Expression and localization of cyclin-dependent kinase 5 in apoptotic human glioma cells[1]

Alaine Catania, Sinisa Urban,[2] Elizabeth Yan, Chunhai Hao, Gerry Barron, and Joan Allalunis-Turner[3]

Cross Cancer Institute, Edmonton, Alberta T6G 1Z2 (A.C., S.U., E.Y., G.B., J.A.-T.); and the Departments of Laboratory Medicine and Pathology (C.H.) and Oncology (A.C., E.Y., J.A.-T.), University of Alberta, Edmonton, Alberta, Canada T6G 2E1

Cyclin-dependent kinase 5 (Cdk5), a member of the cyclin-dependent kinase family, is expressed predominately in mature neurons and is implicated in neurite extension, neuronal migration, and neuronal differentiation. Cdk5 protein expression also has been associated with apoptosis in a number of nonneuronal model systems. In normal brain, substrates for Cdk5 include neurofilament and tau proteins. Because human tumors of glial origin can express neuronal proteins, we examined whether Cdk5 and its activator protein, P35, are present in early passage human glioblastoma multiforme (GBM) cells lines and primary tumor specimens. Here we report the expression of Cdk5 and an "active" proteolytic form of P35 in human GBM cells and demonstrate kinase activity of the holoenzyme. We also show that Cdk5 kinase activity and expression of its activator protein, P35, is increased in the human GBM cell line M059J after exposure to ionizing radiation and that P35 is localized within M059J cells undergoing apoptosis. These results suggest a possible role for Cdk5 in mediating apoptosis in human GBM cells. *Neuro-Oncology 3, 89–98, 2001 (Posted to Neuro-Oncology [serial online], Doc. 00-038, February 15, 2001. URL <neuro-oncology.mc.duke.edu>)*

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[2]Present address: MRC Laboratory of Molecular Biology, University of Cambridge and Trinity College, Cambridge, UK.

[3]Address correspondence and reprint requests to Joan Allalunis-Turner, Experimental Oncology, Cross Cancer Institute, 11560 University Ave., Edmonton, AB, Canada T6G 1Z2.

[4]Abbreviations used are as follows: Cdk5, cyclin-dependent kinase 5; GBM, glioblastoma multiforme; SDS, sodium *n*-dodecyl sulfate.

Cdk5[4] was initially identified on the basis of its sequence similarity to Cdks, key regulators of cell cycle progression (Hellmich et al., 1992; Meyerson et al., 1992). It was also independently isolated as a proline-directed histone H1 kinase from bovine brain (Lew et al., 1992). Despite significant sequence identity to human Cdk1 and Cdk2, Cdk5 does not complement temperature-sensitive budding yeast *cdc2* mutants used to identify kinases that play a *cdc2*-like role in controlling the vertebrate cell cycle (Meyerson et al., 1992). Human cultured cells, including both primary and immortalized cell lines, contain uniformly high levels of Cdk5 protein (Tsai et al., 1993) and transcript (Meyerson et al., 1992). In contrast, the tissue distribution of Cdk5 is quite variable. Cdk5 protein and transcript are expressed at basal levels in most human tissues, in both cycling and noncycling cells (Lew et al., 1995; Tang et al., 1996). Exceptions are the central and peripheral nervous systems in which the expression is several-fold greater (Lew et al., 1994; Meyerson et al., 1992), with highest levels detected in the forebrain (Tsai et al., 1994). Cdk5 is also expressed at intermediate levels in gonads (Ino et al., 1994; Tsai et al., 1993), muscles (Lazaro et al., 1997; Philpott et al., 1997), and fiber and epithelial cells of developing rat lens (Gao et al., 1997). Within the normal brain, Cdk5 protein is expressed in most postmitotic neurons, although there are exceptions, such as cerebellar granule cells (Ino and Chiba, 1996; Ino et al., 1994). In vivo, both mitotically active cells and glial cells such as astrocytes and oligodendrocytes are devoid

of Cdk5 expression (Hayashi et al., 1999; Ino et al., 1994; Nakamura et al., 1998; Tsai et al., 1993).

The human Cdk5 activator, P35, is a 35-kDa protein that displays complete specificity in binding and activating Cdk5 (Tsai et al., 1994). P35 also exists as a 25-kDa proteolytic fragment that retains the Cdk5-activating properties of P35 (Uchida et al., 1994). Although P35 shares minimal sequence homology to cyclins, computer modeling predicts a similar cyclin-like tertiary structure and similar functional sites for kinase activation (Brown et al., 1995; Tang et al., 1997). In contrast to the ubiquitous distribution of Cdk5, the P35 transcript is present predominantly in the forebrain (Ohshima et al., 1996; Uchida et al., 1994) in cells of neuronal lineage (Delalle et al., 1997). Other activators of Cdk5 include 2 neuron-specific proteins, the P39 protein (Tang et al., 1995), and the vesicle trafficking–associated protein, Munc-18 (Shetty et al., 1995). Interestingly, cyclin-D and cyclin-E molecules, which regulate Cdk activity in proliferating cells, bind but do not activate Cdk5 (Guidato et al., 1998; Miyajima et al., 1995; Xiong et al., 1992).

Although Cdk5 is widely expressed, its kinase activity is restricted by its requirement for a regulatory subunit. As a result, Cdk5 kinase activity correlates with P35, not Cdk5 expression (Tsai et al., 1994). The substrate specificity of Cdk5/P35 kinase is similar to that of Cdc2 and Cdk2 and involves a K(S/T)PX(K/R) consensus motif (Beaudette et al., 1993; Songyang et al., 1996). The Cdk5/P35 holoenzyme phosphorylates both histone H1 and the retinoblastoma protein (pRB), 2 substrates frequently used to confirm in vitro activity of Cdks (Lee et al., 1997; Tang and Wang, 1996). In vitro, activated Cdk5 phosphorylates a number of neuron-specific cytoskeletal proteins, including medium molecular weight and high molecular weight neurofilament proteins, tau, mitogen-activated protein-2, the actin-binding protein caldesmon, and brain meromysin 18 (Lee et al., 1997; Lew and Wang, 1995; Tang and Wang, 1996). In addition to structural proteins, Cdk5/P35 phosphorylates proteins associated with synaptic vesicle neurotransmitter release, such as synapsin I and Munc 18 (Matsubara et al., 1996; Shuang et al., 1998). As phosphorylation of cytoskeletal proteins is involved in cytoskeletal dynamics during active process extension of differentiating neurons, Cdk5 has been implicated in neurite extension, neuronal migration, and neuronal differentiation (Nikolic et al., 1996). In neurodegenerative diseases, Cdk5 participates in the hyperphosphorylation of the Lewy bodies of Parkinson's disease (Brion and Couck, 1995), Lewy bodylike inclusions of amyotrophic lateral sclerosis (Nakamura et al., 1997), and neurofibrillary tangles of Alzheimer's disease (Paudel et al., 1993).

Although Cdk5 protein has been detected in a variety of human tumor cell lines, including the GBM cell line T98G, initial reports suggested kinase activity was absent (Tsai et al., 1993). More recently, however, Cdk5 kinase activity has been observed in human leukemia (Chen et al., 2000) and retinoblastoma cell lines (Kato and Maeda, 1999). Here, we report the expression of Cdk5 and an "active" proteolytic form of P35 in human GBM surgical specimens and cell lines and demonstrate kinase activity of the holoenzyme. We show also that Cdk5/P35 protein and kinase activity are associated with apoptosis in human GBM cells.

Materials and Methods

Tissue Samples and Cell Lines

Surgical or autopsy specimens of human nonmalignant brain tissue or astrocytic tumors were used in these studies. All tissues represented excess pathological material and were obtained from the University of Alberta Hospitals, Edmonton, Alberta, Canada, in accordance with procedures approved by the Human Ethics Review Board. The pathology and anatomic location of the nonmalignant brain tissues examined were cortical dysplasia (cortex/subcortical white matter), cortical dysplasia (hippocampal formation), residual ulygyria (hippocampal formation), and hypoxic ischemic encephalopathy (hippocampal formation). Expert neuropathologic review of the primary specimens and of the immunohistochemical and immunofluorescence staining patterns was provided by Drs. Bruce Mielke and Chunhai Hao of the Department of Pathology and Laboratory Medicine, University of Alberta Hospitals.

The isolation, characterization, and culture of the human GBM cell lines used in these studies have been previously described (Allalunis-Turner et al., 1992). The M059J (radiosensitive) and M059K (radioresistant) GBM cell lines used for the apoptosis studies were isolated from different portions of the same human GBM surgical specimen and were irradiated using techniques described previously (Allalunis-Turner et al., 1993).

Immunological Reagents and Immunohistochemistry

Rabbit polyclonal antibodies to Cdk5 and P35, together with relevant control peptides, were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.) and were used for immunohistochemistry, Western blotting, and immunoprecipitation. The biotin-streptavidin–amplified detection system (Super Sensitive Detection System; Santa Cruz Biotechnology) was used according to manufacturer's instructions for immunohistochemical detection of Cdk5 and P35 in paraffin-embedded specimens. Staining was detected using a Fast Red solution system (Biogenex, San Ramon, Calif.), and nuclei were counterstained with Mayer's hematoxylin. All antibodies were diluted in common antibody diluent (Biogenex). Control slides were incubated with rabbit IgG (1 μg/ml) in 1% bovine serum albumin and 0.02% azide.

Cell Lysates

Whole-cell lysates of control or irradiated cells were prepared for Western blotting or kinase assays. For irradiation experiments, to include apoptotic cells detached from the substratum, trypsinized cells were combined with cells floating in the media. For Western blotting, proteins were extracted using radioimmunoprecipitation or SDS lysis buffer with protease inhibitors. For

kinase assays, approximately 1×10^7 cells were lysed in 1 ml lysis buffer (0.1% Triton X, 0.5% sodium deoxycholate, 0.1% SDS, 0.575 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin in phosphate-buffered saline). Protein concentrations were determined by using a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) according to manufacturer's instructions.

Western Blotting

Proteins were separated by running equal amounts of lysate (total cellular protein from 1×10^5 cells) on 12% SDS-polyacrylamide gels. Size markers were run in the gel for all Western blots. Proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-C extra; Amersham, Oakville, Ontario). The loading and transfer of equivalent amounts of protein were confirmed by staining with Ponceau S. The membranes were blocked by overnight incubation in a solution of 5% dry milk in tris-buffered saline and probed with the primary antibody for 2 h, followed by secondary horseradish peroxidase–conjugated goat antirabbit immunoglobulin for 1 h. The immunocomplex was detected by chemiluminescence, and autoradiographs were quantitated by densitometric analysis.

Immunoprecipitation and Kinase Assays

Whole-cell lysates were prepared as described above. Equal amounts of protein were incubated with 1 μg of rabbit IgG or Cdk5 antibody for 15 min at 4°C. Immune complexes were then precipitated by adding 20 μl protein A agarose to each sample and incubating overnight at 4°C. The agarose beads were then washed 4 times with lysis buffer and once with kinase wash (50 mM Hepes, pH 7, 10 mM $MgCl_2$, pH 7, 1 mM dithiothreitol, 1 μM unlabeled ATP in distilled H_2O). Histone H1 kinase reactions were performed by incubating each sample with kinase reaction buffer containing histone H1 (2 μg) or the KSPXX synthetic peptide as substrates and 10 μCi of γ^{32}P-ATP for 20 min at 30°C. Sample buffer (50 μl of 2 × SDS-dithiothreitol) was added; then the samples were boiled for 10 min and analyzed by SDS-polyacrylamide gel electrophoresis. Radioactivity was detected by autoradiography and quantitated by densitometry.

Results

Cdk5/P35 Expression in Astrocytic Tumors

Cdk5 and P35 protein expression were examined by immunohistochemistry in 12 human astrocytic tumor specimens of varying histological grades (Table 1). Because intratumoral heterogeneity is a characteristic feature of GBM, adjacent sections of several regions of each tumor were examined. Cdk5 and P35 protein expression were detected in all 12 tumors. Within positively stained neoplastic cells, Cdk5 and P35 were detected in both the nucleus and the cytoplasm of astroglial cells, with preferential cytoplasmic labeling of both proteins. Other cell types that exhibited positive Cdk5 immunoreactivity included lymphocytes within the vascular proliferative areas, neutrophils, and macrophages.

Cdk5 staining in grades II-IV astrocytomas was consistently stronger than Cdk5 immunoreactivity observed in a single grade I pilocytic astrocytoma. The pattern of immunostaining of both P35 and Cdk5 varied considerably from tumor to tumor and even within a single tumor. Nevertheless, intense Cdk5 staining was preferentially observed in tumor cells surrounding large areas of necrosis in half of the grade IV tumors. This intense Cdk5 staining was not found in pseudopalisading cells surrounding focal necrosis. Although P35 immunoreactivity was observed diffusely without a preferential perinecrotic distribution, intense P35 staining was observed in isolated cells located within these areas.

Four nonmalignant human brain tissue specimens were examined to confirm the specificity of the staining patterns. In all nonmalignant brain tissues, Cdk5 immunoreactivity was absent from glial cells in both the predominately axonal white matter and the predominately neuronal grey matter (Fig. 1). However, neurons of all 6 cortical layers stained positively for Cdk5.

Western blots of whole-cell lysates prepared from human GBM cell lines or normal human diploid fibroblasts were probed with antibodies to Cdk5 and P35. Cdk5 protein was detected in all GBM specimens but was absent from fibroblasts (Fig. 2, upper panels). A single band at approximately 25 kDa, consistent with the known size of an active proteolytic fragment of P35 (P25) (Qi et al., 1995; Tang, et al. 1995) was detected in all GBM, but not fibroblast samples (Fig. 2, lower panels). To exclude the possibility that P25 expression in GBM cell lines was an artifact that arose during in vitro culture, Western blots of whole cell lysates prepared directly from surgical specimens obtained prior to therapy were probed with relevant antibodies. Cdk5 was present in 7 of 7 surgical specimens examined (Fig. 3, upper panel). P35 was detected as an approximately 25-kDa fragment in 6 of 7 samples (Fig. 3, lower panel). In addition, cross-reacting bands were observed at approximately 56 kDa and approximately 90

Table 1. Cdk5 and P35 protein expression in human astrocytic tumors

Grade	Tumor	No. regions examined per tumor	P35	Cdk5	Intense Cdk5 in perinecrotic areas
II	1	2	0 – ++	0 - +	N
	2[a]	7	+ – +++	+ – ++++	N
	3[a]	4	+ – +++	++ – +++	N
III	1	5	++	+++	N
	2	5	0 – +++	++ – +++	N
IV	1[a]	10	+ – ++++	+ – ++++	N
	2[a]	4	0 – ++++	0 – ++++	Y
	3	3	0 – ++++	+ – ++++	N
	4	7	+ – +++	+ – ++++	Y
	5[a]	11	0 – ++++	+ – ++++	N
	6[a]	5	0 – ++++	+ – ++++	Y

[a]Two tumor biopsies taken at separate times from the same patient were included in the analysis. Immunohistochemical staining was graded as follows: 0 = none; + = very weak; ++ = weak; +++ = moderate; ++++ = strong.

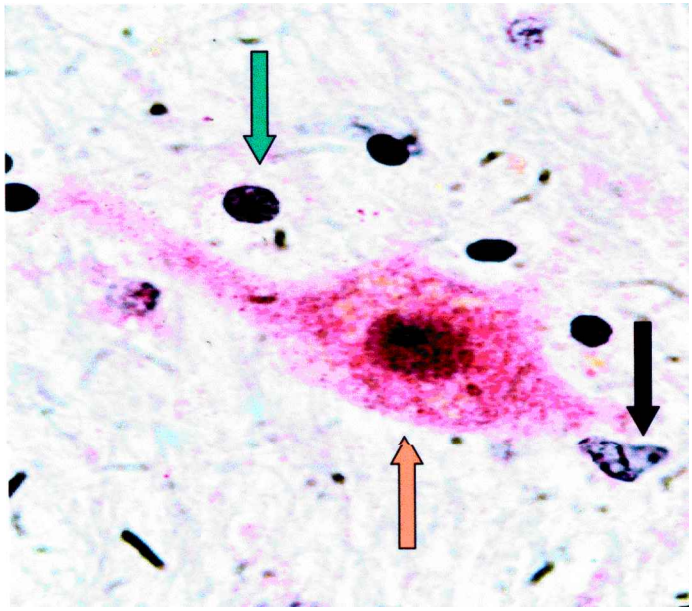

Fig. 1. Cdk5 immunoreactivity in nonmalignant human brain. Positive Cdk5 staining was detected using Fast Red substrate with hematoxylin counterstaining. The high power view of white matter from hypoxic ischemic hippocampal tissue shows a Cdk5-positive pyramidal cell (orange arrow), Cdk5-negative astrocytes (black arrow), and oligodendrocytes (green arrow).

kDa in 6 tumor samples. The identity of these cross-reacting proteins has not been established. For patient M123, surgical specimens were available before treatment (M123), after completion of external beam radiotherapy (M132), and after nitrosourea-based chemotherapy (M147). This allowed assessment of in vivo Cdk5 and P35 protein expression during tumor progression. Both proteins, as well as the cross-reacting bands, were detected in pre- and posttreatment specimens (Fig. 4).

Although equivalent amounts of protein were loaded into each lane, the blots shown in Figs. 2 and 3 were stripped and reprobed with an antibody to the mitotic protein, Cdc2, as an additional loading control. All GBM cell lines (Fig. 5, upper panel) and tumor samples (middle panel) were positive for expression of Cdc2 protein, suggesting that the absence of P35 band in some of the tumor specimens was not the result of protein degradation. The lower panel of Fig. 5 shows a comparison of P35 protein expression in human fibroblasts (GM43, GM34, transformed fibroblasts) versus M059K tumor cells. This blot was subject to a long exposure time to show that GM43 cells do express P35 protein, but at relatively low levels.

Cdk5 Kinase Activity in Human GBM Cells

To determine whether Cdk5 is an active kinase in GBM cells, lysates were immunoprecipitated by incubation with antibodies recognizing Cdk5 or P35. Immunoprecipitates prepared with antibodies against the mitotic kinase Cdc2 or rabbit IgG were used as positive and negative controls, respectively. The resulting immunoprecipitates were analyzed for their ability to phosphorylate histone H1, a known substrate of both Cdc2 and Cdk5 (Lew and Wang, 1995), or a synthetic peptide corresponding to the phosphorylation motif (KSPXX) in high molecular weight neurofilament protein (Shetty et al., 1993). Active kinase complexes were immunoprecipitated with Cdc2, P35, and Cdk5 antibodies (Fig. 6, upper panel). Densitometric analysis indicated that Cdk5 kinase activity was approxi-

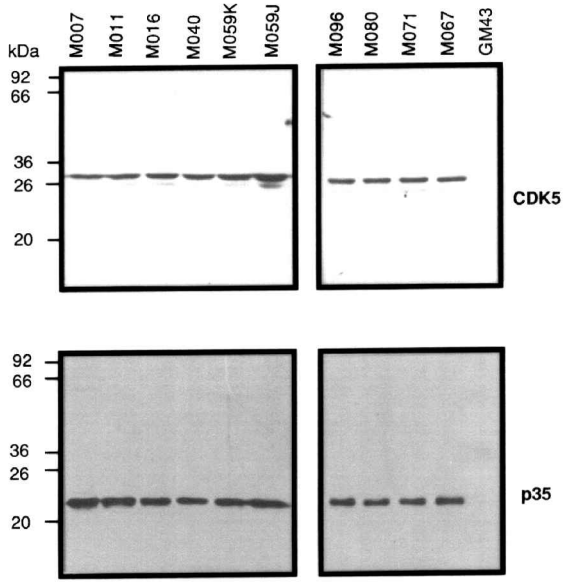

Fig. 2. Cdk5 and P35 protein expression in human GBM cell lines and human diploid fibroblasts. Whole-cell lysates were prepared from 10 human malignant GBM cell lines or normal human fibroblasts (GM43). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibodies to Cdk5 (upper panels) or P35 (lower panels).

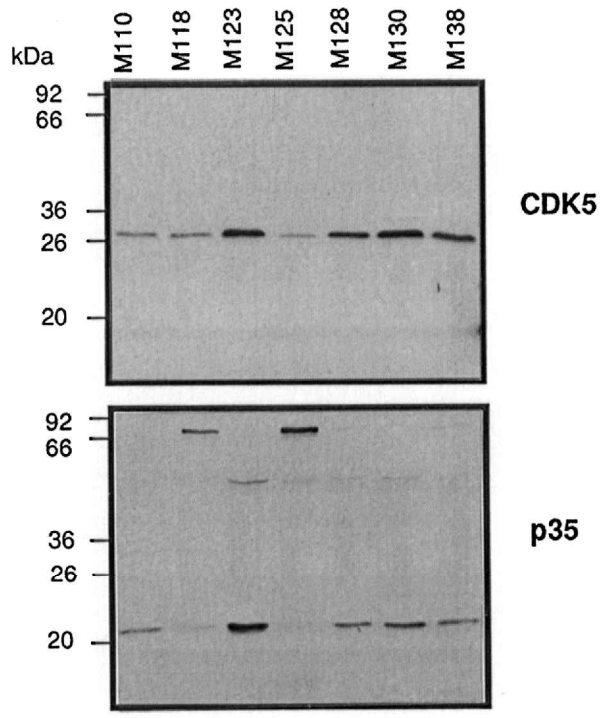

Fig. 3. Cdk5 and P35 protein expression in human GBM surgical specimens. Whole-cell lysates were prepared from tumor specimens resected from 7 patients with proven diagnoses of GBM. Proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with antibodies to Cdk5 (upper panel) and P35 (lower panel).

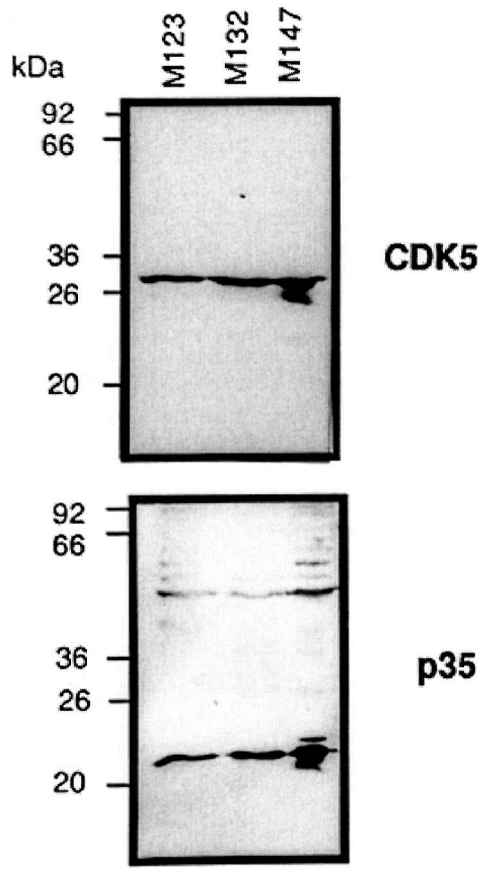

Fig. 4. Cdk5 and P35 protein expression in pre- and posttreatment human GBM surgical specimens. Whole-cell lysates were prepared from tumor specimens resected from a single patient before therapy (M123), after treatment with ionizing radiation (M132), or after treatment with nitrosourea-based chemotherapy (M147). Proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with antibodies to Cdk5 (upper panel) or P35 (lower panel).

mately 4-fold greater than that measured for the Cdc2 sample. Both Cdc2 and P35 antibodies precipitated an active kinase from M006xLo GBM cells (Fig. 6, middle panel) that showed approximately 6-fold greater activity than that of control mononuclear cells. Because the phosphorylation site of high molecular weight neurofilament protein likely represents a more physiologically relevant substrate, we analyzed the ability of Cdk5 kinase complexes to phosphorylate the KSPXX peptide. Our results showed that Cdk5 kinase activity was approximately 12-fold higher in the malignant GBM cells than in normal control cells (Fig. 6, lower panel).

Cdk5/P35 Protein Expression Within Apoptotic GBM Cells

During examination of Cdk5/P35 protein in human astrocytic tumors, we noted that a small subset of cells in GBM tumor sections showed P35 immunoreactivity and intense Cdk5 immunostaining as well as morphologic characteristics consistent with apoptosis. To confirm the apoptotic nature of the Cdk5 positive cells, we analyzed serial sections of the same GBM specimens in situ for evidence of DNA fragmentation. When examined by light microscopy, the DNA fragmentation assay revealed that the subset of cells exhibiting P35 expression and intense focal distribution of Cdk5 protein also showed evidence of chromatin fragmentation characteristic of apoptotic cells (data not shown).

To investigate the involvement of the Cdk5 kinase in apoptosis, we examined Cdk5/P35 protein expression in human GBM cell lines that are sensitive (M059J) or resistant (M059K) to radiation-induced apoptosis. Cdk5 was present at relatively high basal levels in both cell lines (data not shown). Neither cell line showed any change in Cdk5 protein levels after radiation. P35 was detected as a 25-kDa band (P25) in both cell lines (Fig. 7A). In contrast to Cdk5, P25 protein expression was increased at 24 h postirradiation, with highest levels of expression reached in the 36-h sample in both M059K and M059J cells (lane 3). Although P25 levels were slightly reduced at 48 h, they remained higher than those in controls (lane 1).

To quantitatively assess the association between increased P35 protein expression and apoptosis, we examined cytospins prepared from control and irradiated (10 Gy) M059J cells. Because virtually all M059J cells display some degree of P35 immunoreactivity, we counted the number of cells in control and irradiated samples that showed both intense, dark red staining for P35 and morphologic features of apoptosis. Five hundred cells in randomly selected fields were counted for each

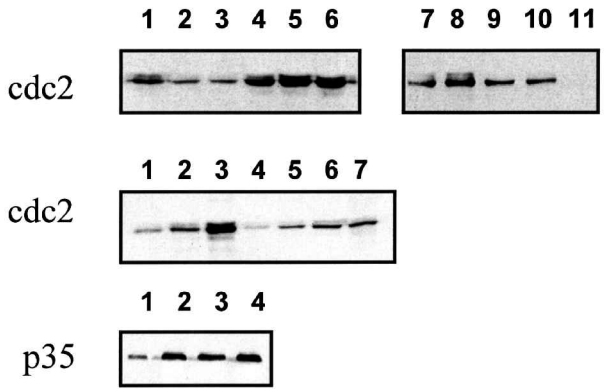

Fig. 5. Western blot analysis of Cdc2 and P35 protein expression. Western blots prepared from human GBM cell lines and surgical specimens shown in Figs. 2 and 3, respectively, were stripped and reprobed with antibodies to the mitotic cell cycle protein cdc2. The upper panels show the GBM cell lines (lane 1, M007; lane 2, M011; lane 3, M016; lane 4, M040; lane 5, M059K; lane 6, M059J; lane 7, M096; lane 8, M080; lane 9, M071; lane 10, M067; lane 11, GM43) . The middle panel shows GBM surgical specimens (lane 1, M110; lane 2, M118; lane 3, M123; lane 4, M125; lane 5, M128; lane 6, M130; lane 7, M138). The lower panel shows a Western blot of P35 protein expression in human fibroblasts versus a GBM cell line. Lane 1, GM43 fibroblasts; lane 2, GM38 fibroblasts; lane 3, SV40-transformed fibroblasts; lane 4, M059K GBM cells.

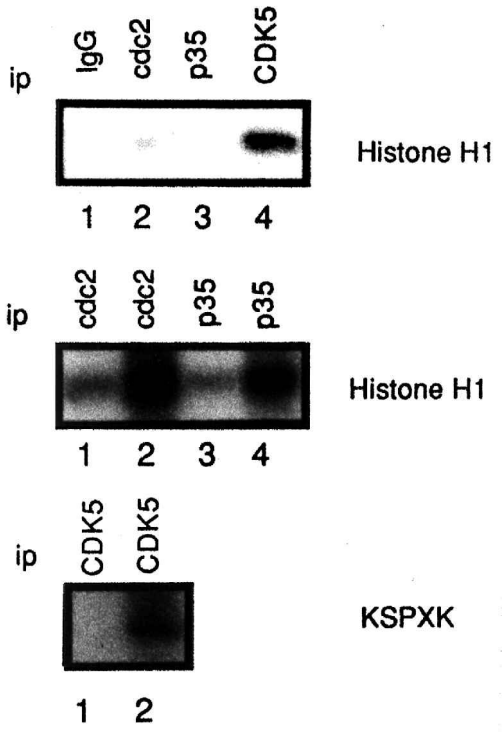

Fig. 6. Kinase activity in human GBM cell lines and normal human peripheral blood mononuclear cells. Cell lysates prepared from GBM cell lines (M006xLo, M016) or mononuclear cells were immunoprecipitated with antibodies to Cdk5, P35, or cdc2 and were analyzed for ability to phosphorylate histone H1 or a synthetic peptide. Upper panel: lanes 1 - 4, M016 cells. Middle panel: lanes 1 and 3, normal human mononuclear cells; lanes 2 and 4, M006xLo cells. Lower panel: lane 1, normal human mononuclear cells; lane 2, M016 cells.

sample. Our results indicate that the proportion of cells with both intense P35 staining and an apoptotic morphology was increased postirradiation (control = 9%; 24 h post 10 Gy = 22%; 36 h post 10 Gy = 35%). The proportion of apoptotic cells counted in this study is consistent with our previous work that determined the incidence of apoptosis in control and irradiated M059J cells by using fluorescence microscopy (control = 12 + 4%; 24 h post 10 Gy = 19 + 2%; 48 h post 10 Gy = 22 + 4%) (J.M. Leithoff and J.A.-T., unpublished data, 1995).

Next we tested whether Cdk5 was present in M059J and M059K cells as an active kinase and whether increased P35 protein levels observed after radiation treatment correlated with increased kinase activity. Cdk5 protein was immunoprecipitated from mock irradiated (control) and irradiated M059J and M059K cells at 5 min and 36 h postirradiation (Fig. 7B). Autoradiographs obtained in 3 separate experiments indicated that average Cdk5 kinase activity was increased in apoptosis-sensitive M059J cells 36 h postradiation compared with unirradiated controls (lane 3 versus lane 1). Cdk5 kinase activity was only slightly increased in apoptosis-resistant M059K cells 36 h after exposure to ionizing radiation.

Discussion

We have identified Cdk5 and its activator protein, P35, in 10 of 10 cell lines derived from patients with GBM. Initial reports suggested that Cdk5/P35 expression was limited to mature, nondividing neurons (Hellmich et al., 1992; Lew and Wang, 1995; Tsai et al., 1994) and that Cdk5 protein and kinase activity were absent from a human GBM cell line, T98G (Tsai et al., 1993). The reason for this discrepancy is not clear; however, a possible explanation may lie in the difference between the cell lines studied. The 10 GBM cell lines used for the present study grew spontaneously from surgical specimens obtained from patients with proven diagnoses of GBM (Allalunis-Turner et al., 1992). In contrast, the precise origin of the T98G cell line is unknown because the cells that grew out of the tumor explant had a different karyotype from that of the original cell culture (Stein, 1979). Furthermore, T98G cells express both normal and transformed aspects of the control of cell proliferation (Stein, 1979).

Interestingly, in our studies, P35 was detected in Western blots as the 25-kDa proteolytic derivative (P25). Previously, Cdk5 was detected in the purest fractions of active kinase from bovine brain as a holoenzyme complex with P25 (Lew et al., 1992). Although P35 is the predominant form found in brain extracts and primary neuronal cultures (Tsai et al., 1994), both P35 and P25 are fully competent for Cdk5 activation (Lee and Johnston, 1997; Lew et al., 1994; Tsai et al., 1994). Their differential expression may be the result of tissue- or species-specific posttranslational regulatory processes.

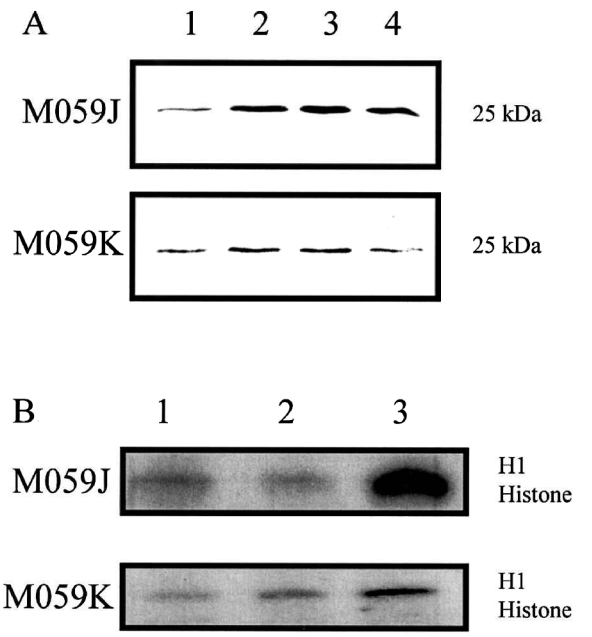

Fig. 7. P35 protein and Cdk5 kinase activity in irradiated GBM cells. A. Western blot analysis of P35 protein expression in M059J and M059K cells at 0 min (lane 1), 24 h (lane 2), 36 h (lane 3), and 48 h (lane 4) postirradiation. B. Cdk5 histone H1 kinase activity in M059J and M059K cells at 0 min (lane 1), 5 min (lane 2), or 36 h (lane 3) postirradiation.

The Cdk5/P35 holoenzyme was present in GBM cell lines as an active kinase as indicated by the ability to phosphorylate histone H1 and a synthetic peptide corresponding to the phosphorylation sites (KSP*X*K) in high molecular weight neurofilament protein (Shetty et al., 1993; Sun et al., 1996). Normal neurons, which express Cdk5 kinase activity, may have been among the cell types present in the initially resected tumor material. However, it is unlikely that they are the source of the kinase activity observed in these GBM cell lines, as the in vitro conditions used for continuous passage of these tumor cell lines are insufficient to maintain the survival of differentiated neurons in culture (Cattaneo and McKay, 1990).

Whereas Cdk5 protein levels were relatively uniform in the GBM cell lines, considerable variation was observed in lysates prepared from fresh tumor specimens. Such differences may in part reflect the presence of varying numbers of infiltrating normal host cells (e.g., lymphocytes) in the tumor specimens. Expression of the Cdk5 regulatory subunit P35 in tumor specimens was of particular interest. In 6 of 7 tumors examined, an approximately 25-kDa protein presumed to correspond to a previously described active proteolytic fragment of P35 (Tang et al., 1995) was detected. Additional immunoreactive species of approximately 56 and 90 kDa were observed. In pre- and posttreatment tumor samples obtained from a single patient, antibodies to P35 detected immunoreactive proteins in all tumor samples, indicating the persistence of these proteins during tumor progression. Although P35 has been shown to bind to and activate Cdk5 in normal neurons, additional Cdk5 regulatory subunits have been described (Shetty et al., 1995; Tang et al., 1995; Tsai et al., 1994; Veeranna et al., 1997), including a 25-kDa proteolytic fragment that has recently been demonstrated to induce cytoskeletal disruption and apoptosis in neurons in the brain of Alzheimer's disease patients (Patrick et al., 1999). The biologic significance of these immunoreactive proteins in GBM has yet to be determined. However, it has been suggested that the activation of Cdk5 kinase activity in nonneuronal tissues may be mediated by isoforms of the P35 family of regulatory proteins (Delalle et al., 1997; Tang et al., 1995).

We next examined whether Cdk5/P35 expression was associated with histologic grade or any pathologic feature of astrocytic tumors. For these studies, surgical specimens of human astrocytic tumors of varying grades were examined for Cdk5/P35 protein expression. Significant intra- and intertumoral variation in Cdk5/P35 protein expression was observed. To our knowledge, no previous study has examined Cdk5/P35 expression in human tumors of varying grades. However, because primary and secondary GBMs are known to develop from a cascade of multiple genetic alterations (Kleihues and Ohgaki, 1999), it is not expected that changes in the expression of a protein kinase would be a singular feature of tumor progression.

Previous studies have established that normal astrocytes are devoid of Cdk5 expression (Hayashi et al., 1999; Ino et al., 1994; Nakamura et al., 1998; Tsai et al., 1993). To confirm these results, and to establish that our observation of Cdk5 expression in GBM cell lines and tumors was not the consequence of nonspecific immunohistochemical staining, human nonmalignant neocortical and hippocampal brain tissue was examined for Cdk5 protein expression by using reagents and techniques identical to those used in the study of tumor specimens. In normal human brain, Cdk5 immunoreactivity was detected in neurons of all 6 cortical layers and the pyramidal cell layer and granular layer of the hippocampus. Within these neurons, Cdk5 was expressed in both dendrites and the cell body, including the cytoplasm and, to a lesser extent, the nucleus, but was absent from axons of the same neurons. Axonal pathways within the white matter and glial cells, including both astrocytes and oligodendrocytes, were devoid of Cdk5 staining. This suggests that our identification of Cdk5 expression in GBM was not a technical artifact.

In cultured primary neurons, activation of Cdk5 kinase activity by binding of the P35 proteolytic fragment, P25, results in tau hyperphosphorylation, microtubule disruption, and apoptosis (Patrick et al., 1999). In addition, Cdk5 expression and kinase activity have been found to be associated with apoptosis in a number of nonneuronal model systems, including atretic ovarian follicular cells, regions of the developing retina and nervous system, and androgen-withdrawn regressing prostate and interdigital zones of developing mouse hindlimbs (Ahuja et al., 1997; Henchcliffe and Burke, 1997; Shirvan et al., 1998; Zhang et al., 1977). In our analysis of Cdk5/P35 protein expression in human primary brain tumor specimens, we observed that positively-stained cells were distributed in the perinecrotic

regions of the tumor. These regions are generally associated with apoptotic tissue. In astrocytic tumors, the incidence of apoptotic cell death in situ has been shown to correlate with tumor grade, with the proportion of cells undergoing apoptosis being greater in anaplastic astrocytomas and GBMs than in lower-grade astrocytomas (Kordek et al., 1996). Apoptosis also occurs more frequently in GBM cells within perinecrotic areas (Kordek et al., 1996; Tachibana et al., 1996). Further, Yin et al. (1999) have observed that Cdk5 protein and kinase activity were increased in human head and neck squamous cell carcinoma xenografts undergoing spontaneous apoptosis.

To examine the role of Cdk5 in apoptosis, we used two human GBM cell lines with differing sensitivities to radiation-induced apoptosis. Consistent with our analyses of human primary brain tumor specimens, up regulation of the P35 proteolytic fragment P25 was detected in apoptosis-sensitive M059J cells by Western blot analysis and immunohistochemistry at times corresponding to the peak incidence of radiation-induced apoptosis. In addition, a concomitant increase in histone H1 Cdk5 kinase activity was observed 36 h postirradiation in both M059J and M059K glioma cells. Previous work from this laboratory established that the peak incidence of radiation-induced apoptosis in these cell lines occurs between 24 h and 48 h postirradiation (J.M. Leithoff and J.A.-T., unpublished data, 1995). The present finding of increased P25 protein expression and Cdk5 kinase activity at 36 h postirradiation is consistent with this. Further, our observations support the notion that Cdk5 kinase activity correlates with the amount of P35/P25 activator protein and not Cdk5 (Tsai et al., 1994).

Although the specific role of Cdk5 kinase in apoptotic cell death remains to be elucidated, we postulate that Cdk5 may mediate its functional role through its association with the microtubule system. Human tumors of glial origin can express neurofilament proteins (Abaza et al., 1998; Bodey et al., 1991) as well as neuronal microtubule-associated proteins (Lopes et al., 1992; Tohyama et al., 1993) that are known in vitro substrates of Cdk5 (Lew and Wang, 1995; Paglini et al., 1998). Rearrangement of the cytoskeleton is a prominent feature of apoptosis frequently resulting in membrane blebbing (Levee et al., 1996; Van Engeland et al., 1997). Studies to elucidate these mechanisms are currently underway.

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