Cdk5 mediates changes in morphology and promotes apoptosis of astrocytoma cells in response to heat shock

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SUMMARY

The cyclin-dependent kinase member, Cdk5, is expressed in a variety of cell types, but neuron-specific expression of its activator, p35, is thought to limit its activity to neurons. Here we demonstrate that both Cdk5 and p35 are expressed in the human astrocytoma cell line, U373. Cdk5 and p35 are present in the detergent-insoluble cytoskeletal fraction of this cell line and Cdk5 localizes to filopodia and vinculin-rich regions of cell-matrix contact in lamellopodia. When exposed to a 46ºC heat shock, U373 cells change shape, lose cell-matrix contacts and show increased levels of apoptosis. To test whether Cdk5 activation might play a role in these events, U373 cells were stably transfected with histidine-tagged or green fluorescent protein-tagged constructs of Cdk5 or a dominant negative mutation, Cdk5T33. Under normal growth conditions, growth characteristics of the stably transfected lines were indistinguishable from untransfected U373 cells and Cdk5 localization was not changed. However, when subjected to heat shock, cells stably transfected with Cdk5-T33 remained flattened, showed little loss of cell-matrix adhesion, and exhibited significantly lower levels of apoptosis. In contrast, cells that overexpressed wild-type Cdk5 showed morphological changes similar to those seen in untransfected U373 cells in response to heat shock and had significantly higher levels of apoptosis. Heat-shocked cells showed changes in p35 mobility and stability of the Cdk5/p35 complex consistent with endogenous Cdk5 activity. Together these findings suggest that endogenous Cdk5 activity may play a key role in regulating morphology, attachment, and apoptosis in U373 cells, and raise the possibility that Cdk5 may be a general regulator of cytoskeletal organization and cell adhesion in both neuronal and non-neuronal cells.

Key words: Cdk5, p35, Adhesion, Heat stress, Cytoskeleton, U373, Astroglioma cells

INTRODUCTION

Cdk5 is a member of the cyclin-dependent kinase family, which is preferentially expressed in terminally differentiated cells, such as neurons (Meyerson et al., 1992; Lew et al., 1992; Tsai et al., 1993), muscle (Lazaro et al., 1997) and lens fibers (Gao et al., 1997). Although expression of Cdk5 is widespread in adult tissues (Meyerson et al., 1992; Ino et al., 1994), constitutive activity seems to be limited to neurons, which also express high levels of the Cdk5-activating proteins, p35 (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994) and p39 (Tang et al., 1995). Several lines of evidence suggest that neuronal Cdk5 plays a role in cytoskeletal regulation (Lew and Wang, 1995). Cdk5 is found in association with cytoskeletal proteins in brain (Humbert et al., 2000; Veeranna et al., 2000), and several cytoskeletal components are known substrates, including the neurofilament proteins, N_H and N_M (Lew et al., 1992; Hisanaga et al., 1995; Pant et al., 1997) and the microtubule associated protein, tau (Kobayashi et al., 1993; Ishiguro et al., 1994). Cdk5 has been shown to be a downstream effector of Rac (Nikolic et al., 1998), a member of the Rho family of small GTPases, which regulates neuronal growth cone motility (Nikolic et al., 1998) and the cytoskeletal rearrangements involved in cell migration and ruffling (Nobes and Hall, 1995). Finally, Cdk5 activity is required for cellular activities that depend on cytoskeletal function, such as neurite extension and proper neuronal migration during development (Nikolic et al., 1996; Ohshima et al., 1996). Elevated levels of neuronal Cdk5 and Cdk5 activity have been reported in pathological conditions (Green et al., 1997; Ma and Haddad, 1999), and may be responsible for neuronal cell death (Alvarez et al., 1999; Lee et al., 1999; Patrick et al., 1999).

Although there is less information about Cdk5 in non-neuronal cells, a number of observations suggest that Cdk5 may also have important non-neuronal functions. For example, Cdk5 activity is correlated with apoptosis in several non-neuronal cell types (Ahuja et al., 1997; Zhang et al., 1997) and may play a role in differentiation of lens (Gao et al., 1997; Philpott et al., 1999), muscle (Lazaro et al., 1997; Philpott et al., 1997), Leydig TM3 cells (Musa et al., 2000) and HL-60 cells (Chen et al., 2000). These observations suggested that Cdk5 might be involved in the response of certain non-

neuronal cells to extracellular signals, such as those responsible for apoptosis or differentiation. To test this possibility, we have examined the possible function of endogenous Cdk5 in a human astroglioma cell line subjected to heat stress.

MATERIALS AND METHODS

Cell culture and heat shock

U373 MG human astroglioma cells (American Type Culture Collection, Rockville, MD) were cultured at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimal essential medium (GIBCO-BRL Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 10 μg/ml gentamicin. Confluent cultures were heat shocked in a water bath at 46ºC for 30 minutes, then returned to 37ºC for 15 minutes, 30 minutes, 2 hours or 4 hours, as indicated.

Constructs and stable transfection

To make pEGFP-Cdk5 and pEGFP-Cdk5T33 constructs, *Bam*HI fragments were directly taken from the pCMV-Cdk5 and pCMV-Cdk5T33 vectors (Nikolic et al., 1996) and cloned into the *Bam*HI site in pEGFP-C1 vector (Clonetech, pEGFP-C1). The cDNAs are C-terminal to GFP and in reading frame. To make the histidine-tagged Cdk5 and Cdk5-T33 constructs, *Bam*HI fragments of pCVM-Cdk5 and pCMV Cdk5-T33 were cloned into the *Bam*HI site in the pcDNA3.1/His C vector (Invitrogen, pcDNA3.1/His C). The cDNAs are C-terminal to the histidine tag and in reading frame. U373 cells were transfected with 10 μg of each of the plasmid constructs using the calcium phosphate precipitation method (Ausubel et al., 1998). Cells carrying the neomycin-resistance marker were selected by addition of G418 at concentration of 500 μg/ml after 3 days of transfection and the stably transfected cells were maintained with G418 at the same concentration.

RNA extraction and RT-PCR

For RNA extraction, 2.0×10^7 U373 cells were harvested and cytoplasmic RNA was isolated as described (Gough, 1988). The RNA was further treated with DNase I (amplification grade; Gibco-BRL), 1 U/μg RNA for 15 minutes at room temperature, followed by heat inactivation for 10 minutes at 65ºC. RT-PCR (reverse transcription and PCR amplification) of p35 was performed according to manufacturer's instructions (Gene Amp RNA PCR core kit; Perkin-Elmer). A total of 1 μg of RNA was used with the following oligonucleotides:

upstream, 5′-CCACCGGCCCAGCCGCCTGCACCCCCGGCC-3′;

downstream, 5′-GCGAGCGGTCCACGCTGCGCAGCCAGAGCA-3′.

The PCR protocol was 5 minutes at 95ºC, followed by 35 cycles of 1 minute at 95ºC, 1 minute at 55ºC, 1 minute at 72ºC, and a final extension of 10 minutes at 72ºC.

Isolation of cytoskeletal fraction

A detergent insoluble cytoskeletal fraction was isolated as previously described (Walker and Menko, 1999), with minor modifications. In brief, cultured U373 cells were extracted on ice in 1% Triton X-100 buffer containing 10 mM imidazole, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 0.5 mM NaF, 0.1 μM okadaic acid, at pH 7.4, with 1 Complete-Mini^{TM} protease inhibitor cocktail tablet per 10 ml (Boehringer Mannheim). Extracts were subjected to centrifugation at 125,000 ***g*** for 20 minutes and 4ºC. Triton X-100 insoluble pellet is referred to as the cytoskeletal-associated fraction; the Triton X-100 soluble supernatant is referred to as the cytoplasmic sample. The Triton X-100 insoluble fraction was washed with 1% Triton X-100 buffer and solubilized directly in RIPA buffer (5 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl, pH 7.4), with 1 Complete-Mini^{TM} protease inhibitor cocktail tablet per 10 ml. Then Laemlli sample buffer was added to each of the extracts followed by SDS PAGE.

Immunoblotting and immunoprecipitation

Cells were lysed in PBSTDS containing one Complete-Mini^{TM} protease inhibitor cocktail tablet per 10 ml, and 5 mM sodium orthovanadate (Sigma, St Louis, MO). Pervanadate (PV) was formed by adding 30% H_2O_2 to above extraction buffer to a final concentration of 50 mM, 10 minutes before use. Immunoblotting and immunoprecipitation were performed as described previously (Gao et al., 1997). Immunoprecipitation of Cdk5 was performed as described above, using 200 μg of cell lysate with anti-Cdk5 rabbit polyclonal IgG (C-8; sc-173, SantaCruz). Control immunoprecipitations without primary antibody were performed with an equivalent amount of cell extract. Immunoblotting was performed on immunoprecipitated proteins or on 25 to 50 μg of total cell extract using anti-Cdk5 mouse monoclonal IgG (DC-17; sc-249, Santa Cruz) or anti-p35 rabbit polyclonal IgG (C-19; sc-820, Santa Cruz). Where indicated, 2-3 μg of p35 blocking peptide (sc-820P, Santa Cruz) was added with the primary antibody. Immunoreactive bands were detected by enhanced chemiluminescence (ECL-Plus; Amersham Life Science) using horseradish peroxidase-linked anti-rabbit IgG (Santa Cruz).

Histology and immunofluorescence

U373 cells were cultured on chamber slides, fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, extracted in 0.25% Triton X-100 for 10 minutes, and incubated for 1 hour at room temperature in a blocking buffer consisting of PBS with 5% goat serum. Subsequent antibody incubations and washes were also performed in blocking buffer. Slides were incubated with anti-Cdk5 (C-8, Santa Cruz) and anti-vinculin (V-4505, Sigma ImmunoChemicals) for 1 hour at room temperature, washed three times and incubated with rhodamine-conjugated goat anti-rabbit IgG (111-295-144, Jackson ImmunoResearch Laboratories). Fluorescein-conjugated goat anti-mouse (115-195-146, Jackson ImmunoResearch Laboratories) were then applied and incubated for 1 hour. Samples were washed and mounted in Aqua Poly mount (18606, Polysciences), then examined with a Zeiss Axioplan 2 photomicroscope equipped with epifluorescence. Images were captured with a CCD camera (OPELCO).

Apoptosis assay

Apoptotic cells were identified using a terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) protocol (Roche Molecular Biochemicals). Typically, U373 cells were air dried, fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for 1 hour at room temperature, washed with PBS and incubated in permeabilization solution (0.1% TritonX-100, 0.1% sodium citrate) for 2 minutes on ice. After three washes in phosphate buffered saline (PBS), samples were blocked overnight at 4°C with 5% sheep serum in PBS. The labeling reaction was started by removing blocking solution and adding 500 μl TdT-labeling mixture containing terminal deoxytransferase and fluorescein-conjugated dUTP, according to the manufacturer's protocol. Samples were incubated for 1 hour in humidified chamber at 37°C. After three washes in PBS, samples were incubated with alkaline phosphatase-conjugated antibody against fluorescein-dUTP for 30 minutes at 37°C in humidified chamber. After extensive washing in PBS, samples were incubated for 10 minutes at room temperature in 100 μl of alkaline phosphatase substrate solution (SK-5100; Vector Laboratories). Samples were mounted, cover-slipped, and viewed under Carl Zeiss Axiovert S100 phase contrast microscope. Images were captured with a Spot II CCD camera (OPELCO) and both labeled and unlabeled cells were counted in at least five fields of 600-700 cells. Statistical analysis was performed using SigmaStat 2.03.

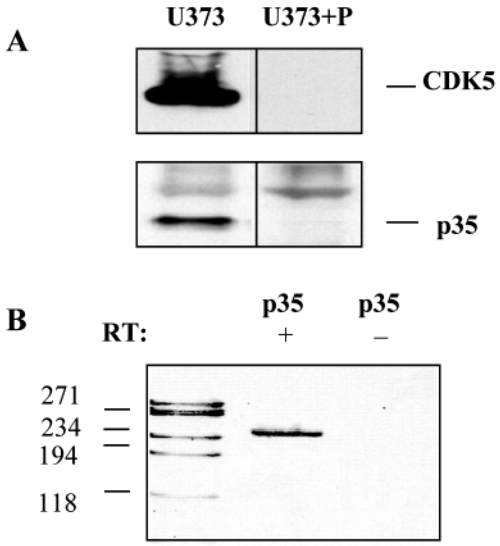


Fig. 1. Expression of Cdk5 and p35 in U373 cells. (A) Protein extracted from confluent cultures of U373 cells was immunoblotted with rabbit polyclonal antibody to Cdk5 or p35. As a negative control, equal amounts of U373 protein were immunoblotted in the presence of 2 μg blocking peptide for the respective antibodies (U373+P). (B) RT-PCR was performed on 1 μg of RNA extracted from confluent cultures of U373 cells. Reverse transcriptase was omitted from the adjacent sample to confirm that the product was derived from RNA.

RESULTS

Cdk5 and its activator, p35, are expressed in U373 cells

To determine whether Cdk5 is expressed in U373 astroglioma cells, proteins were extracted and subjected to immunoblotting with an antibody specific for Cdk5. The antibody detected a single immunoreactive band of the correct molecular weight that was blocked by the antigenic peptide (Fig. 1A) and which co-migrated with authentic brain Cdk5 (see Fig. 9).

To determine whether the Cdk5 activator, p35, is also expressed in U373 cells, cellular proteins were immunoblotted with an antibody to p35. The antibody detected a strong immunoreactive band of approximately 35 kDa that was blocked by the antigenic peptide (Fig. 1A) and co-migrated with authentic p35 from rat brain (see Fig. 9). To determine whether immunoreactive 35 kDa protein interacts with Cdk5, Cdk5 was immunoprecipitated and the precipitated proteins were immunoblotted with p35 antibody. The antibody again detected a 35 kDa protein, indicating that this protein forms a complex with Cdk5 in U373 cells (see Fig. 9).

As an additional test for the expression of p35, we performed RT-PCR, using oligonucleotides specific for p35 mRNA. A single RT-PCR product of the predicted size was detected (Fig. 1B). Sequencing of the isolated DNA band confirmed that it was derived from p35 mRNA. Thus, U373 astroglioma cells appear to express the Cdk5-activating protein, p35, as judged by partial nucleotide sequence, immunoreactivity, co-migration with brain p35, and the ability to bind Cdk5.

Cdk5 localization

The localization of Cdk5 was examined by immunofluorescence.

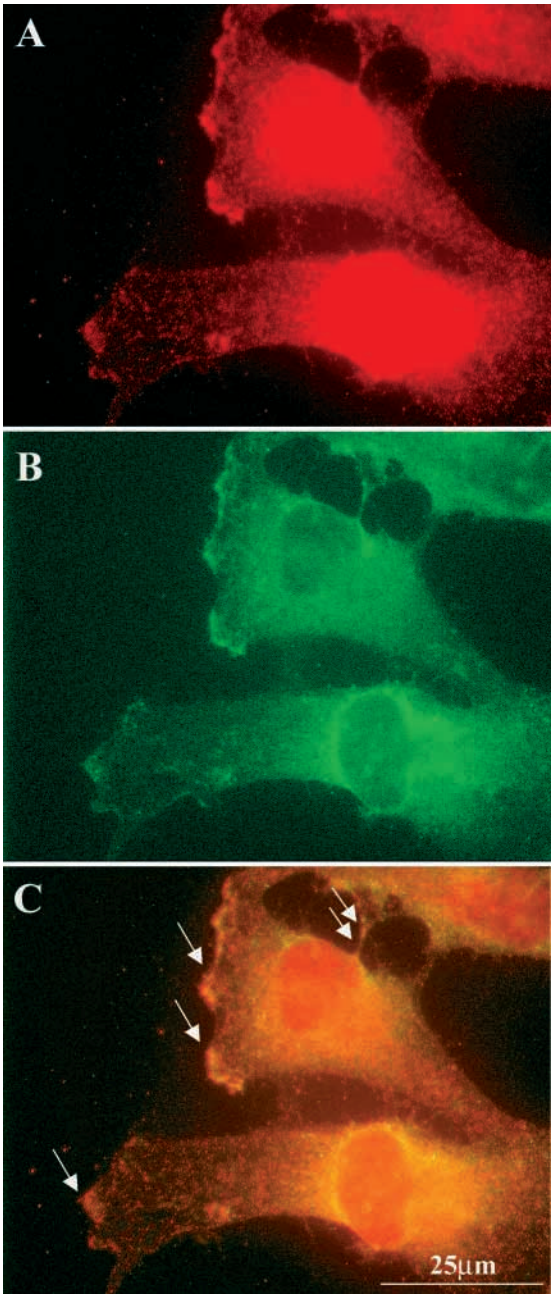


Fig. 2. Immunolocalization of Cdk5 and vinculin. (A,B) U373 cells were fixed and permeabilized as described, then incubated with anti-Cdk5 followed by rhodamine-conjugated goat anti-rabbit IgG (A) or anti-vinculin followed by fluorescein-conjugated goat anti-mouse IgG (B). (C) Superimposed images of Cdk5 and vinculin immunofluorescence. Filopodia (double arrow) and edges of lammelopodia (single arrows) were stained by both antibodies.

Cdk5 staining was observed primarily in the perinuclear region of the cytoplasm. As the intense perinuclear staining would obscure any nuclear staining with the techniques we have used, we can not rule out the possibility that some Cdk5 may also localize to the nucleus. Local regions with strong Cdk5 staining were present in filopodia and along the edges of lamellopodia (Fig. 2A). Staining with vinculin antibody (Fig. 2B) demonstrated that the regions of lamellopodia that stained with Cdk5 antibody were also

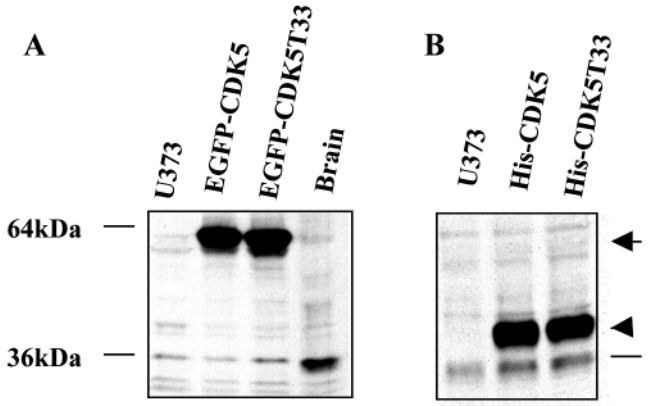


Fig. 3. Expression of Cdk5 and Cdk5T33 in stably transfected cells. Proteins extracted from untransfected U373 cells or from cells stably transfected with EGFP-tagged Cdk5 and Cdk5T33 (A) or his-tagged Cdk5 and Cdk5T33 (B) were immunoblotted with rabbit polyclonal antibody to Cdk5. Arrow indicates position of EGFP-tagged proteins; arrowhead, position of his-tagged proteins; bar, position of endogenous Cdk5. An equal amount of rat brain extract (Brain) was included in A as a positive control.

immunostained by vinculin antibody, suggesting that Cdk5 may be associated with regions of focal adhesion (Fig. 2C).

Dominant negative Cdk5 blocks cellular responses to heat shock

To examine the biological effects of Cdk5, U373 cells were stably transfected with histidine-tagged or EGFP-tagged constructs of either Cdk5 (catalytic subunit) or a dominant negative mutation of Cdk5 (Cdk5T33). These constructs were expressed equally well, at a level that was about 10 times greater than the level of endogenous Cdk5 (Fig. 3A,B). The EGFP-tagged proteins localized to regions of the cell previously shown to contain endogenous Cdk5, such as lamellopodia and filopodia (Fig. 4), demonstrating that overexpression did not affect the normal cellular distribution of this protein. In addition, cell growth curves obtained using the his-Cdk5 and his-Cdk5T33 transfected cell lines were indistinguishable from those of untransfected U373 cells (data not shown), indicating that overexpression of Cdk5 and Cdk5-T33 had no effect on cell growth or survival under normal growth conditions.

To test whether Cdk5 might play a role in the response to stress, we compared the effects of heat shock on untransfected and transfected cells. Phase microscopy revealed that untransfected U373 cells rapidly retracted their boundaries after heat shock, assuming a stellate morphology with a rounded cell body and few lamellopodia (Fig. 5D,G). Similar changes in morphology were seen in cells transfected with a control, histidine-tagged β-galactosidase plasmid (not shown). Cells transfected with Cdk5 also rapidly underwent shape changes after heat shock, but the retraction of cell boundaries was somewhat more pronounced than in untransfected cells (Fig. 5B,E,H). In contrast, heat shock produced only minimal changes in the morphology of cells that expressed the dominant negative construct, Cdk5T33 (Fig. 5C,F,I). Some retraction of cell boundaries was seen in Cdk5T33 cell cultures 30 minutes after heat shock (Fig. 5F), but by 4 hours, the cells had spread again and were indistinguishable from cells that had not been heat shocked (Fig. 5I).

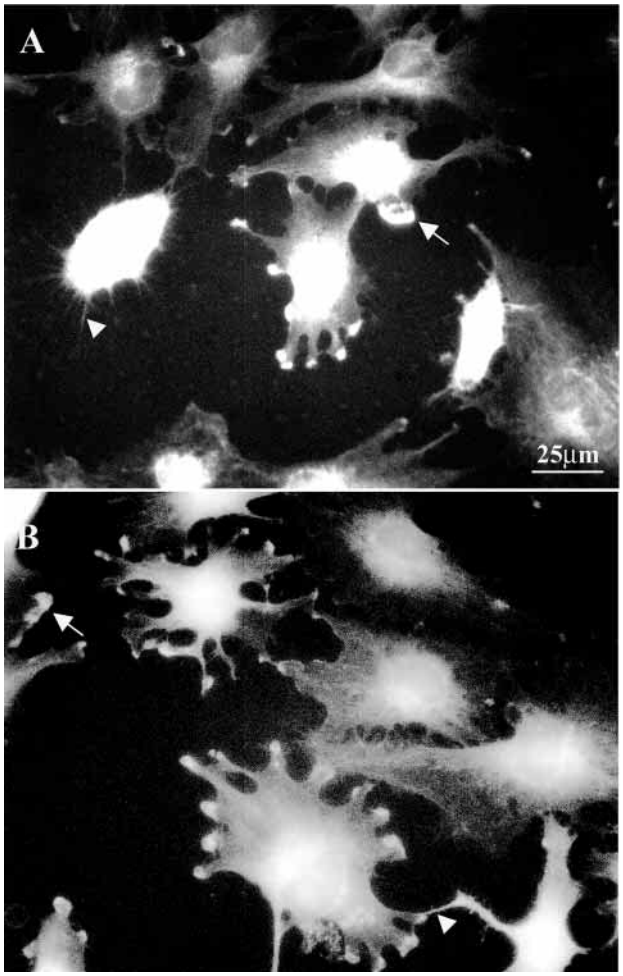


Fig. 4. Localization of EGFP-labeled proteins. U373 cells that were stably transfected with EGFP-Cdk5 or EGFP-Cdk5T33 were examined by fluorescence microscopy to determine the localization of the labeled protein. (A) EGFP-Cdk5 was concentrated in the perinuclear region, in filopodia (arrowhead), and in lamellopodia (arrow). (B) EGFP-Cdk5T33 was similarly localized to the perinuclear region, filopodia (arrowhead) and lamellopodia (arrow).

These differences in cell morphology were mirrored by differences in cell survival, as shown by Trypan Blue staining (Fig. 6). Within 30 minutes of heat shock, untransfected U373 cells showed a sharp increase in the percentage of cells permeable to Trypan Blue. This was accompanied by a 15-20% decrease in total cell number and an increase in cell debris in the supernatant, suggesting that some cells may have lysed. The percentage of cells that could be labeled with Trypan Blue decreased gradually over the next few hours. Although many cells died as a result of heat shock, approximately 75-80% of the cells survived, spread and reattached within 24 hours (not shown). Trypan Blue staining in Cdk5-transfected cells was similar to that in untransfected U373 cells, but somewhat increased (Fig. 6). In contrast, Trypan Blue staining of Cdk5T33-transfected cells was significantly reduced at all times after heat shock, suggesting that Cdk5T33 enhances cell survival (Fig. 6).

As Cdk5 has previously been associated with apoptosis (Ahuja et al., 1997; Zhang et al., 1997), we next tested whether the cell death caused by heat shock (Fig. 6) was due to

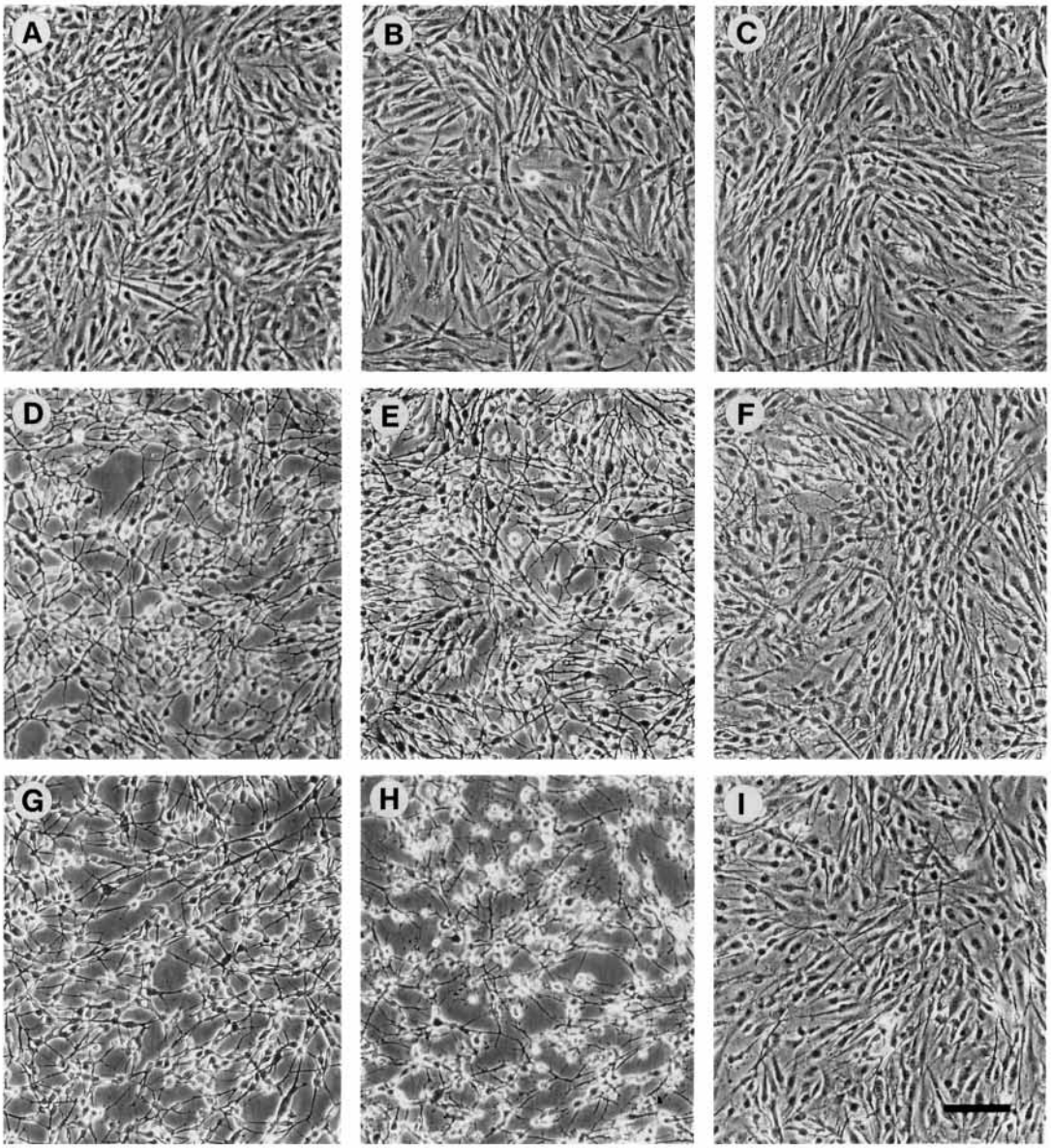


Fig. 5. Effect of heat shock on morphology. Untransfected U373 cells or cells stably transfected with his-Cdk5 or his-Cdk5T33 were grown to confluence and subjected to a 30 minute heat shock at 46ºC. Cell morphology was examined by phase contrast microscopy before heat shock, during the recovery period, and 0.5 hours and 4 hours after heat shock. (A-C) Cells before heat shock: (A) U373, (B) U373-Cdk5, (C) U373-Cdk5T33. (D-F) Heat shocked cells, 0.5 hours of recovery: (D) U373, (E) U373-Cdk5, (F) U373-Cdk5T33. (G-I) Heat shocked cells, 4 hours of recovery: (G) U373, (H) U373-Cdk5, (I) U373-Cdk5T33. Scale bar: 80 μm.

apoptosis. In a preliminary experiment on untransfected U373 cultures (not shown), we used annexin V and BOBO I staining to distinguish necrotic and apoptotic cells. Because the results indicated that most of the cell death 4 hours after heat shock was due to apoptosis, the 4 hour time point was chosen to examine the effect of Cdk5 and Cdk5T33 on apoptotic cell

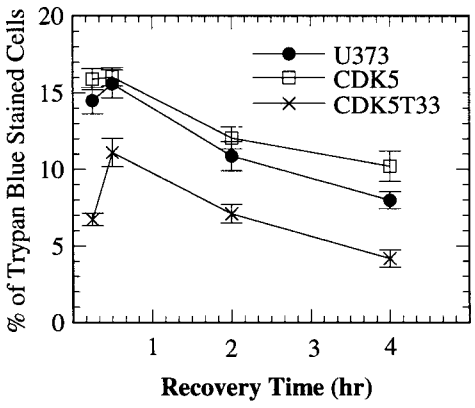
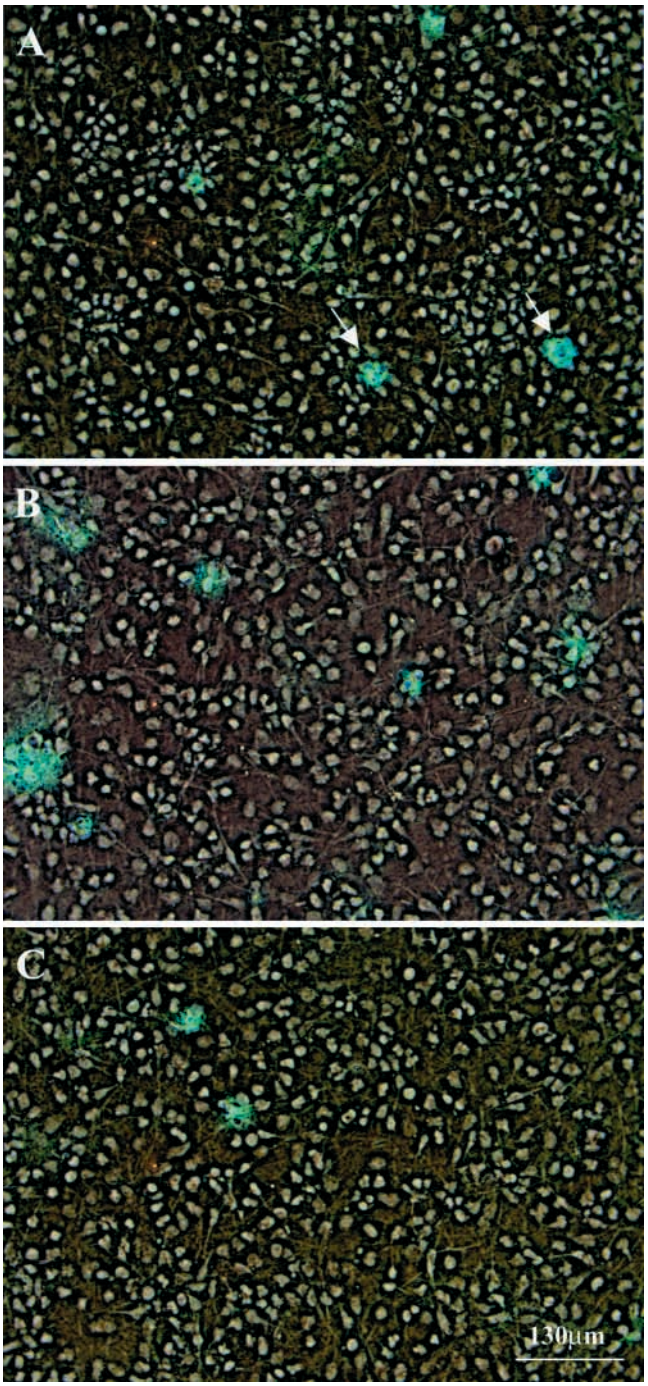


Fig. 6. Cell death in response to heat shock. Cells were stained with Trypan Blue at different times during recovery from heat shock and the percentage of stained cells was calculated. Data plotted are the average of at least three experiments ±s.e.m. The decrease in the percentage of stained cells after the initial peak is correlated with a decrease in total cell number (not shown).



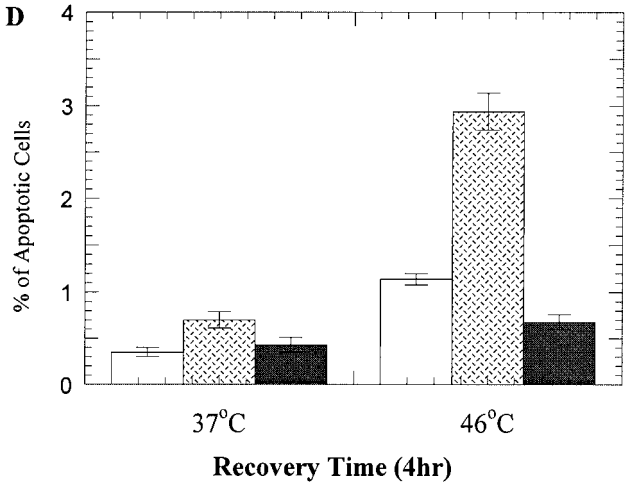


Fig. 7. Effect of Cdk5 and Cdk5T33 on apoptosis. Apoptotic cells present after 4 hours recovery from heat shock were labeled by an in situ TUNEL assay. Colors have been reversed to improve contrast. (A) U373 cells; (B)U373-Cdk5; (C) U373-Cdk5T33. (D) Labeled and unlabeled cells were counted in least five different fields of 600-700 cells and percent labeled was calculated. The bar graph represents an average of all fields counted for each condition. White bar, U373 cells; hatched bar, U373-Cdk5; gray bar, U373-Cdk5T33.

death. Apoptosis was measured in untransfected, Cdk5-transfected and Cdk5T33-transfected U373 cells by terminal transferase end-labeling of nicked DNA (also referred to as TUNEL labeling (Gavrieli et al., 1992)). The results showed that DNA labeling was enhanced in cells transfected with Cdk5 and suppressed in cells transfected with Cdk5T33 (Fig. 7A-D). Both differences were statistically significant (P<0.02). Similar results were obtained using Hoechst staining of condensed chromatin as a measure of apoptosis (data not shown). Together these observations suggest that Cdk5 may regulate U373 cell morphology and apoptosis after heat shock.

Heat shock promotes association of Cdk5 and p35 with the cytoskeletal fraction

The localization of Cdk5 and the biological effects observed in cells transfected with Cdk5 and Cdk5T33 suggested that Cdk5/p35 might play a role in cytoskeletal reorganization. To see whether Cdk5 and p35 might physically associate with the cytoskeleton, we separated U373 homogenates into a detergent-soluble cytoplasmic fraction and a detergent-insoluble cytoskeletal fraction, and examined the distribution of Cdk5 and p35 before and after heat shock (Fig. 8A,B). In confluent U373 cells grown at 37°C, significant levels of Cdk5 and p35 were present in the detergent-insoluble fraction, although both were found primarily in the detergent-soluble fraction. Interestingly, the detergent-insoluble fraction contained a slightly more rapidly migrating form of p35 that was not observed in the detergent-soluble fraction (Fig. 8B). Within 30 minutes of heat shock, the concentration of both proteins in the detergent-insoluble cytoskeletal fraction increased three- to fivefold (Fig. 8A,B). No equivalent shift into the insoluble fraction was seen in immunoblots of glial fibrillary acidic protein (GFAP; Fig. 8C). These findings indicate that Cdk5 and p35 specifically redistribute to the detergent insoluble fraction following heat shock, thus supporting the idea that Cdk5/p35 may play a role in cytoskeletal regulation.

Heat shock is accompanied by changes in p35 mobility and stability

Although the above findings strongly suggested that endogenous Cdk5 may be activated in response to heat stress, attempts to measure kinase activity in U373 Cdk5 immunoprecipitates by conventional techniques (Simanis and Nurse, 1986; Gao et al., 1999) detected only trace levels either before or after heat shock (data not shown). We estimate that Cdk5 activity in heat-shocked U373 cells is less than 2% of the

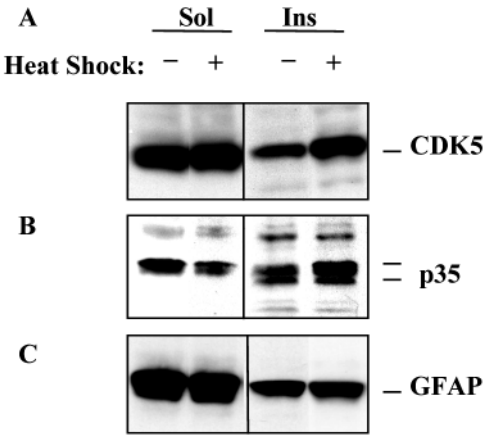


Fig. 8. Effect of heat shock on subcellular distribution of Cdk5. U373 cells were separated into detergent soluble (Sol) and detergent insoluble (Ins) fractions before heat shock or after 0.5 hours recovery from heat shock. Soluble fraction represented approximately 80% of total protein; insoluble fraction, 20%. Soluble and insoluble fractions were examined on the same gel; intervening lanes have been removed for the illustration. Equal amounts of protein from the soluble and insoluble fractions were immunoblotted with rabbit polyclonal antibody to Cdk5 (A)or rabbit polyclonal antibody to p35 (B). We estimate that the apparent molecular weights of the three forms of p35 in the insoluble fraction differ by approximately 100-300 Da.

activity in brain, as judged by direct comparison with rat brain extracts assayed at the same time. Therefore, we attempted to find other indicators of endogenous Cdk5 activity. Studies in neurons have shown that active Cdk5/p35 phosphorylates p35 targeting it for ubiquitin-dependent degradation (Patrick et al., 1998). Therefore, we looked for changes in the mobility and stability of p35 in heat-shocked cells. Cdk5 was immunoprecipitated at 0.5, 2 and 4 hours after heat shock and the precipitated proteins were immunoblotted for p35. Although Cdk5/p35 complexes were present before heat shock and there was no apparent increase in the amount of p35 complexed with Cdk5 after heat shock, a distinct shift in p35 mobility was seen, with the slower migrating forms predominating after heat shock (Fig. 9A). Moreover, 2 and 4 hours after heat shock, the amount of p35 bound to Cdk5 was reduced. These changes in mobility of p35 and stability of the Cdk5/p35 complex provide indirect evidence that endogenous Cdk5 may be transiently activated in response to heat stress.

As elevated levels of p35 and/or Cdk5 might also lead to increased Cdk5 activity, we tested whether either protein is induced by heat shock. Immunoblotting of whole-cell extracts showed that the overall concentrations of Cdk5 and p35 did not change significantly after heat shock (Fig. 9B). As in the Cdk5 immunoprecipitates, slower migrating forms of p35 were more prominent following heat shock. Interestingly, however, there was no apparent loss of p35 from the whole cell extracts, suggesting that the pool of p35 associated with Cdk5 might be a small fraction of the total. To test this possibility, Cdk5 was immunoprecipitated and the supernatant fraction was immunoblotted for both p35 and Cdk5 (Fig. 9B). The results showed that significant levels of p35 remained in the supernatant, while Cdk5 was effectively removed.

DISCUSSION

We have found that the dominant negative construct, Cdk5-T33, suppresses the change of shape, loss of adhesion and apoptosis that characterize the response of U373 glioma cells to heat stress. Cdk5-T33 has no kinase activity, owing to a K to T substitution at amino acid 33 (Nikolic et al., 1996) and exerts its dominant negative effect by sequestering p35. Thus, it is a highly specific inhibitor of Cdk5. Moreover, as comparable levels of wild-type Cdk5 augmented the cellular response to heat shock, the biological effects of Cdk5-T33 seem to depend on its ability to block Cdk5 kinase activity. These findings suggest that endogenous Cdk5 activity may play a key role in regulating cell-substrate attachment, cell shape and apoptosis in U373 cells, and raise the possibility that Cdk5 may be a general regulator of cytoskeletal organization and cell adhesion in both neuronal and non-neuronal cells.

Several additional observations support the possibility that endogenous Cdk5 may be enzymatically active in U373 cells.

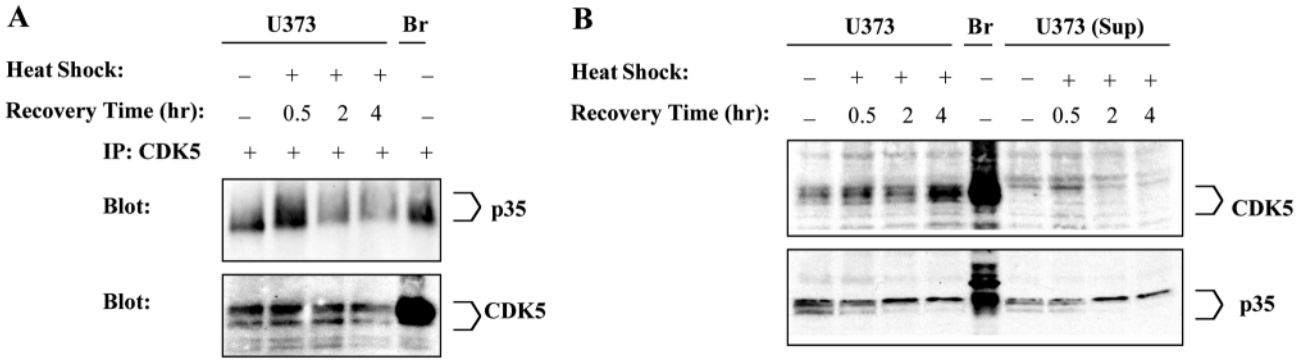


Fig. 9. Effect of heat shock on p35 mobility and stability of the Cdk5/p35 complex. (A) Cdk5 was immunoprecipitated from U373 cells before heat shock or after 0.5, 2 and 4 hours recovery from heat shock using mouse monoclonal antibody to Cdk5. The amount of associated p35 was then assessed by immunoblotting with rabbit polyclonal antibody to p35. No immunoreactive band was present in control immunopreciptates lacking primary antibody (not shown). (B) Top panel: immunoblot with rabbit polyclonal antibody to Cdk5 of whole-cell extracts of U373 cells before heat shock or after 0.5, 2, and 4 hours recovery from heat shock (lanes 1-4); rat brain extract (lane 5); supernatant solution remaining after immunoprecipitation of Cdk5 from U373 cells before heat shock or after 0.5, 2, and 4 hours recovery from heat shock (lanes 6-9). Bottom panel: immunoblot with rabbit polyclonal antibody to p35 of the same samples as above.

First, we have found that the Cdk5-activating protein, p35, is expressed in U373 cells and forms a complex with Cdk5. Like other members of the cyclin-dependent kinase family, Cdk5 can be activated only when complexed with an appropriate activating protein (Lew et al., 1994; Tsai et al., 1994; Ishiguro et al., 1994; Tang et al., 1995). Although p35 expression is often considered to be neuron specific, expression has also been reported in the developing lens (Gao et al., 1997) and in a variety of non-neuronal cell types during apoptosis (Ahuja et al., 1997). Moreover, we have shown that heat shock leads to changes in the mobility of p35 and the stability of the Cdk5/p35 complex. As the active Cdk5/p35 complex has been shown to phosphorylate the p35 subunit, leading to its ubiquitin-dependent degradation (Patrick et al., 1998), such changes in p35 mobility and stability of the Cdk5/p35 complex are expected consequences of Cdk5/p35 activation. Finally, our data indicate that Cdk5 is localized in filopodia and lamellopodia of U373 cells, especially in regions containing focal adhesions. Such localization is consistent with a role in regulation of adhesion and cell shape, possibly through phosphorylation of substrates associated with the cytoskeleton. Many of the known substrates of Cdk5 in neurons are, in fact, cytoskeletal elements or associated proteins. These include neurofilaments (Lew et al., 1992; Hisanaga et al., 1995; Pant et al., 1997), microtubule-associated proteins (Kobayashi et al., 1993; Ishiguro et al., 1994) and PAK-1 (Nikolic et al., 1998). Moreover, p35 has been shown to bind β-catenin and to regulate the association between the actin cytoskeleton and cadherin-catenin dependent contacts at cell-cell junctions (Kwon et al., 2000). While the above findings strongly support a role for endogenous Cdk5 activity in the response of U373 cells to heat shock, we have, nonetheless, been unable to measure Cdk5 kinase activity in heat-shocked U373 cells. Therefore, we can not rule out other possible explanations for the effects of Cdk5T33 on cell adhesion and survival.

Several mechanisms are known from neurons that might lead to the induction of Cdk5 activity in heat-shocked U373 cells. One such mechanism is the cleavage of p35 to p25, which is more stable than p35 (Patrick et al., 1999). However, we did not observe formation of p25 in U373 cells either before or after heat shock. Changes in the amount of p35 can also regulate Cdk5 activity in neurons, as p35 concentration is limiting in neuronal cells (Patrick et al., 1998; Li et al., 2000). Although it is not clear at present how much p35 is present in U373 cells, or whether this amount is sufficient to produce measurable levels of Cdk5 activity, we did not observe an increase in the level of p35 or p35/Cdk5 complexes after heat shock. Furthermore, two observations suggest that p35 may not be limiting in these cells. First, immunoblotting showed significant amounts of p35 in cell extracts that had been depleted of Cdk5 by immunoprecipitation. Secondly, transfection with p35 was not required to produce a biological effect in cells transfected with Cdk5, implying that U373 cells contain excess p35 (or some other activator of Cdk5). Therefore, changes in p35 availability may be less important determinants of Cdk5 activity in U373 cells than in neurons. Nevertheless, we did observe a decrease in the amount of p35 bound to Cdk5 2 to 4 hours after heat shock, suggesting that p35 stability may be important for downregulation of Cdk5 following activation. The possibility that Cdk5 activity might be regulated by the formation of Cdk5/p39 complexes should also be considered, in view of a recent report that astrocytes express p39 in vivo (Honjyo et al., 1999). Finally, Cdk5 activity may be regulated by phosphorylation and dephosphorylation at Tyr17 (Zukerberg et al., 2000) and at Ser159 (Sharma et al., 1999). These amino acids correspond to conserved sites that are important for regulation of all members of the Cdk family (Lees, 1995), although their importance for Cdk5 activity has only recently been demonstrated. As stress-activated kinases are known to play a role in the response of U373 cells to heat shock (Ito et al., 1997), it is possible that post-translational modification of Cdk5 may be a downstream event in the stress-activated kinase cascade.

The present findings suggest that Cdk5 regulates cell survival as well as cell adhesion and morphology. Cell survival depends on survival signals that counteract the effects of pro-apoptotic proteins of the Bcl2 family (Datta et al., 1997; Bonni et al., 1999; Gilmore et al., 2000). Survival signals have been shown to originate from cell-matrix and cell-cell junctions and from growth factor receptor occupancy (Frisch et al., 1996; Watton and Downward, 1999). In many cell types, loss of survival signals from cell-matrix contacts in the absence of growth factors is sufficient to initiate a form of apoptosis termed ‘anoikis’ (Frisch and Francis, 1994; Frisch et al., 1996; Meredith and Schwartz, 1997). Thus, the Cdk5-dependent loss of cell-matrix adhesion would be expected to promote apoptotic cell death. In addition, Cdk5-dependent shape changes would also favor apoptosis (Re et al., 1994). Thus, Cdk5 activity might contribute to the subsequent death of the cells by interrupting essential survival signaling. With the present experimental design, ample growth factors were present in the medium to provide an alternative source of survival signals and most cells ultimately survived. However, under conditions where growth factors or their receptors were limited, Cdk5 activity could be expected to have more severe consequences for cell survival. Thus, the finding that Cdk5 regulates cell adhesion and morphology may help to explain the previously observed correlation between Cdk5/p35 activity and the developmentally programmed death of many non-neuronal cell types (Ahuja et al., 1997; Zhang et al., 1997).

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