p25/Cdk5-mediated retinoblastoma phosphorylation is an early event in neuronal cell death

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Summary

In large models of neuronal cell death, there is a tight correlation between Cdk5 deregulation and cell-cycle dysfunction. However, pathways that link Cdk5 to the cell cycle during neuronal death are still unclear. We have investigated the molecular events that precede p25/Cdk5-triggered neuronal death using a neuronal cell line that allows inducible p25 expression. In this system, no sign of apoptosis was seen before 24 hours of p25 induction. Thus, at that time, cell-cycle-regulatory proteins were analysed by immunoblotting and some of them showed a significant deregulation. Interestingly, after time-course experiments, the earliest feature correlated with p25 expression was the phosphorylation of the retinoblastoma protein (Rb). Indeed, this phosphorylation was observed 6 hours after p25 induction and was abolished in the presence of a Cdk5 inhibitor, roscovitine, which does not inhibit the usual Rb cyclin-D kinases Cdk4 and Cdk6. Furthermore, analyses of levels and subcellular localization of Cdk-related cyclins did not reveal any change following Cdk5 activation, arguing for a direct effect of Cdk5 activity on Rb protein. This latter result was clearly demonstrated by in vitro kinase assays showing that the p25-Cdk5 complex in our cell system phosphorylates Rb directly without the need for any intermediary kinase activity. Hence, Rb might be an appropriate candidate that connects Cdk5 to cell-cycle deregulation during neuronal cell death.

Key words: Apoptosis, Cell cycle, E2F-responsive genes, SKNSH-SY5Y neuroblastoma cells.

Introduction

Cell mechanisms that lead to apoptosis share some pathways with cell proliferation. This feature is even more surprising in differentiated cells such as neurons, in which it was suggested that a reactivation of the cell cycle is a key step towards apoptosis (Copani et al., 2001). In fact, neurons of the adult brain are in G0 phase: they do not divide and are differentiated. Post-mitotic neuronal cells coming out of G0 phase into G1 are usually stopped at the G1/S checkpoint and then undergo into either redifferentiation or apoptosis (Nagy, 2000; Liu and Greene, 2001a). Therefore, the deregulation of cell-cycle proteins may be considered to be pathological. It should be realized that re-expression of G1/S-phase markers is best correlated to the appearance of apoptosis in neurons. It is characterized by the formation of the cyclinD1-Cdk4/6 complex, phosphorylation of Rb, dissociation of the Rb-E2F complex and activation of genes leading to apoptosis (Freeman et al., 1994; Herrup and Busser, 1995; Osuga et al., 2000; Liu and Greene, 2001a).

Among the neuronal death inducers, Cdk5 is of particular interest. Cdk5 is a pro-directed phosphorylation kinase that belongs to the cyclin-dependent-protein-kinase family. However, its usual activators do not share any cyclin consensus sequence and are referred to as p35 and p39. Interestingly, p35 is usually anchored to the membrane because it has a myristoylation site in its N-terminus. The p35-Cdk5 complex

is abundant in the adult brain and Cdk5 activity increases in neurons during development. p35 can be proteolysed by calpains following changes in calcium homeostasis into a cytosolic C-terminal fragment referred to as p25 that is more stable than p35 and binds more tightly to Cdk5, leading to a hyperactive, mislocalized p25-Cdk5 complex (for review, see Dahavan and Tsai, 2001). Such cleavage of p35 to p25 has been reported in disorders such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (for reviews, see Patzke and Tsai, 2002; Shelton and Johnson, 2004). Furthermore, exposure of primary cortical neurons to various insults like Aβ peptide, H_2O_2 or glutamate also leads to p25 formation and cell death (Patrick et al., 1999; Lee et al., 2000; Kusakawa et al., 2000). However, no mechanisms have been determined by which this kinase complex triggers its neurotoxicity. It is worth noting that, in neurodegenerative disorders and cellular models of neuronal death in which p25-Cdk5 is probably involved, aberrant cell-cycle deregulation has been reported (Vincent et al., 1996; Vincent et al., 1997; Vincent et al., 1998; Nagy et al., 1997; Busser et al., 1998; Copani et al., 1999; Giovanni et al., 1999; Husseman et al., 2000; Zhu et al., 2000; Ranganathan et al., 2001; Yang et al., 2003). In addition, a tight correlation has been established between Cdk5 deregulation and expression of cell cycle regulatory proteins (Nguyen et al., 2002; Nguyen et al., 2003). Nevertheless, pathways linking Cdk5 to cell cycle remain obscure.

In order to address this question, we used a recently reported stable neuronal cell line that expresses an inducible p25-Cdk5 activity (Hamdane et al., 2003a). This constitutes a suitable cell model to follow the sequence of events preceding neuronal death when there is formation of the p25-Cdk5 complex.

Materials and Methods

Cell culture

p25-inducible SH-SY5Y cells that constitutively express the Tau isoform with three microtubule-binding domains (2+3–10–) (Tau-SY5Y) were used as the basis for a tetracycline-regulated mammalian expression T-Rex system (Invitrogen). Cells were transfected with inducible expression vector alone (mock cells) or with p25-encoding cDNA (p25 cells). Individual stable clones were generated and those that exhibited the weakest basal expression of p25 were selected. For induction of p25 expression, cells were maintained in medium with tetracycline at 1 µg ml^{-1} (Hamdane et al., 2003a).

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 1 mM nonessential amino acids, 50 U ml^{-1} penicillin/streptomycin, 5 µg ml^{-1} Blasticidin and 100 µg ml^{-1} Zeocin (Invitrogen, France) in a 5% CO_2 humidified incubator at 37°C. Cells were differentiated for 7 days in Dulbecco's modified Eagle's medium or Ham's F12 medium supplemented with 2 mM L-glutamine, 50 U ml^{-1} penicillin/streptomycin, 7 µg ml^{-1} progesterone, 1% insulin-transferrin-selenium (Invitrogen), 10 ng ml^{-1} NGF (2.5S subunit, Sigma). Medium was replenished every 2 days. Roscovitine was added for 6 hours as described previously (Hamdane et al., 2003a).

Antibodies

Anti-Cdk5 monoclonal antibody (J-3; Santa Cruz Biotechnology, Tebu-Bio, France), polyclonal antibody against the p35 C-terminus (C-19; Santa Cruz Biotechnology), antibody against neuron-specific γ-enolase (NSE) (Santa Cruz Biotechnology), rabbit polyclonal antibody against lamin-B (H-90; Santa Cruz Biotechnology), rabbit polyclonal antibody against p27^{Kip1} (C-19; Santa Cruz Biotechnology), rabbit polyclonal antibody against p21^{Cip1} (C-19, Santa Cruz Biotechnology), rabbit polyclonal antibody against caspase-3 (Cell Signaling Technology, Ozyme, France), mouse monoclonal antibody against Cdc2-p34 (17; Santa Cruz Biotechnology), rabbit polyclonal antibody against cyclin D that recognizes cyclin D1 and cross-reacts with cyclin D2 (Cell Signaling Technology), rabbit polyclonal antibody against cyclin D3 (C-16, Santa Cruz Biotechnology), rabbit polyclonal antibody against cyclin A (C-19, Santa Cruz Biotechnology), affinity-purified rabbit polyclonal antibody against cyclin B1 (H-20, Santa Cruz Biotechnology), mouse monoclonal antibody against cyclin E (HE12; Cell Signaling Technology), rabbit polyclonal antibody against Cdk2 (M2; Santa Cruz Biotechnology), rabbit polyclonal antibody against Cdk4 (C-22; Santa Cruz Biotechnology), rabbit polyclonal antibody against Cdk6 (C-21; Santa Cruz Biotechnology), affinity-purified rabbit polyclonal IgG against phospho-Rb (Ser807/811) and IgG against phospho-Rb (Ser795) (Cell Signaling Technology), mouse monoclonal IgG1 against Rb (IF8; Santa Cruz Biotechnology), rabbit affinity-purified antibody against the C terminus of Rb (C-15, Santa Cruz Biotechnology).

Western blotting

Cells were harvested in ice-cold RIPA modified buffer [50 mM Tris, pH 7.4, 1% Nonidet P-40 (NP-40), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA] with protease inhibitors (Complete Mini, Roche Applied Science) and 125 nM phosphatase inhibitor okadaic acid (Sigma), sonicated and stirred for 1 hour at 4°C. Cell lysate was recovered in supernatant after centrifugation at 12,000 g, 4°C for 20 minutes. Protein concentration was determined using the BCA protein assay kit (Pierce Perbio Science, France). Samples were mixed with an equal volume of 2× Laemmli sample buffer and dithiothreitol, and heated for 5 minutes at 100°C; then, 10-20 µg were loaded onto Nu-PAGE Novex gels (Invitrogen). After transfer, membranes were blocked in TBS pH 8, 0.05% Tween-20 with 5% skimmed milk and incubated with primary antibody. Horseradish peroxidase (HRP) conjugated antibody (Sigma) was used as secondary antibody and HRP activity was detected with the ECL detection kit (Amersham Biosciences, France). For immunoblot analyses of immunoprecipitated complexes and in vitro kinase assays, HRP-conjugated antibody TrueBlot™ (eBioscience) was used. Restore™ western-blot stripping buffer (Perbio Pierce) was used before reprobing membranes.

Cytoplasmic and nuclear fractionation

Cells were harvested in ice-cold buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.15% NP-40) supplemented with protease inhibitors (Complete Mini) and incubated for 10 minutes on ice. After centrifugation at 1000 g for 10 minutes, the supernatant was collected as the cytoplasmic fraction. Nuclei in the pellets were washed three times and then lysed in ice-cold RIPA modified buffer with protease inhibitors (Complete Mini) in an equal volume to that of buffer A. Centrifugation at 12,000 g at 4°C for 20 minutes allowed the recovery of the supernatant as the nuclear fraction. The BCA protein assay kit determined protein concentration.

Immunoprecipitation

Cells (after a 6 hour tetracycline treatment) were harvested in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) supplemented with protease inhibitors, and cell lysates were prepared as described above. Protein concentration was determined using the BCA protein assay kit. Cell lysates (800 µg at 4 µg ml^{-1}) were incubated with 1 µg immunoprecipitating antibody for 1 hour at 4°C and then incubated overnight at 4°C with 20 µl anti-rabbit-IgG beads (TrueBlot Ig IP Beads, eBioscience). Immunoprecipitated complexes were washed four times with lysis buffer (centrifugation at 1000 g at 4°C for 5 minutes), recovered in 40 µl 2× Laemmli buffer with 50 mM of fresh dithiothreitol and boiled for 5 minutes, and then equal volume of samples were loaded onto SDS-PAGE gel and analysed by immunoblotting.

In vitro kinase assays

For immunopurified complexes, immunoprecipitation was performed as described above. Immunoprecipitated complexes were washed twice in NP-40 lysis buffer and once in Hepes buffer (40 mM Hepes, pH 7.2, 8 mM $MgCl_2$) and then used to phosphorylate 2 µg Rb-C fusion protein (containing Rb residues 701-928 fused to maltose-binding protein; Cell Signaling Technology) in 30 µl kinase buffer [40 mM Hepes, pH 7, 8 mM $MgCl_2$, 125 nM okadaic acid, protease inhibitors (EDTA-free Complete Mini, Roche Applied Science), 10 mM ATP]. After incubation for 1 hour at 37°C, the reactions were stopped by adding 2 Laemmli buffer (1 volume) and boiling for 5 minutes.

For radiolabelled in vitro phosphorylation, kinase assays were carried out as above by substituting cold ATP with 10 µCi (γ^{32}P)-ATP. Samples were loaded (equal volumes) onto SDS-PAGE gel. The gel was then dried and results were visualized using a phosphorImager

For crude cell lysates, cells were harvested in 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors (EDTA-free Complete Mini) and lysates were prepared as described above. Lysates (200 µg) were then used to perform in vitro kinase assays under the same

experimental procedure as for immunopurified complexes. When kinase inhibitors (roscovitine at 10 μM, PD98059 at 50 μM, SB203580 at 10 μM and SP600125 at 50 μM; Calbiochem) were used, they were mixed with cell lysates before adding kinase buffer and Rb-C fusion protein.
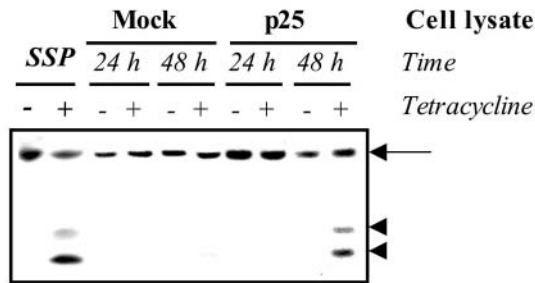
Results

Deleterious effect of p25-Cdk5 kinase in differentiated SY5Y cells

p25-inducible cells were generated from SH-SY5Y cells, which constitutively overexpress Tau protein (Tau-SY5Y) (Delobel et al., 2002; Delobel et al., 2003). As previously shown, p25 protein was not found in mock Tau-SY5Y cells. Conversely, p25 non-induced cells displayed a low basal expression of transgene protein, whereas tetracycline treatment induced high p25 expression, and an active p25-Cdk5 complex is formed, as shown by coimmunoprecipitation and in-vitro-phosphorylation experiments (Hamdane et al., 2003a). In this Tau-SY5Y cell system, Tau overexpression might counteract

toxicity linked to the basal level of p25, because no stable viable cells were obtained from native SH-SY5Y cells. Similarly, it has been reported that neurofilament proteins might serve as a 'phosphorylation sink' for p25-Cdk5 complex, hence sequestering it away from other death-inducing substrates (Couillard-Després et al., 1998; Nguyen et al., 2001; Patzke and Tsai, 2002).
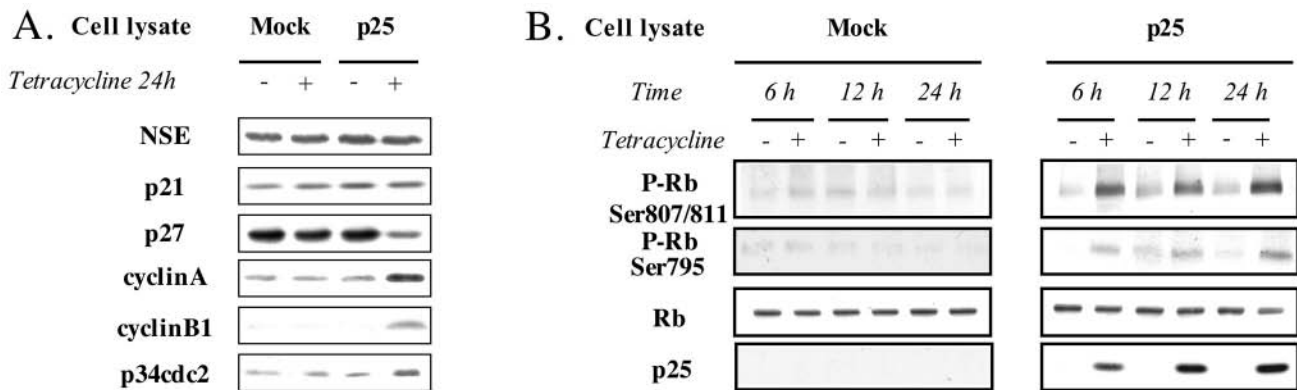
In the present cell model, NGF-differentiated cells showed morphological changes including neurite retraction and the appearance of rounded cells only after 48 hours of p25 expression (Hamdane et al., 2003a). p25-Cdk5 toxicity was also investigated at the molecular level in differentiated cells. Caspase-3 cleavage was analysed at different times of p25 expression (with tetracycline) compared with p25 non-induced cells (without tetracycline). Caspase-3 activation was observed after 48 hours of p25 induction, whereas no caspase activity was detected after 24 hours (Fig. 1). Thus, in order to investigate the molecular events that precede p25-induced neuron death, all experiments were first performed after 24 hours of p25 induction.

Cell-cycle expression in p25-inducible Tau-SY5Y cells

Several reports show that the reactivation of the cell cycle is an early marker of neuron death. Thus, we investigated the expression of cell-cycle proteins in NGF-differentiated neuronal p25-inducible cells. First, the Cdk inhibitors p21 and p27 were analysed by western blotting in NGF-differentiated mock and p25 cells. After 24 hours of p25 induction, no change in p21 level was observed, whereas a significant decrease of p27 immunoreactivity was seen (Fig. 2A). Similarly, cyclin-A and -B1 levels were increased. Finally, the immunoreactivity of the kinase Cdc2-p34 was also increased. None of these changes was observed in mock cells treated with tetracycline compared with untreated ones. These results indicated that there is a deregulation of cell-cycle-regulatory proteins in p25-induced cells before death. These data are reminiscent of those observed in neuronal apoptosis and neurofibrillary degeneration.

Fig. 1. Representative western-blot analysis of caspase-3 cleavage in a time-course experiment of p25 expression. NGF-differentiated (7 days) mock and p25 cells were treated (+) or not (–) with tetracycline for 24 hours or 48 hours, and lysates were analysed using anti-caspase-3 antibody that recognizes the full-length protein (35 kDa, arrow) and the large fragments resulting from its cleavage (19/17 kDa, arrowheads). As positive control, lysates derived from cells treated (+) or not (–) with 1 μM staurosporine (SSP) were used.

Fig. 2. (A) Representative immunoblot analyses of cell-cycle-regulatory proteins. NGF-differentiated mock and p25 cells were treated (+) or not (–) with tetracycline for 24 hours and lysates were analysed using antibodies against p21^{CIP1} (p21), p27^{KIP1} (p27), cyclin A, cyclin B1 and p34^{Cdc2}. Equal amounts of proteins were loaded, and visualized with an antibody against neuron-specific γ-enolase (NSE). (B) Analysis of Rb phosphorylation in a time-course experiment of p25 expression. Lysates from NGF-differentiated mock and p25 cells treated (+) or not (–) with tetracycline for 6 hours, 12 hours and 24 hours were first immunolabelled with antibodies against Ser807/811-phosphorylated and Ser795-phosphorylated Rb. After stripping, phosphorylation-independent antibody (IF-8) was used to visualize the amount of loaded Rb (Rb). Concomitant with this, p25 expression was analysed during the kinetics by antibody against the p35 C-terminus.
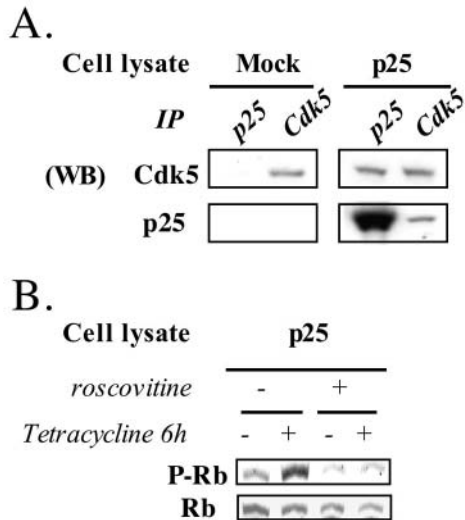
Fig. 3. (A) Analysis of p25-Cdk5 complex. Cell lysates from NGF-p25 and NGF-mock cells (used as negative control), treated for 6 hours with tetracycline, were subjected to immunoprecipitation (IP) by antibodies against either the p35 C-terminus or Cdk5. Immune complexes were then analysed by western blotting (WB) with antibodies against the p35 C-terminus and Cdk5. (B) Analysis of roscovitine's effect on earlier Rb phosphorylation. NGF-differentiated p25 cells were induced (+) or not (–) with tetracycline for 6 hours in the absence or the presence of 5 μM roscovitine (–/+). Lysates were immunoblotted with antibody against phospho-Ser807/811 Rb (P-Rb) followed, after stripping, with the phosphorylation-independent antibody (IF-8) against Rb protein (Rb).

Cell-cycle reactivation was likely mediated through Rb phosphorylation

Among these cell-cycle genes, some are E2F-responsive genes encoding proteins including cyclin A and p34-Cdc2. In this respect, Rb might be of particular interest because its activity is involved in neuronal cell death and linked to E2F. In dividing cells, E2F forms an inhibited complex with hypophosphorylated form of Rb in G0/G1 phase. Inactivation of Rb by phosphorylation leads to its release from E2F, allowing transcription of E2F-responsive genes and G1/S-phase transition. In neuronal cells, Rb protein plays a crucial role in cell survival because its phosphorylation is tightly correlated to neuronal death (Galderisi et al., 2003; Greene et al., 2004).

In the present cell model of p25-Cdk5 neurotoxicity, Rb phosphorylation status was examined during a time-course experiment of p25 induction (6 hours, 12 hours and 24 hours of tetracycline treatment). Rb phosphorylation was visualized using phosphorylation-dependent antibodies (P-RbSer795 and P-RbSer807/811). Total Rb was then detected using an antibody recognizing Rb protein independently of its phosphorylation form (Rb). In controls, no change in Rb-phosphorylation status was observed between the two conditions: mock cells treated with tetracycline (+) compared with untreated ones (–). Conversely, a strong Rb phosphorylation was present after p25 induction. This phosphorylation was found as early as 6 hours after p25 induction (Fig. 2B). The different cell cycle markers studied
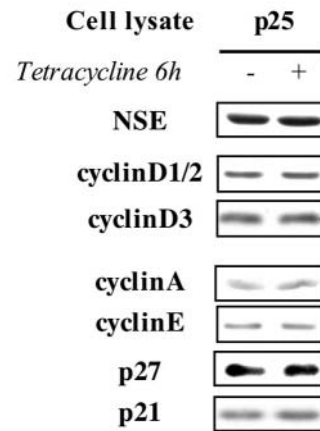
Fig. 4. Immunoblot analyses of cell-cycle-regulatory proteins at the earliest stage of p25 expression. NGF-differentiated p25 cells were treated (+) or not (–) with tetracycline for 6 hours and lysates were analysed using the indicated antibodies. Anti-NSE antibody was used after stripping the membranes to estimate the total amount of loaded proteins.

above (Fig. 2A) were then analysed following 6 hours of p25 induction. None of them displayed any change in immunoreactivity at this earlier time of p25 expression (data not shown).

As shown above, p25 expression was detected as early as 6 hours after tetracycline treatment. We then checked for formation of p25-Cdk5 complex at this earlier time. As showed by immunoprecipitation assay (Fig. 3A), Cdk5 is found associated with p25 protein in 6-hour tetracycline-treated cells. Furthermore, Rb phosphorylation was analysed in p25-induced cells in the presence of roscovitine, which specifically inhibits Cdk1, Cdk2 and Cdk5 but not cyclin-D-dependent kinases (Meijer et al., 1997; Meijer et al., 1999). In the present cell system, Rb phosphorylation was abolished by roscovitine (Fig. 3B), suggesting that it was specifically triggered by p25-Cdk5 kinase activity.

These data suggest that Rb phosphorylation in our model was an early event in p25-Cdk5 induced neuronal death. Nonetheless, it remains to be established whether p25-Cdk5 complex directly phosphorylates Rb or requires activation of intermediary kinases.

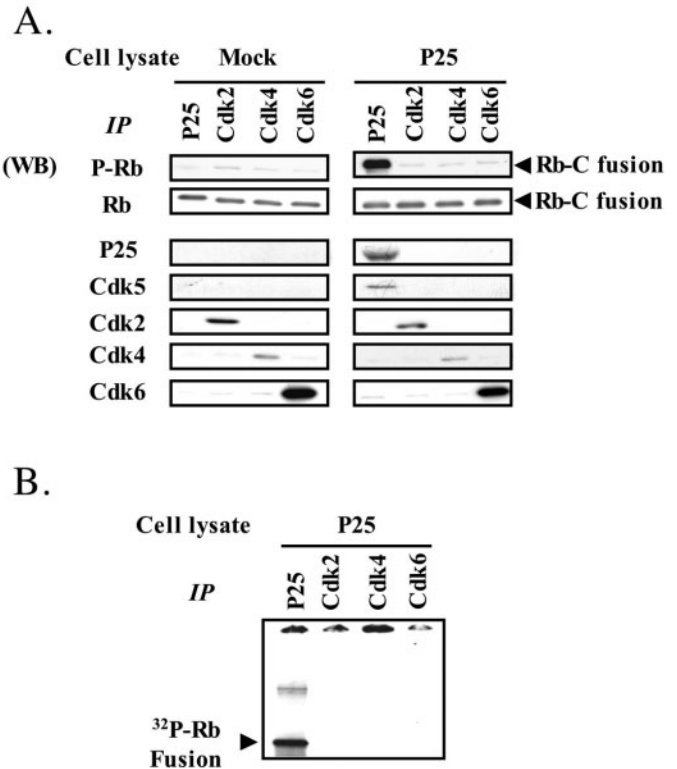## Rb phosphorylation and early cell-cycle-regulatory proteins

Rb phosphorylation is mainly regulated by cyclin-D-dependent kinases (Cdk4 and Cdk6). An increase in cyclin-D1 level is often considered to be necessary to leave G0 phase to G1 in neurons. Hence, the expression of cyclin D was investigated in the present cellular model from earlier time of p25 expression (Fig. 4). No variation in cyclin D1, D2 or D3 levels was observed following 6 hours of p25 expression (Fig. 4, compare + with –). Similarly, western-blot analyses did not show any increase in the immunoreactivity of cyclins E and A, the Cdk2 regulatory proteins, and the levels of related inhibitory proteins p21 and p27 did not decrease following p25-induced expression (Fig. 4). These data suggest that Rb phosphorylation is probably not associated with an increase in
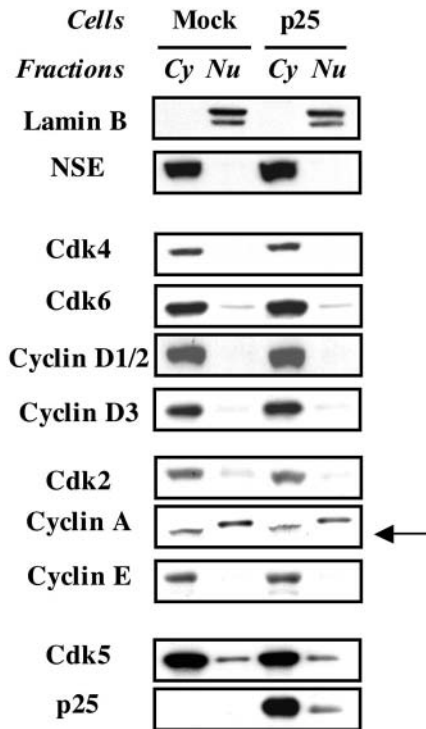
Fig. 5. Cell fractionation analysis. Cytoplasmic and nuclear fractions were prepared from NGF-mock and NGF-p25 cells, both treated for 6 hours with tetracycline. For each cell line, 10 µg cytoplasmic fraction (Cy) and an equivalent volume of the nuclear fraction (Nu) were analysed by western blotting. Subcellular distribution of the specified proteins was visualized using specific antibodies. The arrowhead shows cyclin-A immunoreactivity (lower bands), as determined by comparison with the SDS-PAGE pattern of immunoprecipitated cyclin A (not shown). Stripped membranes were reprobed with antibodies against NSE and lamin B to evaluate the purity of the cytoplasmic and nuclear fractions, respectively.
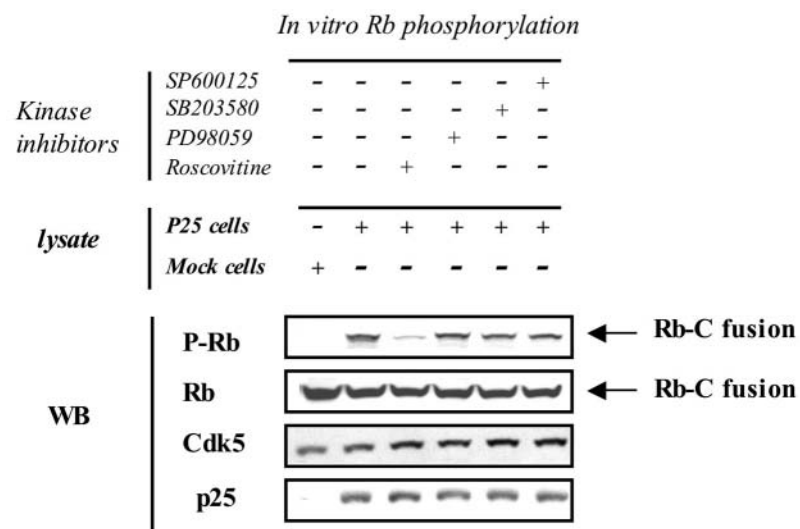

Fig. 6. Immunoprecipitation and in vitro kinase assays. Lysates from NGF-mock and NGF-p25 cells, both treated for 6 hours with tetracycline, were subjected to immunoprecipitation (IP) with the indicated antibodies. (A) Western-blot analyses. Following in vitro kinase assays, Rb-C fusion protein (68 kDa) phosphorylation was evaluated by immunoblotting using P-Rb Ser807/811 and P-Rb Ser795 antibodies that give similar results (representative immunoblot with P-Rb Ser807/811 is shown). To evaluate the amount of loaded Rb-C fusion protein, blots were stripped and reprobed with a phosphorylation-independent antibody (C-15) directed against the C terminus of Rb (Rb). The lower sections of blots were also probed with specific antibodies to check the presence of the following proteins: p25, Cdk5, Cdk2, Cdk4 and Cdk6. (B) In vitro kinase assays in the presence of (γ-^{32}P)ATP. Arrowhead indicates Rb-C fusion protein with incorporated ^{32}P, which is only seen in in vitro kinase assay with purified p25-Cdk5 complex.

the levels of Cdk4 and Cdk6, and Cdk2-associated cyclins. However, because Cdk4, Cdk6 and Cdk2 activation could be linked to a change in cellular redistribution of related cyclins, western blotting was performed on fractionated cytoplasmic (Cy) and nuclear (Nu) extracts from mock and p25 cells, treated with tetracycline for 6 hours. As shown in Fig. 5, proper fractionation was checked by analysis of cellular distribution pattern of NSE and lamin B (cytoplasmic and nuclear markers, respectively).

Results showed that cyclins D1, D2 and D3, as well as their associated kinases Cdk4 and Cdk6, were mainly detected in the cytoplasm and their expression patterns were similar between p25-expressing cells and mock cells. Furthermore, p25 expression did not induce any cellular redistribution of cyclin A, cyclin E and their associated kinase Cdk2 (Fig. 5). Taken together, these data strongly suggest that Cdk4, Cdk6 and Cdk2 activities could not be involved in p25/Cdk5-induced Rb phosphorylation. Interestingly, analysis of cellular fractionation showed that p25 and Cdk5 could be detected in the nuclear fraction of p25-expressing cells (Fig. 5), arguing for a possible direct Rb phosphorylation by p25-Cdk5 complex.

Rb phosphorylation and the p25-Cdk5 kinase complex

p25 expression did not trigger any change in either the levels of Cdk4-, Cdk6- and Cdk2-related cyclins or their cellular localization following 6 hours of tetracycline treatment. Because, at this time, the only active Cdk was the complex p25-Cdk5, we asked whether Cdk5 could directly phosphorylate Rb. To address this question, in vitro kinase assays using Rb-C fusion protein as substrate were performed by p25-Cdk5 complex from p25-expressing cells.

Monitoring of P-Ser807/811 and P-Ser795 (P-Rb) immunoreactivities showed that an immunoprecipitated p25-Cdk5 complex from p25-expressing cells phosphorylates Rb (Fig. 6A). Conversely, western-blot analyses of in vitro Rb phosphorylation by immunoprecipitated Cdk2, Cdk4 and Cdk6 revealed no significant activity from either mock cells or p25 ones (Fig. 6A). These experiments were confirmed by in vitro kinase assays in the presence of (γ^{32}P)-ATP (Fig. 6B). These

In vitro Rb phosphorylation

Kinase inhibitors						
SP600125	−	−	−	−	−	+
SB203580	−	−	−	−	+	−
PD98059	−	−	−	+	−	−
Roscovitine	−	−	+	−	−	−

lysate						
P25 cells	−	+	+	+	+	+
Mock cells	+	−	−	−	−	−

WB

P-Rb ← Rb-C fusion

Rb ← Rb-C fusion

Cdk5

p25

Fig. 7. Western blotting of in vitro kinase assays with crude cell extracts. Lysates from NGF/p25-expressing cells (6 hours of tetracycline treatment) were used for Rb-C fusion protein phosphorylation, alone and in the presence of the kinase inhibitors roscovitine, PD98059, SB203580 and SP600125. As negative control, an in vitro kinase assay was performed with lysate from NGF-mock cells treated for 6 hours with tetracycline. Phosphorylation of Rb-C fusion protein (P-Rb) was monitored by P-Rb Ser807/811 and Ser795 antibodies, which showed similar data. After stripping, membranes were probed with a phosphorylation-independent antibody against Rb (C-15) to evaluate the amount of loaded Rb-C fusion protein (Rb). The lower side of membrane was analysed by immunoblotting for the levels of p25 and Cdk5, which attests to the presence of equivalent amounts of crude lysate in each kinase reaction.

data clearly demonstrate that, in the present cell model, Cdk2, Cdk4 and Cdk6 were not involved in Rb phosphorylation. It is worth noting that analyses of immunoprecipitated complexes showed that p25 protein was associated with Cdk5 and not any other Cdks (Fig. 6A), confirming that Rb phosphorylation is exclusively linked to the p25-Cdk5 complex. To confirm this and to exclude the involvement of other non-studied kinases, in vitro kinase assays were performed using crude lysate from 6-hour p25-expressing cells in the presence of kinase inhibitors including roscovitine, PD98059 (MEK1 inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor). Rb phosphorylation was monitored by western blotting using anti-phospho-Rb antibodies. As shown in Fig. 7, lysate from p25-expressing cells (unlike that from mock cells) could phosphorylate Rb protein. Interestingly, this phosphorylation was abolished in the presence of the Cdk inhibitor roscovitine, whereas no effect was shown with the other inhibitors. Hence, all these data clearly demonstrate that the p25-Cdk5 complex in the present cell model is involved in a direct phosphorylation of Rb independent of cyclin-D-associated kinases.

Discussion

A broad range of neuronal cell death models have showed coexistence between deregulation of Cdk5 activity and expression of cell-cycle-regulatory proteins (Nguyen et al., 2002), and a close link between these two events was established by a recent study (Nguyen et al., 2003). Furthermore, the p25-Cdk5 complex was shown to be harmful to neurons, as evidenced by cytoskeleton disruption, neuritic retraction and expression of apoptotic markers (Ahlijanian et al., 2000; Bian et al., 2002; Patrick et al., 1999; Hamdane et al., 2003a). Interestingly, deregulation of Cdk5 activity by association with its activator p25 is likely to be involved in AD pathogenesis (Lee et al., 1999; Patrick et al., 1999; Patrick et al., 2001; Cruz et al., 2003; Noble et al., 2003). Concurrently, evidence indicates an involvement of cell-cycle-regulatory elements in neurodegeneration. Indeed, both G1/S- and G2/M-phase markers are found in neurons undergoing neurofibrillary degeneration during AD (Vincent et al., 1996; Nagy et al., 1997; Vincent et al., 1997; Vincent et al., 1998; Busser et al.,

1998; Husseman et al., 2000; Zhu et al., 2000; Dranovsky et al., 2001; Ranganathan et al., 2001; Hamdane et al., 2003b; Yang et al., 2003).

We recently showed that the p25-Cdk5 complex induced the appearance of mitotic epitopes in differentiated neuronal cells (Hamdane et al., 2003a), which are considered to be early markers of neurofibrillary degeneration in AD (Vincent et al., 1998; Augustinack et al., 2002). In this cell system and prior to neuronal death, p25-Cdk5 kinase induced expression of regulatory proteins of both G1/S and G2/M phases. The present data provide an additional support of a tight correlation between Cdk5 deregulation and expression of cell-cycle markers during neuronal cell death. Time-course experiments of p25 expression showed that Rb phosphorylation was an early event in p25/Cdk5-induced neurotoxicity. This result is of particular interest because the pathways that mediate the deleterious effects of p25-Cdk5 kinase in neurons remain unclear. In neuronal cells, Rb phosphorylation leads to cell death, probably by subsequent transactivation of E2F-responsive genes involved in regulation of neuron survival (Park et al., 2000; Liu and Greene, 2001b; Galderisi et al., 2003; Greene et al., 2004). Aberrant expression of other E2F-targeted genes, like some that encode cell-cycle-regulatory proteins, might also occur. Our results showed an increase in the levels of E2F-responsive genes, including those encoding cyclin A and Cdc2-p34, following Rb phosphorylation. This might explain why both G1/S- and G2/M-phase markers of the cell cycle are found in degenerating neurons during AD (Vincent et al., 1997; Husseman et al., 2000).

Some data from the literature suggested that Rb phosphorylation could be mediated through Cdk5 pathway. First, Ser residues at positions 807/811 of Rb protein are located in a consensus sequence with basic residue at position +3 preferentially targeted by Cdk5 (Songyang et al., 1996; Sharma et al., 1999). In addition, it has been reported that p25-Cdk5 complex is able to interact with Rb protein in vitro and to phosphorylate it (Lee et al., 1997). Because Rb has been shown to associate with partners such as cyclin D through the common peptide sequence motif LXCXE, Cdk5 binding might be mediated by a related sequence motif (LXCXXE) found in p25 (Lee et al., 1997). Furthermore, p25-Cdk5 is detected in

the nucleus of degenerating neurons (Patrick et al., 1999) and interacts with nuclear substrates (Zhang et al., 2002; Gong et al., 2003). In agreement with that, our experiments showed a nuclear localization of p25 and Cdk5. Moreover, p25-Cdk5 complex isolated from the present cell system leads to Rb phosphorylation in vitro.

It is worth noting that an increase in cyclin-D1 level, which is necessary to exit from G0 phase to G1 in neurons, is often characterized as an early event leading to Rb phosphorylation and then neuronal apoptosis. Our data revealed that this pathway could be a shortcut through the p25-Cdk5 complex that allows direct Rb phosphorylation. Indeed, in our time-course experiments, Rb phosphorylation was correlated with p25 expression. At that time, no variation in the levels or the subcellular localization of cyclins D1, D2 and D3 was observed. Besides, Rb phosphorylation was abolished in the presence of roscovitine. This latter is a Cdc2, Cdk2 and Cdk5 inhibitor but not an inhibitor of cyclin-D-dependent kinases (Cdk4/Cdk6). More importantly, analysis of Cdk4, Cdk6 and Cdk2 activities by in vitro kinase assays did not reveal any change following p25 expression. Finally, in vitro kinase assays using p25 cell lysate in the presence of kinase inhibitors clearly demonstrate that Rb protein was directly phosphorylated by p25-Cdk5 in the current cell system.

Mechanisms by which Cdk5 triggers its deleterious effects during neurodegeneration are not well elucidated. On one hand, these effects were related to Tau phosphorylation, leading to microtubule destabilization (Ahlijanian et al., 2000; Nguyen et al., 2001; Bu et al., 2002). On the other hand, several reports, including ours, show a link between Cdk5 and cell-cycle proteins during neuron death. Activation of cell-cycle signalling might constitute a crucial step in the neuronal death pathway inherent to p25-Cdk5 kinase complex. These pieces of evidence led us to hypothesize that deregulation of Cdk5 triggers the aberrant phosphorylation of different substrates, involving pathways that will concomitantly promote neurodegeneration. It will be interesting to perform more investigations on available models of neuronal death to identify relevant players for neurodegeneration, allowing an improved understanding of this process.

In conclusion, our data argue for the involvement of p25-Cdk5 in the deregulation of cell-cycle-regulatory proteins that occurs during neuronal death and identify Rb as having a function in the survival of neuron as an early target of this kinase complex.

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