has been implicated in neurodegenerative diseases, such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Patrick *et al.* 1999, Lee *et al.* 2000, Nguyen *et al.* 2001).

Cdk5 regulates the dynamics of the neuronal cytoskeleton, which is comprised of actin, neurofilaments, and microtubule networks (Smith 2003). Actin filaments are major cytoskeletal components of the head and neck regions of dendritic spines, the dendritic spine periphery, and filopodia/lamellipodia of growth cones. The process of actin polymerization is a key component for the formation of dendritic spines, synaptic plasticity, and the guidance- and path -finding of growth cones (Matus 2000, Kalil & Dent 2005). Monomeric actin or globular actin (G-actin) assembles into long filamentous polymers (F-actin), whose dynamics are under tight regulation by over 150 actin-associated proteins and signaling molecules (Smith 2003). A number of molecules that regulate actin dynamics have been identified as Cdk5 substrates or interacting molecules, such as Pak1 (p21-activated serine/threonine kinase) (Nikolic *et al.* 1998), α-actinin-1, CaMKII (Calcium/calmodulin-dependent protein kinase II) (Dhavan *et al.* 2002), Cables (Cdk5 and Abl enzyme substrate) (Zukerberg *et al.* 2000), Synapsin I (Matsubara *et al.* 1996), p27 (Lee *et al.* 1996b), cofilin (Kawauchi *et al.* 2006), WAVE1/WASP (Kim *et al.* 2006), and neurabin 1 (Causeret *et al.* 2007). However, it is unknown whether the actin can also regulate Cdk5 kinase activity or not. We have shown that Cdk5/p35 activity is negatively correlated with co-precipitated actin in the mouse brain (Sato *et al.* 2008), suggesting that actin may negatively regulate Cdk5 kinase activity. In this report, we show evidence indicating a direct association of Cdk5 with G-actin and the inhibitory regulation of Cdk5 activity by G-actin.

Materials and Methods

Antibodies

The following antibodies were purchased: anti-p35 (C-19, rabbit polyclonal), anti-Cdk5 (C-8, rabbit polyclonal), anti-α-actinin-1 (H-2, mouse monoclonal), anti-GST (B-14, mouse monoclonal) from Santa Cruz Biotechnology, anti-β-actin (AC-15, mouse monoclonal from Sigma), anti-β III tubulin (G712, mouse monoclonal) from Promega Corporation

Reagents

Non-muscle actin (>99% pure) and α-actinin-1 (>90%) were purchased from Cytoskeleton Inc. Cytochalasin D and protease inhibitor cocktail were purchased from Sigma. Jasplakinolide was purchased from EMD Chemicals. Roscovitine was purchased from Calbiochem. Alexa Fluor® 594 conjugated DNase I was purchased from Invitrogen. The γ-^{32}P-ATP (3,000 μCi/mmol) was a product of Perkin Elmer.

Recombinant protein production

Recombinant proteins GST, N-terminally GST-tagged Cdk5, p35, and p35 fragments (plasmids kindly provided by Qi and Wang) (Lim *et al.* 2004, Hou *et al.* 2007) were expressed in *Escherichia coli* BL21 (DE3) and were purified as reported (Lim et al. 2004, Qu *et al.* 2002). C-terminally 6xHis-tagged Cdk5, p35 and p25 were purified by Ni-beads (Qiagen) from Sf9 cells infected with baculovirus encoding the respective genes as described and were of high purity (Supplemental Fig. S1) (Sakaue *et al.* 2005, Saito *et al.* 2003). Recombinant 6xHis-tagged human tau protein (htau 40,2N4R, 441 amino acid residues) with purity greater than 90% was purified as reported previously (Sato *et al.* 2006).

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F-actin co-sedimentation assay using purified recombinant proteins

The F-actin co-sedimentation assay was performed as described previously (Banerjee & Wedegaertner 2004). Recombinant proteins (100 ng each) were ultracentrifuged at 4°C (100,000 × *g*, 20 min) with an Optima TLX Ultracentrifuge (Beckman Coulter) to remove contaminated aggregates before the experiments and incubated with F-actin (polymerized *in vitro* with 5 μM G-actin in the presence or absence of 10 nM α-actinin-1) in 30 μL binding buffer (10 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM EGTA, 0.2 mM DTT, 1 mM ATP and protease inhibitor cocktail) at 30°C for 30 min. Subsequently, samples were centrifuged at 100,000 × *g* for 20 min and both supernatant and pellet fractions were fractionated with 12% SDS-PAGE and immunoblotted with anti-β-actin, anti-p35, anti-Cdk5, anti-α-actinin-1 and anti-GST antibodies.

GST pull-down assay using purified recombinant proteins

The GST pull-down assay was performed according to the published method with minor modifications (Kesavapany *et al.* 2003). Briefly, 10 nM GST or GST-tagged p10, p25, p35 and Cdk5 were incubated with 50 nM or 1.0 μM G-actin in the presence of equimolar cytochalasin D in 20 μL kinase buffer (20 mM Tris- HCl, pH 7.6, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, and protease inhibitor cocktail) at 30°C for 30 min, followed by the addition of 20 μL 50% slurry high affinity glutathione sepharose beads (GE Healthcare) and rotated at 4°C for 3 h. The beads were collected after spinning at 500 × *g* for 5 min and washed four times with TEE buffer (20 mM Tris-HCl, pH 8, 5 mM EDTA, and 5 mM EGTA). Subsequently, the beads were boiled for 5 min in 30 μL Laemmli sample buffer (BIO-RAD), and the supernatants were subjected to 12% SDS-PAGE and immunoblotting.

Cdk5 kinase activity assay using purified recombinant proteins

The Cdk5 kinase activity assay with recombinant proteins was conducted as reported (Lee *et al.* 1996a). 10 nM 6xHis-tagged Cdk5 was incubated with 10 nM 6xHis-tagged p35 or p25 at 30°C for 30 min in 10 μL kinase buffer. Phosphorylation was initiated by adding 5 μg histone H1 (HH1) (Axxora LLC) and 10 μCi γ-^{32}P-ATP, and incubated for 30 min at 30°C. The reaction was terminated by the addition of Laemmli sample buffer and heating at 65°C for 10 min, followed by 12% SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. Radioactivity exposed to a Phosphor Screen (Molecular Dynamics) was scanned by a Typhoon system (GE Healthcare), and the intensities of phosphorylated HH1 (p-HH1) bands were quantified with ImageQuant software (GE Healthcare).

Immunoblotting

Proteins were subjected to electrophoresis on 12% SDS-PAGE and then transferred to Immobilon-P membrane (Millipore), blocked by blocking buffer (SuperBlock, Pierce, with 5% skim milk), then probed with specific primary antibodies and HRP-conjugated anti-mouse/rabbit secondary antibodies. Chemiluminescence was performed with ECL Plus reagent (GE Healthcare) and the images were scanned by a Typhoon system. Chemiluminescent quantification of immunoreactive bands was done with ImageQuant software.

Kinase activity assay of Cdk5 complexes treated with F-actin

Equimolar (10 nM) Cdk5 and p25 or Cdk5 and p35 were incubated with or without 5 μM F-actin (in the presence or absence of 10 nM α-actinin-1) in 10 μL kinase buffer at 30°C for 30 min. HH1 (5 μg) and 10 μCi γ-^{32}P-ATP were added and incubated at 30°C for an additional 30 min. The remaining procedures were the same as for the Cdk5 kinase activity assay detailed above.

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Kinase activity assay of Cdk5 complexes treated with G-actin

Equimolar (10 nM) Cdk5 and p25 or Cdk5 and p35 were incubated with or without 0.5 μM G-actin (in the presence of 0.5 μM cytochalasin D to prevent actin polymerization), and in the presence or absence of 10 nM α-actinin-1 in 10 μL kinase buffer at 30°C for 30 min. HH1 (5 μg) or purified htau40 (5 μg) and 10 μCi γ-^{32}P- ATP were added and incubated at 30°C for an additional 30 min. The remaining procedures were the same as for the Cdk5 kinase activity assay detailed above.

Cdk5 kinase activity assay using immunoprecipitates from mouse brain extracts

Immunoprecipitation of Cdk5 complexes from mouse brains (kindly provided by Dr. Li-Huei Tsai) was conducted as described previously (Wang *et al.* 2003). Hemibrains of 4-month old wild type ($p35^{+/+}$, N=2) and p35 knockout ($p35^{-/-}$, N=3) littermates were weighed and homogenized in four volumes of ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 2 mM EDTA, and protease inhibitor cocktail, and spun at 14,000 × *g* at 4°C for 20 min. After determination of the protein concentration of the supernatants using a BCA assay (Pierce), 2 mg protein extracts were mixed with 4 μg anti-Cdk5 (C-8), 4 μg anti-p35 (C-19) rabbit polyclonal antibody or 4 μg normal rabbit serum (NRS) in 500 μL of lysis buffer and rotated head-to-end overnight at 4°C. Subsequently, 100 μL protein A/G-sepharose beads (Santa Cruz Biotechnology) were added and mixed by head-to-end rotation at 4°C for an additional 3 h. The beads were collected by brief centrifugation at 8,000 rpm for 30 sec, and the immunoprecipitates were washed four times with lysis buffer and once with 1 mL kinase buffer. The immunoprecipitates (100 μL) were incubated with kinase buffer containing 0, 0.1 μM, or 1.0 μM G-actin (supplemented with equimolar cytochalasin D to inhibit actin polymerization) or 20 μM roscovitine for 30 min at 30°C. HH1 (5 μg) and 10 μCi γ-^{32}P-ATP were added and incubated at 30°C for 60 min. The remaining procedures were the same as for the Cdk5 kinase activity assay detailed above.

Primary neuron culture and confocal microscopy

Human cortical neurons were dissociated human from fetal brain tissue (gestational age 13–16 weeks) from electively aborted specimens in full compliance with the University of Nebraska Medical Center and National Institutes of Health (NIH) ethical guidelines and cultured as reported previously (Peng *et al.* 2006). The purity of the neuron culture is more than 90% in general. At day 7 of *in vitro* differentiation, cells were treated with either membrane-permeable cytochalasin D (1 μM) or jasplakinolide (1 μM) for 5 hours, or 0.1% dimethyl sulfoxide (DMSO) as a control. Subsequently, cells were washed with PBS (pH 7.4) twice, fixed with 4% paraformaldehyde/PBS for 15 min at room temperature, washed with PBS five times, and permeabilized with 0.5% Triton-X-100 in PBS. Cells were incubated with 5% BSA and 5% normal donkey serum in PBS for 1 h at room temperature to block non-specific binding, followed by incubation with p35 rabbit polyclonal antibody (1:50, C-19, Santa Cruz), β III-tubulin mouse monoclonal antibody (1:1000, G712, Promega) for 1 h at room temperature and washed tree times with 0.1% Triton X-100 in PBS (PBST). The samples were incubated at room temperature for 30 min with appropriate fluorescent-labeled secondary antibodies (for p35, Alexa Fluor 488 donkey anti-rabbit IgG; for β III-tubulin, Alexa Fluor 647 donkey anti-mouse IgG, Invitrogen) diluted 1:500 in PBS. In addition, cells were incubated with Hoechst 33342 (1: 1000, Invitrogen) together with either Alexa Fluor 594-conjugated phalloidin (1:40, Invitrogen) or Alexa Fluor 594-conjugated DNase I (0.3 μM, Invitrogen) at room temperature for 20 min. Images were acquired using a Zeiss 510 Meta Confocal Laser Scanning Microscope (Carl Zeiss).

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Results

F-actin binds to the p10 region of p35 independently of α-actinin-1

We examined whether purified Cdk5, p25, p35, or p10 interact with F-actin by a co-sedimentation assay. Since Cdk5 and the p10 region of p35 can interact through α-actinin-1 (hereafter abbreviated as actinin), a known actin cross-linking molecule (Dhavan et al. 2002), we also investigated whether actinin plays a role in the interaction. F-actin polymerized from G-actin in the presence or absence of actinin was incubated with the various recombinant proteins, followed by ultracentrifugation. F-actin-bound molecules were found in the pellet and unbound molecules were found in the supernatant. As a negative control, GST protein did not co-sediment with F-actin (Fig. 1a). Co-incubated p35 and p10 co-sedimented with F-actin in the pellet fractions (Fig. 1b, c), while p25 and Cdk5 were mostly in the supernatant fractions (Fig. 1d, e). Pre-incubation of actinin with F-actin had no effect on the interaction of F-actin with any of the molecules. The ability of F-actin to bind p35 and p10 is independent of the presence of actinin, indicating that the N-terminal p10 region of p35 interacts directly with F-actin.

Effect of F-actin on the kinase activity of Cdk5/p35 and Cdk5/p25

We next determined how F-actin and actinin affected the kinase activity of Cdk5/p35 and Cdk5/p25 complexes. To optimize the experimental conditions, Cdk5/p35 or Cdk5/p25 complexes were incubated with substrate histone H1 (HH1) and γ-^{32}P-ATP at different time points. The Cdk5 kinase assay with p35 or p25 showed a time-dependent linear increase of HH1 phosphorylation with approximately 2-fold more phosphorylation occurring with p25 than p35 after 120 min (Supplemental Fig. S2). Based on this study, we incubated the recombinant kinase mixture with HH1 for 30 min, the intermediate time point for the linear increase of phosphate transfer to the substrate. Pre-incubation of Cdk5/p35 or Cdk5/p25 complexes with F-actin for 30 min before the kinase assay resulted in a 13% or 38% reduction in kinase activity compared to HH1 phosphorylation without actin, respectively (Fig. 2c, d), and the presence of actinin had no effect. The inhibition levels were unexpected, since p35 but not p25 binds to F-actin, and thus we predicted that F-actin would have a greater effect on Cdk5/p35 complexes than Cdk5/p25 complexes. To explore the possibility that complex formation of p35 or p25 with Cdk5 alters their binding activity to F-actin, we next looked at co-sedimentation of the complexes with F-actin. F-actin was polymerized from G-actin in the presence or absence of actinin and incubated with either Cdk5/p35 or Cdk5/p25 complexes, followed by the co-sedimentation assay (Fig. 3a, b). When Cdk5/p35 complexes and F-actin were incubated together, the majority co-sedimented in pellets, and actinin had no effect (Fig. 3a). Incubation of Cdk5/p25 complex with F-actin resulted in more Cdk5/p25 present in the supernatants than the Cdk5/p35 samples, and actinin had no effect. Since we observed little change in Cdk5/p35 complex kinase activity in the presence of F-actin, and greater inhibition occurred under conditions where more Cdk5/p25 complexes are found in the supernatants, these results suggest that F-actin-bound Cdk5 complexes maintain their activity. Since G-actin is present in small amounts in the F-actin preparations, these data suggest that inhibition of Cdk5 activity is mediated by G-actin on Cdk5 complexes in the supernatants that are free from F-actin.

G-actin binds to Cdk5 or p35 without disrupting complex formation of Cdk5/p35 or Cdk5/p25

To test this hypothesis, we examined the interaction of G-actin with p35, p25, p10, and Cdk5 by affinity precipitation and immunoblotting. Our preliminary study revealed that Ni-beads and anti-6xHis antibodies had non-specific interactions with G-actin, and thus 6xHis-tagged recombinant molecules were inapplicable for the study. GST-tagged recombinant molecules were used for these experiments. N-terminally GST-tagged p35, p25, p10, and

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Cdk5 were incubated with G-actin (in the presence of equimolar cytochalasin D to prevent actin polymerization), and the GST fusion proteins were precipitated with glutathione-conjugated sepharose beads, followed by extensive washings and immunoblotting. GST, a negative control, did not bind to G-actin (Fig. 4a). The relative amounts of G-actin bound to Cdk5, p35, and p25 were in the order Cdk5 > p35 ≫ p25 (Fig. 4a), which is distinctly different from the F-actin interaction with these molecules. We next examined the possibility that G-actin may disrupt Cdk5/p35 or Cdk5/p25 complex formation. GST-tagged p35 or p25 was pre-incubated with 6xHis-tagged Cdk5 in the presence or absence of G-actin for 30 min at 30°C, followed by the GST pull-down and immunoblotting. G-actin did not disrupt either Cdk5/p35 or Cdk5/p25 complex formation (Fig. 4b, c), suggesting that G-actin does not compete with p35 or p25 for binding to Cdk5.

G-actin potently inhibits Cdk5/p35 and Cdk5/p25 activities

We next tested G-actin for its ability to inhibit the kinase activity of Cdk5 complexes. Kinase assays were performed with complexes reconstituted *in vitro* from purified recombinant proteins with or without G-actin. The results showed that incubation with 0.5 μM G-actin strongly suppressed the activity of Cdk5/p35 and Cdk5/p25 (82% and 90% inhibition of HH1 phosphorylation, respectively, Fig. 5a–d), while actinin had no effect on the inhibition. We also examined G-actin-HH1 interaction by immunoprecitation and immunoblotting. Histone H1 does not bind to G-actin, ruling out the possibility that G-actin may bind to histone H1 to suppress Cdk5 kinase activity (data not shown). In addition, this inhibitory effect is not substrate-dependent, since potent inhibition of phosphorylation of another known substrate of Cdk5, tau protein (2N4R isoform, 441 amino acids), was also observed (Supplemental Fig. S3). The dose-response analysis of G-actin inhibition results in IC_{50} values of 37.0 and 36.5 nM against Cdk5/p35 and Cdk5/p25 complexes, respectively (Fig. 6). The similarity of the IC_{50} values against the two different Cdk5 complexes suggests that G-actin directly binds to and inhibits Cdk5independently of p35.

G-actin strongly inhibits kinase activity of Cdk5/p35 immunoprecipitated from mouse brain

Finally, we have tested whether G-actin regulates the activity of endogenous Cdk5 complexes in mouse brain, and if p35 is involved in the regulation. For that purpose, Cdk5 complexes were immunoprecipitated from the brains of $p35^{+/+}$ and $p35^{-/-}$ mice using anti-Cdk5 or anti-p35 antibodies. The complexes were incubated with increasing concentrations of G-actin (0.1 or 1 μM) or roscovitine, a Cdk5-specific inhibitor, for 30 min at 30°C, followed by the Cdk5 kinase assay. Immunoprecipitates of $p35^{-/-}$ mice using either anti-Cdk5 antibodies or anti-p35 antibodies displayed significantly lower Cdk5 kinase activity compared with that of $p35^{+/+}$ littermates (Fig. 7), consistent with a previous study (Chae *et al.* 1997). Roscovitine inhibited most of the Cdk5 activity from $p35^{+/+}$ brain, indicating that the kinase activity of immunoprecipitated samples is specifically from Cdk5. G-actin potently inhibited the activity of Cdk5 complexes immunoprecipitated from $p35^{+/+}$ and $p35^{-/-}$ mice (Fig. 7). This indicates that G-actin inhibits Cdk5 activity independent of p35 in mouse brain. We also found that endogenous actin co-immunoprecipitated with p35 using anti-p35 antibody in $p35^{+/+}$ but not $p35^{-/-}$ mouse brains (Fig. 7, arrow). Actin was, however, absent in anti-Cdk5 immunoprecipitates. This result is consistent with the findings here that p35 but not Cdk5 binds to F-actin (Fig. 1c, d), as well as with the previous study that p35 but not Cdk5 co-immunoprecipitates with actin (Sato et al. 2008).

P35/p25 colocalized with F-actin and G-actin in cultured neuronal cells

Since p35 is highly expressed in the cerebral cortex (Delalle *et al.* 1997) and plays a critical role in cortical lamination (Chae et al. 1997), we have chosen primary cultured cortical neurons to examine if alterations in intracellular actin polymerization can affect endogenous p35/p25 distribution. For that purpose, primary cultured neurons were treated with

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cytochalasin D (disrupts actin filaments) or jasplakinolide (stabilizes actin filaments) for 5 hr, followed by confocal microscopy imaging of p35/p25 in conjunction with either F-actin visualized by Alexa 594-tagged phalloidin (Fig. 8) or G-actin visualized by Alexa 594-conjugated DNase I (Fig. 9). We did not observe any overt change in cellular distribution pattern of p35/p25 upon treatments with cytochalasin D or jasplakinolide (compare Fig. 8c,g and k; Fig. 9c,g and k). P35/p25 immunocytochemistry results show that in control cells (treated with 0.1% DMSO), there are clear colocalizations of p35/p25 and F-actin in growth cones (arrow in Fig. 8d), soma and neurites (arrows, Fig. 8m). However, disruption of F-actin by cytochalasin D resulted in the formation of large F-actin clusters (arrows, Fig. 8f), consistent with previous reports (Humbert *et al.* 2000, White *et al.* 2001, Litman *et al.* 2000). P35/p25 co-localization with clumped F-actin became more apparent (arrows, Fig 8n). In jasplakinolide-treated neurons, the phalloidin signal was greatly reduced due to the competitive binding of jasplakinolide and phalloidin to F-actin (Bubb *et al.* 1994). Therefore, we also stained the cells with Alexa Fluor 594-conjugated DNase I which specifically stains G-actin (Zhang *et al.* 2004). In control neurons, p35/p25 colocalized with G-actin in cytoplasm and neurites (Fig. 9d). As expected, cytochalasin D-treated neurons showed increased DNase I staining concomitant with more p35/p25 co-localization. Jasplakinolide-treated neurons showed greatly reduced DNase I staining, and co-localization of p35/p25 was diminished also (Fig. 9l). There were no changes in βIII-tubulin staining, ruling out nonspecific effectsof exposure to cytochalasin D and jasplakinolide.

Discussion

Cdk5 is regulated by two central mechanisms, namely protein-protein interaction and phosphorylation (Lim *et al.* 2003). A majority of the Cdk5-interacting proteins that have been identified interact with Cdk5 through p35, while a small group of proteins interact directly with Cdk5 (Lim et al. 2003). For instance, casein kinase 2 inhibits Cdk5 activity by inhibiting Cdk5/p35 complex formation by binding to both Cdk5 and p35 independently (Lim et al. 2004). To our knowledge, this is the first report that a cytoskeletal protein (G-actin) binds to Cdk5 and inhibits its kinase activity directly, without disrupting Cdk5/p35 or Cdk5/p25 complex formation.

F-actin specifically interacts with the p10 region of p35 in an actinin-independent manner, presumably through interactions with the enriched basic residues found in the p10 sequence (Hou et al. 2007). Our study suggests that the binding of F-actin to p35 does not hinder Cdk5/p35 complex formation or inhibit its activity, because Cdk5 kinase binding and activating domains do not reside in the p10 region (Poon *et al.* 1997). F-actin also directly binds to Cdk5/p35 complexes in our study, suggesting that F-actin may serve as an anchoring cytoskeleton for Cdk5/p35 complexes in the neuronal periphery. How F-actin modestly interacts with Cdk5/p25 complexes as compared to Cdk5/p35 is unknown, since neither Cdk5 nor p25 interacts with F-actin. Perhaps Cdk5 partially stabilizes a weak association of p25 with F-actin, or the association of Cdk5 with p25 may create a novel binding pocket for F-actin.

It has been suggested that p25 has more potency than p35 to activate Cdk5 due to its longer (5~10- fold) half-life in cells (Patrick et al. 1999). However, to the best of our knowledge, this is the first report that, when Cdk5 complexes were prepared from individually purified Cdk5, p35 or p25, Cdk5/p25 complexes had higher catalytic activity compared with Cdk5/p35 complexes. Cdk5/p25 complexes indeed showed a greater rate of phosphorylation of HH1 than Cdk5/p35 complexes. This implies that there are differences in the conformation of the Cdk5 molecule between the two complexes. Perhaps the substrate-binding site is more accessible in the Cdk5/p25 complex than in the Cdk5/p35 complex, allowing for faster turnover. Hashiguchi et al showed that recombinant co-expressed complexes of Cdk5/p25-

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His_6 have significantly higher intrinsic activity (6-fold) on tau and HH1 than Cdk5/p35-His_6 complexes (Hashiguchi *et al.* 2002). However, a recent study reported that there is no difference in kinetics of tau or histone phosphorylation by co-expressed complexes of His_6-Cdk5/GST-p35 versus His_6-Cdk5/GST-p25(Peterson *et al.* 2010). These contradicting results might be attributed to differences in experimental paradigm, i.e, Cdk5 complexes purified from cells with co-expressed Cdk5 and its activators may include additional interacting molecules.

The modest inhibitory effects of Cdk5/p25 activity observed in *in vitro* kinase assays with F-actin are most likely due to the presence of residual G-actin after monomer-polymer equilibrium and its interaction with Cdk5/p25 complexes. The reason why we see more inhibition of Cdk5/p25 as compared to Cdk5/p35 is unknown. Considering the significantly higher amount of Cdk5/p25 complexes in the supernatant as compared to Cdk5/p35 after co-sedimentation with F-actin, it is possible that F-actin-free Cdk5 complexes are more susceptible to G-actin inhibition. Since G-actin can directly bind to Cdk5, one of the potential mechanisms is that F-actin might hinder the G-actin binding of Cdk5 and thus may protect Cdk5 complex activity. Alternatively, F-actin may efficiently incorporate G-actin to the F-actin polymer and thus dissociate it from Cdk5.

We have shown that G-actin is a potent inhibitor of the kinase activity, with an IC_{50} value of about 37 nM. This potency suggests that G-actin can suppress Cdk5 activity in the cytoplasm, especially in actin-rich regions, such as neuronal periphery, dendritic spine, and growth cone. Thus, our study suggests that G-actin may play a key role in Cdk5 activity in these regions. Indeed we observed co-localization of Cdk5 with both G-actin and F-actin in neuronal soma and neurites by confocal microscopy (data not shown). The functional implications for a differential effect of F-actin versus G-actin on Cdk5 kinase activity are not clear. Within a dendritic spine, there is a stable pool of F-actin in the spine core, while a dynamic pool of F-actin and G-actin rests in the spine shell (Racz & Weinberg 2006, Honkura *et al.* 2008), as well as the growth cone tip of the axon (Dent & Gertler 2003). Since enhancement of F-actin polymerization leads to a reduction of G-actin, the activity of F-actin-associated Cdk5 complexes would be sustained. This would lead to further actin polymerization in the growth cone tip, where Cdk5 interacts with and phosphorylates a plethora of proteins to regulate cytoskeletal dynamics. These observations are not restricted to purified molecules, since our observations were replicated using native Cdk5 complexes freshly immunoprecipitated from mouse brain. In addition, G-actin inhibits Cdk5 complex activity independent of p35 based on our $p35^{-/-}$ mouse study. These data also substantiate our point that G-actin inhibits Cdk5 activity through its direct interaction with Cdk5. While it was reported previously that $p35^{-/-}$ mice showed no Cdk5-associated kinase activity (Chae et al. 1997), we found that a lower level of Cdk5 kinase activity still exists in anti-Cdk5 immunoprecipitates from $p35^{-/-}$ mouse brain. Perhaps p39, and recently identified Cyclin I, are involved in the residual Cdk5 activity in the $p35^{-/-}$ mouse brain. In addition, p39 was also associated with the actin cytoskeleton, suggesting similar regulation by actin (Humbert et al. 2000).

It has been reported that p35 binds directly to α/β-tubulin and microtubules, which block p35 interaction with Cdk5 and therefore inhibit Cdk5/p35 activity (Hou et al. 2007). In addition, p35 induces microtubule assembly and bundling, modulating microtubule dynamics *in vitro* (Hou et al. 2007). On the other hand, Kaminosono *et al.* reported that Cdk5/p35 disrupts microtubule formation, thereby suppressing aggregation of mutant Huntington proteins (Kaminosono *et al.* 2008). This discrepancy may be due to differences in experimental paradigm since microtubule stabilization was observed with purified p35 *in vitro*, while microtubule-destabilization was observed in cultured cell lines possessing the Cdk5/p35 complex. However, in neuronal cells Cdk5 and p35 immunoreactivity coincides

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best with peripheral regions rich in actin filaments, such as the leading edges of growth cones, while tubulin immunoreactivity is mostly in the cell soma and axons (Nikolic *et al.* 1996). We have shown that p35/p25 colocalized with both F-actin and G-actin in soma and neurites in cultured cortical neurons. These co-localizations were enhanced upon F-actin clumping as a result of cytochalasin D exposure and weakened upon masking of phalloidin-stained F-actin due to competitive binding of jasplakinolide. Nonetheless, the overall cellular distribution of p35/p25 did not alter after toxin treatment, which might be due to the fact that p35 also binds to microtubules (Hou et al. 2007). Indeed, we found that p35 colocalized with βIII-tubulin better than with F-actin in control neurons (data not shown).

Our model of actin/p35/Cdk5 interaction (Fig. 10) will best apply to the regulation of Cdk5 activity in actin-enriched cell compartments, such as growth cone tips and synaptic dendrites. Indeed, Cdk5 and p35 are highly enriched in these areas and promote actin polymerization and neurite outgrowth through regulation of Rac/Pak1 signaling (Xie *et al.* 2006,Nikolic et al. 1998). Cdk5/p35 complexes can anchor to F-actin via direct interaction of p35 with F-actin or through α-actinin, and promote actin polymerization on site (Fig. 9). G-actin is also highly concentrated in this region and inhibits Cdk5 activity if it is not attached to F-actin, leading to spatial restriction of the Cdk5 activity to Cdk5/p35 complexes attached to F-actin. This tight regulation of Cdk5/p35 activity may be important for unidirectional extension of filopodia, and further extension of the F-actin will accumulate Cdk5/p35 complexes along the actin cytoskeleton. Cdk5/p25, on the other hand, lacks an association with F-actin. Thus its activity is not tightly regulated along F-actin, leading to its activation in the rest of the cytoplasm. F-actin polymerization is also essential for synaptic hypertrophy (or maturation), and Cdk5/p35 activity will also be tightly regulated along F-actin in dendrites, while G-actin will inhibit Cdk5 complexes not attached to actin filaments.

Abnormalities in cytoskeletal organization and/or dynamics are a common feature of many neurodegenerative disorders, including AD (Mendoza-Naranjo *et al.* 2007). Pathological actin in the form of F-actin has been found throughout Hirano bodies, which are cytoplasmic inclusions found in several neurodegenerative diseases (Galloway *et al.* 1987). Actin is also a component of cofilin-actin rods, inclusion-like structures described in hippocampal and cortical neurons of post-mortem AD brains (Minamide *et al.* 2000). However, how actin cytoskeletal alterations lead to neurodegeneration is unclear (Minamide et al. 2000). Intriguingly, tau-induced neurodegeneration is associated with the accumulation of F-actin and the formation of actin-rich rods in Drosophila and mouse models of tauopathy. Additionally, human amyloid-β peptide synergistically enhances the ability of wild-type tau to promote alterations in the actin cytoskeleton and mediate neurodegeneration (Fulga *et al.* 2007). In light of our novel discovery of the actin cytoskeleton as a modulator of Cdk5, it is possible that chronic activation of Cdk5 leads to abnormal F-actin accumulation in the cell soma and depletion of G-actin. This would result in the loss of G-actin-mediated Cdk5 inhibition and the abnormal activation of Cdk5 in the periphery, leading to synaptic degeneration. Further study will be necessary to address how Cdk5 dysregulation leads to synaptic loss in the context of actin regulation.

In summary, we have determined that G-actin but not F-actin potently suppresses Cdk5/p35 and Cdk5/p25 activities without disrupting the complexes. This effect is not substrate-selective, as G-actin inhibited phosphorylation of both HH1 and tau proteins. G-actin mainly binds to Cdk5 and p35 but not p25, whereas F-actin binds to p35 but not Cdk5 or p25. G-actin-mediated inhibition of Cdk5 complexes is not dependent on p35, suggesting its inhibition is through its direct interaction with Cdk5. Finally, actinin had no effect on actin-Cdk5 complex interaction or G-actin-mediated inhibition of Cdk5 complex activity. This actin-mediated regulation of Cdk5 complex activity will be relevant to understanding

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neuronal development, synaptogenesis, and neurodegenerative disorders where Cdk5 plays a key role in their mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
Cables	Cdk5 and Abl enzyme substrate
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBB	Coomassie Brilliant Blue
Cdk	cyclin-dependent kinase, GST, glutathione S-transferase
DTT	dithiothreitol
HH1	histone H1
HRP	horseradish peroxidase
NRS	normal rabbit serum
Pak1	p21-activated serine/threonine kinase
TTBK1	tau-tubulin kinase 1

References

Banerjee J, Wedegaertner PB. Identification of a novel sequence in PDZ-RhoGEF that mediates interaction with the actin cytoskeleton. Mol Biol Cell. 2004; 15:1760–1775. [PubMed: 14742719]

Brinkkoetter PT, Olivier P, Wu JS, Henderson S, Krofft RD, Pippin JW, Hockenbery D, Roberts JM, Shankland SJ. Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells. J Clin Invest. 2009

Bubb MR, Senderowicz AM, Sausville EA, Duncan KL, Korn ED. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J Biol Chem. 1994; 269:14869–14871. [PubMed: 8195116]

Causeret F, Jacobs T, Terao M, Heath O, Hoshino M, Nikolic M. Neurabin-I is phosphorylated by Cdk5: implications for neuronal morphogenesis and cortical migration. Mol Biol Cell. 2007; 18:4327–4342. [PubMed: 17699587]

Chae T, Kwon YT, Bronson R, Dikkes P, Li E, Tsai LH. Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron. 1997; 18:29–42. [PubMed: 9010203]

Delalle I, Bhide PG, Caviness VS Jr, Tsai LH. Temporal and spatial patterns of expression of p35, a regulatory subunit of cyclin-dependent kinase 5, in the nervous system of the mouse. J Neurocytol. 1997; 26:283–296. [PubMed: 9192293]

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Dent EW, Gertler FB. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron. 2003; 40:209–227. [PubMed: 14556705]

Dhavan R, Greer PL, Morabito MA, Orlando LR, Tsai LH. The cyclin-dependent kinase 5 activators p35 and p39 interact with the alpha-subunit of Ca2+/calmodulin-dependent protein kinase II and alpha-actinin-1 in a calcium-dependent manner. J Neurosci. 2002; 22:7879–7891. [PubMed: 12223541]

Dhavan R, Tsai LH. A decade of CDK5. Nat Rev Mol Cell Biol. 2001; 2:749–759. [PubMed: 11584302]

Fulga TA, Elson-Schwab I, Khurana V, Steinhilb ML, Spires TL, Hyman BT, Feany MB. Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo. Nat Cell Biol. 2007; 9:139–148. [PubMed: 17187063]

Galloway PG, Perry G, Gambetti P. Hirano body filaments contain actin and actin-associated proteins. J Neuropathol Exp Neurol. 1987; 46:185–199. [PubMed: 3029338]

Hashiguchi M, Saito T, Hisanaga S, Hashiguchi T. Truncation of CDK5 activator p35 induces intensive phosphorylation of Ser202/Thr205 of human tau. J Biol Chem. 2002; 277:44525–44530. [PubMed: 12226093]

Honkura N, Matsuzaki M, Noguchi J, Ellis-Davies GC, Kasai H. The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines. Neuron. 2008; 57:719–729. [PubMed: 18341992]

Hou Z, Li Q, He L, Lim HY, Fu X, Cheung NS, Qi DX, Qi RZ. Microtubule association of the neuronal p35 activator of Cdk5. J Biol Chem. 2007; 282:18666–18670. [PubMed: 17491008]

Humbert S, Dhavan R, Tsai L. p39 activates cdk5 in neurons, and is associated with the actin cytoskeleton. J Cell Sci. 2000; 113 (Pt 6):975–983. [PubMed: 10683146]

Kalil K, Dent EW. Touch and go: guidance cues signal to the growth cone cytoskeleton. Curr Opin Neurobiol. 2005; 15:521–526. [PubMed: 16143510]

Kaminosono S, Saito T, Oyama F, Ohshima T, Asada A, Nagai Y, Nukina N, Hisanaga S. Suppression of mutant Huntingtin aggregate formation by Cdk5/p35 through the effect on microtubule stability. J Neurosci. 2008; 28:8747–8755. [PubMed: 18753376]

Kawauchi T, Chihama K, Nabeshima Y, Hoshino M. Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. Nat Cell Biol. 2006; 8:17–26. [PubMed: 16341208]

Kesavapany S, Lau KF, Ackerley S, et al. Identification of a novel, membrane-associated neuronal kinase, cyclin-dependent kinase 5/p35-regulated kinase. J Neurosci. 2003; 23:4975–4983. [PubMed: 12832520]

Kim Y, Sung JY, Ceglia I, et al. Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. Nature. 2006; 442:814–817. [PubMed: 16862120]

Lai KO, Ip NY. Recent advances in understanding the roles of Cdk5 in neuronal plasticity. Biochim Biophys Acta. 2009

Lee KY, Rosales JL, Tang D, Wang JH. Interaction of cyclin-dependent kinase 5 (Cdk5) and neuronal Cdk5 activator in bovine brain. J Biol Chem. 1996a; 271:1538–1543. [PubMed: 8576150]

Lee MH, Nikolic M, Baptista CA, Lai E, Tsai LH, Massague J. The brain-specific activator p35 allows Cdk5 to escape inhibition by p27Kip1 in neurons. Proc Natl Acad Sci U S A. 1996b; 93:3259–3263. [PubMed: 8622924]

Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature. 2000; 405:360–364. [PubMed: 10830966]

Lim AC, Hou Z, Goh CP, Qi RZ. Protein kinase CK2 is an inhibitor of the neuronal Cdk5 kinase. J Biol Chem. 2004; 279:46668–46673. [PubMed: 15342635]

Lim AC, Qu D, Qi RZ. Protein-protein interactions in Cdk5 regulation and function. Neurosignals. 2003; 12:230–238. [PubMed: 14673210]

Litman P, Amieva MR, Furthmayr H. Imaging of dynamic changes of the actin cytoskeleton in microextensions of live NIH3T3 cells with a GFP fusion of the F-actin binding domain of moesin. BMC Cell Biol. 2000; 1:1. [PubMed: 11112983]

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Matsubara M, Kusubata M, Ishiguro K, Uchida T, Titani K, Taniguchi H. Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. J Biol Chem. 1996; 271:21108–21113. [PubMed: 8702879]

Matus A. Actin-based plasticity in dendritic spines. Science. 2000; 290:754–758. [PubMed: 11052932]

Mendoza-Naranjo A, Gonzalez-Billault C, Maccioni RB. Abeta1–42 stimulates actin polymerization in hippocampal neurons through Rac1 and Cdc42 Rho GTPases. J Cell Sci. 2007; 120:279–288. [PubMed: 17200137]

Minamide LS, Striegl AM, Boyle JA, Meberg PJ, Bamburg JR. Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. Nat Cell Biol. 2000; 2:628–636. [PubMed: 10980704]

Nguyen MD, Lariviere RC, Julien JP. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. Neuron. 2001; 30:135–147. [PubMed: 11343650]

Nikolic M, Chou MM, Lu W, Mayer BJ, Tsai LH. The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. Nature. 1998; 395:194–198. [PubMed: 9744280]

Nikolic M, Dudek H, Kwon YT, Ramos YF, Tsai LH. The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev. 1996; 10:816–825. [PubMed: 8846918]

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature. 1999; 402:615–622. [PubMed: 10604467]

Peng H, Erdmann N, Whitney N, Dou H, Gorantla S, Gendelman HE, Ghorpade A, Zheng J. HIV-1-infected and/or immune activated macrophages regulate astrocyte SDF-1 production through IL-1beta. Glia. 2006; 54:619–629. [PubMed: 16944452]

Peterson DW, Ando DM, Taketa DA, Zhou H, Dahlquist FW, Lew J. No difference in kinetics of tau or histone phosphorylation by CDK5/p25 versus CDK5/p35 in vitro. Proc Natl Acad Sci U S A. 2010; 107:2884–2889. [PubMed: 20133653]

Poon RY, Lew J, Hunter T. Identification of functional domains in the neuronal Cdk5 activator protein. J Biol Chem. 1997; 272:5703–5708. [PubMed: 9038181]

Qu D, Li Q, Lim HY, Cheung NS, Li R, Wang JH, Qi RZ. The protein SET binds the neuronal Cdk5 activator p35nck5a and modulates Cdk5/p35nck5a activity. J Biol Chem. 2002; 277:7324–7332. [PubMed: 11741927]

Racz B, Weinberg RJ. Spatial organization of cofilin in dendritic spines. Neuroscience. 2006; 138:447–456. [PubMed: 16388910]

Saito T, Onuki R, Fujita Y, Kusakawa G, Ishiguro K, Bibb JA, Kishimoto T, Hisanaga S. Developmental regulation of the proteolysis of the p35 cyclin-dependent kinase 5 activator by phosphorylation. J Neurosci. 2003; 23:1189–1197. [PubMed: 12598607]

Sakaue F, Saito T, Sato Y, Asada A, Ishiguro K, Hasegawa M, Hisanaga S. Phosphorylation of FTDP-17 mutant tau by cyclin-dependent kinase 5 complexed with p35, p25, or p39. J Biol Chem. 2005; 280:31522–31529. [PubMed: 15994305]

Sato S, Cerny RL, Buescher JL, Ikezu T. Tau-tubulin kinase 1 (TTBK1), a neuron-specific tau kinase candidate, is involved in tau phosphorylation and aggregation. J Neurochem. 2006; 98:1573–1584. [PubMed: 16923168]

Sato S, Xu J, Okuyama S, et al. Spatial learning impairment, enhanced CDK5/p35 activity, and downregulation of NMDA receptor expression in transgenic mice expressing tau-tubulin kinase 1. J Neurosci. 2008; 28:14511–14521. [PubMed: 19118186]

Smith D. Cdk5 in neuroskeletal dynamics. Neurosignals. 2003; 12:239–251. [PubMed: 14673211]

Wang J, Liu S, Fu Y, Wang JH, Lu Y. Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci. 2003; 6:1039–1047. [PubMed: 14502288]

White SR, Williams P, Wojcik KR, Sun S, Hiemstra PS, Rabe KF, Dorscheid DR. Initiation of apoptosis by actin cytoskeletal derangement in human airway epithelial cells. Am J Respir Cell Mol Biol. 2001; 24:282–294. [PubMed: 11245627]

Xie Z, Samuels BA, Tsai LH. Cyclin-dependent kinase 5 permits efficient cytoskeletal remodeling--a hypothesis on neuronal migration. Cereb Cortex. 2006; 16(Suppl 1):i64–68. [PubMed: 16766709]

Zhang H, Vutskits L, Calaora V, Durbec P, Kiss JZ. A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells. J Cell Sci. 2004; 117:93–103. [PubMed: 14627627]

Zukerberg LR, Patrick GN, Nikolic M, et al. Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron. 2000; 26:633–646. [PubMed: 10896159]

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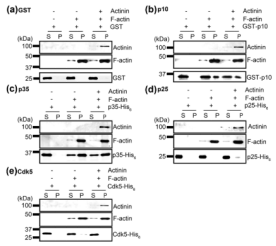

Fig. 1.

The interaction of F-actin with the components of Cdk5 complexes. The actin co-sedimentation assay was performed with F-actin polymerized from 5 μM G-actin *in vitro*. Recombinant GST (a), GST-p10 (b), p35 (c), p25 (d) and Cdk5 (e) were incubated with F-actin in the presence or absence of 10 nM actinin. After sedimentation at 100,000 × *g* for 30 min, both the supernatants (S) and the pellets (P) were fractionated by 12% SDS-PAGE and immunoblotted with anti-GST, anti-p35, anti-Cdk5, anti-β-actin, and anti-actinin antibodies.

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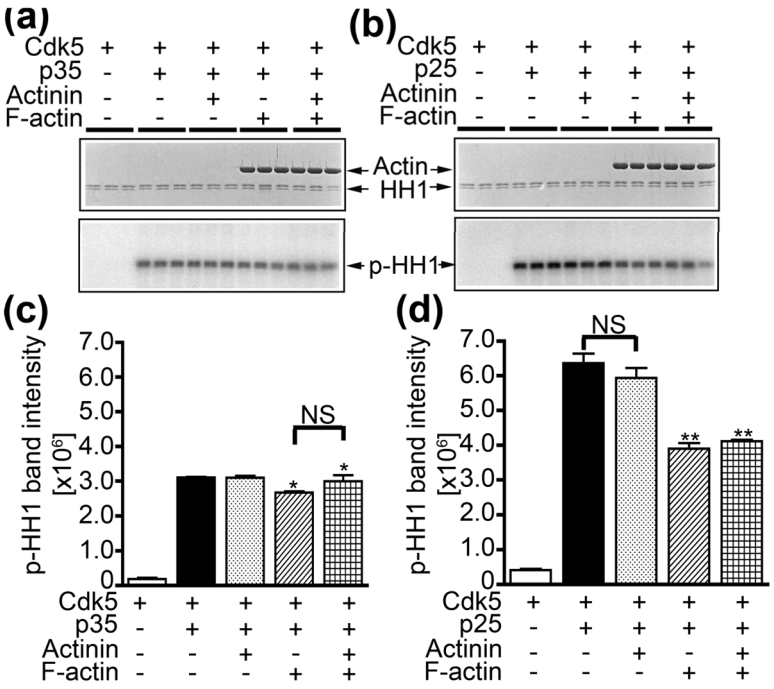

Fig. 2.

Effect of *in vitro*-prepared F-actin on the kinase activity of Cdk5/p35 and Cdk5/p25 complexes. 6xHis-tagged Cdk5 (10 nM) was incubated with equimolar p35 (a) or p25 (b) with or without F-actin (polymerized from 5 μM G-actin in the presence or absence of 10 nM actinin) for 30 min at 30°C. Histone H1 (HH1, 5 μg) and 10 μCi γ-^{32}P-ATP were added and incubated at 30°C for 30 min. The phosphorylation reaction was terminated by adding Laemmli sample buffer and heating at 65°C for 10 min, fractionated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (HH1, top). The autoradiogram of HH1 (p-HH1, bottom) was scanned and intensities of the p-HH1 bands were quantified with ImageQuant (c and d) and analyzed by one-way ANOVA. NS denotes no statistically significant difference by Student's *t* test, * denotes $p<0.05$ against Cdk5/p35, ** denotes $p<0.001$ against Cdk5/p25.

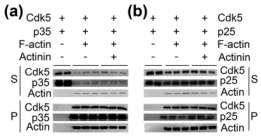

Fig. 3.

The interaction of F-actin with Cdk5 complexes. Equimolar (10 nM) 6xHis-tagged proteins Cdk5 and p35 (a) or Cdk5 and p25 (b) were incubated for 30 min at 30°C alone or with F-actin (polymerized from 5 μM G-actin in the presence or absence of 10 nM actinin), followed by centrifugation at 100,000 × *g* for 30 min, and the supernatants (S) and pellets (P) were immunoblotted with anti-Cdk5, anti-p35, and anti-β-actin antibodies.

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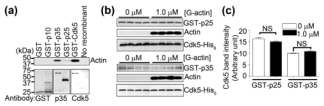

Fig. 4.

The interaction of G-actin with the components of Cdk5 complexes. (a), GST or GST-tagged p10, p25, p35, Cdk5 or no recombinant protein control were incubated with 1.0 μM G-actin at 30°C for 30 min, followed by addition of high affinity GST-resins to retrieve GST-tagged proteins. Washed resins were boiled with Laemmli sample buffer and the supernatants were immunoblotted with anti-β-actin, anti-GST, anti-p35 and anti-Cdk5 antibodies. GST-p25 (b, top panel) or GST-p35 (b, bottom panel) was incubated with Cdk5-His_6 without G-actin or with 1.0 μM G-actin, followed by addition of high affinity GST-resins for retrieval of GST-tagged proteins; the resins were subjected to intensive washing and boiled with Laemmli sample buffer and the supernatants were analyzed with Western blotting for Cdk5. (c), densitometry results of Cdk5 bands, NS denotes no statistically significant difference by Student's *t* test.

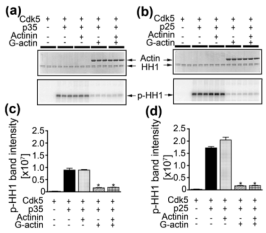

Fig. 5.

The effect of G-actin on Cdk5 kinase activity. 6xHis-tagged Cdk5 (10 nM) was incubated with equimolar p35 (a) or p25 (b) with or without 0.5 μM G-actin in the presence or absence of 10 nM actinin for 30 min at 30°C, followed by the addition of 5 μg histone H1 (HH1) and 10 μCi γ-^{32}P-ATP and incubated at 30°C for 30 min. The phosphorylation reaction was terminated by adding Laemmli sample buffer and heating at 65°C for 10 min, fractionated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (HH1, top). The autoradiogram of HH1 (p-HH1, bottom) was scanned and the intensities of p-HH1 bands were quantified with ImageQuant (c and d) and analyzed by one-way ANOVA, * denotes $p<0.001$against Cdk5 + p35 or Cdk5 + p25.

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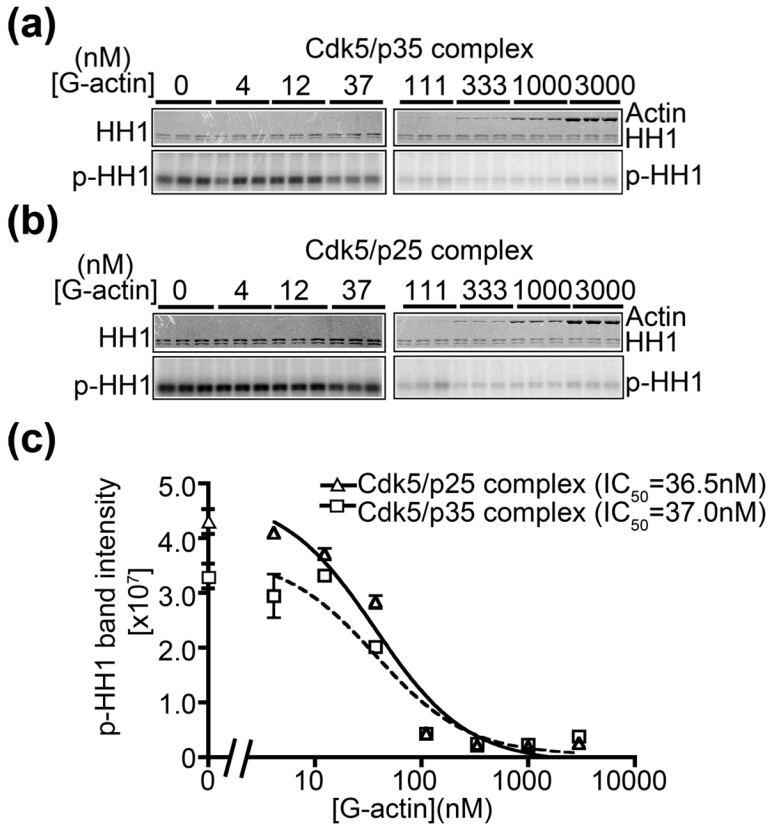

Fig. 6.

Determination of the IC_{50} for G-actin. 10 nM 6xHis-tagged Cdk5 and equimolar p35 (a) or p25 (b) were incubated with G-actin of increasing concentrations as indicated for 30 min at 30°C, followed by the addition of 5 μg histone H1 (HH1) and 10 μCi γ-^{32}P-ATP and incubated at 30°C for 30 min. The phosphorylation reaction was stopped by adding Laemmli sample buffer and heating at 65°C for 10 min, fractionated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (HH1, top). The autoradiogram of HH1 (p-HH1, bottom) was visualized and the intensities of p-HH1 bands were quantified with ImageQuant and plotted against G-actin concentration (c). The results were representative of three independent experiments.

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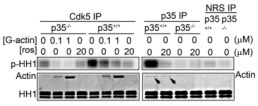

Fig. 7.

The effect of G-actin on Cdk5 complexes immunoprecipitated from mouse brain. Brain lysates (2 mg each) of $p35^{+/+}$ or $p35^{-/-}$ mice were immunoprecipitated with 4 μg Cdk5 antibody (C-8), p35 antibody (C-19) or normal rabbit serum (NRS). The immunoprecipitates were incubated with increasing concentrations of G-actin (0, 0.1, or 1 μM) or 20 μM roscovitine for 30 min at 30°C, followed by addition of 5μg histone H1 (HH1) and 10 μCi γ-^{32}P-ATP and incubated at 30°C for 60 min. The phosphorylation reaction was stopped by adding Laemmli sample buffer and heating at 65°C for 10 min, fractionated by 12% SDS-PAGE, and stained with Coomassie Brilliant Blue R-250 (HH1, bottom panels). The autoradiogram of HH1 (p-HH1, top panels) was scanned using the Typhoon system. The results were representative of three independent experiments.

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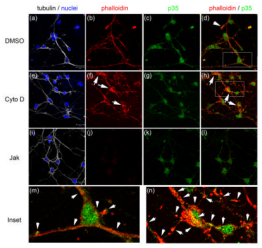

Fig. 8.

Co-localization of p35/p25 and F-actin in primary cultured cortical neurons. Human cortical neurons (day 7 *in vitro*) were treated with 0.1% DMSO (a), 1 μM cytochalasin D (Cyto D) (e), or jasplakinolide (Jak) (i) for 5 h. Cells were fixed and immunostained with p35/p25 (c,g,k) and β III-tubulin antibodies, and nuclei were stained with Hoechst 33342 (a,e,i). F-actin was visualized with Alexa 594 Fluor-conjugated phalloidin (b,f,j). Overlays of phalloidin and p35 staining are displayed in (d, h, i). Magnified views of framed fields in (d) and (h) are shown in (m) and (n) respectively, with arrows pointing to co-localized signals. Original magnification: 63x objective.

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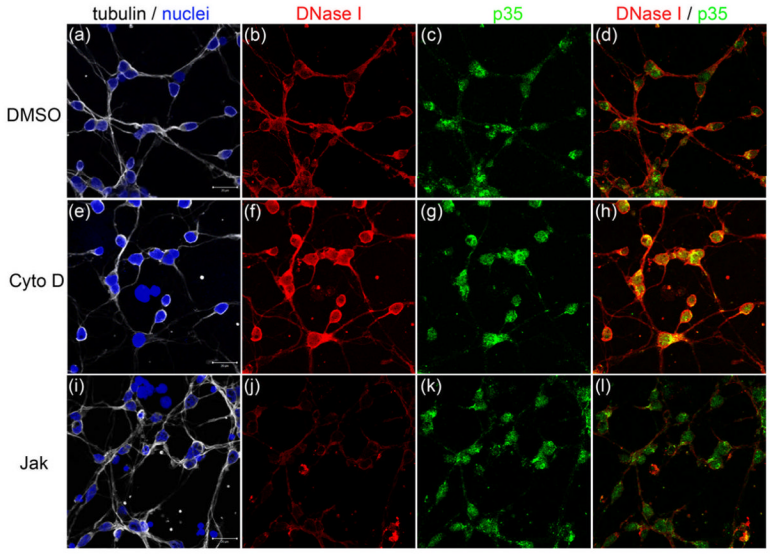

Fig. 9.

Co-localization of p35/p25 and G-actin in primary cultured cortical neurons. Human cortical neurons (day 7 *in vitro*) were treated with 0.1% DMSO (a), 1 μM cytochalasin D (Cyto D) (e), or jasplakinolide (Jak) (i) for 5 h. Cells were fixed and immunostained with p35/p25 (c,g,k) and β III-tubulin antibodies and nuclei were stained with Hoechst 33342 (a,e,i). G-actin was visualized with Alexa 594 Fluor-conjugated DNase I (b,f,j). Overlays of DNase I and p35 staining are displayed in (d, h, i). Original magnification: 63x objective.

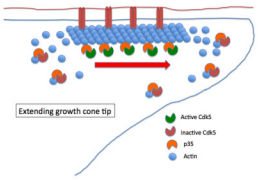

Fig. 10.
Schematic diagram of actin/p35/Cdk5 interaction and regulation of Cdk5 activity in an extending growth cone tip.

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