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The Presence of Active Cdk5 Associated with p35 in Astrocytes and Its Important Role in Process Elongation of Scratched Astrocyte

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KEY WORDS
Cdk5/p35; astrocyte; injury; hypertrophy; scratch

ABSTRACT
Cyclin-dependent kinase 5 (Cdk5) is a unique member of the Cdk family; its kinase activity requires association with its activator, p35 or p39. p35 is the strongest and best characterized activator. Previous studies showed that p35 is a neuron-specific protein that restricts Cdk5 activity in neurons. However, a high expression level of Cdk5 is found in astrocytes, which raises the possibility that astrocytic Cdk5 is functional. Here we show the presence of functional Cdk5 associated with p35 in astrocytes and demonstrate its important role in process elongation of scratched astrocytes. We found that p35 and glial fibrillary acidic protein (GFAP) were co-localized in primary cultured and acute isolated brain cells. Cdk5 could form an immunocomplex with p35 and its activity was shown in pure primary cultured astrocytes. p35 was upregulated in astrocytes injured by scratching, concomitantly with upregulation of Cdk5 kinase activity. Pretreatment of the scratched astrocytes with a Cdk5 inhibitor, roscovitine, could delay wound healing by inhibiting the reorganization of tubulin, GFAP, and the extension of hypertrophic processes. Moreover, overexpression of dominant negative Cdk5 could shorten the length of extending protrusion of reactive astrocytes. Thus, our findings demonstrated that functional Cdk5, associated with p35, was expressed in astrocytes and its activity could be upregulated in reactive astrocytes, a new role of Cdk5 that has never been reported in the nervous system. The present study may provide new insight for understanding the multifunctional protein complex Cdk5/p35 in the nervous system.

INTRODUCTION

Cdk5 is a unique member of the cyclin dependent kinase family of Cdks; activation of Cdk5 kinase activity requires association with its specific activators, p35 and p39 (Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994) or their truncated forms, p25 and p30 (Lew et al., 1994; Tang et al., 1995). Presently, the most important activator of Cdk5 is p35 (Chae et al., 1997; Kwon et al., 1999). However, these cofactors, which are believed to provide substrate specificity, differ markedly from other Cdk activating subunits called cyclins in both their primary sequence and regulation (Hellmich et al., 1992; Lew et al., 1994; Miyajima et al., 1995; Tsai et al., 1993). Previous studies show that, unlike Cdk5, which is expressed in numerous tissues including astrocytes, the expression of p35 is relatively restricted to terminally differentiated neurons, where it regulates neuronal functions such as migration, neurite outgrowth, exocytosis, microtubule organization, and synaptic vesicle endocytosis (Delalle et al., 1997; Lew et al., 1994; Tsai et al., 1994). However, a yeast two-hybrid system, which was employed to identify p35-interacting proteins from a human brain cDNA library, indicated that one of the isolated clones encodes a fragment of GFAP, which is a glial-specific protein (Qi et al., 1998). This gives a hint that p35 might also be expressed in astrocytes.

As we know, the astrocyte is the major glial cell type in the central nervous system (CNS), and astrocytes also play a pivotal role in scar formation following traumatic brain injury. In response to chemical or pathological tissue damage, astrocytes acquire characteristic functional and morphological features, referred to as reactive gliosis (Aono et al., 1988; Eddleston and Mucke, 1993; Eng et al., 1992; Reier et al., 1986; Ridet et al., 1997). Hence, we focused a great deal of attention on astrocytes to determine whether Cdk5 may have functions in these cells.

MATRIALS AND METHODS

Primary Culture of Astrocytes

Astrocyte cultures were prepared from the cerebral cortex of newborn ICR mice as previously described (Yu et al., 1993). Disassociated brain cells were seeded in 35-mm Falcon culture dishes (Becton Dickinson & Company, San Jose, CA) and maintained in 2 mL Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (Hyclone, Logan, UT) at 37°C in 5% CO_2.

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Cultures were fed twice a week and were used for experiments after they reached 4 weeks.

Acute Isolation of Brain Cells

Acute isolated brain cells were prepared from the cerebral cortex of newborn mice (ICR). Meninges-free cerebral cortices were cut into small pieces and vortexed in DMEM. Dissociated brain cells were seeded on poly-D-lysine (100 μg/mL, sigma)-coated glass coverslips (12 mm in diameter) in 35-mm culture dishes and maintained in 2 mL DMEM with 10% (v/v) fetal calf serum (Hyclone, Logan, UT) at 37°C in 5% CO_2 for 2 h to allow for attachment. Cells on coverslips were washed with PBS three times and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by immunofluorescent staining.

Scratch Wound Model

Confluent cultures were subjected to scratch wounds according to Yu et al. (1993). In brief, the cultures were scratched with a sterile plastic pipette tip according to the pattern of a 26-line grid to produce 40% injury (i.e., a removal of ~40% of the protein) (Lau and Yu, 2001; Yu et al., 1993). Immediately following scratching, culture dishes were washed twice with DMEM with 10% fetal calf serum, and then incubated in a CO_2 incubator until use.

Primary Culture of Cortical Neurons and Coculture of Astrocytes and Neurons

Cultures of male Sprague-Dawley (SD) rat cerebral cortical neurons (embryonic Day 18) were prepared as previously described (Rosenstein et al., 2003). In brief, cortices were dissected from the brains and subjected to trypsin digestion (0.25%; Sigma, USA) and mechanical trituration. The dissociated cells were harvested by centrifugation (1,000*g*) and resuspended in neurobasal growth medium containing B27 supplement (Invitrogen, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.5 mM glutamine. Cells were seeded to a density of 2×10^5 cells/cm^2 in dishes previously coated with poly-D-lysine (100 μg/mL, Sigma). After incubation for 24 h, the culture medium was replaced with freshly prepared serum-free growth medium. The cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

Astrocyte-neuron co-cultures were prepared using a protocol adopted from Guillemin et al. (2005). Primary rat cortical astrocytes and neurons were prepared from cerebral cortices of embryonic SD rats (E18-19) and incubated in sterile 10 mM HEPES buffer. Then these cortices were digested with 0.25% trypsin (Sigma, USA) and the digestion was ended by addition of fetal bovine serum. The suspension was centrifuged at 1,000*g* for 5 min and the precipitated cells were resuspended in DMEM containing 10% fetal bovine serum (HyClone, USA), 2 mM glutamine, 200 IU/mL penicillin G, and 200 μg/mL streptomycin sulfate (Sigma, USA). The cell suspension was filtered through a 70-μm nylon mesh filter and was plated at a density of 5×10^5 cells/cm^2 into poly-D-lysine-pretreated dishes. Cells were maintained in a 5% CO_2 incubator at 37°C. The medium was changed after 3 days, and then every 3–4 days.

RNA Extraction and RT-PCR

Total RNA of cultures was isolated using TRIzol (Invitrogen; 1 mL/100 mg tissue) according to the protocol provided by the manufacturer. The integrity of the RNA was verified by electrophoresis through 1% agarose gels. First-strand cDNA was synthesized by reverse transcription using oligo-dT primers and MMLV-reverse transcriptase (Progema, USA). The total PCR reaction mixture was 50 μL, containing 10× PCR buffer, 5 μL; 25 mM $MgCl_2$, 4 μL; 10 mmol/L dNTP, 1 μL; 10 mmol/L forward primer, 1 μL; 10 μmol/L reverse primer, 1 μL; cDNA, 10 μL; and Taq DNA polymerase (Progema, USA; 5 U/μL), 0.25 μL. PCR was carried out beginning with a single cycle of 94°C for 2 min, followed by 32 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. This was followed by a single cycle of 72°C for 10 min to facilitate final extension. The primers utilized were as follows: p35 forward primer, AGC AAG CTT CCA TGG GCA CGG TGC TGT CCC TG; and p35 reverse primer, AGC GGA TCC CCG ATC GAG CCC CAG GAG GAG. Final PCR products were confirmed with sequencing performed by Shanghai Sangon Biological Engineering & Technology and Service (Shanghai, China).

Protein Extraction and Western Blot Analysis

Cells on culture dishes were harvested by washing with ice-cold phosphate-buffered saline (PBS) three times and then scraping in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 10 mM EDTA, 0.5% NP-40, 1 μg/mL leupeptin, 1 mM PMSF, 4 mM NaF). The homogenates were sonicated 6 s for three times on ice, then centrifuged at 12,000*g* for 5 min at 4°C to yield the total protein extract in the supernatants. Naïve SD rats were deeply anesthetized with chlorate hydrate (300 mg/kg, i.p.) and the brains were dissected out and immediately homogenized in ice-chilled RIPA buffer. The homogenates were centrifuged at 12,000*g* for 10 min at 4°C to yield the total protein extract in the supernatants. Concentration of protein was measured with a BCA assay kit (Pierce). Then samples (50 μg) were denatured, subjected to SDS-PAGE using 12% running gels and transferred to nitrocellulose membranes. After blocking with 5% milk powder for 1 h at room temperature, the membranes were incubated with primary antibody, rabbit polyclonal anti-p35 antibody (1:100; Santa Cruz Biotechnology; sc-820) or monoclonal anti-Cdk5 antibody (1:1,000; Upstate; 05-364) overnight at 4°C.

The membranes were then washed and incubated with HRP-conjugated secondary antibody (1:2,000, goat anti-rabbit or anti-mouse; Bio-Rad Laboratories) and incubated for 1 h at room temperature. All washes were done using TBS T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 3 × 10 min. Finally, blots were detected with the Lightening Chemiluminescence Kit (Santa Cruz Biotechnology; sc-2048).

Cdk5 Kinase Assay and Immunoprecipitation

Cell lysates were immunoprecipitated with anti-Cdk5 antibody (1:50, Santa Cruz Biotechnology, CA, USA. sc-173) or rabbit control IgG (Santa Cruz Biotechnology, CA, USA) at 4°C for 3 h. Protein A-Sepharose CL-4B resin (Amersham Biosciences, Sweden) was added to the samples and the incubation was continued for a further 12 h, after which samples were washed four times with TBS/0.1% Triton X-100 and two times with assay buffer [20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 0.1 mM dithiothreitol]. The final pellet was resuspended in reactive buffer [20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 1 μg/μL Histone H1 protein (Sigma, St. Louis, MO, USA. H5505), 0.2 μCi/μL ^{32}P ATP] to yield a total volume of 40 μL. The mixture was incubated at 30°C for 30 min, and the reaction was terminated by the addition of SDS-PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed to X-ray film for autoradiography. For immunoprecipitation, cells lysates were immunoprecipitated with rabbit anti-Cdk5 antibody, rabbit anti-P35 antibody, or rabbit control IgG (Santa Cruz Biotechnology, CA) at 4°C for 3 h. Protein A-Sepharose was added to the samples and the incubation was continued for a further 12 h, after which samples were washed six times with TBS/0.1% Triton X-100 and the final pellet was boiled with SDS-PAGE sample buffer and were subjected to Western blot analysis with mouse anti-Cdk5 antibody used for immunoblotting. Rabbit IgG staining by an HRP-conjugated secondary antibody (1:5,000, goat anti rabbit; Bio-Rad Laboratories) was used as a loading control.

Immunofluorescent Staining

Cells on culture dishes were washed with PBS three times, fixed with 4% paraformaldehyde for 10 min, washed with PBS three times for 5 min, permeabilized with 0.3% Triton X-100 for 30 min and washed with PBS three times for 5 min. After blocking of nonspecific binding with 10% goat serum in PBS for 1 h at room temperature, the cells were incubated with primary antibody (rabbit anti-p35 antibody 1:100, Santa Cruz Biotechnology, sc-820; mouse anti-α-tubulin antibody 1:1,000, Sigma, T6199; and mouse anti-GFAP antibody 1:1,000, Sigma, G3893) overnight at 4°C, washed with PBS three times for 5 min, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies for 1 h, and washed with PBS three times for 5 min. Finally, the cells were mounted on a slide.

Negative controls were incubated with mixture of primary antibody and blocking peptide. The stained section was examined with a Leica fluorescence microscope (Nussloch, Germany) or a Leica TCS 4D confocal microscope using an omnichrome air cooled helium/neon laser tuned to produce beams of 488 and 568 nm.

Plasmids Construction and Transfection

For mammalian expression, the open reading frame of Cdk5 was amplified by PCR and subcloned into pEGFP-N1 (Clontech). The dominant-negative (kinase inactive) Cdk5 (N144) (Nikolic et al., 1996) was prepared by mutating an aspartic acid in the 144 position to an asparagine in the pEGFP-N1 Cdk5 plasmid. Mutagenesis was performed with the Quick Change Site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by sequencing performed by Shanghai Sangon Biological Engineering and Technology and Service (Shanghai, China).

Plasmid was transiently transfected into confluent primary astrocyte cultures using Lipofectamine™ 2000 (Gibco, USA) with serum-free DMEM and the culture medium was replaced with 10% FBS after 4 h. Scratch wounds were made at 24 h after transfection.

Morphology Study

After scratching, primary astrocyte cultures were observed under a Leica phase contrast microscope. Images from the same field of view were captured and analyzed at different time intervals following the scratching by a CCD camera system (SPOT, Diagnostic Instruments). To observe the variation in wounds after scratching at different time points and with different treatments, the edge of the cell free area was outlined in black. The total area and the length of the cell free area were measured. The area divided by the length is the average width of the cell free area. The difference in the width of the cell free area before and after treatment was obtained by the width after treatment subtracted from that of before treatment. In transfection experiments, images of transfected cells at scratched edge were evaluated. To calculate the length of protrusion of reactive astrocytes, the distance from the centre of nucleus to the furthest tip of protrusion was measured.

Statistical Analysis

Data are expressed as means ± SEM and were analyzed using one-way ANOVA followed by Newman-Keuls test.

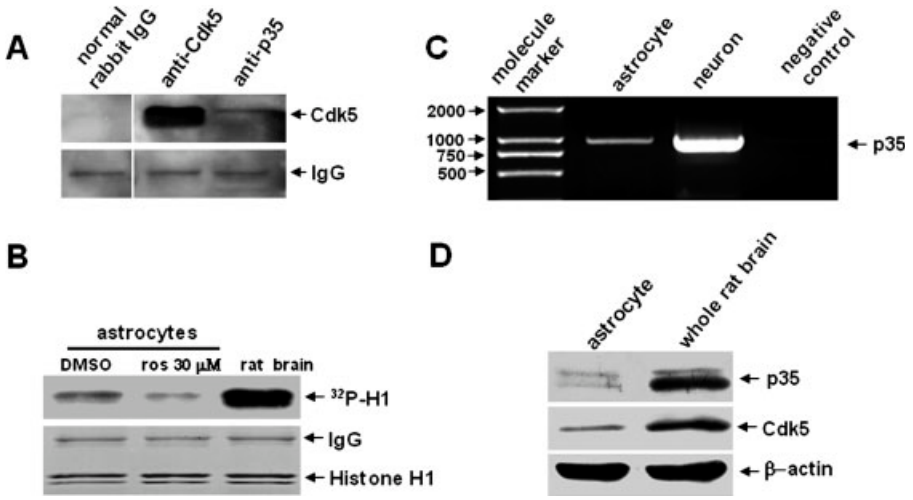

Fig. 1. The presence of active Cdk5 associated with p35 in astrocytes. (**A**) Cell lysates from primary astrocytes were immunoprecipitated (IP) with normal IgG, Cdk5, or p35 antibodies, and then were immunobloted with Cdk5 antibody. (**B**) Cdk5 was immunoprecipitated from the lysates of primary astrocytes or rat brain and the immunocomplex was then subjected to a kinase assay with or without treatment of the inhibitor for Cdk5, roscovitine. The autoradiograph for ^{32}P-labeled Histone H1 (upper) and the corresponding Coomassie blue-stained gel of IgG and Histone H1 (lower) are shown. 30 μM ros: 30 μM roscovitine. (**C**) RT-PCR was performed with the total RNA from primary astrocytes or primary neurons. A PCR was performed as a negative control in which RNA (preparation for reverse transcription) in stead of cDNA was added. A full-length p35 was amplified in primary astrocytes. (**D**) Presence of p35 in astrocytes. Cell lysates from primary astrocytes or rat brain homogenate were subjected to 12% SDS-PAGE and detected by western blot. β-actin was served as a loading control.

RESULTS
p35-Associated Cdk5 Kinase Activity is Present in Astrocytes

Although it has been reported that Cdk5 is expressed in astrocytes, no information has been shown about whether it has kinase activity in astrocytes. Thus, we performed a kinase assay to evaluate this issue. Figure 1A shows that the endogenous Cdk5 complex was immunoprecipitated with a Cdk5 antibody (Fig. 1A, Lane 2) and this Cdk5 complex had an associated kinase activity, which was measured with Histone H1 as a substrate (Fig. 1B, Lane 1), although the level was markedly lower than in whole rat brain (Fig. 1B, Lane 3). Since Histone H1 can also be a substrate for other kinases such as Cdk1 and Cdk2, we analyzed the specificity of Cdk5 activity by using the Cdk5 inhibitor roscovitine in identical kinase assays. We observed an approximately 80% reduction in phosphorylated Histone H1 levels after adding 30 μM roscovitine as compared with controls (Fig. 1B, Lane 2). It is reported that roscovitine also depresses Cdk1 and Cdk2 activity (Meijer et al., 1997). However, 4 week astrocyte primary cultures were used in the present study, and during this period, astrocytes showed less proliferation and fully differentiation due to cell to cell contacts (Etienne-Manneville, 2006). In addition, Tikoo et al. (1997) reported that no activity of Cdk2 was found in astrocyte extracts, which suggested that 4 weeks mature astrocytes were lacking of Cdk1 and Cdk2 activities. All the aforementioned results suggested that most of the kinase activity presents in Cdk5 immunoprecipitates was derived from Cdk5, and astrocytes could express active Cdk5.

As we mentioned earlier, the activation of Cdk5 requires the presence of its activators, a 35-kd protein distantly related to cyclins, known as p35, its isoform, p39 (Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994), or their truncated forms, p25 and p30 (Lew et al., 1994; Tang et al., 1995). Presently, the most important activator of Cdk5 is p35 (Chae et al., 1997; Kwon et al., 1999). To test whether p35 was present in astrocytes, we performed the following experiments: First, we did the co-immunoprecipitation of p35 with Cdk5 in a primary astrocyte culture, where the results showed that p35 antibody brought down with it a 33-kd protein for Cdk5 (Fig. 1A, lane 3). This result determined that Cdk5 could bind to p35 in astrocytes. Normal rabbit IgG was served as a negative control (Fig. 1A, lane 1). Second, we determined the level of expression of mRNA encoding p35 in primary cultures of astrocytes. RNA was isolated and RT- PCR was performed to detect the presence of mRNA encoding p35. To ensure the authenticity of PCR products, we designed specific primers for obtaining about a 1 kb full-length fragment. As determined by electrophoresis, PCR products from primary cultures of astrocytes and neurons were of the correct size, indicating that they were derived from intended target. PCR products were also verified by sequencing. To eliminate genomic DNA as a possible source of contamination, a PCR was performed as a negative control in which RNA (preparation for reverse transcription) in stead of cDNA was added. In addition, a positive control was detected from cDNA of primary neuronal cultures (Fig. 1C). RT-PCR data demonstrated that p35 was expressed in primary cultures of astrocytes. Third, considering that gene expression does not translate into protein in some cases and to determine whether p35 may be the functional activator of Cdk5 in astrocytes, we investigated p35 protein expression by Western blot analysis. Astrocytes were found to express a 35-kd protein for p35, but not

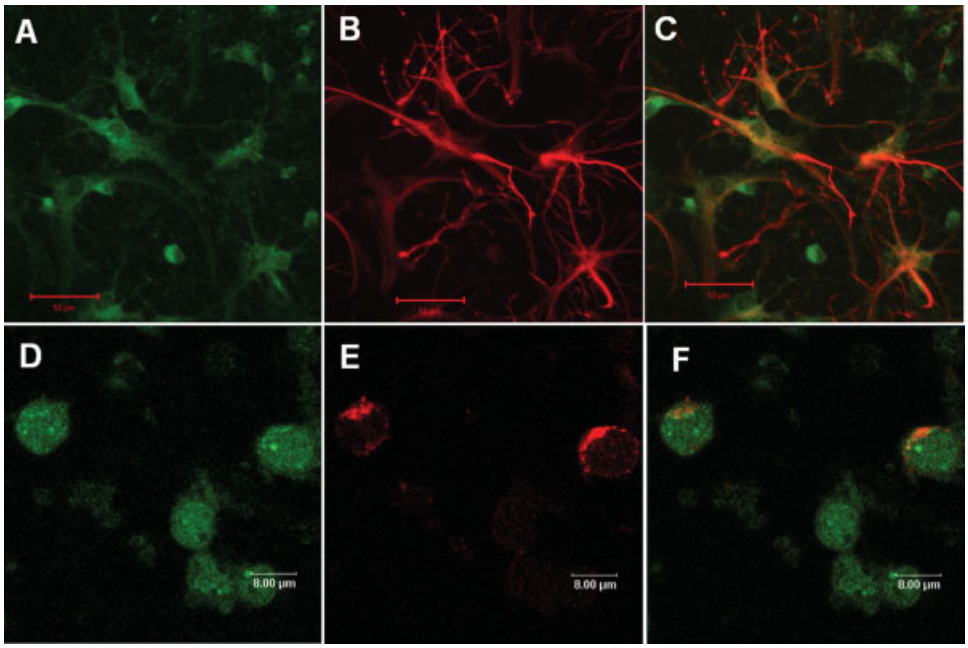

Fig. 2. Presence of p35 in astrocyte-neuron mixed primary cultures and acutely isolated brain cells. Immunofluorescence double-staining for p35 (green) and GFAP (red) was performed. Stained astrocyte-neuron mixed primary culture cells (**A–C**) or acutely isolated brain cells (**D–F**) were mounted and examined by confocal microscopy. (A, D) are staining for p35 (green); (B, E) are staining for GFAP (red); (C, F) are merges of A and B or D and E, respectively. GFAP could be co-locolized with p35 in astrocyte-neuron mixed primary cultures and acutely isolated brain cells. Scale bars represent 50 μm (A–C) or 8 μm (D–F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

for p25 (the cleavage product of p35). A brain tissue sample was served as a positive control (Fig. 1D). The specificity of the antibody for p35 has been confirmed by many laboratories (Chen and Studinski, 2001). Together, these data provide strong evidence that active Cdk5 is associated with p35 in astrocytes.

p35 Is Present in Astrocytes of Astrocyte-Neuron Cocultures and in Acute Isolated Brain Astrocytes

To further confirm that p35 was expressed in astrocytes *in vivo*, we investigated double immunofluorescent staining of p35 in astrocytes from astrocyte-neuron cocultures and from acute isolated brain cells of new-born mice. To test the specificity of the p35 antibody, samples were incubated with mixture of antibody and a blocking peptide of p35. No immunofluorescent signal was visualized (data not shown), indicating the antibody for p35 was specific and suitable for the further verification of expression in astrocytes. The results showed that some p35 (green) was partially located in GFAP (red)-positive cells and suggested that p35 was present in astrocytes *in vivo* (Fig. 2).

Both Cdk5 Kinase Activity and p35 Were Upregulated in Astrocytes After Scratching Injury

The aforementioned results showed that active Cdk5, associated with p35, was present in astrocytes, although the expression level was lower than that of whole brain. We then asked whether the lower active Cdk5 in astrocytes has a functional effect. Scratch-wound assays have been used to study different aspects of repair processes during scar formation along lesion sites (Nobes and Hall, 1999; Kornyei et al., 2000). In the following experiments, we used the scratch-wound model to determine the function of active Cdk5 in astrocytes. Astrocytes at about 40% confluence were scraped away with a yellow pipette. The kinase activity of Cdk5 in the astrocytes was detected by kinase assay at different time points (0, 3, 6, and 24 h) after scratching. As compared with untreated astrocytes, Cdk5 activity was upregulated markedly at 3 and 6 h, and sustained for at least 24 h after scratching (Fig. 3A). Since the activity of Cdk5 requires the presence of its activators, we then asked whether p35 in astrocytes contributes to the elevated activity of Cdk5 in wounded astrocytes. Therefore, p35 was measured at the same time points by western blot analysis (Fig. 3B). The results indicated that p35 was upregulated with a similar fashion as that of Cdk5 activity, no significant difference of Cdk5 level was shown among different time points. To avoid the influence of a variety of skeletal proteins, we chose Coomassie brilliant blue R250 staining instead of β-actin as the loading control.

To confirm the elevation of p35 in astrocytes after scratching, we employed immunofluorescent staining of p35. Since a remarkable increase in p35 expression occurred at 3 h after scratching, the image at 3 h after scratching is shown in Figs. 3C,D. The results indicated that brighter p35 staining was present at the edge of scratch wound, while staining was relative faint in the astrocytes far away from the edge of scratch wound. Moreover, in the confluent, nonmigrating cells (far away from the edge of scratch wound), p35 was localized in the cytoplasm and mainly associated with perinuclear membranes (Fig. 3D, white arrowhead). In migrating astrocytes, p35 was still visible in perinuclear membranes, but p35 fluorescence was additionally enhanced at the very tip of the process, apparently associated with

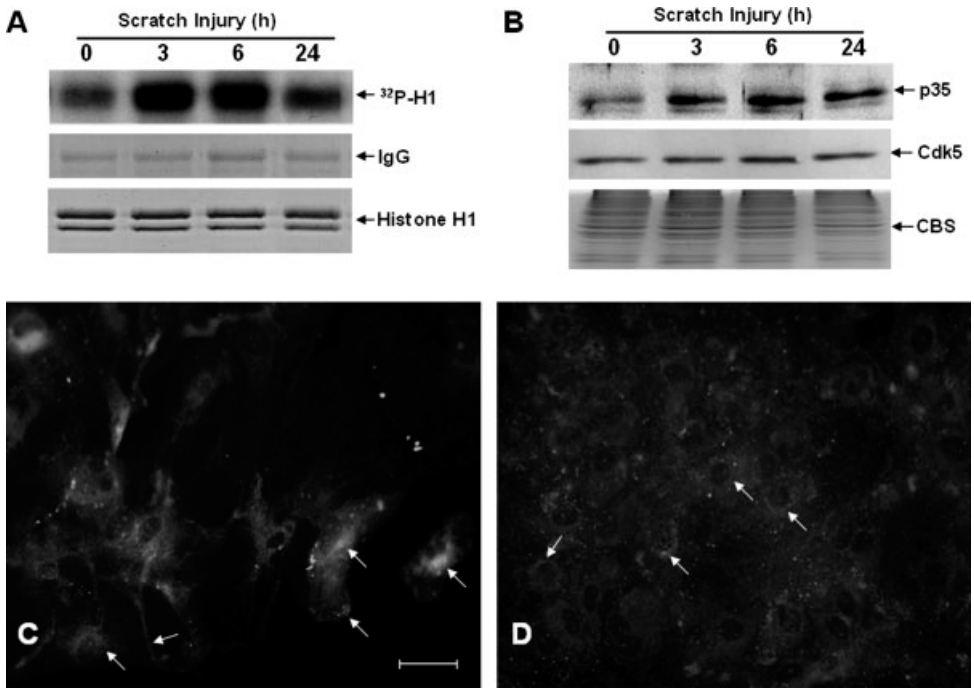

Fig. 3. Both p35 and Cdk5 kinase activity were upregulated in the astrocyte primary cultures after scratching. (**A**) Cdk5 kinase activity was detected at indicated time points. The autoradiograph (upper) and the corresponding Coomassie blue-stained gel of IgG (middle) and Histone H1 (lower) are shown. The panel shows an autoradiograph for ^{32}P-labeled Histone H1 and demonstrated that Cdk5 kinase activity was up regulated in astrocytes after scratching. (**B**) Scratch wounds were made in astrocyte primary cultures at different time points (0, 3, 6, and 24 h), the cells were harvested, and total protein extracts of different time points were subjected to SDS-PAGE and detected with p35 and Cdk5 antibodies, respectively. Coomassie brilliant blue R250 staining (CBS) (lower panel) was served as loading control. (**C**, **D**) 3 h after scratching, cells were fixed and stained with an antibody to p35. Stained cells were mounted and examined by immunofluorescence microscopy. (C) Immunofluorescence staining for p35 at the edge of a scratch wound. p35 shows a bright staining pattern; (D) Immunofluorescence staining for p35 far away from edge of a scratch wound. p35 staining is faint. Scale bars represent 40 μm. All experiments were repeated three times independently.

the plasma membrane at the leading edge (Fig. 3C, white arrowhead). These findings suggested that Cdk5 kinase activity was upregulated in astrocytes after scratching, which may be due to upregulation of p35, especially those located at the scratch wounded edge.

Inhibition of Cdk5 Activity Suppressed the Protrusion of Scratched Astrocytes and Restrained the Elongation of Astrocytes at the Edge of the Scratch

Our results demonstrated that Cdk5 activity was upregulated in the wounded astrocytes that may be due to upregulation of p35, especially those located at the scratch wounded edge; therefore, active Cdk5 might play an important role in the migration of astrocytes. To study the function of Cdk5/p35 in scratched astrocytes, roscovitine, an inhibitor of Cdk5, was applied. We incubated the cultures with roscovitine or DMSO at different concentrations (20 min before scratching). Twenty-four hours after the scratching, the astrocytes treated with 100 μM roscovitine healed much slower than those treated with DMSO ($P < 0.001$), and astrocytes treated with 10 μM roscovitine healed slight slower than those treated with DMSO, although no statistical significance was shown ($P > 0.05$). The astrocytes treated with 30 μM roscovitine showed intermediate effects ($P < 0.001$) (Fig. 4B). Quantification of the remaining denuded area revealed that, compared with that of scratched control cultures treated with DMSO, elongation of astrocytes at the edge of the scratch was strongly inhibited in cells treated with the Cdk5/p35 inhibitor in a concentration-dependent manner (Fig. 4A). To localize the nuclei of the cells covering the wound area and to exclude an apoptotic effect, we performed Hoechst 33342 staining. No obvious difference in nucleus staining of scratched astrocytes among these groups (Fig. 4A, right column) was observed.

Cytoskeletal Characteristics in Wounded Atrocytes after Roscovitine Treatment

Cell migration requires coordinated changes in cytoskeletal dynamics, which govern the protrusion of intermediate filament GFAP and microtubule-containing lamellipodia and filopodia at the front and retraction at the back (Ridley et al., 2003). We were then interested in whether there are cytoskeletal changes in wounded astrocytes after treatment with Cdk5 kinase inhibitor roscovitine. As in previous scratch experiments, we gen-

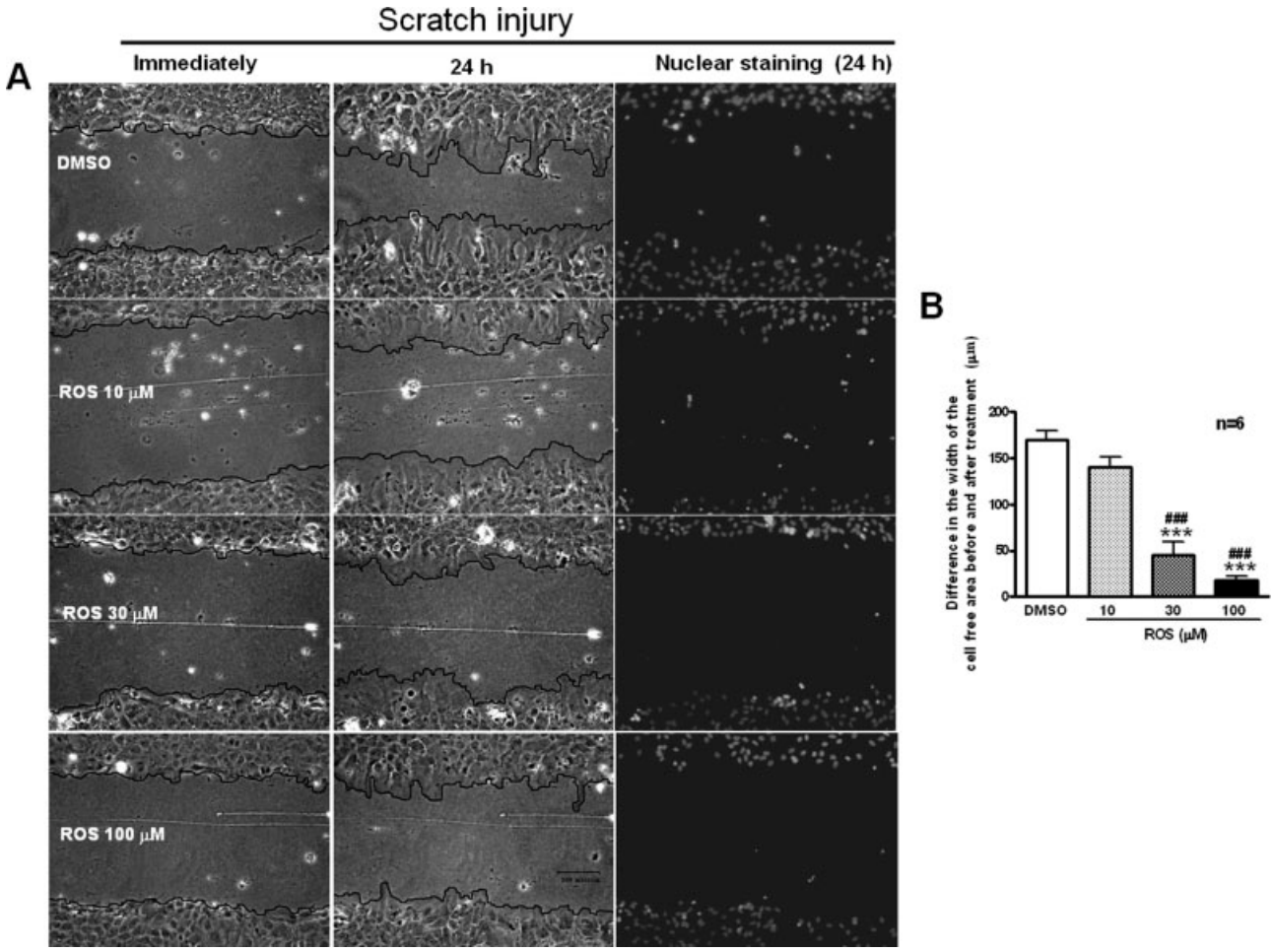

Fig. 4. Roscovitine restrained scratch-wound closure in astrocyte primary cultures in a dose-dependent fashion. Scratch wounds were made in confluent astrocyte primary cultures. (**A**) Cell cultures were treated with DMSO (A, upper panel), or 10 μM (A, middle panel), 30 μM (A, lower panel), or 100 μM (A, lowest panel) roscovitine 20 min before scratching. Twenty-four hours after scratching, images from the same fields were evaluated (middle column). The culture treated with 100 μM roscovitine healed slower than that treated with DMSO; the cultures treated with 10 or 30 μM roscovitine displayed the intermediate phenotype. No obvious difference was observed in nucleus staining of scratched astrocytes by Hoechst 33342 staining (right column). Scale bars represent 100 μm. Pictures shown are representative of three independent experiments. (**B**) To observe the variation in wounds after scratching at different time points and with different treatments, the edge of the cell free area was outlined in black. The total area and the length of the cell free area were measured. The area divided by the length is the average width of the cell free area. The difference in the width of the cell free area before and after treatment was obtained by the width after treatment subtracted from that of before treatment and the difference among different treatments was compared. ***$P < 0.001$, compared with DMSO group; ###$P < 0.001$, compared with the 10 μM roscovitine group.

erated wounds and applied Cdk5 kinase inhibitor roscovitine (Figs. 5A,B) or DMSO (Figs. 5C,D) to the cultures at a concentration of 30 μM. Cells were fixed at 24 h after scratching. We found that, in contrast to cells cultured in the absence of roscovitine, which adopted an elongated shape with long microtubule-containing protrusions toward the wound, cells cultured in the presence of roscovitine displayed a microtubule meshwork that was not orientated and appeared disorganized (Figs. 5A,C). These results were further supported by the staining of astrocyte-specific intermediate filament GFAP (Figs. 5B,D). The phenotype was observed throughout scratch-induced migration, demonstrating that microtubule morphology and rearrangement were altered in cells lacking Cdk5.

In addition to GFAP and α-tubulin staining, we performed Hoechst 33342 staining to localize the nuclei of the cells covering the wound area and rule out an apoptotic effect. The result showed no differences in the number of nuclei with apoptotic nucleus morphology between control and roscovitine-treated astrocytes (Fig. 5), thus excluding the possibility that the decreased migration of roscovitine-treated astrocytes was due to differences in cell death.

Overexpression of Dominant Negative Cdk5 in Wounded Atrocytes Shorten the Length of Protrusion

The aforementioned results indicated that inhibition of endogenous Cdk5 activity by pretreatment with roscovitine suppressed the protrusion of scratched astrocytes and restrained the elongation of astrocytes at the

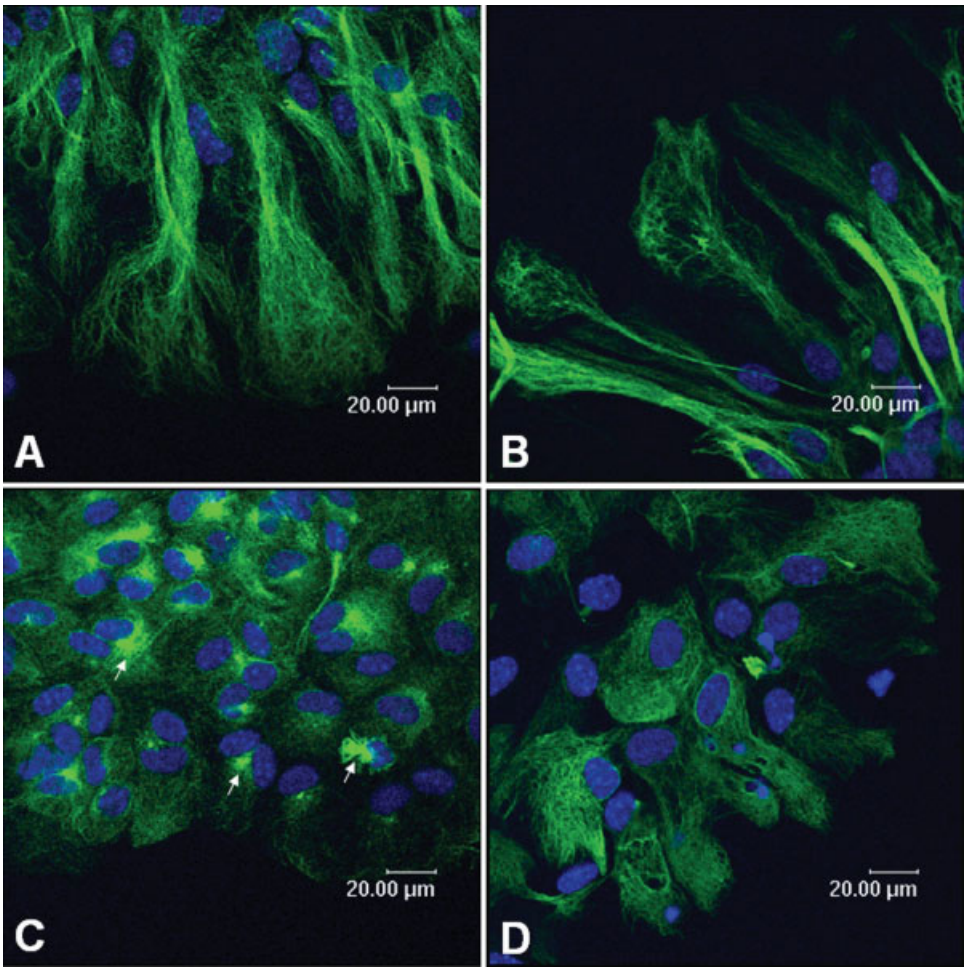

Fig. 5. Roscovitine inhibited the reorganization of α-tubulin and GFAP in reactive astrocytes. Astrocyte primary cultures were treated with DMSO (**A**, **B**) or 30 μM roscovitine (**C**, **D**) 20 min before scratching. Twenty-four hours after scratching, cells were fixed and stained with antibodies to α-tubulin (A, C) or to GFAP (B, D). α-Tubulin and GFAP at the edge of scratch wounds in the reactive astrocytes treated with roscovitine were diffused into the cytoplasma and α-tubulin was condensed perinuclearly (white arrow), while they showed filiform distribution in the DMSO group. In addition, extension of hypertrophic processes from these reactive astrocytes was inhibited by roscovitine. No obvious difference was observed in nucleus staining of scratched astrocytes by Hoechst 33342 (blue). Scale bars represent 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

edge of the scratch. As we mentioned above that besides Cdk5 activity, roscovitine could suppress the activities of Cdk1 and Cdk2. In addition, mature astrocytes will show both proliferative (which may due to the activation of Cdk1 and Cdk2) and protruding response to scratched injury. Thus, to further elucidate the roles of Cdk5 activity in protrusion of scratched astrocytes, we transfected a dominant negative Cdk5 with a GFP tag that could specifically inhibit the activity of Cdk5 into confluent primary astrocyte cultures transiently. Wild-type Cdk5 and empty vector were used as controls. Twenty-four hours after transfection, cells were scratched, and then the cells were fixed and stained with antibody to α-tubulin or nucleus marker to Hoechst 33342 at 24 h after scratching. Cells that only expressed GFP or GFP fusion protein and localized at the wound edge were evaluated. To calculate the length of protrusion of reactive astrocytes, the distance from the centre of nucleus to the furthest tip of protrusion was measured. The results indicated that the protruding length of the cells transfected with dominant negative Cdk5 was shorter than that of GFP or Cdk5-GFP group (Figs. 6A–D). (** $P < 0.01$, as compared with the GFP or Cdk5-GFP group.) Cells transfected with wild-type Cdk5 or empty vector adopted an elongated shape with long microtubule-containing protrusions toward the wound (Figs. 6A,B). No significant difference was found between Cdk5-GFP and GFP group (Figs. 6A,B).

Taken together, impairment of Cdk5/p35 signaling in astrocytes resulted in both reduced process extension towards the scratch and a decreased migratory activity of astrocytes, contributing to a faster response to *in vitro* injury.

DISCUSSION

The present study shows that astrocytic Cdk5 has kinase activity that is associated with p35. Furthermore, functional astrocytic Cdk5 played an important role in process elongation of scratched astrocytes. This is the first report showing the function of Cdk5/p35 in the astrocytes.

Previous data showed that Cdk5 was expressed extensively in various tissues or cells, even in astrocytes. However, p35, the best studied activator of Cdk5, was reported to be neuronal-specific (Tsai et al., 1993). Moreover, Zheng et al. (1998) showed that p35 mRNA is at the highest level in post-mitotic neurons but almost absent in proliferative cells, which restricts Cdk5 activa-

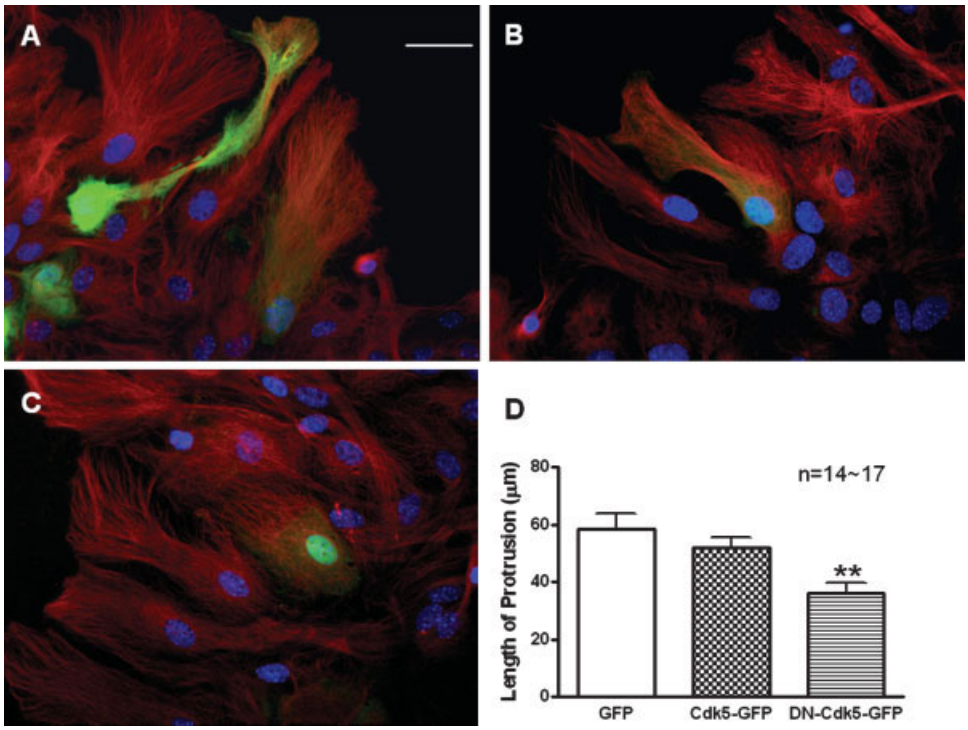

Fig. 6. Effects of overexpressed Cdk5 or dominant negative Cdk5 on reactive astrocytes. GFP, Cdk5-GFP, or dominant negative Cdk5-GFP was transiently transfected into confluent primary astrocyte cultures using Lipofectamine 2000 with serum-free DMEM, scratch wounds were made after 24 h, 24 h after scratching, and then the cells were fixed and stained with antibody to α-tubulin (red) or nucleus marker to Hoechst 33342 (blue). Images of transfected cells at scratched edge were evaluated. To calculate the length of protrusion of reactive astrocytes, the distance from the centre of nucleus to the furthest tip of protrusion was measured. The protrusion length of cells transfected with dominant negative Cdk5 (**C**) was shorter than that of GFP group (**A**) and Cdk5 goup (**B**) at the edge of wounds. Scale bars represent 20 μm. (**D**) Quantitative analysis of the difference in the length of protrusion of reactive astrocytes in the different groups is shown. $^{*}P < 0.01$, compared with the GFP or Cdk5-GFP group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion to neurons rather than proliferative cells. Recent studies proved that p35 is not only a brain-specific activator of Cdk5, but is also expressed and functions in Cdk5 activation outside the nervous systems, such as in testis, human keratinocytes, corneal epithelial cells (Gao et al., 2004; Nakano et al., 2005; Rosales et al., 2004), etc. Therefore, the cellular expression of p35 in the brain needs to be further investigated, in particular whether astrocytes, as an important cell type in the brain, have functional Cdk5 related to p35.

In the present study, we showed that p35 (the best-characterized activator of Cdk5) and GFAP (a marker of astrocytes) co-localized in the same cells both in primary cultures and in acute isolated astrocytes, which indicated that p35 was indeed present in astrocytes. However, the mRNA level of p35 in astrocyte primary cultures was significantly less than that of neurons and the protein level of p35 in astrocyte primary cultures was less than that of rat brain homogenate as well. Thus, the previous negative results in astrocytes might be due to the lower expression level of p35 in astrocytes. Moreover, previous studies were mostly carried out using *in vivo* models, which might make it difficult to exclude the interference of neurons.

Although the presence of Cdk5 activity and p35 in astrocytes was at low level under normal conditions, the small amount of Cdk5 activity and p35 was functional, especially in pathologic situations. As we know, astrocytes contributing to scar-formation along lesion sites acquire a characteristic "reactive" morphology and display a variety of functional changes (Eddlestone and Mucke, 1993; Ridet et al., 1997).

To study the process of glial activation, several *in vitro* models have been proposed (Wu et al., 1998). In a set of these model systems, the *in vivo* traumatic events were simulated by exposing cells to direct mechanical injury, namely by scratching confluent astrocytic monolayers with blades, needles, or pipette tips (Hou et al., 1995; Mukhin et al., 1998; Yu et al., 1993). In response to "scratch-wound injury" some well-known signs of the *in vivo* reactivation of astroglial cells were recognized in the scratch-area, such as elongation of "hypertrophic" processes, hypertrophy of astrocytic nuclei, enhanced expression of extracellular matrix molecules, and an increase in GFAP content (Yu et al., 1993). The realignment and expansion of the intermediate filament network are the most characteristic responses of astrocytes to injury *in vivo*. On the basis of the appearance of the above markers, the models were used to predict the effects of a variety of molecular factors on glial activation (Faber-Elman al., 1995; Hou et al., 1995).

Thus, we employed astrocyte primary cultures, in which more than 95% of the cells were GFAP-positive and lacked neuronal components, to establish the scratch-wound model. By Western blot analysis, it was indicated that p35 protein expression in astrocytes was upregulated after scratching that was accompanying with upregulation of Cdk5 activity. Moreover, we found that the p35 signal at the scratch wound edge was much stronger than was present far away from the scratch wound edge, as detected by immunofluorescence. Roscovitine, an inhibitor of Cdk5, could restrain wound closure in a dose-dependent manner. Further, both roscovitine and dominant negative Cdk5 could repress the protru-

sion of wounded astrocytes. These results indicated that the activity of Cdk5 was upregulated at the edge of scratch wounds due to the upregulation of p35 which played an important role in astrocyte activation resulting from mechanical wounds.

Figures 5 and 6 showed that the inhibition on protrusion of wounded astrocyte by dominant negative Cdk5 was weaker than that of roscovitine. As we mentioned above that, besides Cdk5, Cdk1, and Cdk2 activity, which plays an essential role in the cell division cycle, can also be inhibited by roscovitine (Meijer et al., 1997). Although the extract from astrocytes was lacking of Cdk2 kinase activity in mature astrocytes (Tikoo et al., 1997), when fully differentiated astrocytes (4 week primary cultures) encounter mechanical injury, they will show proliferative response which might relate to the activation of Cdk1 and Cdk2. Thus, the reasons for the weaker effect of dominant negative Cdk5 might be due to the following aspects: one might be the incomplete repression of Cdk5 activity by dominant negative Cdk5; another might be the double inhibition of roscovitine on proliferation (inhibition of Cdk1 and Cdk2 activity) and protrusion (inhibition of Cdk5 activity) of wounded astrocytes. A recent study has reported that the application of the Cdk inhibitor flavopiridol (a broad-spectrum Cdk inhibitor), roscovitine, and olomoucine (which mainly inhibits Cdk2 and Cdk5) to astrocyte primary cultures can reduce astrocyte proliferation. Among these, flavopiridol is the most effective. In rats with traumatic brain injury (TBI), flavopiridol can reduce the formation of astroglial scars and microglial activation (Di Giovanni et al., 2005). These *in vivo* results support our *in vitro* findings. However, there is also the inconsistent result of the application of roscovitine, which indicated that Cdk5/p35 activation retards corneal debridement wound closure in mice and suppresses the construction of the cytoskeleton (Gao et al., 2004). These inconsistent results might be caused by differences between tissue or cell types, or a variety of other mechanisms.

Since active Cdk5 was present in astocytes and Cdk5/p35 played an important role in astrocyte activation resulting from mechanical wounds, what mechanisms contributed to Cdk5/p35-mediated astrocyte scratch wound closure? The combined *in vivo* and *in vitro* data indicate that Cdk5 is necessary and sufficient for neuronal cell cycle arrest and subsequent differentiation (Cicero and Herrup, 2005), suggesting that the promotion of scratched astrocyte elongation via activation by Cdk5/p35 was not mediated by cell proliferation. Furthermore, we showed here that scratching a confluent monolayer of primary mouse astrocytes leads to polarization of cells at the leading edge and directed cell protrusion and migration, specifically occurring perpendicularly to the scratch. Roscovitine, the inhibitor of Cdk5 and the dominant negative Cdk5, suppressed this phenomenon, indicating that Cdk5 activation by scratching might be involved in the protrusion and migration of scratched astrocytes. The mechanisms of Cdk5 involvement in protrusion and migration of scratched astrocytes needs to be further investigated. In addition, the role of astrocytic Cdk5/p35 in neuron and the brain is worthy to be further elucidated.

In the present study, we indicated that active Cdk5 associated with p35 was present in astrocytes, although it was at a lower level under normal conditions. Cdk5 kinase activity was upregulated in astrocytes injured by scratching, concomitantly with upregulation of p35 indicated that Cdk5/p35 may play a key role in the activation of astrocytes after mechanical injury. The presence of functional Cdk5/p35 in astrocytes may provide new insight for understanding the multifunctional protein complex Cdk5/p35 in the nervous system.

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