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cdk5 Modulates β- and δ-Catenin/Pin1 Interactions in Neuronal Cells

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Abstract The cdk5/p35 complex has been implicated in a variety of functions related to brain development, including axonal outgrown and neuronal migration. In this study, by co-immunoprecipitation and pull-down experiments, we have shown that the cdk5/p35 complex associates with and phosphorylates the neuronal δ-catenin. Immunocytochemical studies of δ-catenin and the cdk5-activator p35 in primary cortical neurons indicated that these proteins co-localize in the cell body of neuronal cells. In addition, cdk5 co-localized with β-catenin in the cell–cell contacts and plasma membrane of undifferentiated and differentiated N2A cells. In this context, we identified Ser^{191} and Ser^{246} on β-catenin structure as specific phosphorylation sites for cdk5/p35 complex. Moreover, Pin1, a peptidyl–prolyl isomerase (PPIase) directly bound to both, β- and δ-catenin, once they have been phosphorylated by the cdk5/p35 complex. Studies indicate that the cdk5/p35 protein kinase system is directly involved in the regulatory mechanisms of neuronal β- and δ-catenin. J. Cell. Biochem. 100: 738–749, 2007.

Key words: cdk5; β-catenin; δ-catenin; Pin1; neurons

cdk5 is a proline-directed serine/threonine protein kinase that form an active complex with p35 in differentiated cells, such as neurons, muscle, germinal, and blood cells [Hellmich et al., 1992; Hirooka et al., 1996; Lazaro et al., 1997; Chen and Studzinski, 2001; Godet et al., 2003]. This enzyme plays a major role in neuronal migration and differentiation, as well as in neuronal plasticity [Muñoz et al., 2000; Maccioni et al., 2001]. cdk5 phosphorylates cytoskeletal proteins, such as tau, MAP-1b, neurofilament proteins as well as proteins linked to cell adhesion and intraneuronal transport processes [Kobayashi et al., 1993; Shetty et al., 1995; Pigino et al., 1997; Shuang et al., 1998; Humbert et al., 2000]. Through these activities cdk5 contributes to modulate cytoskeletal organization and dynamics [Ledda et al., 2002; Sasaki et al., 2002]. The regulation of its kinase activity is determined by its interaction with the activators p35 and p25, a truncated form of p35, which contains the proline rich region and the cdk5 binding domains [Tang et al., 1997]. The 10-kDa N-terminal domain contains myristylation signals that are crucial for p35 binding to the plasma membrane, as well as the signals for proteosomal degradation [Patrick et al., 1999]. The p25 moiety, p35 and p39 are only expressed in the nervous tissue, particularly in differentiating neurons, together with the short half lives of these proteins are factors that determine that cdk5 is mainly active in the nervous system. Therefore, substrate specificity and intracellular localization of cdk5 are precisely regulated by these activators [Patrick et al., 1998; Humbert et al., 2000]. Antisense oligonucleotides that block the expression of cdk5 produce a marked decrease in neuritogenesis [Pigino et al., 1997]. Similar effects were produced after transfection of neurons with a plasmid that

Abbreviations used: cdk5, cyclin-dependent kinase 5; Pin1, peptidyl–prolyl isomerase 1.

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codifies a dominant negative of cdk5 [Paglini et al., 1998]. Overexpression of p35 and cdk5, as well as GDNF and semaphorin 3A induces neuritic growth.

Neuritogenesis involves re-organization of actin and microtubules cytoskeletal networks, a process that contributes to the changes required, in neuronal plasticity, for lamellipodia and filopodia formation. cdk5 connects cytoskeleton organization regulating proteins that participate in actin remodeling, such as β-catenin, Pak1, Rac1, and MAPs [Kobayashi et al., 1993; Hosoi et al., 1995; Nikolic et al., 1998; Humbert et al., 2000; Zhong et al., 2003]. Defects in axonal paths and fasciculation were observed in mice deficient in cdk5 and p35. This reveals a role of cdk5 in axonal guidance phenomena [Ohshima et al., 1996, 1999; Chae et al., 1997; Ko et al., 2001]. In addition, cdk5 knockout mice display alterations in the anatomical organization of hippocampal and cortical neurons [Gupta et al., 2002]. However, p35 knockout mice are not lethal, possibly due to its compensation for the presence of p39, but exhibit serious disturbance in axonal migration [Chae et al., 1997; Kwon et al., 1999].

Double hybrid studies have shown that p35 interacts with β-catenin [Kwon et al., 2000], a protein of great importance in cell migration and adhesion phenomenon. Interestingly, cortical neurons generate aggregates mediated by N-cadherin interactions between different neuronal cells, when treated with the cdk5 inhibitor roscovitine [Kwon et al., 2000; Kesavapany et al., 2001]. β-catenin contains an N-terminal domain of around 130 aminoacids followed by 12-repeats in tandem and a C-terminal domain of around 100 aminoacids [Huber et al., 1997]. This protein interacts with transmembrane cadherin and can link cadherin–cadherin complex with actin and microtubule cytoskeletons [Barth et al., 1997; Levenberg et al., 1998]. Furthermore, β-catenin participates as a transactivator in the control of genes involved in cell differentiation and proliferation [Polakis, 2000]. The levels of β-catenin are regulated by the Wnt/β-catenin signaling. The Wnt/*frizzled*/LRP complex signals to disheveled and blocks Gsk3β, a protein kinase that phosphorylates β-catenin and it is responsible for its proteosomal degradation [Mao et al., 2001]. When β-catenin is phosphorylated in tyr645, interacts with TCF/LEF transcription factor, translocating into the nucleus [Willert and Nusse, 1998], an association that controls the activity of genes, such as c-jun, c-myc, and cyclin D1, key proteins in the control of cell proliferation [He et al., 1998; Tetsu and McCormick, 1999].

The protein β-catenin is expressed in high levels in neuritic and synaptic processes at early stages of neuronal development. Its association with Wnt appears to be important in modeling brain architecture. The brains of transgenic mice expressing stabilized β-catenin development enlarged brain, increased cerebral cortical surface area, and regulates the formation of neuronal precursors. A loss in Wnt function causes malformations in different brain areas [Chenn and Walsh, 2002, 2003; Zechner et al., 2003]. The mechanisms by which β-catenin increases neuronal precursors is linked to its capacity to re-enter to the cell cycle [Chenn and Walsh, 2002].

δ-catenin belongs to the family of proteins with the *armadillo* domain, and contains 10-repeats of this domain [Lu et al., 1999a], this region present high homology with β-catenin in the Arm repeats, interacts with N-cadherin and participates in the formation of adhesion complexes. The expression of this protein is related to the stage of brain development. Thus, at early stages of neuronal migration, δ-catenin is homogeneously expressed in precursor cells, and their levels decrease once neuronal migration occurs. At advanced stages of neuronal migration re-expression of δ-catenin seems to be important for the formation of N-cadherin mediated cell–cell contacts between neuronal cells [Ho et al., 2000]. In the adult nervous tissue, δ-catenin is expressed in dendrites and soma of neuronal cells, participating in sprouting of these processes [Kim et al., 2002; Lu et al., 2002; Martinez et al., 2003]. Thus, an overexpression of δ-catenin in hippocampal cells induces formation of neurites [Kim et al., 2002]. Additionally, it has been reported that δ-catenin triggers the recruitment of Arp2/3 and actin polymerization, thus favoring neurites elongation [Martinez et al., 2003]. In this study, we found that δ-catenin interacts with the cdk5/p35 complex, an association that determines specific phosphorylation of this catenin at residues Ser^{191} and Ser^{246} by the protein kinase. Interestingly, δ-catenin phosphorylation as well as the modification of β-catenin by the cdk5/p35 system appears to increase its affinity for isomerase peptidyl–prolyl isomerase 1 (Pin1), actions that seem to be involved in its ability to

promote dendrites formation during neuronal development.

MATERIALS AND METHODS

Cell Lines and Cells Transfections

N2A neuroblastoma cells and Cos7 cells were grown in Petri dishes with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were grown under humidity, at 37°C with 5% CO_2. Neurite outgrowth of N2A cells was promoted by changing the cells to a DMEM medium plus 0.25% serum, and the addition of 5 mM db-cAMP. After 48 h treatment, cells exhibited a differentiated morphology with long neuritic processes.

PC12 cells expressing δ-catenin were generous gifts from Dr. K. Kosik at Harvard University. The PC12 cells expressing δ-catenin were grown in DMEM 10% FBS/5% HS, 100 U/ml penicillin, 100 U/ml streptomycin, 25 μg/ml hygromicine, and 100 μg/ml geniticine with 5% CO_2 and at 37°C [Lu et al., 2002]. Transients transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cos7 cells or N2A cells were transfected with 2 μg of plasmid DNA for 10 h. The medium was changed and cells were maintained for additional 36 h.

Primary Cultures

Primary rat cortical neurons from Sprague–Dawley rats at embryonic day 18, were dissected in Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (Invitrogen) with 10 mM Hepes, pH 7.4, and 0.5% glucose, and rinsed twice with HBSS by allowing the tissue to settle to the bottom of the tube. After the second wash, the tissue was re-suspended in HBSS containing 0.25% trypsin (Invitrogen) and incubated for 5 min at 37°C. After three rinses with HBSS, the tissue was mechanically dissociated in MEM-10 solution from Invitrogen (MEM, Earle's salt, sodium bicarbonate buffered, 600 mg/L glucose) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 μM sodium pyruvate, and 2 μM L-glutamine, by gentle passage through Pasteur pipettes. Undisrupted tissue fragments were allowed to settle, cells in suspension were transferred to a new tube, and viable cells were counted using 0.2% Tripan blue. Cells were initially plated in MEM-10 media and maintained at 37°C in a humid atmosphere with 5% CO_2. Two hours after plating, media MEM-10 was removed and serum-free medium N2/Neurobasal (Invitrogen) was added.

Immunoprecipitation and Immunoblot Analysis

The whole brain tissue of adult or 18-day-old rat embryos and cells lines was homogenized in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). The homogenate was centrifuged at 20,000*g* at 4°C for 1 h.

Recombinants Proteins

pGex-cdk5, pGex-p35, and pGex-p25 were gifts from Dr. Harish Pant (NIH). His-Pin1 were gifts from Dr. M. Vincent (Universite Laval, Quebec, Canada). pBluescript-β-catenin (mouse full length) and 1–10 ARM δ-catenin-GFP were gifts from Dr. K. Kosik (Harvard University). To produce pGex-β-catenin we digested pBluescript-β-catenin with *Bam*H1 and then subcloned the *Bam*H1 fragment in pGex (Promega). GST fusion proteins and His-Pin1 fusion proteins were expressed in *E. Coli* BL21 cells and purified with glutathione–Sepharose beads (Amersham Pharmacia Biotech, UK) or histidine-binding beads (Novagen) according to the manufacturer's instructions. Recombinant δ-catenin protein was obtained of PC12 cells expressing δ-catenin [Lu et al., 2002]. The cells were lysated with RIPA buffer, and δ-catenin was immunoprecipitated with a monoclonal antibody (MDC Signal Transduction BD), and used as a substrate of GST-cdk5/GST-p35 complex.

In Vitro Kinase Assay

For in vitro kinase assay GST-cdk5, GST-p35, and GST-p25 were pre-incubated for 30 min at 4°C with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, pH 7.5) plus 1 μM ATP. Then, recombinant GST-fusion proteins or immunoprecipitated protein was incubated with GST-cdk5/p35 or GST-cdk5/p25 complex in kinase buffer, [γ-^{32}P]ATP and 1 μM ATP for 60 min at 30°C, 50 μl final volume. The samples were analyzed by SDS–PAGE and Molecular Imager FX (Bio-Rad).

Immunoprecipitation and Pull-Down Assay

One milligram of brain protein extract or total cellular protein was pre-cleared with 10 μl of A-Sepharose CL-4B beads for 1 h at 4°C (Sigma, St. Louis, MO). Specific antibody for cdk5 (C-8, Santa Cruz Biotechnology) or δ-catenin (Signal Transduction BD) was added, and the extract was incubated overnight at 4°C, followed by 2 h of additional incubation with protein A-Sepharose CL-4B beads, and then centrifuged at 500*g* for 10 min. The immunoprecipitate was washed three times with RIPA extraction buffer. The samples were analyzed by SDS–PAGE and Western blot or used for the kinase activity assay.

Brain rat lysates or Cos7 cells transfected with 1–10 ARM δ-catenin-GFP and pEGFP constructs lysates were incubated with 50 μl Sepharose bead containing GST-cdk5, GST-p35, or GST at 4°C for 2 h. The bound proteins were washed with RIPA buffer and subjected to Western blot. To detect interactions between the catenin and Pin1, 50 μl Sepharose bead containing GST-β-catenin fusion protein or δ-catenin obtained of PC12 cells were incubated with GST-cdk5/GST-p35 complex in the presence of 1 mM of ATP in kinase buffer. Then, Sepharose bead containing phosphorylated protein was incubated with His-Pin1 for 2 h. The binding proteins were washed with RIPA buffer and subjected to Western blots analyses. Roscovitine (10 μM) was used for specific cdk5 inhibition.

Immunofluorescence Studies

For immunofluorescence analysis, cortical neurons or N2A cells were fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 1 min. Immunostaining was done as described by Muñoz et al. [2000]. Cells were incubated with the cdk5 antibody (C8 Santa Cruz), p35 (C18 Santa Cruz), β-catenin antibody (Santa Cruz), δ-catenin antibody (Signal Transduction BD), tubulin antibody (Sigma), or V5 tag antibody (Invitrogen). Cells were then incubated with FITC or rhodamine-conjugated secondary antibodies (Sigma). Coverslips were mounted in FluorSave (Calbiochem). Images were acquired with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany).

Site-Directed Mutations

β-catenin mutants S192A, S246A, and S604A were generated from the pcDNA 3.1-V5 plasmid containing human β-catenin (Invitrogen-GeneStore H-X87838M), using a GeneTailor Kit (Invitrogen). After confirmation of the sequence, the mutants were transfected in Cos7 or N2A cells.

RESULTS

δ-Catenin Is Associated With cdk5/p35 System

First of all, we investigated the expression of β- and δ-catenin in neuronal cells from the brain cortex, maintained in primary cultures for 1 through 10 days. The studies revealed that β-catenin expression did not change during the course of neuronal development (Fig. 1A, upper panel). However, the levels of δ-catenin increased progressively within a period of 2 days of development, attaining a maximum at the third day, and then remained unchanged (Fig. 1A, lower panel). Therefore, it was interesting to analyze the levels of expression of cdk5 and p35 in cortical neurons. The data showed a time-dependent increase in both cdk5 and p35 expression (Fig. 1B).

The intracellular distribution of the cdk5 activator p35 and δ-catenin in cortical neurons was evaluated. Double immunofluorescence of cortical neurons stained with p35 and δ-catenin antibodies are shown in Figure 2. Immunoreactivity toward p35 was present in both dendritic and axonal processes, as well as in the cell body (Fig. 2), while δ-catenin tagged mainly the cell soma and dendrites, co-localizing with p35 epitopes (Fig. 2).

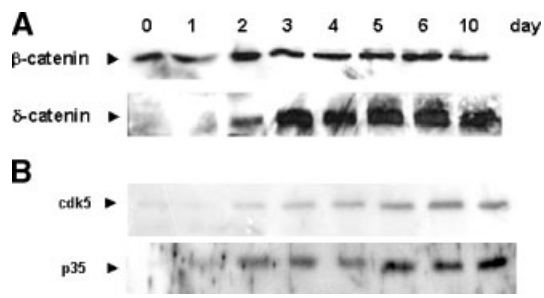

Fig. 1. Western blots of catenins, cdk5, and p35 in cortical neurons. **A**: Immunodetection of β- and δ-catenin in primary cortical neurons cultured during the time intervals indicated. **B**: Immunodetection of cdk5, p35 of cortical neuronons as related with the age of primary cultures.

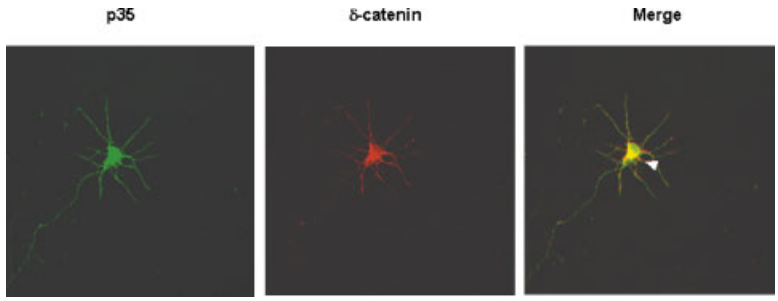

Fig. 2. Co-localization of δ-catenin and p35 in cortical neurons. Intracellular distribution of p35 and δ-catenin in cortical neurons. Immunoreactivity towards p35 (left) was present both in the neuronal processes and in the cell body (green), while δ-catenin (center) tagged mainly the soma and dendrites (red). The merge image (right) shows co-localization of δ-catenin with p35 epitopes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

To analyze the association of δ-catenin with the cdk5/p35 complex, co-immunoprecipitation experiments were carried out. cdk5 was immunoprecipitated from rat brain extracts and the results showed that δ-catenin was present in the immunoprecipitates (Fig. 3A, right panel). On the other hand, when immunoprecipitation was done with anti-δ-cantenin, the protein kinase cdk5 was detected in the immunoprecipitates (Fig. 3A, left panel). Similar findings on N2A cells corroborated this observation (Fig. 3A). Mouse IgGs instead of the immunoprecipitant antibody was used as a negative control (data not shown).

At this stage it was important to assess the interaction of δ-catenin with the cdk5/p35 system. For this purpose, in pull-down experiments we used the GST fusion proteins of both cdk5 and p35 in order to precipitate δ-catenin from adult rat brain extracts (Fig. 3B). GST coupled to glutathione–Sepharose beads was used as a negative control. p35 fusion protein precipitated δ-catenin, as shown by the presence of a 160-kDa δ-catenin band in the pull-down precipitate (Fig. 3B, upper panel). Interestingly, as a difference with p35, δ-catenin was not pulled down with GST-cdk5. Thus, based on this assays we suggest that the direct interaction of cdk5 with δ-catenin, in the absence of p35, is weaker than cdk5/p35 complex interaction with δ-catenin (Fig. 3B, lower panel). We next examined the association of the ARM domain of δ-catenin with p35. Cos7 cells were transfected with the full 1–10 domain of ARM region of δ-catenin cloned in pEGFP. Lysates from transfected Cos7 cells were used for pull-down experiments. As shown in Figure 3C, GST-p35, but not the GST control, specifically precipitated 1–10 ARM δ-catenin from Cos7 transfected cells.

cdk5 Phosphorylates δ-Catenin

To examine if cdk5/p35 complex could phosphorylate δ-catenin, we carried out an in vitro kinase assay with the cdk5/p35 GST fusion protein. The fusion GST proteins with cdk5, p35, and p25 or GST alone were incubated in the presence of [γ-^{32}P] ATP with δ-catenin, isolated by immunoprecipitation from PC12 transfected cells. cdk5/p35 and cdk5/p25 complexes were able to phosphorylate δ-catenin (Fig. 4). This phosphorylation was inhibited in the presence of 10 μM roscovitine.

cdk5/p35 Complex Associates With and Phosphorylates β-Catenin

The distributions of endogenous cdk5 and β-catenin were studied in neuroblastoma N2A cells. β-catenin staining localized in cell–cell contacts in adherents junctions and plasma membrane of undifferentiated and 48 h differentiated N2A cells, while cdk5 was mainly found in cell bodies and neurites. The merge shows that only a fraction of cdk5 co-localized with β-catenin in both cell–cell contacts and plasma membrane domains of undifferentiated and differentiated N2A cells (Fig. 5A). We also showed that β-catenin interacted with the cdk5/p35 complex by immunoprecipitation from embryonic and adult rat brain extracts (Fig. 5B). Similar observations were reported in brain cortical cultures [Kesavapany et al., 2001].

Kinase assay showed that cdk5/p35 GST and cdk5/p25 GST fusion proteins also phosphorylates

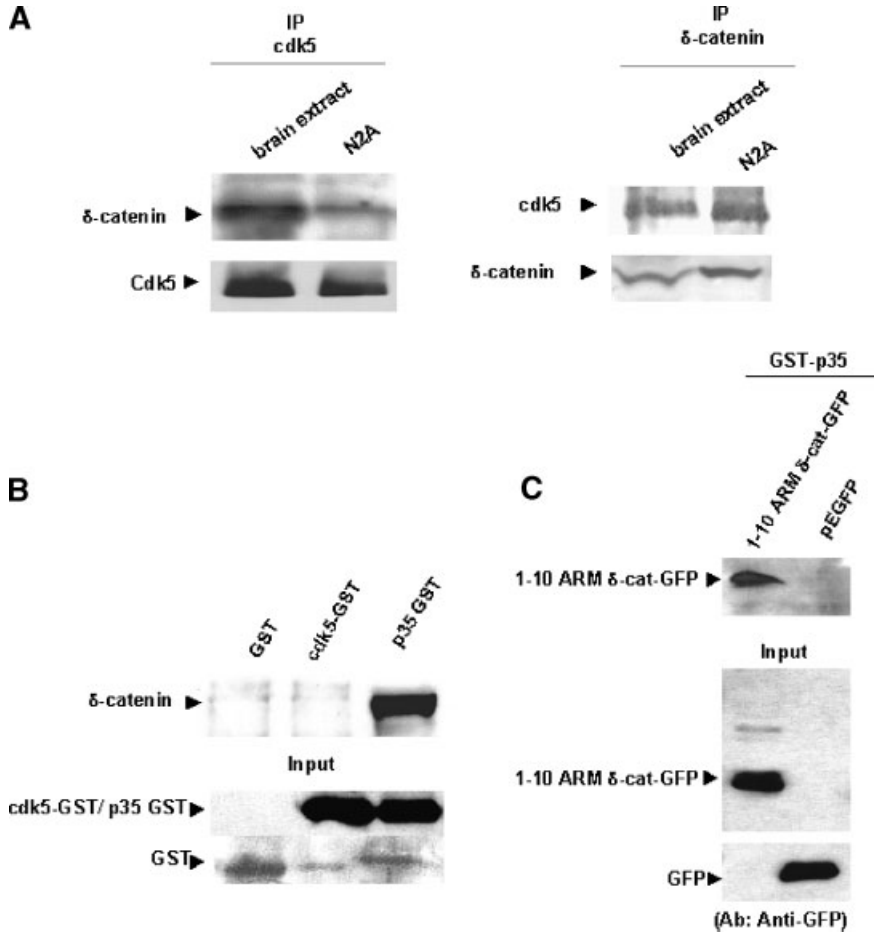

Fig. 3. cdk5/p35 interacts with δ-catenin. **A**: (**Left panel**) cdk5 was immunoprecipitated from rat brain and N2A cells extracts and the immunoprecipitated was probed with δ-catenin antibody. (**Right panel**) δ-catenin was immunoprecipitated from rat brain and N2A cells extracts and the immunoprecipitated were probed with cdk5 antibody. **B**: GST-p35 fusion protein precipitated δ-catenin by pull-down assays. GST, GST-cdk5, or GST-p35 proteins immobilized on glutathione–Sepharose beads were incubated with rat brain extracts, and the proteins bound to the resin were immunoblotted with δ-catenin antibody. **C**: Glutathione–Sepharose beads containing GST-p35 were incubated with extracts of Cos7 cells transfected with 1–10 ARM δ-catenin GFP or pEGFP. Afterward, the bound proteins were immunoblotted with GFP antibody.

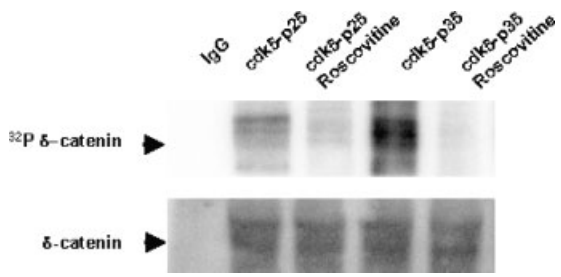

Fig. 4. cdk5/p35 complex phosphorylates δ-catenin in vitro. (**Upper panel**) δ-catenin isolated by immunoprecipitation from PC12 cells expressing δ-catenin was incubated with GST-cdk5/GST-p35 or GST-cdk5/GST-p25 complex in presence of [γ-^{32}P] ATP. The in vitro kinase reactions were carried out in absence or presence of roscovitine 10 μM. Mouse IgG was used as negative control of the immunoprecipitation. (**Bottom panel**) the levels of δ-catenin were determined by immunoblot analysis using δ-catenin antibody.

β-catenin (Fig. 5C), phosphorylation that was inhibited in presence of 10 μM roscovitine. Even though some data have been reported on β-catenin phosphorylation [Kesavapany et al., 2001], no information exists on the precise phosphorylation sites and the identity of the cdk5/p35 phosphorylation sites on β-catenin. In the present study, three β-catenin point mutations were generated in which Ser^{191}, Ser^{246}, or Ser^{604} potential phosphorylation sites were substituted to alanine in the pcDNA β-catenin-V5 plasmid. Cos7 cells were transfected with pcDNA β-catenin-V5 wild-type and mutant. Lysates from transfected Cos7 cells were used for immunoprecipitation and extraction of β-catenin-V5 wild-type and mutant. Then, we carried out in vitro kinase assays using [γ-^{32}P]

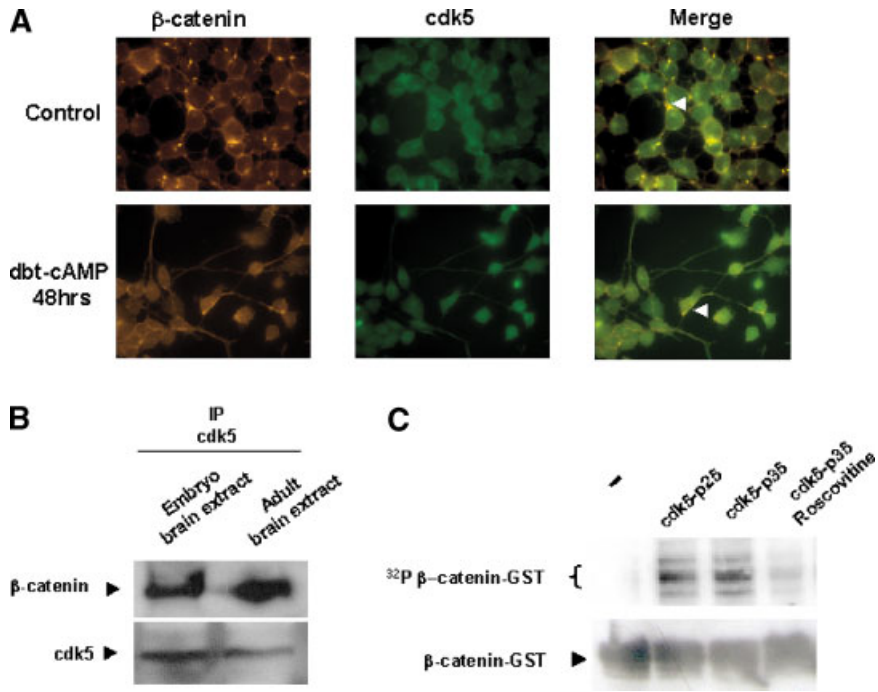

Fig. 5. cdk5/p35 complex associates and phosphorylated β-catenin in vitro. **A**: Immunofluorescence of cdk5 and β-catenin in neuroblastoma N2A cells undifferentiated and differentiated by 46 h with db-cAMP. Arrows shows co-localization of cdk5 and β-catenin in cell-to-cell contacts and plasma membrane. **B**: Embryonary and adult rat brains were immunoprecipitated (IP) with IgG and cdk5 antibody and then probed with β-catenin antibody. **C**: In vitro phosphorylation of GST-β-catenin. Recombinant GST-β-catenin was incubated with GST-cdk5/GST-p35 or GST-cdk5/GST-p25 complex in the presence of [γ-^{32}P] ATP. The in vitro kinase reactions were carried out in absence or presence of roscovitine 10 μM. (−) denotes the negative control of kinase activity without cdk5/p35 complex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ATP and cdk5/p35 to assess phosphorylation of the mutants. Data showed that the intensity of phosphorylation in S191A and S246A decrease in 50% ($P < 0.05$) in β-catenin-V5, thus indicating that cdk5/p35 phosphorylates β-catenin on residues Ser^{246} and Ser^{191} (Fig. 6A, left panels). The histograms (Fig. 6A, right) indicate the levels of phosphorylation as compared with WT controls. In addition, wild-type and mutant β-catenin were transfected into N2A cells. Immunofluorescence experiments using antibodies against V5 tag showed that β-catenin is localized in the cell-to-cell contact domains and plasma membrane of the mutant, similarly of what it was observed in the wild-type (Fig. 6B).

cdk5 Increases Association of β- and δ-Catenin With Pin1

Studies have previously shown that the prolyl isomerase Pin1 interacts with β-catenin once this protein is phosphorylated in Ser^{246}, thus contributing to its translocation to the cell nucleus as well as to a decrease in its degradation mechanisms [Ryo et al., 2001]. Thus, with the interest to test the hypothesis that cdk5 could be the protein kinase that contributes to β-catenin/Pin1 interaction in neuronal cells, we evaluated the interaction between β-catenin phosphorylated with cdk5/p35 and Pin1 by pull-down experiments. The β-catenin GST fusion protein bound to glutathion–agarose beads, when the affinity matrixes were incubated in presence of 1 mM of ATP with and without cdk5/p35 recombinant proteins (Fig. 7). Pin1 recombinant protein fused to His was used to evaluate the Pin1 interaction with β-catenin phosphorylated with cdk5. Pin1-His bound to β-catenin after it had been phosphorylated by the cdk5/p35 system (Fig. 7A, left panel). In order to corroborate the specificity of this cdk5 interaction, co-incubation of β-catenin plus cdk5/p35 complex and ATP, in presence of the cdk5 inhibitor 10 μM roscovitine, induced a marked reduction in the interaction of Pin1 with β-catenin (Fig. 7A, left and right panels). Similar findings were observed in studies in which δ-catenin, from stably transfected PC12 cells, was used as cdk5/p35 substrate (Fig. 7B).

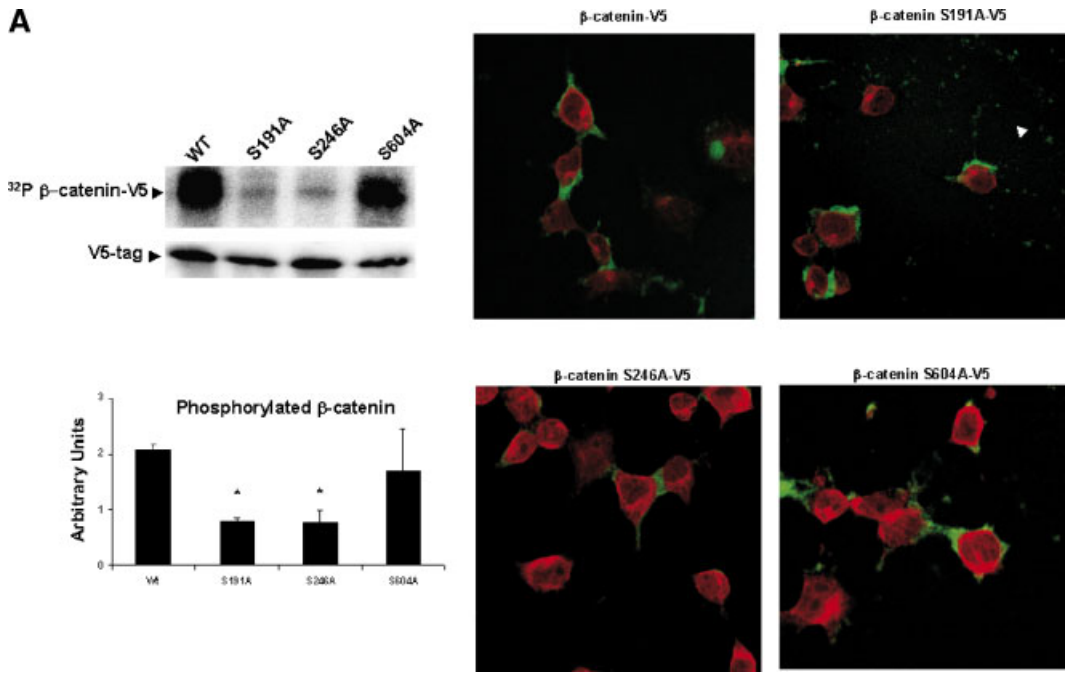

Fig. 6. β-catenin is phosphorylated on Ser^{191} and Ser^{246} by cdk5/p35 in vitro. **A**: (**Left panel**) Cos7 cells were transfected for 48 h with the expression construct of the wild-type β-catenin-V5 and β-catenin-V5 point mutants Ser^{191}, Ser^{246}, and Ser^{604}, and the lysates were subjected to immunoprecipitation with anti-V5 antibody. β-catenin-V5 immunoprecipitates were incubated with recombinant GST-cdk5/GST-p35 complex in the presence of [γ-^{32}P] ATP. Phosphorylated proteins were analyzed in a Molecular Imager FX (Bio-Rad). (**Right panel**) Densitometric analyses of β-catenin-V5 phosphorylation. The band density was quantified and normalized to the amount of β-catenin-V5. Asterisks indicate $P < 0.05$ calculated using Student's test. **B**: Immunofluorescence studies of wild-type β-catenin-V5 and β-catenin-V5 point mutants transfected in neuroblastoma N2A cells. Arrows show β-catenin-V5 in plasma membrane and cell-to-cell contacts. Green staining to the β-catenin-V5 and red staining corresponds to tubulin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

These analyses of data suggest that the cdk5/p35 system could be associated with the regulation of β- and δ-catenins. β- and δ-catenins are proteins that participate in the formation of adhesion complexes mediated by cadherins. Catenins posses *armadillo* domains, which are critical for the protein–protein interactions [Kemler, 1993]. Important changes in the cell-to-cell adhesion mechanisms occur during the process of cell differentiation, leading to neurites formation. δ-catenin is a protein that is expressed in the soma and dendrites of neuronal cells, and participates in the formation of focal adhesions through its association with N-cadherin [Lu et al., 1999a].

We observed that δ-catenin co-localizes with p35 in dendrites and neuronal soma of cortical cells. Moreover, our results show an in vitro association of these proteins, and that the *armadillo* region of δ-catenin is possibly involved in the direct δ-catenin/p35 interaction. Additionally, cdk5/p35 phosphorylates δ-catenin. When cortical neuronal cultures were differentiated after incubation for 10 days, we observed an increase in δ-catenin after the second day of differentiation, result that correlated with the significant increase in the number of dendrites.

The function of δ-catenin seems to be regulated by changes in the endogenous phosphorylation patterns. The post-translational modifications of this protein could contribute to maintain the balance between elongation and ramification of neurites [Martinez et al., 2003]. During elongation δ-catenin interacts with cortactin, triggering the assembly of Arp2/3 complex and re-organization of the actin cytoskeleton. However, when δ-catenin is phosphorylated in tyrosine residues by *Src* or *Abl* kinases, the action of δ-catenin on actin filaments nucleation is blocked [Martinez et al., 2003].

cdk5 and p35 appears to be distributed in both axons and dendrites during neuronal

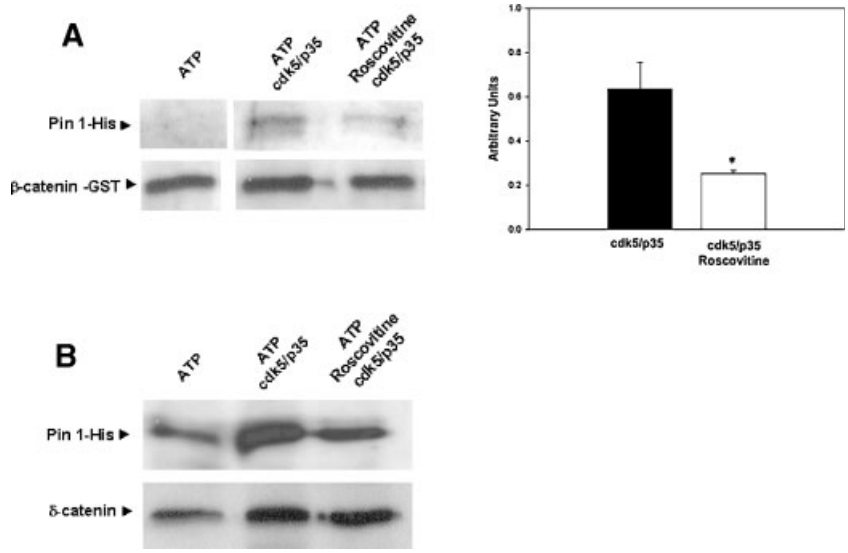

Fig. 7. Pin1 binds β- and δ-catenin phosphorylated by cdk5/p35. **A**: (**Left panel**) Glutathione agarose bead containing GST-β-catenin was phosphorylated with purified GST-cdk5/GST-p35 complex in the presence or absence of Rocovitine 10 μM. Then, the samples were incubated with His-Pin1. After washing the bound protein, bound proteins were subjected to immunoblotting analysis with anti-Pin1 antibody. ATP denotes the reaction mix only, in the absence of the GST-cdk5/GST-p35 complex. (**Right panel**) Densitometric scans of the His-Pin1 protein bond to phosphorylated GST-β-catenin in the presence or absence of roscovitine 10 μM in the reaction. Asterisk indicates $P < 0.05$ calculated using Student's test. **B**: δ-catenin isolated by immunoprecipitation from PC12 cells expressing δ-catenin was incubated with GST-cdk5/GST-p35 complex. The in vitro kinase reactions were carried out in absence or presence of roscovitine 10 μM, after the samples were incubated with His-Pin1. The bound protein were subjected to immunoblotting analysis with anti-Pin1 antibody.

development. Furthermore, an increment of the cdk5 activity is concomitant with an increase in the Abl activity. Therefore, δ-catenin phosphorylation by cdk5/p35 could be functionally involved with the Abl activity and of this way to potentiate the branching of the dendritic network. It has been reported that both overexpression and tyrosine phosphorylation of δ-catenin in cortical neurons lead to an increase in dendrites ramification [Lu et al., 2002; Martinez et al., 2003]. Thus, δ-catenin roles act at three different levels: (i) modulating cell–cell interactions during events prior to cell migration; (ii) after neuronal progenitors initiate migration, δ-catenin decrease, thus contributing to changes in cell's adhesion required for the higher dynamics associated with neuronal cytoskeleton; and (iii) in adult neurons, δ-catenin is critical for dendrites branching, required to generate novel synaptic connections [Ho et al., 2000].

Additionally, transfection of 3T3 cells and hippocampal cells with an δ-catenin constructs induces formation of dendritic like processes [Kim et al., 2002]. Our data have shown association of δ-catenin with the cdk5/p35 system, complex that is associated both with microtubules and the actin cytoskeleton [Smith, 2003]. Thus, it is likely that cdk5/p35 may be involved in modulating cytoskeleton dynamics and therefore the equilibrium from cell adhesion and neurites formation.

By double hybrid strategies it has been also reported that p35 interacts with β-catenin, an association that appears to be involved in the changes of the adhesion of cortical neurons [Kwon et al., 2000]. cdk5 inhibition induces cell aggregation and decreases of β-catenin/N-cadherin interaction [Kwon et al., 2000].

This information taken together with data indicating that β-catenin is a substrate of cdk5 favor the notion that the complex cdk5/p35 may participate in modulating cell adhesion phenomena in brain neurons [Kwon et al., 2000; Kesavapany et al., 2001]. In this study, we have identified two sites on β-catenin Ser^{191} and Ser^{246} phosphorylated by cdk5. The site Ser^{246} is localized in the *armadillo* domain of β-catenin, within the region for the interaction with APC, a protein involved in β-catenin degradation mechanisms [Rubinfeld et al., 1993]. The phosphorylation of aminoacids in this region favor β-catenin degradation, and therefore phosphorylation in Ser^{246} by cdk5/p35

could be involved in regulating the intraneuronal levels of this protein [Graham et al., 2000].

On the other hand, it has been reported that the outgrowth of neurites is regulated by APC protein, a protein associated with microtubules plus ends and that forms clusters in the neuritic tips of unpolarizated neurons [Zhou et al., 2004]. Recent studies have shown that δ-catenin is localized in the APC cluster and negativity regulates neurites outgrowth. Moreover, regulation of APC by β-catenin in the neuritic tips is independent of transcriptional activity [Votin et al., 2005]. Our results show that β- and δ-catenin are associated with cdk5 and p35, a protein that is myristoylated and bind to plasma membrane. This association and protein phosphorylations may participate in the regulation of neuronal outgrown mediated by remodeling actin and microtubule cytoskeleton in neuritic tips. Thus, the links of both cdk5/p35 system and APC protein appears to be related with neuritic development and cytoskeleton organization.

In this study, we found that the isomerase Pin1 interacts with δ-catenin, and suggest that the cdk5/p35 complex favor this interaction. The functional effect of this interaction seems to be related with the regulation in the subcellular distribution of δ-catenin. In addition, we have shown that Pin1, a prolyl-isomerase interacts with β-catenin phosphorylated by cdk5/p35. Possibly Pin1 inhibits β-catenin degradation. Previous reports have described that phosphorylation of β-catenin in Ser^{246} by an unknown kinase, increases its stability upon its interaction with Pin1, and nuclear translocation to activate genes involved in cell proliferation [Ryo et al., 2001, 2003]. It is possible that in neuronal cells, catenin-Pin1 interactions increase the catenin function in the process that regulates the cytoskeletal dynamics and cell adhesion during differentiation.

Regulation of β-catenin by cdk5 appears to participate in cytoskeletal re-organization during neuritogenesis. Overexpression of β-catenin in cortical neurons increases neurites branching in a process that is independent of the activity of β-catenin as a transactivator [Yu and Malenka, 2003]. We have to point out on the possible changes in the control of cdk5/β-catenin interaction in neurodegenerative events. It is known that β-catenin decreases its levels in Alzheimer's patients. Furthermore, β-catenin levels decrease neuronal cultures treated with β-amyloid while cdk5 increases its activity [Zhang et al., 1998; Alvarez et al., 1999, 2001]. Therefore, it is likely that abnormally active cdk5 phosphorylates β-catenin in epitopes involved in its APC association and further degradation in the proteosomes. In this context, the present data on the contribution of Pin1 to β-catenin regulation are noteworthy. Pin1 produces isomerization of catenin region with Ser^{246} phosphorylation, thus preventing interaction with APC and therefore degradation by β-catenin [Ryo et al., 2001]. In Alzheimer's brains, Pin1 is trapped in the PHFs, thus decreasing intraneuronal levels of this protein and therefore its functions [Lu et al., 1999b; Hamdane et al., 2003]. On this basis, it is reasonable to postulate that in Alzheimer's disease, β-catenin decrease would be potentiated by an increase in cdk5 that phosphorylates a regulatory site on Ser^{246} β-catenin as well as a decrease in Pin1, protein that appears to block β-catenin degradation in the proteosome.

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