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**University of Alberta**

# THE EXPRESSION AND LOCALIZATION OF CDK5/p35 KINASE IN HUMAN MALIGNANT GLIOMA CELLS

by

**Alaine Catania** 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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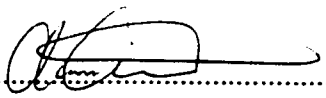
(Signed) 

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Date: 30 Sept 99

**DEDICATION:**

For everything that I have achieved, I owe to my parents, Peter and Josie. Without their unrelenting love, support and hard work, I would not be at this point in my life. Thankyou, for making me who I am today.

## ABSTRACT

Cdk5 is a cell cycle related protein found in many tissue types, with its highest expression in the brain. For activation, Cdk5 requires binding to another protein, p35, which is predominantly found in neuronal cells. Although it is well established that normal astrocytes are devoid of Cdk5 expression, previous studies from our laboratory showed that both Cdk5 and p35 proteins were aberrantly expressed in both human malignant glioma cell lines and human glioma biopsy specimens.

To study the expression and localization profiles of Cdk5 and p35 protein in human malignant glioma cells, we have examined the *in vivo* distribution of Cdk5 and p35 protein in human glioma biopsy tissue as well as their subcellular localization in a human glioma cell line. To evaluate the functional role of Cdk5/p35 kinase in apoptotic cell death, the association of radiation-induced apoptosis with Cdk5 and p35 protein expression and kinase activity was examined in human malignant glioma cells.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AP-1 | activating protein 1 |
| AP-2 | activating protein 2 |
| APC | adenomatous polyposis coli |
| ATP | adenosine triphosphate |
| ALS | amyotrophic lateral sclerosis |
| BCA | bicinchonic acid |
| BSA | bovine serum albumin |
| BDGF | brain-derived growth factor |
| cdc | cell division control (yeast Cdk homologue) |
| CNS | central nervous system |
| Cs | Cesium |
| cDNA | complementary deoxyribonucleic acid |
| cAMP | cyclic adenosine monophosphate |
| Cdk | cyclin-dependent kinase |
| C | cytosine |
| CAAT | cytosine/adenine/adenine/thymine sequence promoter element |
| DTT | dithiothreitol |
| dUTP | deoxyuridine triphosphate |
| DNK5 | dominant negative *Cdk5* |
| E | embryonic day |
| ECM | extracellular matrix |
| GFAP | glial fibrillary acidic protein |
| GCI | glial cytoplasmic inclusions |
| GDNF | glial-derived neurotrophic factor |
| GBM | glioblastoma multiforme |
| Gy | gray |

| | |
|---|---|
| G | guanine |
| HRP | horseradish peroxidase |
| NF-H | high-molecular weight neurofilament protein |
| h | hour |
| IgG | immunoglobulin G |
| kb | kilobase |
| kDa | kilodaltons |
| -/- | gene knock-out |
| LB | Lewy body |
| LI | Lewy body-like inclusion |
| NF-L | low-molecular weight neurofilament protein |
| K | lysine |
| $MgCl_2$ | magnesium chloride |
| NF-M | medium-molecular weight neurofilament protein |
| MAP 1B | microtubule-associated protein 1B |
| MAP2 | microtubule-associated protein 2 |
| mM | millimolar |
| M | molar |
| NGF | nerve growth factor |
| NSF | *N*-ethylmaleimide-sensitive fusion protein |
| NFT | neurofibrillary tangle |
| OD | optical density |
| PHF | paired helical filament |
| PNS | peripheral nervous system |
| PBS | phosphate-buffered saline |
| PMSF | phenylmethylsulfonyl fluoride |
| P | proline |
| P | postnatal |
| C-terminus | protein carboxy terminus |

| | |
|---|---|
| N-terminus | protein amino terminus |
| rpm | revolutions per minute |
| S | serine |
| SDS | sodium *n*-dodecyl sulfate |
| SDS-PAGE | sodium *n*-dodecyl sulfate polyacrylamide gel electrophoresis |
| SNAP | soluble *N*-ethylmaleimide-sensitive fusion attachment protein |
| SNARE | soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor |
| TdT | terminal deoxynucleotidyl transferase |
| TUNEL | TdT-mediated deoxyuridine triphosphosphate nick end labeling |
| TATA | thymine/adenine repeat promoter element |
| TBS | Tris-buffered saline |

# I. INTRODUCTION

## 1.1 Astrocytic Tumors and Their Precursor Astrocyte Cells

As supporting cells of the nervous system, astrocytes function in close association with neurons in the brain. Astrocytic functions include: (1) provision of structural support for nerve cells, (2) production of neurotrophic factors required for neuronal survival and maintenance such as nerve growth factor (Goss *et al.*, 1998), brain-derived growth factor (Inoue, *et al.*, 1997) and glial-derived neurotrophic factor (Moretto *et al.*, 1996), (3) aiding in rapid nerve impulse conduction by regulating the neuronal microenvironment through rapid uptake of ions, neurotransmitters and their metabolites (Travis, 1995; Nicholls, 1992), (4) generation of slow waves of potassium and calcium transient currents (Dietzel *et al.*, 1989; Nicholls, 1992; Parpura *et al.*, 1995; Nedergaard *et al.*, 1994), and lastly, (5) interacting with neighboring cells via gap junctions (Travis, 1995).

Within the vertebrate CNS, astrocytes are classified into two principal groups: fibrous astrocytes and protoplasmic astrocytes. Fibrous astrocytes contain filaments such as the intermediate filament, GFAP, and are more prevalent among bundles of myelinated nerve fibers within the white matter of the brain (Nicholls, 1992). Two subtypes of fibrous astrocytes (type I and type II) have been identified in rat optic nerve which vary with respect to morphology, embryological origin and immunological reaction in culture. Type II astrocytes arise from the same precursor as oligodendrocytes but appear later in development. They associate with

oligodendrocytes at the neuronal nodes of Ranvier which are the focal areas of synaptic transmission. Type I astrocytes arise from a different precursor and are found in the mature optic nerve where they envelop and influence capillaries. It is unclear whether this subtype classification of fibrous astrocytes are present in other areas of the brain. Protoplasmic astrocytes contain less fibrous material and are more abundant in the grey matter surrounding neurons. Both protoplasmic and fibrous astrocytes make contact with neurons and capillaries in the brain (Nicholls, 1992).

Under normal circumstances, astrocytes have small, round nuclei and no visible cytoplasm. Fibrous astrocytes extend short cytoplasmic processes from the cell which contain GFAP. In reaction to brain injury, astrocytes develop enlarged, visible cytoplasms with an increase in filaments. Their cytoplasmic processes become thicker and elongated. These enlarged, reactive astrocytes are termed gemistocytes (Levin, 1996).

Astrocytic tumors, the most common primary CNS tumor in humans, retain the general appearance of normal astrocytes from which they are derived. The prognosis and survival of patients with astrocytic tumors is dependent on the tumor's cellular type and histologic grade, both of which cover a wide pathologic spectrum. Two general categories of astrocytic tumors are diffuse infiltrative astrocytomas and low grade, non-infiltrative astrocytomas. Diffuse infiltrating neoplasms of the cerebral hemispheres are derived from either protoplasmic astrocytes of the cerebral cortex or, more commonly, from fibrillary astrocytes of the white matter. Astrocytomas of protoplasmic astrocytic origin arise within the cortex itself and are relatively filament-poor. They have short, delicate processes and weak GFAP

immunoreactivity. Astrocytomas derived from fibrillary astrocytes arise in the subcortical white matter and produce highly variable amounts of GFAP often depending on the grade of the tumor. By immunohistochemistry, highly anaplastic gliomas express less GFAP than do well-differentiated gliomas (Deck *et al.*, 1978)

Three major variants of fibrillary astrocytic neoplasms are recognized by the World Health Organization (WHO): low grade astrocytomas, anaplastic astrocytomas and glioblastoma multiforme (GBM). These tumors show a range of features that become more prominent with increasing grades of malignancy. These features include degree of cellularity, nuclear and cytoplasmic pleomorphism, mitotic activity, vascular changes and tumor necrosis. Other histologic features that suggest tumor malignancy but are not considered for histologic grading are cortical tumor invasion, presence of microcysts or gemistocytes, and pial or subpial invasion (Levin, 1996).

Low grade astrocytomas show increased cellularity compared to normal white matter, and mild to moderate pleomorphism with slightly enlarged nuclei. No mitotic figures are seen, vessels appear normal and necrosis is not evident. Microcysts may be a distinctive feature and may contain proteinaceous fluid (Levin, 1996).

Anaplastic astrocytomas express a moderate degree of cellularity and nuclear and cytoplasmic pleomorphism. Frequent mitotic figures are also evident. Although necrosis is generally absent, any proliferative vascular change within the tumor warrants a grade of anaplastic astrocytoma. In addition, any astrocytoma in which 20-60% of the cells are gemistocytic is considered anaplastic and is designated a gemistocytic astrocytoma (Levin, 1996).

Glioblastoma multiforme (GBM) is the highest malignant grade of astrocytoma and represents 15-23% of intracranial tumors and approximately 50% of all astrocytomas (Levin, 1996). The most prominent histologic feature of GBM is the presence of necrotic areas within the tumor. Tumor necrosis is accompanied by peripheral pseudopalisading of neoplastic cells around the foci of necrosis. This palisading is a common feature of GBM but is not required for the diagnosis of GBM. As in anaplastic astrocytomas, microvascular proliferation, mitotic activity and nuclear and cytoplasmic pleomorphism are evident (Schold *et al.*, 1997).

Although specific histologic features are used for grading the malignancy of astrocytomas, most GBMs display variable histological features from one area of a tumor to another area. This tumor heterogeneity is thought to arise from the presence of different transformed clones of astrocytes in different areas of the neoplasm during tumor growth. Due to its highly heterogeneous nature, glioblastomas have been appropriately designated, "multiforme" (Levin, 1996).

Pilocytic astrocytomas, the most common non-infiltrative astrocytoma, are widely assumed to be exclusively pediatric tumors with a restricted site of origin. However, they occur throughout all decades of life and in all areas of the CNS including the optic pathway, cerebral hemisphere, cerebellum, brain stem, spinal cord and thalamus/hypothalamus. The designation *pilocyte* reflects the presence of elongated, spindle astrocytes with spindle-shaped nuclei and long, thin eosinophilic cytoplasmic processes extending from the cell. Although pilocytic astrocytomas are diverse in their histologic appearance, a common lesion is a spongy, microcyst-rich tissue located amongst GFAP-positive elongated cells and Rosenthal fibers. Unlike

fibrillary astrocytic tumors, proliferative vascular changes and necrosis are occasionally present in pilocytic astrocytomas but neither are considered evidence of anaplasia (Schold *et al.*, 1997).

Treatments for astrocytomas include surgical resection, radiation, chemotherapy, and immumotherapy. Prognostic factors estimating a patient's outcome with fibrillary astrocytic neoplasms are histologic grade, age at diagnosis and clinical performance status. The median survival period following diagnosis decreases with histologic progression to a higher-grade tumor. In addition, younger patients have a better outcome in all histologic groups. Last of all, patients with extensive neurologic symptoms probably have larger tumors or tumors located in critical regions of the brain (Schold *et al.*, 1997).

GBM is a lethal malignancy with a poor prognosis (Halperin *et al.*, 1988). Treatment of GBM is palliative rather than curative. Although metastases from these tumors are rare, they frequently show extensive infiltration of malignant cells into the normal brain tissue (Halperin *et al.*, 1988). This infiltration renders complete surgical resection almost impossible and increases the probability of tumor recurrence (Halperin *et al.*, 1988). Surgical resection palliates symptoms and prolongs survival but few patients survive more than three years following diagnosis (Leibel *et al.*, 1975; Ramsey and Brand, 1973; Wara, 1985). This has lead to an increased interest in the biology and treatment of malignant gliomas.

## 1.2 Cyclin-Dependent Kinases

Eukaryotic cell proliferation is regulated by the coordinated activity of a family of serine/threonine protein kinases, each acting at a discrete transition point in the cell cycle. Members of this kinase family are heterodimeric proteins comprised of a catalytic subunit [a cyclin-dependent kinase (Cdk 1-9)] and a regulatory subunit [a member of the cyclin family (cyclin A-H)] (Morgan, 1995). As the name suggests, cyclins are proteins whose levels oscillate with specific phases of the cell cycle and thus determine the timing of Cdk activation as well as their substrate specificity (Dirks and Rutka, 1997). The activity of these kinases is further regulated by an intricate system of protein-protein interactions and phosphorylation/ dephosphorylation events (Morgan, 1995).

Although members of the Cdk family share a high level of amino acid sequence identity (40-70%) (Morgan, 1995), cyclins are a family of structurally related proteins with low sequence identity. Homology among cyclins is limited to a relatively conserved domain of approximately 100 amino acids in the center of the molecule. This domain is responsible for Cdk binding and activation and is referred to as the 'cyclin box' (Nugent, 1991).

Human Cdk5 was initially identified as a structurally similar cdc2 (Cdk1)-related kinase with 58% and 52% sequence identity to human Cdk1 and Cdk2, respectively (Meyerson *et al.*, 1992). Despite its high degree of homology, Cdk5 was found to be functionally different to Cdk1 and Cdk2. Cdk5 did not complement temperature sensitive budding yeast cdc-2 mutants used to identify kinases that play a

cdc2-like role in controlling vertebrate cell cycle (Meyerson *et al.*, 1992). Independently, Cdk5 was discovered and purified from bovine brain as a proline-directed kinase (Lew *et al.*, 1992). It was also identified as a neuronal cdc2-like kinase by screening a rat brain cDNA library (Hellmich *et al.*, 1992) and isolated and characterized as a cdc2-like kinase which phosphorylates KSPXK motifs in neurofilaments (Shetty *et al.*, 1993). Further analysis identified Cdk5 as the catalytic subunit of tau protein kinase II (Kobayashi et al., 1993).

Active Cdk5 is purified from brain extracts as a heterodimer consisting of a catalytic subunit, Cdk5, and a 25-kDa regulatory subunit (Ishiguro *et al.*, 1992; Lew et al., 1992). The molecular cloning of the 25-kDa regulatory subunit of tau protein kinase II revealed it to be a proteolytic product of a larger 35-kDa protein, p35 (Ishiguro *et al.*, 1994; Tsai *et al.*, 1994; Lew *et al.*, 1994). p35 is a novel protein that displays very limited homology to members of the cyclin family and no homology to any known protein. p35 shows only 8% overall identity to the cyclin box and contains only one of five highly conserved residues in this region (Lew *et al.*, 1994). Therefore, despite its sequence homology to other Cdk's, Cdk5 does not require association with a classic cyclin regulatory protein for activation.

## 1.3 Molecular Biology of Cdk5 and p35

### 1.3.1 Cdk5 and p35 Genes

Human (Meyerson *et al.*, 1992), rat (Hellmich *et al.*, 1992), mouse (Tsai *et al.*, 1993) and *Drosophila* (Hellmich *et al.*, 1994) *CDK5* homologs share high overall

sequence identity, indicating substantial conservation during evolution. The human *Cdk5* gene maps to chromosome 7q36 (Demetrick *et al.*, 1994) and encodes a ~1.4 kb transcript (Meyerson *et al.*, 1992). The mouse *Cdk5* gene contains 12 exons and 11 introns (Ohshima *et al.*, 1995). Several potential sequence motifs for positive and negative transcription regulatory factors have been identified in the G+C-rich promoter region of the mouse *Cdk5* gene, including AP-1, AP-2 and a cAMP responsive element (Ishikuza *et al.*, 1995). This suggests a complex control of the regulation of *Cdk5* transcription.

Similarly, *p35* is highly conserved among vertebrates (Ohshima *et al.*, 1996a). Mouse *p35* is located on chromosome 11 which shows conserved linkage to human chromosome 17 (Ohshima *et al.*, 1996a). The human *p35* gene has a continuous coding region with absence of introns and encodes a ~4.0 kb transcript. The G+C-rich promoter region lacks canonical TATA and CAAT motifs but contains several potential transcription regulatory sequence motifs (Ohshima *et al.*, 1996a). This indicates stringent regulation of the *p35* gene (Delalle *et al.*, 1997).

### 1.3.2 Structural Properties of Cdk5 and p35 Proteins

The Cdk5 protein is 293 amino acids in length (Ohshima *et al.*, 1995) and has a molecular weight of 33 kDa (Lew *et al.*, 1992). It shares conserved secondary structural elements with other protein kinases at important functional sites such as those involved in ATP-binding and catalysis. Similar to other protein-serine/threonine kinases, Cdk5 contains eleven conserved subdomains (I-XI). Through homology modeling using the protein structure of Cdk2, Cdk5 is predicted

to fold into a two-lobed structure. The N-terminus, which includes subdomains I-IV, is organized predominantly into anti-parallel β-sheets. This lobe is responsible for anchoring and orienting the nucleotide, ATP. The C-terminus, including subdomains VI-XI, is predominantly an α-helical structure. The C-terminus is involved in substrate binding and the phosphotransfer process. Subdomain V, which spans and forms a cleft between the two lobes, is the site of catalysis (Moorthamer *et al.*, 1998).

The human Cdk5 activator, p35, is a 35 kDa protein composed of 307 amino acids that also exists as a 25 kDa proteolytic derivative. p25 is generated by cleavage of the first 98 amino acids at the N-terminus but 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 (Poon *et al.*, 1997). Two functionally important domains of p35 include residues 150-200 involved in Cdk5 binding *in vitro* and residues 279-229 which are critical for Cdk5 activation (Poon *et al.*, 1997).

### 1.3.3 Localization of Cdk5 and p35 Expression

Human cultured cell lines, including both primary and immortalized cell lines, contain uniformly high levels of Cdk5 protein (Tsai *et al.*, 1993) and transcript (Meyerson *et al.*, 1992). Although the *in vivo* tissue distribution of Cdk5 protein is quite variable (Lew and Wang, 1995), Cdk5 protein and transcript are expressed at basal levels in most human tissues, both in cycling and non-cycling cells (Meyerson

*et al.*, 1992; Lew and Wang, 1995; Tang *et al.*, 1996). Of exception are the central and peripheral nervous systems in which the expression of Cdk5 is several-fold greater (Meyerson *et al.*, 1992; Lew *et al.*, 1994). Cdk5 protein distribution in mice is similar to humans where highest levels are detected in the forebrain (Tsai *et al.*, 1994). Murine and rat models have indicated Cdk5 is also expressed at intermediate levels in the gonads (Tsai *et al.*, 1993; Ino *et al.*, 1994), muscle cells (Lazaro *et al.*, 1997; Philpott *et al.*, 1997) and fiber and epithelial cells of the developing lens (Gao *et al.*, 1997).

Within the normal brain, Cdk5 protein is expressed in most postmitotic neurons, although there are some exceptions such as cerebellar granule cells (Ino *et al.*, 1994). *In vivo*, both mitotically active cells and glial cells in normal brain, such as astrocytes and oligodendrocytes, are devoid of Cdk5 expression (Tsai *et al.*, 1993; Ino *et al.*, 1994; Nakamura *et al.*, 1998; Hayashi *et al.*, 1999). Recently however, Cdk5 expression has been reported in a variety of oligodendrocyte cultured cell lines (Tang *et al.*, 1998; Tikoo *et al.*, 1997 ; Casaccia-Bonnefil *et al.*, 1997). Lack of an *in vivo* correlation of these findings indicates that they may be artifacts of *in vitro* tissue culturing.

At a subcellular level, there are contradictory reports regarding exclusive axonal protein localization *vs.* protein localization within the cell body of neurons (Tsai *et al.*, 1993; Ino *et al.*, 1996; Terada *et al.*, 1998; Nikolic *et al.*, 1996). Interestingly, the subcellular level of Cdk5 protein has been found to vary from cell body to axon in various neurons during neuronal differentiation (Matsushita *et al.*,

1995). In addition, redistribution of Cdk5 from the cytoplasm to the nucleus was detected in differentiating myoblasts during early myogenesis (Lazaro *et al.*, 1997).

In contrast to the ubiquitous distribution of Cdk5, the p35 transcript is present predominantly within neurons of the forebrain (Ohshima *et al.*, 1996a; Uchida *et al.*, 1994; Delalle *et al.*, 1997). Within the developing forebrain, p35 is excluded from the ventricular zone, composed predominantly of proliferating cells, but is concentrated within areas containing post-mitotic neurons such as the cortical plate (Tsai *et al.*, 1994). p35 expression in the peripheral nervous system (PNS) displays interspecies variation. No p35 protein is detectable in embryonic mouse PNS tissue (Tsai *et al.*, 1994). In contrast, p35 protein is expressed in both the peripheral dorsal root ganglion and sciatic nerve of adult rat (Terada *et al.*, 1998).. At a subcellular level, p35 is found in both the nucleus and cytoplasm as well as along the entire length of neurites (Nikolic *et al.*, 1996; Terada *et al.*, 1998; Hayashi 1999).

Recent reports of the association of Cdk5/p35 with apoptotic cell death in various developmental and experimental systems broadens the tissue distribution of Cdk5 and p35 expression. These systems include atretic ovarian follicles, prostate regression after androgen withdrawal and embryonic mouse limb (Ahuja *et al.*, 1997; Zhang *et al.*, 1997). The role of Cdk5/p35 in apoptosis in these systems will be discussed in a later section.

### 1.3.4 Post-Translational Regulation of Cdk5 Activity

Cdk5 kinase activity is regulated by various mechanisms including an association with an activator protein, phosphorylation/dephosphorylation events and

proteolytic degradation of the regulatory subunit. 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 the presence of p35, not Cdk5 (Tsai *et al.*, 1994). Due to a limited tissue distribution of p35 protein, Cdk5 kinase activity is predominantly found in the nervous system, both central and peripheral, where it is restricted to postmitotic neural cells (Tsai *et al.*, 1993; Terada *et al.*, 1998).

In addition to its dependence on a regulatory protein for activity, Cdk5 has been shown to be regulated by post-translational phosphorylation mechanisms similar to those of other Cdks. Most Cdks require phosphorylation of $Thr^{160}$/$Thr^{161}$ and dephosphorylation $Thr^{14}$ and $Tyr^{15}$ for full activation (Morgan, 1995). Identical residues for all three sites are conserved in Cdk5 (Qi *et al.*, 1995, Lazaro *et al.*, 1996). Although these phosphorylation events are not required for Cdk5 activity (Lew *et al.*, 1994), tyrosine dephosphorylation has been found to correlate with Cdk5 kinase activity during cerebellum neurogenesis (Lazaro *et al.*, 1996). Furthermore, mutation of the $Tyr^{15}$ residue in Cdk5 results in reduced binding to p35 (Lazaro *et al.*, 1996). A two step mechanism for Cdk5 kinase activation has been proposed. The first step is binding of tyrosine-phosphorylated Cdk5 to p35. The second step is tyrosine dephosphorylation resulting in full kinase activation (Lazaro *et al.*, 1996).

A third regulatory mechanism for Cdk5 activity is the proteolytic degradation of p35. As discussed above, the availability of p35 appears to be the primary determinant of Cdk5 activation. Within primary cultured rat neuronal cells, p35 has a short half-life of approximately 20 to 30 min. This rapid turnover is mediated in part by a ubiquitin-dependent proteosomal pathway (Patrick *et al.*, 1998). The

degradation process occurs as a negative feedback response in which the active Cdk5/p35 kinase complex is rapidly inactivated by autophosphorylation of p35 at four consensus Cdk phosphorylation sites. This leads to ubiquitination of p35 and ATP-dependent degradation of the p35 activator (Patrick *et al.*, 1998). Interestingly, p25, the proteolytic fragment of p35, lacks one of the Cdk phosphorylation motifs within the N-terminus of p35 and is a more stable protein. This suggests that the cleaved N-terminus is required for degradation and may mediate protein interactions with the ubiquitination machinery (Patrick *et al.*, 1998).

### 1.3.5 Association of Cdk5 with Other Cellular Proteins

Although p35 and its proteolytic derivative are the predominant regulatory proteins of Cdk5, two other Cdk5 activators have been identified. p39 is a neuron-specific Cdk5 activator which shows 57% amino acid identity to p35 (Tang *et al.*, 1995). p39 is also expressed exclusively in post-mitotic neurons in rat embryonic brain. In contrast to p35, p39 is expressed at higher levels in the spinal cord than in the brain (Zheng *et al.*, 1998).

The second potential activator of Cdk5 enzymatic activity is Munc-18. Munc-18 is a 67-kDa neuron-specific protein which is an essential component of the synaptic vesicle fusion protein complex mediating the secretory process (Hata *et al.*, 1993; Shuang *et al.*, 1998). Although Munc-18-stimulated Cdk5 kinase activity has been demonstrated *in vitro*, *in vivo* studies using rat brain lysates do not confirm these observations (Shetty *et al.*, 1995; Shuang *et al.*, 1998).

Interestingly, cyclin D and cyclin E (molecules which regulate Cdk activity in proliferating cells) bind to, but do not activate Cdk5 (Xiong *et al.*, 1992; Guidato *et al.*, 1998). Cyclin D may act as a negative modulator of Cdk5/p35 activity (Guidato *et al.*, 1998). Also, Cdk5 and cyclin D may form protein complexes with other cellular proteins *in vivo*. For example, p27, a Cdk inhibitor, can recognize a Cdk5/cyclin D2 complex without affecting the activity of the Cdk5/p35 complex (Lee *et al.*, 1996b). Although no kinase activity has been detected with Cdk5 bound to cyclin D or cyclin E, it could be argued that the cyclin D/Cdk5 or cyclin E/Cdk5 complexes may phosphorylate substrates that are yet unknown.

Cdk5/p35 kinase activity is also modulated by several molecules which function as kinase inhibitors. One such protein is Cdk5i, a kinase-defective Cdk5 isoform. When complexed as a heterodimer with p35, Cdk5i displays appreciably weaker kinase activity than the wild type Cdk5/p35 complex (Moorthamer *et al.*, 1998). In addition, Cdk5i/p35 cannot autophosphorylate either subunit as does Cdk5/p35 (Moorthamer *et al.*, 1998). Therefore, expression of Cdk5i may affect wild-type function by acting in the manner of a dominant negative mutant (Van de Heuvel and Harlow, 1993). Recently, two novel Cdk5 kinase inhibitors have been identified: L34 and dbpA. Both L34, the 60S ribosomal protein (Moorthamer and Chaudhuri, 1999), and dbpA, a DNA-binding protein (Moorthamer *et al.*, 1999), potently inhibit p35-activated Cdk5. This suggests a possible cellular role of Cdk5 in both transcriptional and translational regulation.

### 1.3.6 Substrates of Cdk5/p35

The substrate specificity of Cdk5/p35 kinase is similar to that of cdc2 and Cdk2. Phosphorylation occurs exclusively within a K(S/T)P*X*(K/R) consensus motif (Beaudette *et al.*, 1993; Songyang *et al.*, 1996). The Cdk5/p35 complex can phosphorylate both histone H1 and the retinoblastoma protein, two substrates frequently used to confirm *in vitro* activity of Cdks (Tang and Wang, 1996; Lee *et al.*, 1997a). Activated Cdk5 phosphorylates a number of neuron-specific cytoskeletal proteins *in vitro* including neurofilaments NF-M and NF-H, the microtubule-associated proteins tau and MAP2, the actin-binding protein caldesmon, and brain meromyosin II-B (Lew and Wang, 1995; Tang and Wang, 1996; Lee *et al.*, 1997b; Pato *et al.*, 1996). In addition to these structural proteins, Cdk5/p35 also phosphorylates proteins associated with synaptic vesicle neurotransmitter release such as synapsin I and Munc-18 (Matsubara *et al.*, 1996; Shuang *et al.*, 1998).

## 1.4 Physiological Function of Cdk5/p35 Kinase

### 1.4.1 Neuronal Differentiation and Neurogenesis

The vertebrate embryonic cerebral wall is composed of five basic cellular zones. These zones, from the inner ventricular surface to the outer pial surface are the ventricular zone, the intermediate zone, the subplate, the cortical plate and the marginal zone. The early cerebral wall is a 2-layered structure composed of the ventricular zone and the preplate (Caviness, 1982) with radial glial cells running the length of the wall from the ventricular to the pial surface (Nicholls *et al.*, 1992). The

ventricular zone is the principal source of neurons and glia from where, following terminal mitoses, neurons migrate to their final destinations and become postmitotic (Rakic and Caviness, 1995; Nicholls *et al.*, 1992). The preplate is composed of pioneer neurons and Cajal-Retzius cells in the marginal zone. Following migration of early cortical plate neurons along radial glial fibers within the intermediate zone, the preplate is split into two contingents: the marginal zone above and the subplate below (Lambert de Rouvroit and Caviness, 1998). Cortical plate neurons are precursors to most of the cortex which is composed of six cortical layers (I-VI) . As subsequent neurons migrate to the cortical plate, they move past neurons generated earlier and take more superficial positions in the cortical plate (Fig. 1). This migration process results in an "inside-out" layering order of sequentially generated cortical neurons (Angevine and Sidman, 1961; Caviness, 1982; Luskin and Shatz, 1985). In mice, neuronal differentiation occurs largely between E11 and E17 while glial formation takes place throughout the early postnatal period.

Various neurological mutants displaying cortical lamination defects have provided insight into the mechanisms by which cortical patterning is regulated. Reelin is an extracellular-matrix glycoprotein secreted most intensely by Cajal-Retzius cells in the marginal zone (D'Arcangelo *et al.*, 1997). It is believed to act at a distance on target cells, such as cortical plate neurons, in a cell-repulsive fashion (Lambert de Rouvroit and Goffinet, 1998). *Reelin* knockout mice exhibit a *reeler* phenotype characterized by an inversion of cortical layering, failure of preplate separation into marginal zone and subplate, and a lack of cortical layer I (Fig. 1) (Caviness, 1982; Rakic and Caviness, 1995). *Scrambler* and *yotari* mice which have

a splicing defect in the transcript for *mdab1*, display very similar cortical phenotypes to those exhibited by *mdab1* knockout mice (Howell *et al.*, 1997a; Goldowitz *et al.*, 1997; Sheldon et al. 1997; Ware *et al.*, 1997). *mdab1* encodes a cytoplasmic protein that is expressed in reelin-responsive neurons (Sheldon et al. 1997; Howell et al. 1997a). It is postulated that through its function as a tyrosine kinase adaptor, mDab1 is a key component in a reelin-initiated transduction cascade involved in regulation of neuronal migration (Howell et al. 1997b; Goffinet, 1997).

Targeted disruption of the *Cdk5* and *p35* genes in mice results in *reeler*-like cortical lamination defects, neuronal pathology and lethality. *Cdk5* knockout mice exhibit accumulation of neurofilament proteins. These unique neuronal proteins are suggestive of abnormal cytoskeletal architecture. Late embryonic and perinatal death are associated with these lesions (Ohshima *et al.*, 1996b). In contrast, brain malformations are more subtle in *p35* knock-out mice. These mice display seizures and sporadic adult lethality (Chae *et al.*, 1997). *Reeler*, *mdab1*, *Cdk5* and *p35* mutant mice all share an abnormal "outside-in" stratification of cortical neurons. As mentioned above, cortical development arrests at the preplate stage in *reeler* and *mdab1* mutant mice resulting in absence of preplate division. In contrast, cortical development in *Cdk5* (-/-) and *p35* (-/-) mice is blocked at a later stage after separation of the preplate and migration of early cortical plate neurons (Gilmore *et al.*, 1998; Kwon and Tsai *et al.*, 1998). As Fig. 1 depicts, later-born neurons do not pass the subplate and settle at a deeper level within the subplate and the periphery of the intermediate zone than normal. This results in a disruption of the 'inside-out' cortical layered pattern.

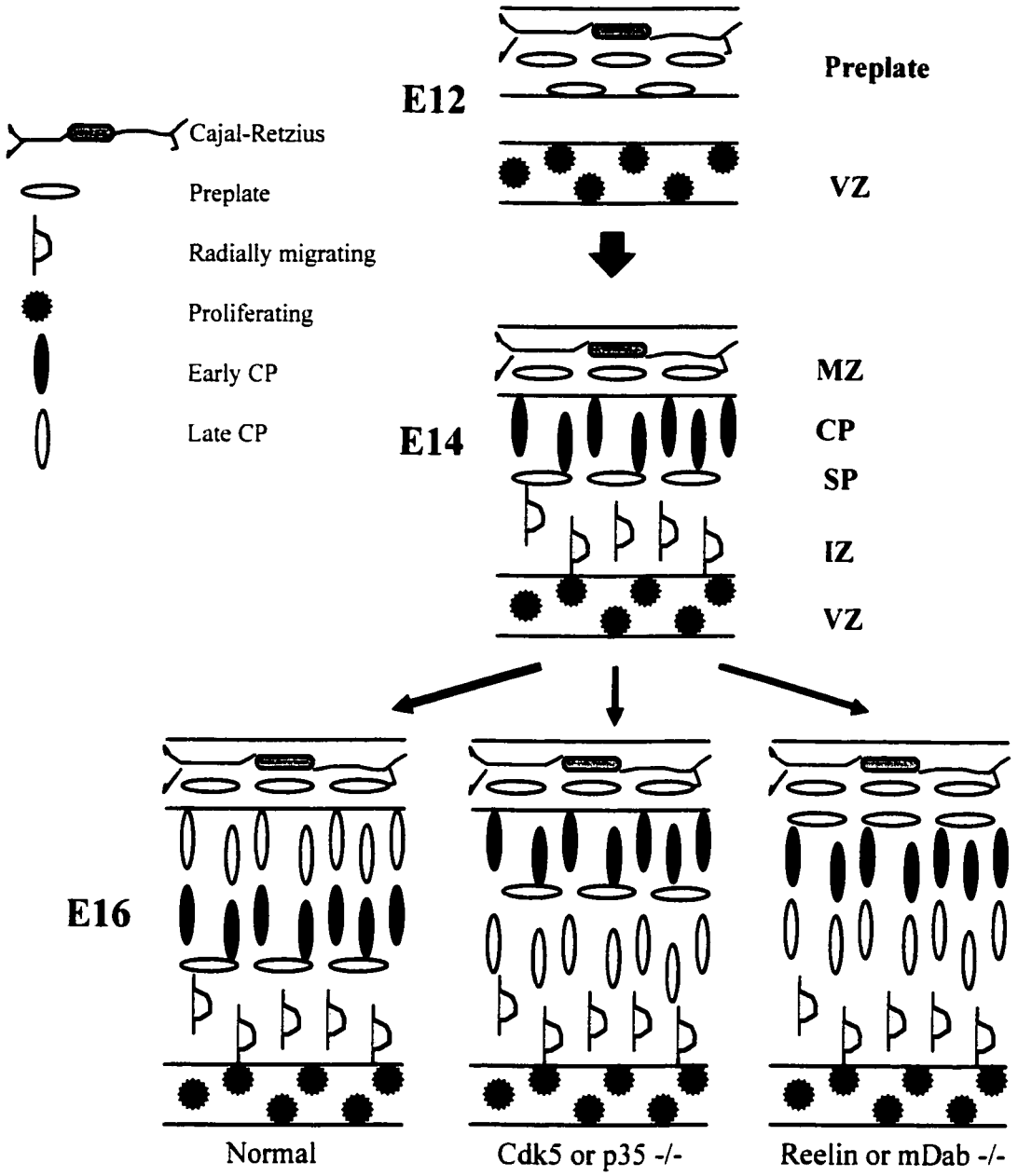


Fig 1. Model of cortical development. In the developing cerebrum, CP neurons separate the preplate into two populations: MZ with Cajal-Retzius cells and SP. Later migrating CP neurons migrate past earlier cells for an 'inside-out' formation. *Reelin* and *m-dab1* null mutants display defective CP organization during early cortical development while disruption of the CP organization occurs later in *Cdk5* and *p35* mutants. Abbr.: ventricular zone (VZ), marginal zone, (MZ), cortical plate (CP), subplate (SP), intermediate zone (IZ), ventricular zone (VZ). (Adapted from Lambert de Rouvroit and Goffinet, 1998)

A tentative model of Cdk5 involvement in cortical development has been proposed by Lambert de Rouvroit and Goffinet (1998). Early Cajal-Retzius cells within the marginal zone secrete reelin. This protein is incorporated into the local extracellular matrix and functions as a stop signal for migrating cortical plate neurons. Through an unknown cell-surface/transducer system, an intracellular cascade involving mDab1 tyrosine kinase is initiated within reelin-responsive cortical neurons. This results in detachment of the neurons from their radial guides and formation of a well-organized cortical plate. As later-migrating neurons must pass through previously settled cortical layers, they require a stronger association with the glial scaffolding. Fine control of migratory mechanics including regulation of the cytoskeletal structure, is also required for this migration. Through its cytoskeletal regulatory function, Cdk5/p35 kinase may be required for later-generated neurons to migrate past pre-existing neurons and reside within more superficial layers.

Temporal and spatial patterns of Cdk5 and p35 expression also suggest a functional role for this kinase in cortical development. As mentioned previously, Cdk5 and p35 are expressed in post-mitotic neurons and are excluded from the ventricular zone (Tsai *et al.*, 1993; Ino *et al.*, 1994; Tsai *et al.*, 1994). In mice, Cdk5 and its associated kinase activity gradually increase in parallel during neuronal development from E11 to E15 with maximal expression and activity between E15 and P0 (Tsai *et al.*, 1993). p35 expression gradually increases from E12 with a rapid rise from 18 days post-coitum to two weeks after birth. It then declines at three weeks (Tomiwaza *et al.*, 1996). In adult brain, Cdk5 levels remain constant. In contrast, p35 expression gradually declines in adult brain, but remains high in highly plastic

areas of the forebrain including the hippocampal formation, the olfactory cortex, and the pyramidal layer of the neocortex (Nikolic *et al.*, 1996). The developmental pattern of Cdk5 and p35 therefore correlate with terminal neuron differentiation.

Neuronal differentiation is morphologically characterized by the development of neurite extensions. Expression of dominant negative mutants of *Cdk5*, or transfection of an antisense *p35* construct in primary cultured cortical rat neurons, results in neurite outgrowth inhibition. This inhibition can be rescued by co-expression of the wild-type proteins (Nikolic *et al.*, 1996). These data suggest a critical role for the Cdk5/p35 kinase in neurite outgrowth.

### 1.4.2 Differentiation of Non-Neuronal Cells

Several observations indicate Cdk5/p35 kinase involvement in muscle development. Although present at lower levels than in the developing nervous system in mice, both Cdk5 and p35 are detected in the myotome at E12 and continue to be expressed in the premuscle mass and mature muscle in later stages (Zheng *et al.*, 1998). In addition, cultured mouse myoblasts show Cdk5 histone H1 kinase activity which increases with induced differentiation (Lazaro *et al.*, 1997). Cdk5 kinase activity has also been shown to be required for muscle development in *Xenopus* where a *Cdk5* homologue and a *p35* homologue (*Xp35.1*) are expressed in developing somites. Overexpression of *Xp35.1* or a *Xenopus Cdk5* dominant-negative mutant (DNK5) disrupts somatic muscle patterning. Expression of the myogenic determination factors, MyoD and MRF4, is also suppressed in the presence of DNK5

(Philpott *et al.*, 1997). The presence of both Cdk5 and p35 and their associated kinase activity during myogenesis indicates a role for Cdk5/p35 in muscle development.

Another non-neuronal cell type in which Cdk5-associated kinase activity may contribute to differentiation is oligodendrocytes. Oligodendrocytes are the myelinating cells of the central nervous system and like neurons, their presursors arise from the ventricular zone where they migrate to populate the entire brain. Once migration is completed, postmitotic oligodendrocytes activate myelin-specific genes and extend multiple processes to ensheath neuronal axons. Cdk5 is expressed in various oligodendrocytic cell lines (Tikoo *et al.*, 1997; Casaccia-Bonnefil *et al.*, 1997; Tang *et al.*, 1998). Although Cdk5 protein levels are equal in precursor and postmitotic rat primary oligodendrocytes, Cdk5 activity is shown to increase two-fold as oligodendrocytes differentiate (Tang *et al.*, 1998). As mentioned earlier, these results do not correlate with immunohistochemical studies using mouse, rat, or human tissue. No Cdk5 or p35 expression is detected *in vivo* within glial cells of the nervous system (Tsai *et al.*, 1993; Ino *et al.*, 1994; Nakamura *et al.*, 1998; Hayashi *et al.*, 1999). Therefore the role of Cdk5/p35 activity in glial cells *in vivo* remains undetermined.

### 1.4.3 Synaptic Vesicle Release

Membrane targeting and synaptic vesicle neurotransmitter release involve a fundamental set of interactions between key proteins which is termed the SNARE hypothesis. This hypothesis is based on the formation of a complex composed of soluble cytosolic proteins (NSF and SNAPs) and proteins integral to the synaptic

vesicle and the target membrane (SNAREs). The soluble proteins include the ATPase *N*-ethylmaleimide-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) which are required for membrane attachment and NSF activation. SNAREs are SNAP-receptors found on both the synaptic vesicle (v-SNAREs) and the target membrane (t-SNAREs). During the secretory process, the synaptic vesicle is targeted to the membrane via the interaction between appropriate v-SNAREs and t-SNAREs. This protein complex then further recruits the cytosolic SNAPs followed by NSF (Bennett and Scheller, 1993; Sollner *et al.*, 1993; Sughof *et al.*, 1993; Rothman, 1994).

A number of factors regulate the secretory machinery either positively or negatively. One such factor is Munc-18, a human homologue of *Saccharomyces cerevisiae* Sec1p protein (Hata *et al.*, 1993). Munc-18 is both essential and inhibitory to the secretory process. It is essential since Sec1p was reported to be important for vesicle trafficking (Hata *et al.*, 1993; Cowles *et al.*, 1994; Pevsner, 1996). However, the interaction between Munc-18 and syntaxin, a membrane-associated t-SNARE, blocks the interaction of syntaxin with vesicle SNARE proteins (Hata *et al.*, 1993; Pevsner *et al.*, 1994). Syntaxin is an essential component of the protein complex mediating vesicle fusion (Rothman and Warren, 1994; Bennett and Scheller, 1993; Sudhof *et al.*, 1993). As a result, any factor that regulates the interaction between Munc-18 and syntaxin may provide an important mechanism of controlling the secretory process (Fletcher *et al.*, 1999).

Cdk5 is a candidate regulator of secretory responsiveness through its interactions with Munc-18 and syntaxin. Cdk5 copurifies with Munc-18 from rat

nervous system (Shetty *et al.*, 1995; Shuang *et al.*, 1998). Cdk5 phosphorylates Munc-18 *in vitro* within a preformed Munc-18-syntaxin 1A complex. This results in disassembly of this complex (Fletcher *et al.*, 1999). Cdk5 can also bind syntaxin 1A and a complex of Cdk5/p35/Munc-18/syntaxin 1A can be formed in the absence of ATP (Shuang *et al.*, 1998). Stimulation of secretion from neuroendocrine cells produces a translocation of cytosolic Cdk5 to a particulate fraction and a corresponding increase of Cdk5 kinase activity (Fletcher *et al.*, 1999). In addition, in chromaffin cells, transfection and expression of the Cdk5 activator, p25, results in increased evoked norepinephrine secretion whereas inhibition of Cdk5 activity decreases nicotinic agonist-induced secretion (Fletcher *et al.*, 1999). These data suggest a model depicted in Fig. 2 whereby p35-activated Cdk5 regulates the secretory process by localizing to the syntaxin 1A-Munc18 complex through its affinity for both proteins. Cdk5 then phosphorylates Munc-18 allowing syntaxin 1A to interact with upstream proteins of the secretory mechanims (Shuang *et al.*, 1998).

### 1.4.4 Apoptosis

Over the past few years, Cdk5 involvement in apoptotic cell death has been demonstrated in both experimental and physiological model systems. For example, the pattern of Cdk5 protein expression in oxidative stress-induced apoptosis in sympathetic neurons changes as a function of the apoptotic process and coincides with the commitment of cells to die (Shirvan *et al.*, 1998). Cdk5 protein expression has been found to be correlated with apoptosis in a number of non-neuronal model systems. These include atretic ovarian follicular cells, regions of the developing

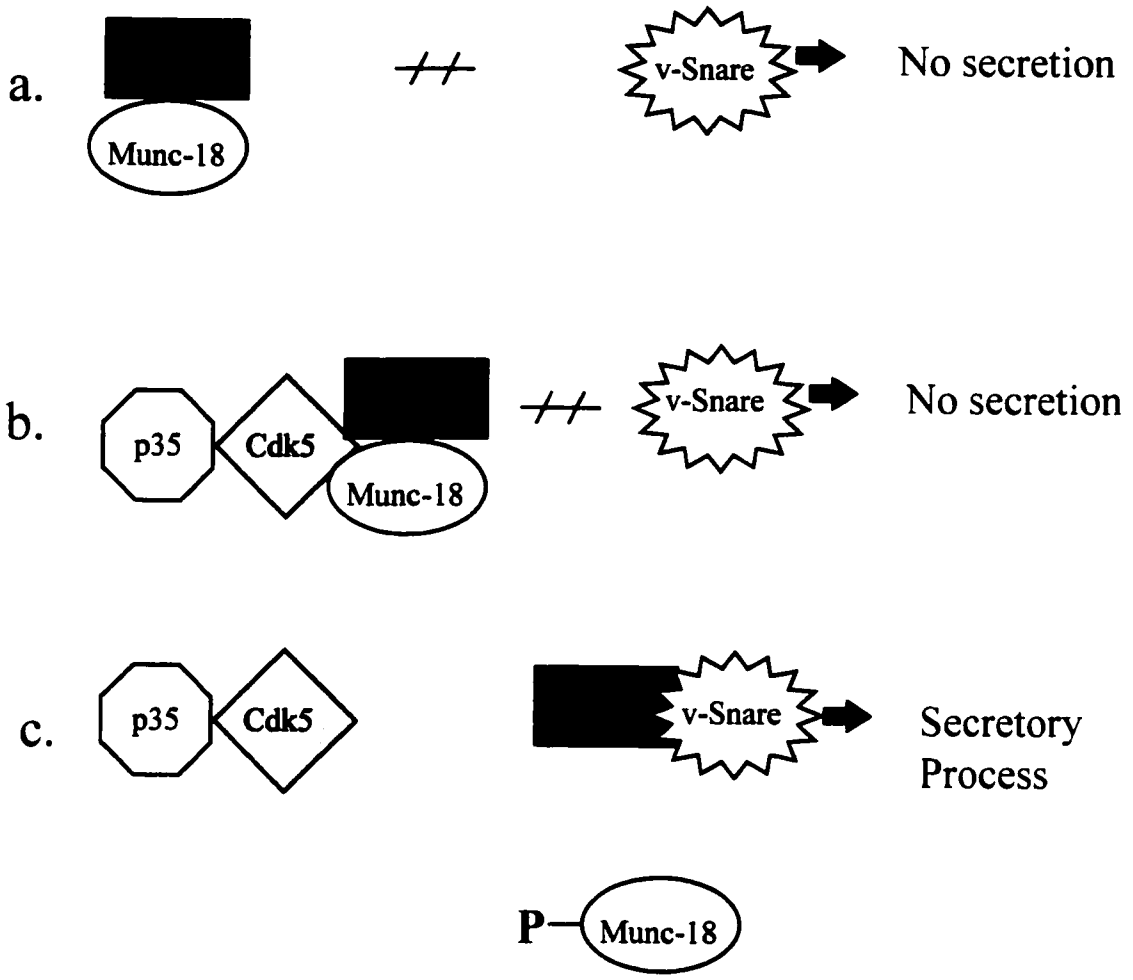


Fig 2. Model of Cdk5 regulation of exocytosis. (a) High affinity binding between Munc-18 and syntaxin 1a (Synt) inhibits the association of vesicle SNAREs (v-Snare) with syntaxin. (b) The p35-Cdk5 heterodimer becomes localized to the Munc-18-syntaxin 1a complex by its affinity for both proteins. (c) Phosphorylation of Munc-18 by p35-activated Cdk5 permits interaction of syntaxin 1a with upstream protein effectors of the secretory process.

retina and nervous system, androgen-withdrawn regressing prostate and interdigital zones of developing mouse hindlimbs (Zhang *et al.*, 1997; Henchcliffe and Burke, 1997; Ahuja *et al.*, 1997). Association of Cdk5 kinase activity with apoptotic cell death was demonstrated in mouse embryonic hindlimbs where the increase in apoptosis between E12.5 and E14.5 coincides with a concomitant 3.4-fold increase in Cdk5 kinase activity (Zhang *et al.*, 1997). Interestingly, within the developing mouse limb, both Cdk5 and p35 protein levels, not transcript, were found to be associated with dying cells. This indicates that the regulation of Cdk5 and p35 in apoptotic cells occurs at the level of mRNA transport or translation (Ahuja *et al.*, 1997).

A specific function for Cdk5 in dying cells has not been determined. Given its association with cytoskeletal proteins, it is hypothesized to mediate pathological cytoskeletal processes. Rearrangement of the cytoskeleton is a distinct feature of apoptosis (Brancolini *et al.*, 1995; Levee *et al.*, 1996; Brown *et al.*, 1997; Van Engeland *et al.*, 1997). Cdk5 kinase activity may be responsible for, or involved in, the formation of apoptotic bodies (Ahuja *et al.*, 1997).

## 1.5 Role of Cdk5/p35 in Pathological Systems

### 1.5.1 Neurodegenerative Diseases

Dynamic changes of the neurocytoskeletal structure underlie many histopathological features exhibited in neurodegenerative diseases. Cdk5 phosphorylates several cytoskeletal components *in vitro* and has been implicated in

the regulation of cytoskeletal dynamics in both physiological as well as pathological conditions.

The occurrence of Lewy bodies (LB) in Parkinson's disease and Lewy body like inclusions (LI) in the anterior horn cells of ALS patients are major histological hallmarks of these neurodegenerative diseases. LB's are eosinophilic inclusions composed of aggregates of fibrils. These fibrils are made up of abnormally phosphorylated neurofilament proteins. LI's are round or rod-shaped eosinophilic cytoplasmic inclusions formed with granule-associated filaments in the core and are surrounded by a halo of aberrantly phosphorylated neurofilament proteins.

Neurofilament proteins (NF), members of the intermediate-filament family, are neuron-specific proteins that constitute the major cytoskeletal infrastructure in axons and include NF-H, NF-M and NF-L subunits. The long C-terminal domains of human NF-H and NF-M contain repeated KSP motifs which are phosphorylated *in vivo*. This phosphorylation is thought to (1) influence the interactions between NF's and other components of the axoplasm (Carden *et al.*, 1987), (2) control neurofilament transport (De Waegh *et al.*, 1992) and, (3) determine axon caliber (De Waegh *et al.*, 1992; Nixon *et al.*, 1994).

Purified Cdk5 phosphorylates KSP motifs in the C-terminus of NF-H and NF-M *in vitro* (Hellmich *et al.*, 1992, Lew *et al.*, 1992, Shetty *et al.*, 1993; Sun *et al.*, 1996). Cdk5 protein expression has been observed in both LB's of patients with Parkinson's disease (Brion and Couck, 1995) and LI's of ALS patients (Nakamura *et al.*, 1997). p35 expression is also detected within LB's of the same patients (Nakamura *et al.*, 1997). It is hypothesized that Cdk5's involvement in

phosphorylation of the C-terminus of NF-H and NF-M in LB of Parkinson's disease may be a crucial step for the conversion of neurofilament proteins into insoluble fibrils. In addition, Cdk5 protein levels and activity are associated with ALS-like neurofilament pathology in canine motor neuron disease (Green *et al.*, 1998). Cdk5 may play a critical role in the development of neurofilament-associated inclusions in different neurodegenerative diseases.

Cdk5 is implicated the regulation of cytoskeletal dynamics in pathological features of Alzheimer's disease. Tau is a neuron-specific, microtubule-associated protein which plays a role in the establishment and maintenance of neuronal polarity (Goedert *et al.*, 1991). Abnormally hyperphosphorylated tau is the main component of the paired helical filaments (PHF) that constitute neurofibrillary tangles (NFT) in Alzheimer's diseased brains (Ihara *et al.*, 1986). This hyperphosphorylated tau (PHF-tau) loses its ability to promote and stabilize microtubules, resulting in neuronal dysfunction (Yoshida and Ihara, 1993). Cdk5 can phosphorylate tau protein in an Alzheimer-like state *in vitro* (Ishiguro *et al.*, 1992, Baumann *et al.*, 1993). The *in vitro* phosphorylation sites correspond to those hyperphosphorylated in Alzheimer's diseased brains (Ishiguro *et al.*, 1992). Pre-phosphorylation of tau by Cdk5 was shown to stimulate subsequent kinase-induced hyperphosphorylation of tau (Sengupta *et al.*, 1997). Immunohistochemical analysis of Alzheimer's brain specimens indicates a preferential and consistent labeling of intraneuronal NFT's (Yamaguchi *et al.*, 1996; Pei *et al.*, 1998) particularly in neurons bearing early stage NFT's (Pei *et al.*, 1998). These data suggest that Cdk5 may be involved in the *in vivo* generation of hyperphosphorylated tau within NFT's at a relatively early stage in the neocortex.

Ischemic brain injury has profound effects on the cytoskeletal components of neurons. Levels of cytoskeletal proteins including the microtubule associated proteins, tau protein (Dewar and Dawson, 1995), MAP1 (Matesic and Lin, 1994), and MAP2, (Matesic and Lin, 1994) decrease in the neurons of ischemic brain. Phosphorylation of various proteins at specific sites can induce depolymerization of these cytoskeletal proteins (Yanigihara *et al.*, 1990; Schulze *et al.*, 1987). It has been proposed therefore that Cdk5 may be involved in the neurocytoskeletal degradative processes seen in ischemic brain (Green *et al.*, 1997; Hayashi *et al.*, 1999). Both Cdk5 and p35 immunoreactivity is increased in neurons within middle cerebral artery occlusion-induced ischemic areas of rat brain (Hayashi *et al.*, 1999). In a post-decapitation rat model of complete ischemia, Cdk5 tau phosphorylating activity increased following ischemia (Green *et al.*, 1997). This rat model of ischemia mimics the ischemic brain injury that occurs under various clinical conditions including cardiac arrest, brain edema due to massive head injury, sepsis and severe blood loss (Green *et al.*, 1997). Thus, Cdk5 kinase activity may induce depolymerization of neurocytoskeletal proteins in the brain after ischemia resulting in neuronal cell death.

Through its potential to phosphorylate various neurocytoskeletal components *in vivo*, Cdk5 and its associated kinase activity is implicated in the abnormal phosphorylation of cytoskeletal proteins in various neuronal pathologies. As a result, Cdk5 may play an essential role in the cytoskeletal disorganization and neuronal cell death associated with various neurodegenerative diseases.

### 1.5.2 Glial Cell Pathologies

Glial cytoplasmic inclusions (GCI) are histological features found in the oligodendrocytes of multiple system atrophy patients. They are composed of loosely packed tubular filaments 20 to 30 nm in diameter (Murayama *et al.*, 1992; Abe *et al.*, 1992) and are associated with major microtubule components such as tubulin subunits ($\alpha$ and $\beta$) (Papp and Lantos, 1989), MAP1B (Abe *et al.*, 1992) and tau proteins (Abe *et al.*, 1992). Due to its tau- (Baumann *et al.*, 1993) and MAP2- (Lew and Wang, 1995) associated kinase activity *in vitro*, Cdk5 is suggested to be involved in the development of GCI's (Nakamura *et al.*, 1998). Although Cdk5 is not normally expressed in glial cells in normal brain tissue (including oligodendrocytes) (Tsai *et al.*, 1993; Ino *et al.*, 1994; Nakamura *et al.*, 1998; Hayashi *et al.*, 1999), it was detected in GCI's in brains of multiple system atrophy patients (Nakamura *et al.*, 1998). Considering the close association of GCI's with the microtubular cytoskeleton and Cdk5 phosphorylating activity of tau and MAP2 proteins *in vitro*, ectopic expression of Cdk5 may result in the abnormal phosphorylation of microtubule-associated proteins. This would result in GCI formation in the oligodendrocytes of multiple system atrophy patients (Nakamura *et al.*, 1998).

# II. EXPERIMENTAL OBJECTIVES

## 2.1. What are the Expression Patterns of Cdk5 and p35 in Human Glioma Cells?

### 2.1.1. Cdk5 and p35 Protein Expression in Human Glioma Biopsy Specimens

Analysis of normal human, rat and mouse brain tissue reveals an exclusive neuronal expression pattern for Cdk5 and p35. These proteins are not normally found in supporting glial cells including oligodendrocytes and astrocytes (Tsai *et al.*, 1993; Ino *et al.*, 1994; Nakamura *et al.*, 1998; Hayashi *et al.*, 1999). However, previous studies have indicated that Cdk5 and p35 proteins were expressed in human glioma cell lines and that the p35 protein was unexpectedly present in the 'active' proteolytic form, p25. In addition, p35 was further detected by immunohistochemistry in 7 of 7 human glioma biopsy specimens, suggesting that its expression in glioma cells was not an artifact of *in vitro* tissue culture.

Using a variety of non-malignant brain tissue samples and a spectrum of cerebral astrocytic tumors of various histological grades, we wished to (1) confirm the absence of Cdk5 and p35 proteins from normal astrocytes, and (2) determine if the aberrant expression of Cdk5 or p35 in astrocytoma cells was associated with any histopathological features of astrocytic tumors.

### 2.1.2. Localization of Cdk5 and p35 Protein in Cultured Glioma Cells

The subcellular localization of Cdk5 and p35 has been extensively examined in neuronal cells. Both Cdk5 and p35 display unique expression patterns in the brain as well as interspecies variation. Following the novel finding of the *in vivo* expression of Cdk5 and p35 proteins in human glioma cells, we wished to determine the subcellular distribution of Cdk5 and p35 in cultured glioma cells and to examine (1) if Cdk5 and p35 were nuclear or cytoplasmic proteins, and (2) if Cdk5 and p35 proteins displayed any unique expression patterns within human glioma cells.

## 2.2. Is Cdk5/p35 Kinase Associated with Apoptotic Cell Death in Human Glioma Cells?

Although the exact functional properties of Cdk5 remain to be determined, a strong correlation between Cdk5/p35 protein expression and apoptotic cell death has previously been reported in a number of developmental and experimental systems (Aruja *et al.*, 1997; Zhang *et al.*, 1997). Apoptotic cell death is a feature of anaplastic astrocytomas and glioblastoma multiforme (Kordek *et al.*, 1996; Tachibana *et al.*, 1996; Ellison *et al.*, 1995) and has been suggested to increase with the degree of anaplasia in astrocytic tumors (Kordek *et al.*, 1996; Ellison *et al.*, 1995). Using a panel of human malignant glioma biopsy specimens of varying histopathological grades, we wished to examine the *in vivo* association of Cdk5/p35 protein expression with apoptotic cell death.

Human glioma cell lines which exhibit significant differences in susceptibility to radiation-induced apoptosis provide a useful model for examining the role of Cdk5 in apoptosis. The human malignant glioma cell lines, M059J and M059K (isolated from different portions of the same glioblastoma specimen), have an approximately 30-fold difference in their inherent radiation sensitivities as measured by surviving fraction at 2 Gy (SF2): M059K = 0.64 and M059J = 0.02 (Allalunis-Turner *et al.*, 1993). This variance in radiosensitivities is, in part, the consequence of differences in sensitivity to radiation-induced apoptosis (Leithoff *et al.*, 1995). We used M059J and M059K cells to evaluate the functional role of Cdk5/p35 in apoptotic cell death and to determine if Cdk5 and p35 protein expression and kinase activity was associated with apoptosis in these cells.

## 2.3 Is p35 Subject to Rapid Turnover in Human Glioma Cells?

Recent studies examining p35 protein stability in cultured neurons have shown p35 to be a short-lived protein with a half-life of approximately 20-30 min (Patrick *et al.*, 1998; Saito *et al.*, 1998). To determine whether the half-life of p35 protein in human glioma cells was similar to that determined for neuronal p35, M059J cells were treated with cycloheximide for various times and analyzed for p35 expression.

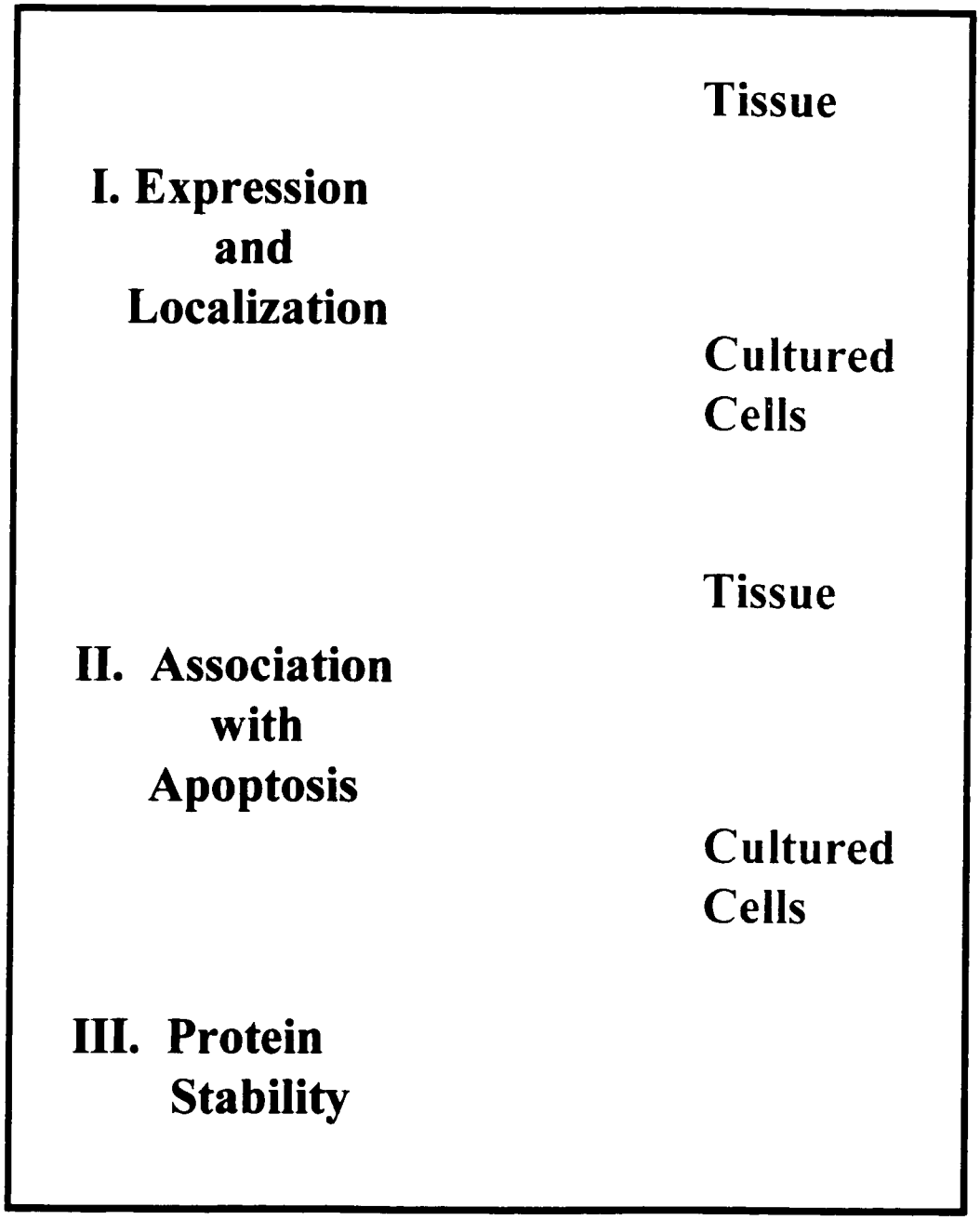


Fig 3. Overview of experimental objectives.

# III. MATERIALS AND METHODS

## 3.1 Glioma Model Systems

### 3.1.1 Human Glioma Cell Lines

The M059J and M059K cell lines were isolated from different portions of the same human glioblastoma multiforme surgical biopsy specimen (Allalunis-Turner *et al.*, 1993). M059J cells are approximately 30-fold more sensitive to ionizing radiation than are M059K (surviving fractions at 2 Gy equal 0.02 and 0.64, respectively) and provide the only human example of a cell line lacking DNA-dependent protein kinase activity (Lees-Miller *et al.*, 1995). M059J and M059K cells in exponential growth phase were used for immunohistochemical staining of cytospin preparations, western blot analyses and kinase assays.

### 3.1.2 Human Normal Brain and Brain Tumor Specimens

Paraffin-embedded histological sections of human astrocytoma and human glioblastoma multiforme tumors were obtained from Dr. Bruce Mielke at the University of Alberta Hospital. Paraffin-embedded histological sections representing non-malignant brain and brain tumors were a gift from Dr. Chunghai Hao of the University of Alberta Hospital. Brain tumor tissue sections included specimens obtained from patients with medullablastoma and pilocytic astrocytoma. Drs. Bruce Meilke and Chunghai Hao also provided neuropathological review of all the immunohistochemical staining patterns.

## 3.2 Cell Culture

Cells were grown as monolayer cultures at 37°C in an atmosphere of 5% $CO_2$ in air and 90% relative humidity. Stock cultures were maintained in Dulbecco's modified Eagles medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 20 units/ml penicillin (Gibco BRL) and 500 μM L-glutamine (Gibco BRL). Cells were passaged by regular trypsinization of confluent cultures. A Coulter Z2 electronic particle counter was used for cell number determinations.

## 3.3 Immunological Reagents

Affinity-purified rabbit polyclonal antibodies to Cdk5 (C-8, Santa Cruz Biotechnology, sc-173) raised against a peptide corresponding to an amino acid sequence at the C-terminus of human Cdk5 origin was used for immunohistochemistry (1 : 100), western blotting (1 : 1000), immunoprecipitation (1 μg) and confocal microscopy (1 : 300). Cdk5 peptide (Santa Cruz Biotechnology, cat #sc-173 P, 10 X volume of antibody) was used as a control for confocal microscopy. A mouse monoclonal Cdk5 antibody (Upstate Biotechnology, 1 : 333) raised against a recombinant full length human Cdk5 protein was also used for confocal microscopy.

Affinity-purified rabbit polyclonal antibodies raised against a peptide corresponding to amino acids 2-21 mapping to the N-terminus of p35 of human origin (N-20, Santa Cruz Biotechnology, #sc-821) was used for immunohistochemistry (1: 100) and western blotting (1 : 200). A second p35 rabbit polyclonal antibody raised against an epitope mapping to the C-terminus of human p35 (C-19, Santa Cruz Biotechnology, #sc-820, 1 : 40) and a p35 control peptide (Santa Cruz Biotechnology, # sc-820 P, 10 X volume of antibody) were used for confocal microscopy. A

monoclonal anti-α-tubulin antibody (DM 1A. Sigma. . # T 9026. 1 : 1000) was used for confocal analysis of co-localization with p35.

Secondary antibodies used include: peroxidase-conjugated. affinity-purified goat anti-rabbit IgG (H+L) (Jackson Laboratories. #111-035-144. 1 : 50 000); peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology. # sc-2004. 1 : 50 000); Alexa 488-conjugated highly cross-absorbed goat anti-rabbit IgG (Molecular Probes. A-11034. 1 : 250); Cy3-conjugated goat anti-rabbit IgG (H+L) (Jackson Laboratories. # 111-165-144. 1 : 200) and Cy5-conjugated goat anti-mouse IgG (H+L) (Jackson Laboratories. #115-175-062. 1 : 250). Normal rabbit purified IgG (Santa Cruz Biotechnology. # sc-2027) was used as a negative control for immunohistochemistry (1 μg/ml) and immunoprecipitation (1 μg).

## 3.4 Irradiation Experiments

M059J and M059K cells were trypsinized. replated at the density required per procedure. and returned to the incubator for 1 to 4 days to allow for cell adherence to occur and to re-establish exponential growth. A dose of 10 Gy γ-rays was then delivered using a $^{137}Cs$ source (Sheperd Irradiator) at a dose rate of 1.41 Gy/min. Control cultures were mock-irradiated. Following irradiation. cells were immediately placed on ice and transported to the laboratory.

## 3.5 Cytospin Preparation of M059J and M059K Glioma Cells

M059J and M059K human glioma cells were plated at a density of $4 \times 10^5$ cells per 75 $cm^2$ flask. Cells were mock-treated or exposed to 10 Gy γ-rays as described above. Following irradiation. flasks were returned to the incubator for 15 min. 30 min. 1 h. 3 h. 12 h. 24 h. 36 h or 48 h. Following incubation cells were

trypsinized, counted and diluted in complete medium to yield a concentration of 8 x $10^4$ cells/ml. Cytospins were prepared by adding 250 µl (2 x $10^4$) of the cell suspension to the cytospin slide holder loaded with a poly-l-lysine-coated (Sigma) glass slide and filter paper (Shandon). Slides were centrifuged at 400 rpm for seven minutes at low acceleration (Cytospin 3, Shandon). All slides were immediately fixed in 4% paraformaldehyde (Fisher Scientific), permeabilized in 0.1% Triton X-100 (Sigma), air dried and stored at –20°C until further analysis.

## 3.6 Immunohistochemical Analysis of Cdk5 and p35 Protein Expression in Human Glioma Cells

The biotin-streptavidin amplified (B-SA) detection system was used for immunohistochemical detection (Super Sensitive Detection System, Santa Cruz Biotechnology) of Cdk5 and p35 protein expression in paraffin-embedded human gliomblastoma multiforme biopsy specimens and cytospin preparations of irradiated and mock-treated M059J and M059K cells. Paraffin-embedded human biopsy specimens were de-waxed by three washes in xylene and rehydrated by passage through a series of ethanol solutions (three times in 100% ethanol, once in 80% ethanol, and once in 70% ethanol). Cytospin preparations and paraffin-embedded tissue specimen slides were incubated for 30 min in 2.1% citric acid for antigen unmasking. After two washes with 1 x PBS, slides were blocked for 30 min with 10% goat serum (Jackson Laboratories). Slides were placed in a humidified chamber at room temperature, primary antibodies were added to cover the entire specimen and incubated for 2 h. After two washes with PBS, slides were incubated with

biotinylated goat anti-rabbit IgG antibody (diluted 1:15) for 20 min, washed twice in PBS and incubated with streptavidin-conjugated to alkaline phosphatase (diluted 1:15) for 20 min. Staining was detected using a Fast Red solution system (Biogenex) and nuclei were counterstained with Harris Hematoxylin (Fisher Scientific). All antibodies were diluted in common antibody diluent (Biogenex). For control, the slides were incubated with rabbit IgG in 1% BSA and 0.02% azide.

## 3.7 Confocal Microscopy Analysis of Cdk5 and p35 Protein Distribution in Human Malignant Glioma Cells

For cellular localization of p35, Cdk5 and tubulin in glioma cells, M059J cells were seeded onto coverslips in rectangular eight-well dishes (Nunc Nalge) at a concentration of 6 x $10^4$ cells/slide in phenol red-free media (Gibco BRL). Cells were grown for one day and then fixed in 4% paraformaldehyde, rinsed in 1 x PBS and permeabilized in 0.5% Triton X-100 (Sigma). Following two washes in 1 x PBS, cells were incubated with 25 µl of a mixed primary antibody solution on a parafilm-covered glass plate for 1 h at room temperature. After 3 washes in 0.1% Triton X-PBS, cells were incubated in 25 µl of a mixed secondary fluorochrome-labeled antibody solution for 1 h at room temperature. For nuclear staining, cells were incubated in Hoechst 33258 (0.1 µg/ml, Molecular Probes, # H-3569) for 2 min at room temperature. Coverslips were mounted using 90% glycerol ( BDH) and analyzed at 63x magnification using a Zeiss LSM 510 confocal microscope.

For analysis of p35 protein expression and apoptosis, paraffin-embedded glioblastoma multiforme biopsy specimens were dewaxed by three washes in xylene and rehydrated by passage through a series of ethanol solutions (three times in 100% ethanol, once in 80% ethanol, and once in 70% ethanol). After incubation in 1 x PBS

for 10 min, sections were treated with 20 μg/ml proteinase K (Gibco BRL) for 10 min at room temperature. Sections were rinsed twice in 1 x PBS and then permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate (BDH) for 1.5 min on ice. Following two washes in 1 x PBS, sections were incubated in the TUNEL reaction mixture (In Situ Cell Death Detection Kit, Boehringer Manheim) containing fluorescein-labeled d-UTP and TdT, for 30 min at 37°C. After three washes in 1 x PBS, the procedure for immunofluorescence staining was followed as described above. For negative controls, the sections were incubated without addition of TdT.

### 3.8 Whole Cell Lysate Preparation

Whole cell lysates of irradiated or mock-treated M059J and M059K cells were prepared at various times after irradiation (0, 30 min, 1 h, 3 h, 14 h, 24 h, 36 h, 72 h) for western blot analyses and kinase assays. In order to include apoptotic cells detached from the substratum after irradiation, trypsinized cells were combined with cells floating in the media. Cells were counted using a Coulter counter. For western blotting, cells (1 x $10^4$/ml) were lysed in RIPA lysis buffer (1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 μg/ml PMSF, 2 μg/ml aprotinin, 10 μg/ml sodium orthovanadate). Lysate products were collected after passage through a 21 gauge needle and centrifugation at 13 000 rpm for 20 min at 3 °C using a microcentrifuge. For kinase assays, approximately 1x $10^7$ cells were lysed in 1 ml lysis buffer (0.1 % Triton X, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.575 mM PMSF and 1μg/ml aprotinin in PBS). Cell lysates were collected after passing through a 21 gauge needle and centrifugation at 3000 rpm for 15 min at 3 °C. Protein concentrations of each sample were determined by using a BCA protein assay (Pierce) according to manufacturer's instructions.

## 3.9 Western Blot Analysis of Cdk5 and p35 Protein Expression in Human Malignant Glioma Cells

Proteins were separated by running equal amounts of lysate products (total cellular protein from $1x10^5$ cells or equal protein) on 12% SDS-polyacrylamide gels at 200 V for approximately 50 min. Proteins were then transferred (1 h at 100 V) to a nitrocellulose membrane (Hybond-C extra, Amersham). The loading and transfer of equivalent levels of protein was confirmed by staining with Ponceau S (Sigma). The membranes were blocked by overnight incubation in a solution of 5% dry milk in TBS (2 M Tris HCl, pH 7.5), probed with the primary antibody for 2 h, followed by secondary HRP-conjugated goat anti-rabbit immunoglobulin for 1 h. The immunocomplex was detected by chemiluminescence (ECL, Amersham or Supersignal, Pierce) and autoradiography (Kodak XAR film). Autoradiographs were quantitated by densitometric analysis.

## 3.10 Densitometric Analysis

Quantitation of western blots and kinase assays were performed by densitometry using *The Discovery Series* (*pdi*, Huntington Station, NY) workstation and *Quantity One* (Version 2.2, *pdi*) scanning and one-dimensional image analysis software. The scanner was calibrated using an optical density step tablet. Background subtraction was achieved by subtracting the average interlane optical density for kinase assays and the average lane optical density for western blots. Band width was set at 5 mm and bands were detected automatically using default detection parameters. Band quantitation (O.D. x mm) was achieved by integrating the average pixel optical density across the sample width and values were normalized relative to the control. All blots were analyzed at a resolution of 176 x 176 pixels.

### 3.11 Immunoprecipitation and Histone H1 Kinase Assay

Cell lysates were prepared as described for the western blot analyses. Equal amounts of protein were incubated with 1 μg of rabbit IgG (Santa Cruz Biotechnology) or IgG anti-Cdk5 (C-8) for 15 min at 4 °C. A rotating platform was used to ensure adequate mixing. Immune complexes were then precipitated by adding 20 μl Protein A-agarose to each sample (Santa Cruz Biotechnology) overnight at 4°C with constant rotating. The agarose beads were washed by collecting the pellet complex by centrifugation for 5 min at 2500 rpm at 4 °C and resuspending the pellet in 1 ml lysis buffer. The beads were washed four times with lysis buffer and washed once with kinase wash (50 mM Hepes pH 7, 10 mM $MgCl_2$ pH 7, 1 mM DTT, 1 μM unlabeled ATP in distilled $H_2O$). Pellets were centrifuged and excess liquid removed using a micropipette.

Histone H1 kinase reactions were performed by incubating each sample with 45 μl kinase reaction buffer containing ATP (0.5 μM), histone (2 μg) (as a substrate) and 5 μCi of $\gamma$ $^{32}$P-ATP (10 μCi/(l) for 20 min at 30 °C. Sample buffer (50 μl of 2x SDS-DTT) was added to each sample which was then boiled for 10 min. The reactions were flash spun (5 s), loaded on a polyacrylamide gel (35 μl/ sample) and phosphorylated histone H1 was separated by 12 % SDS-PAGE. Radioactivity was detected by autoradiography and quantitated by densitometry.

### 3.12 p35 Protein Stability Experiments

M059J human glioma cells were plated at a density of 5 x $10^5$ cells per 75 $cm^2$ plate and grown for three days. Test cultures were treated with cycloheximide (Sigma, final concentration of 75 μg/ml) for 4 h, 8 h, 24 h, or 36 h. Following treatment, cells were lysed as described in Section 3.9 and proteins separated by

running equal amounts of protein on 12% SDS-polyacrylamide gels at 200 V for approximately 50 min. Proteins were then transferred (1 h at 100 V) to a nitrocellulose membrane (Hybond-C extra, Amersham). The loading and transfer of equivalent levels of protein was confirmed by staining with Ponceau S (Sigma). The membranes were blocked by overnight incubation in a solution of 5% dry milk in TBS (2 M Tris HCl, pH 7.5) and then probed with the p35 antibody for 2 h, followed by HRP-conjugated goat anti-rabbit immunoglobulin for 1 h. The immunocomplex was detected by chemiluminescence (ECL, Amersham or SuperSignal, Pierce) and autoradiography (Kodak XAR film). Autoradiographs were quantitated by densitometric analysis.

# IV. RESULTS

## 4.1 Expression and Localization of p35 and Cdk5

### 4.1.1 Immunohistochemical Analysis of Cdk5 in Non-Malignant Human Brain

The Cdk5 protein expression in four human non-malignant brain tissue specimens was examined by immunohistochemical staining. The formalin-fixed, paraffin-embedded tissues were obtained from Dr. Chunhai Hao, Department of Laboratory Medicine - University of Alberta Hospitals, and represented excess pathological material. Acquisition of this material was in accordance with the guidelines established by the Human Ethics Review Board. The pathological description of these tissues is provided in Table 1.

| Pathology | | |
|---|---|---|
| cortical dysplasia | surgical | cortex / subcortical white matter |
| cortical dysplasia | surgical | hippocampal formation |
| residual ulygyria | surgical | hippocampal formation |
| hypoxic ischemic encephalopathy | autopsy | hippocampal formation |

Table 1. Summary of non-malignant tissue analyzed for Cdk5 immunoreactivity.

Cdk5 immunoreactivity was detected in the form of fine dark red grains in all samples examined. Upon examination of the hippocampal formation in three non-malignant brain sections at higher magnification, Cdk5 immunoreactivity was detected in the pyramidal cell layer in CA1, CA2, CA3 and CA4 as well as the granular layer of the dentate gyrus (shown in Fig. 4a, top right corner). Cdk5 was absent from white matter (Fig. 4a, bottom left corner). At a subcellular level, both cell bodies and dendrites of pyramidal cells (Fig. 4c) and granular cells were positively stained for Cdk5. In contrast, no positively stained axons in either the grey matter or white matter of the hippocampus were detected.

The expression pattern of Cdk5 in the neocortex and subcortical white matter revealed comparable Cdk5 immunoreactivity in neurons of all six cortical layers. Cdk5 was localized to cell bodies (including both the cytoplasm and nucleus), and dendrites, but was absent from the subcortical white matter composed predominantly of axonal pathways. The lateral geniculate nucleus, a component of the thalamus, observed in the autopsy slide of hypoxic ischemic encephalopathy, displayed a similar Cdk5 staining pattern where neurons of all six layers were positively stained and axon bundles were negative.

In all non-malignant brain tissue specimens examined, Cdk5 immunoreactivity was absent from glial cells in both the predominantly axonal white matter and the predominantly neuronal grey matter. As Fig. 4b shows, no oligodendrocytes or astrocytes displayed positive Cdk5 staining in the neocortex, subcortical white matter or hippocampus.

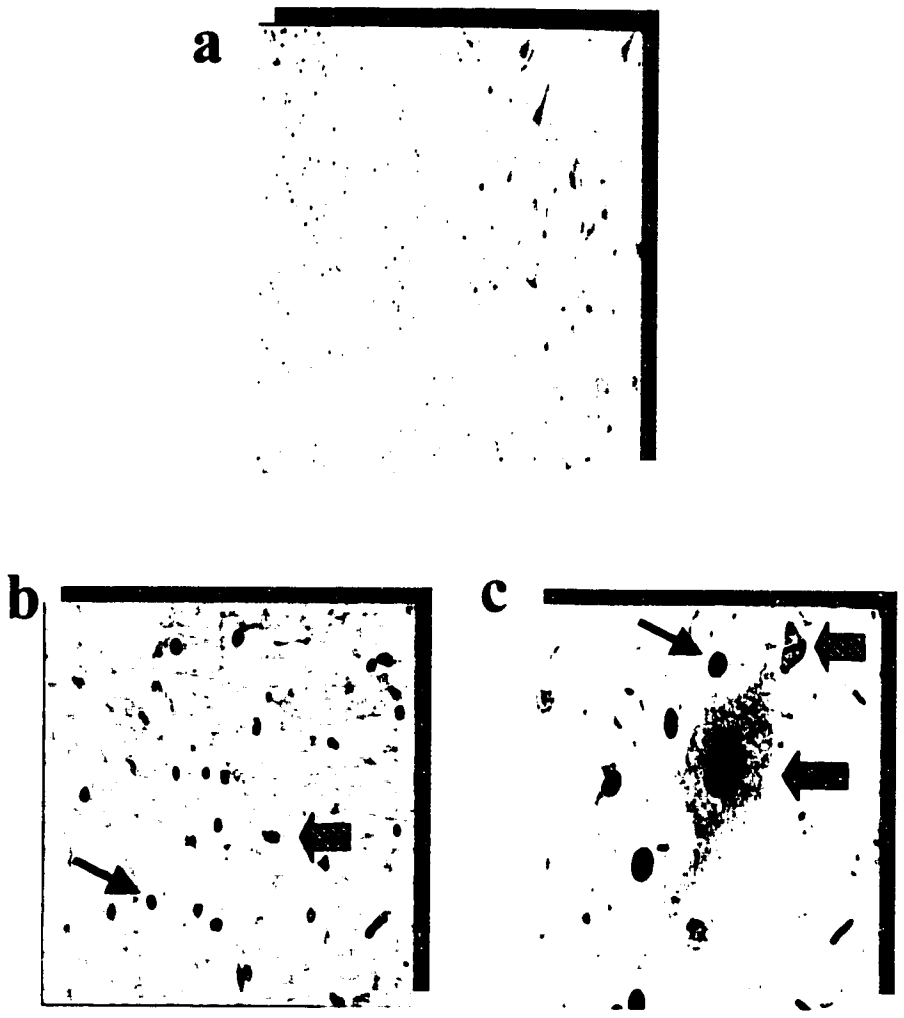


Fig. 4. Cdk5 immunoreactivity in non-malignant brain tissue. Paraffin-embedded tissue sections were stained using Fast red substrate (red stain) and counterstained with hematoxylin (purple stain). (a) Low power view of residual ulygyria hippocampus demonstrates positively stained grey matter (top right corner) and negatively stained white matter (bottom left corner). (b) High power magnification of white matter from hypoxic ischemic hippocampal tissue indicates both astrocytes (striped arrow) and oligodendrocytes (thin arrow) are devoid of Cdk5 immunoreactivity. (c) High power magnification of Cdk5-positive pyramidal cells (green block arrow) and Cdk5-negative astrocytes (striped arrow) and oligodendrocytes (line arrow) from the same hypoxic ischemic hippocampal tissue section as in (b).

### 4.1.2 Immunohistochemical Analysis of Cdk5 and p35 in Human Astrocytic Tumors

The expression profiles of Cdk5 and p35 proteins were examined by immunohistochemistry in 12 human astrocytic tumor specimens of varying histological grades (one Grade I, three Grade II, four Grade III and six Grade IV). Because of intra-tumoral heterogeneity, adjacent sections of several regions of the same tumor were examined for Cdk5 and p35 immunoreactivity as described in Table 2.

Cdk5 and p35 expression was detected in all 12 tumors examined. Within positively stained neoplastic cells, Cdk5 and p35 were detected in both the nucleus and the cytoplasm of astroglial cells, with preferential cytoplasmic labeling for both Cdk5 and p35. In general, Cdk5 staining was much more intense than p35 staining. Other cell types which exhibited positive Cdk5 immunoreactivity included lymphocytes within the vascular proliferative areas, neutrophils and macrophages.

Cdk5 and p35 immunoreactivity produced a weak correlation with the tumor grade. However, Cdk5 staining in grade II-IV astrocytomas (Fig. 5b) was consistently stronger than Cdk5 immunoreactivity in a Grade I pilocytic astrocytoma (Fig. 5a). Although the pattern of immunoreactivity of both p35 and Cdk5 varied considerably from neoplasm to neoplasm and even within a single tumor, intense Cdk5 staining was preferentially observed in tumor cells surrounding large areas of necrosis in half (3/6) of the Grade IV glioblastoma specimens (reviewed in Table 2). This intense Cdk5 staining was not found in pseudopalisading cells surrounding focal

| Grade | Tumor | Sex | Age | # regions examined per tumor | p35 staining | Cdk5 staining | Intense Cdk5 in peri-necrotic |
|---|---|---|---|---|---|---|---|
| I | 1 | F | 7 | 1 | - | none to very weak | N |
| II | 1 | F | 24 | 2 | none to weak | very weak to weak | N |
| | 2 | *M | 7 | 7 | very weak to medium | very weak to strong | N |
| | 3 | *M | 7 | 4 | very weak to medium | weak to medium | N |
| III | 1 | F | 73 | 5 | weak | medium | N |
| | 2 | M | 39 | 5 | none to medium | weak to medium | N |
| IV | 1 | ×F | 40 | 10 | very weak to strong | very weak to strong | N |
| | 2 | ×F | 41 | 4 | absent to strong | absent to strong | Y |
| | 3 | M | 43 | 3 | absent to strong | very weak to strong | N |
| | 4 | M | 69 | 7 | weak to medium | weak to strong | Y |
| | 5 | +M | 45 | 11 | absent to strong | very weak to strong | N |
| | 6 | +M | 45 | 5 | absent to strong | very weak-strong | Y |

Table 2. Summary of Cdk5 and p35 protein expression profiles in human glioma biopsy specimens. Each symbol (*, ×, +) indicates two tumor biopsies taken at different times from the same patient.

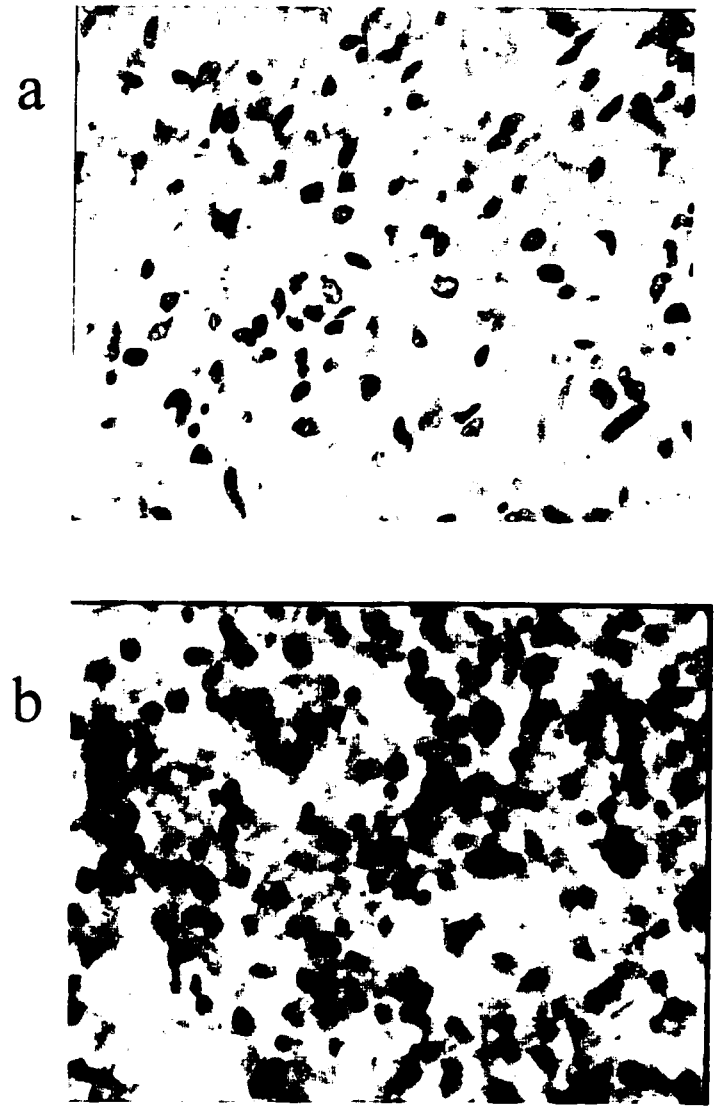


Fig. 5. Cdk5 immunoreactivity in varying grades of human astrocytomas. Paraffin-embedded biopsy tissue sections of (a) a pilocytic astrocytoma and (b) a Grade III anaplastic astrocytoma were immunostained with anti-Cdk5 (C-8) antibody.

necrosis. Although p35 immunoreactivity was observed diffusely without preferential perinecrotic distribution, intense p35 staining was observed in rare, isolated cells located within these areas.

### 4.1.3 Subcellular Localization of p35 and Cdk5 in M059J Glioma Cells

M059J glioma cells were seeded onto glass coverslips and examined 24 h later for p35 and Cdk5 protein distribution using confocal microscopy. p35 protein was detected by indirect immunofluorescence labeling using a polyclonal antibody which recognized an epitope in the C-terminus of p35 (C-19), followed by anti-rabbit IgG-Alexa 488. The resulting staining pattern displayed a lattice-like distribution similar to that of cytoskeletal proteins in both extended glioma cells (Figs. 6 & 7) and mitotic cells (Fig. 8). p35 immunoreactivity was predominantly cytoplasmic (Figs. 6,7 & 8) but was absent from short glial processes (Fig. 8). p35 was also detected in the nucleus of some cells as seen in a cross sectional view through the nucleus of a p35 immunostained M059J cell (Fig. 9). The staining pattern generated by this antibody was disrupted by neutralization with purified peptide prior to staining (Fig. 10).

Cdk5 immunoreactivity in cultured M059J cells was examined by indirect immunofluorescence using two different antibodies: (i) a polyclonal antibody corresponding to an epitope in the C-terminus of Cdk5 (C-8) followed by anti-rabbit IgG-Cy3 (red) and (ii) a monoclonal antibody raised against a recombinant full-length human Cdk5 protein (Upstate Biotechnology) followed by anti-mouse IgG-Cy5 (red). Although both antibodies detected Cdk5 as small granules within the cytoplasm and nucleus, they differed with respect to the relative fluorescent intensities between the cytoplasm and the nucleus. The monoclonal antibody produced stronger staining in the cytoplasm (Fig. 11). While Cdk5 was detected within the nuclear structure, it was absent from nucleoli (Fig. 12). On the other hand, the staining with polyclonal antibody was ubiquitous throughout the cytoplasm and nucleus (Fig. 13) with nuclear

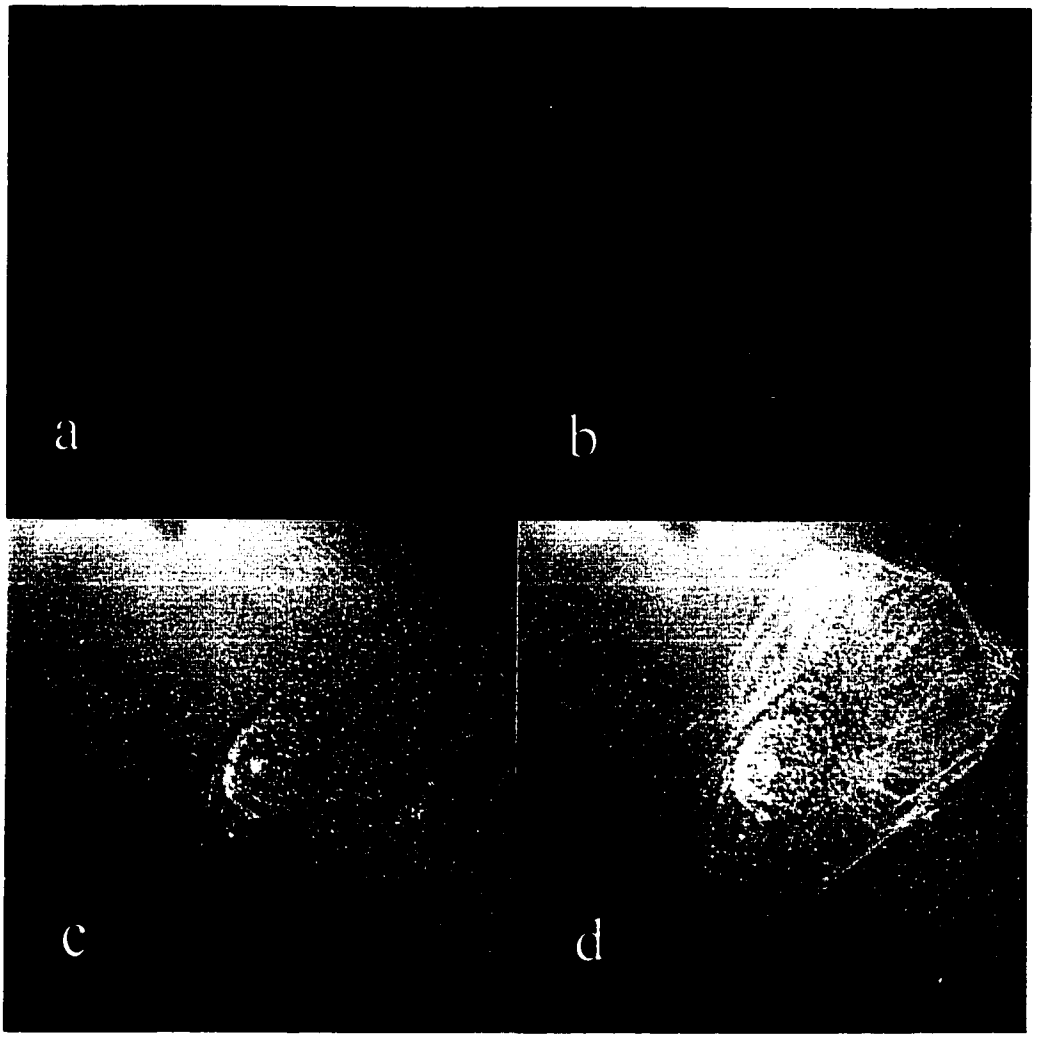


Fig. 6. Confocal analysis of the subcellular distribution of p35 protein in mature M059J glioma cells. Cells were double immunolabeled using (a) Hoechst 33258 DNA stain (blue) and (b) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

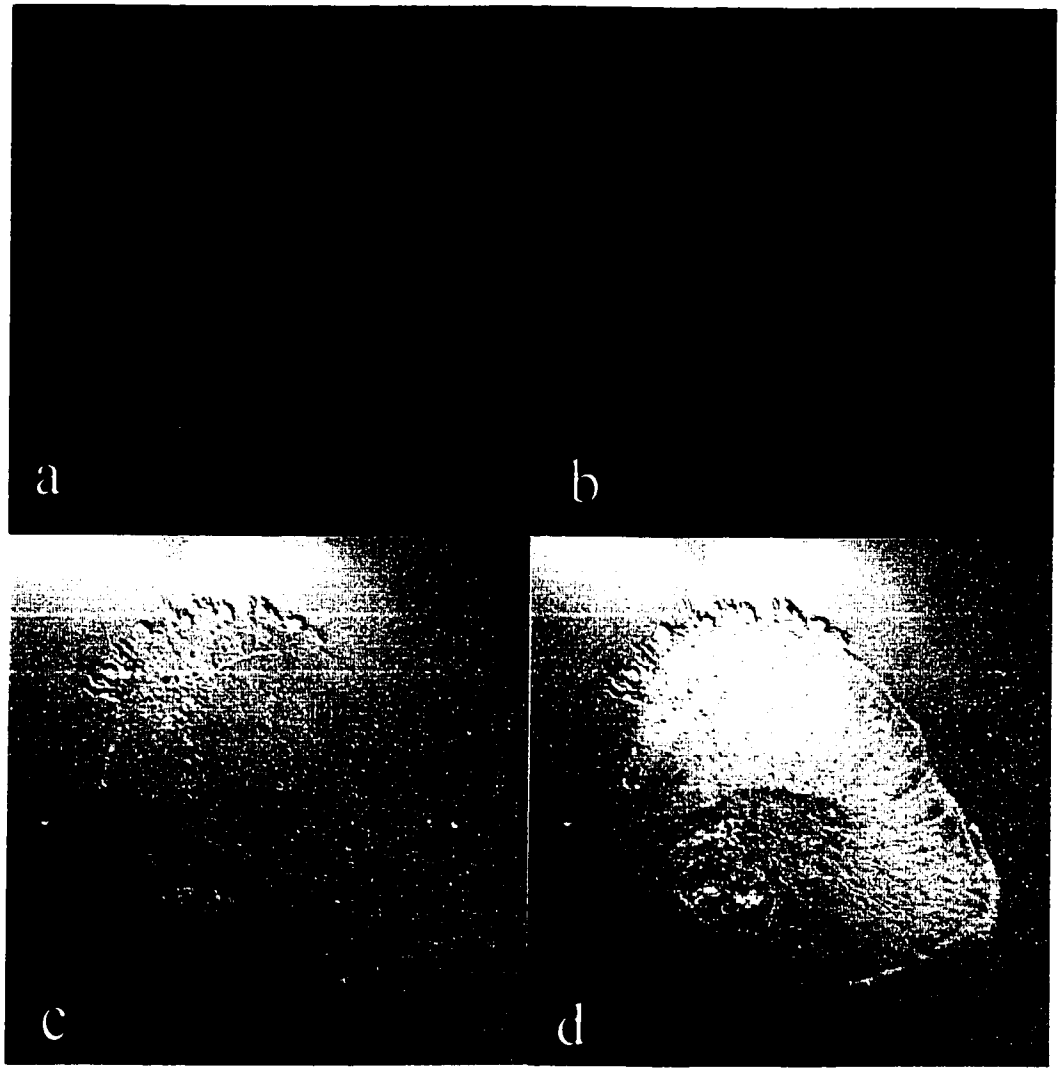


Fig. 7. Confocal analysis of the subcellular distribution of p35 protein in mature M059J glioma cells. Cells were double immunolabeled using (a) Hoechst 33258 DNA stain (blue) and (b) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

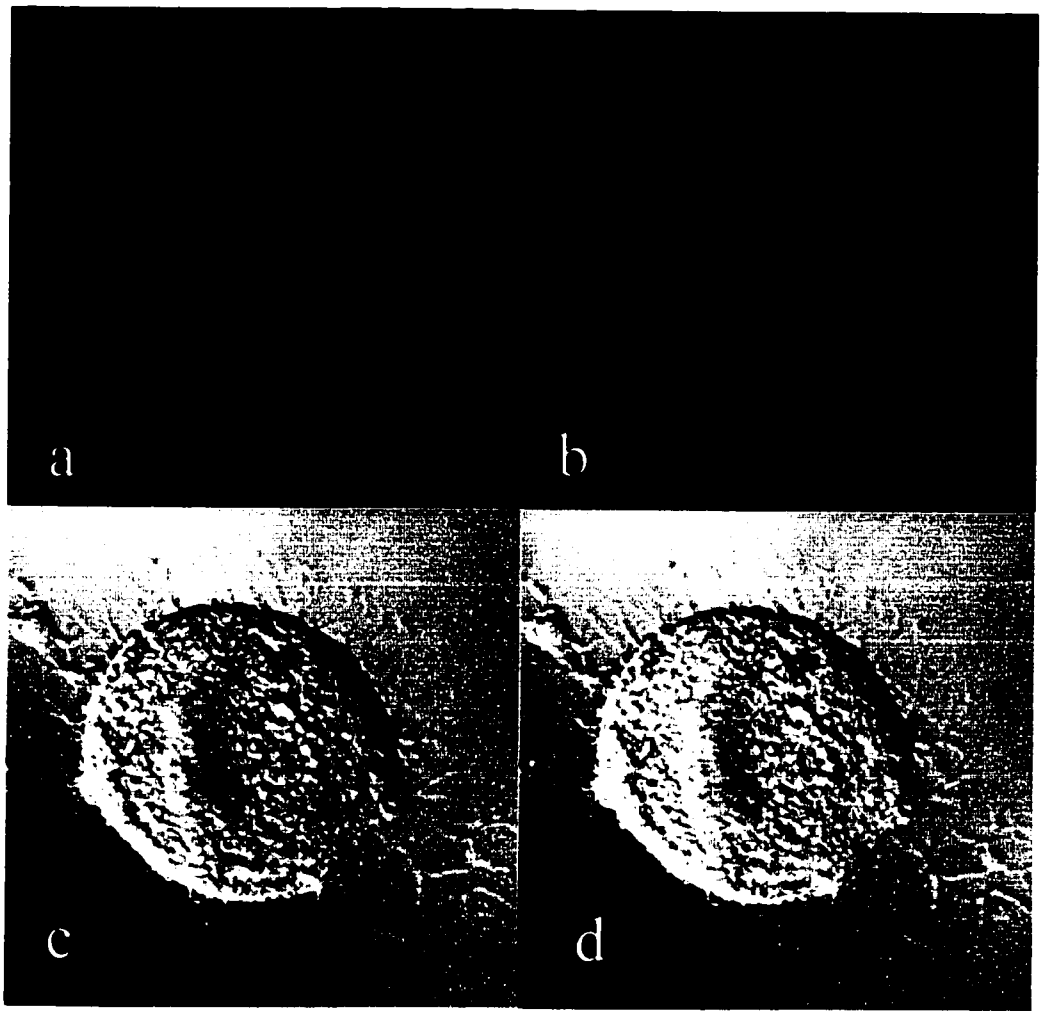


Fig. 8. Confocal analysis of the subcellular distribution of p35 protein in mitotic M059J glioma cells. Cells were double immunolabeled using (a) Hoechst 33258 DNA stain (blue) and (b) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b,and c) is shown in (d).

Fig. 9. p35 nuclear immunostaining of M059J glioma cells. This figure depicts the cross-sectional view through the cell. p35 staining (green) is present within both the nuclear structure (red stain) and the cytoplasm as indicated by the arrow.

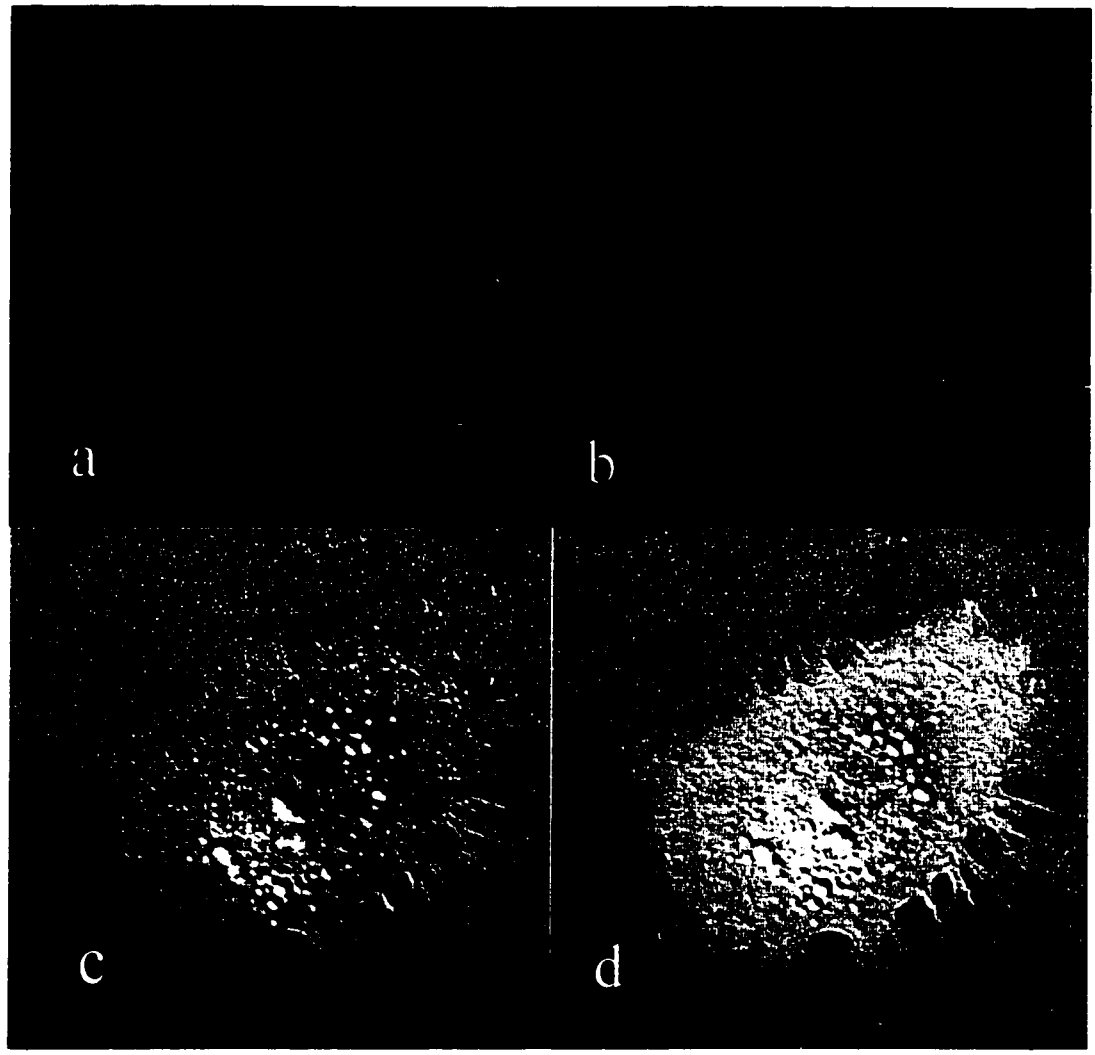


Fig. 10. Effect of control peptide on p35 immunostaining in M059J glioma cells. Following preincubation of antibodies with control peptide, cells were double immunolabeled using (a) Hoechst 33258 DNA stain (blue) and (b) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

concentration observed in some cells. The staining intensity with C-8 antibody was significantly reduced by pre-incubation with purified control peptide (Fig. 14) indicating the specificity of the antibody towards the targeted epitope.

To colocalize p35 and Cdk5 proteins in the same glioma cell, M059J cells were stained using p35 (C-19) and Cdk5 (Upstate) antibodies, followed by anti-rabbit-Alexa 488 (green) and anti-mouse-Cy5 (red) secondary antibodies, respectively. As expected, both p35 and Cdk5 proteins were detected within the same glioma cells and retained the staining patterns observed with single labeling experiments in mature, extended cells (Fig. 15) and in mitotic cells (Fig. 16). Fig. 17 shows a high powered view of the glial process from the same cell in Fig. 15 and illustrates p35 and Cdk5 protein expression along the length of glial extensions as well as the tip of these processes.

During early cytokinesis, as cells are beginning to separate, Cdk5 staining was diffusely cytoplasmic (Fig. 18b) whereas p35 produced a focal staining point located at the periphery of the hilum of the kidney-shaped nuclei (Fig. 18d). This staining pattern was visualized in all rounded cells of M059J cultured cell preparations. Strong Cdk5 staining in the midbody was apparent later during cytokinesis (Fig. 19a), with very faint p35 immunoreactivity along the process separating the two cells (Fig. 19c).

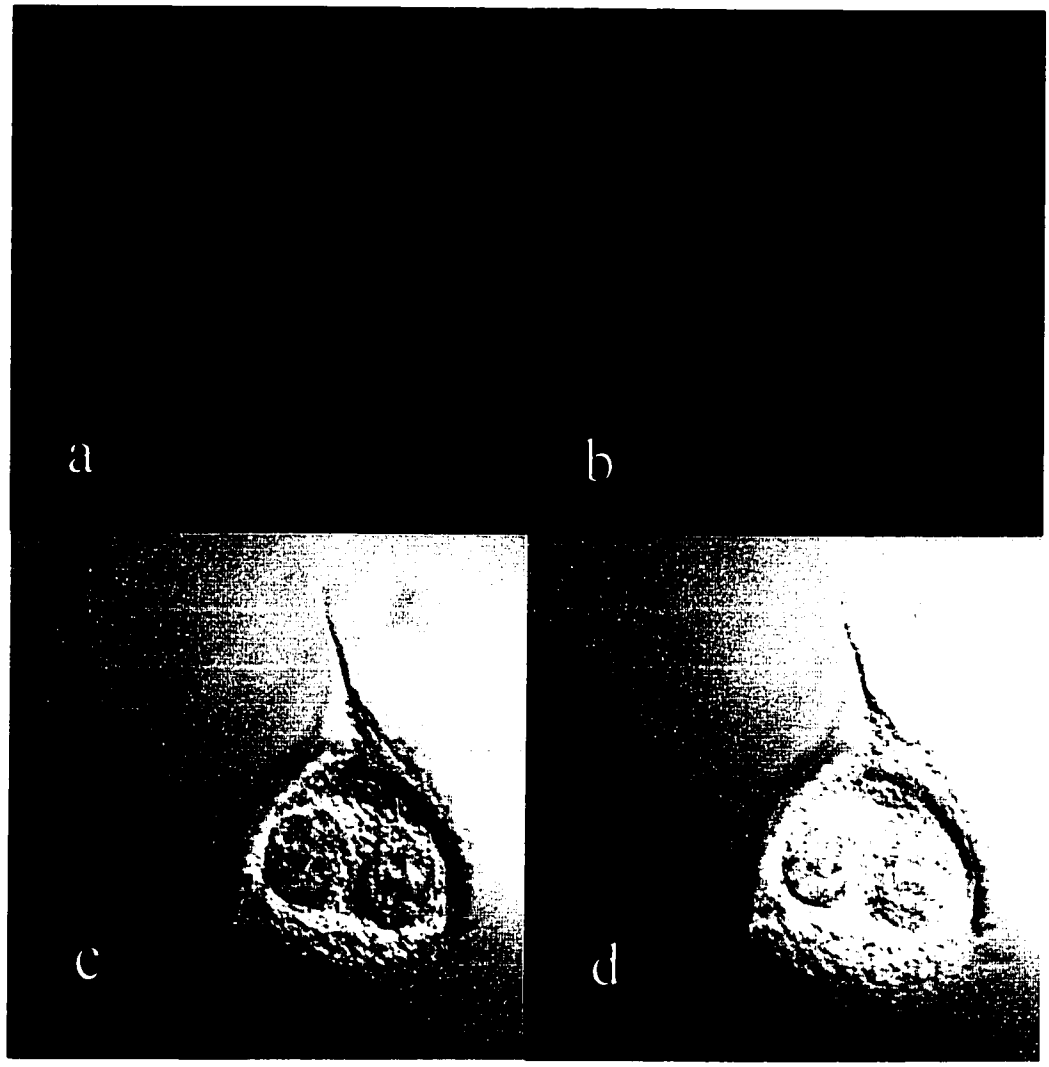


Fig. 11. Confocal analysis of the subcellular distribution of Cdk5 protein in M059J glioma cells. Cells were double immunolabeled using (a) anti-Cdk5 (Upstate) antibodies (red) and (b) Hoechst 33258 DNA stain (blue). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

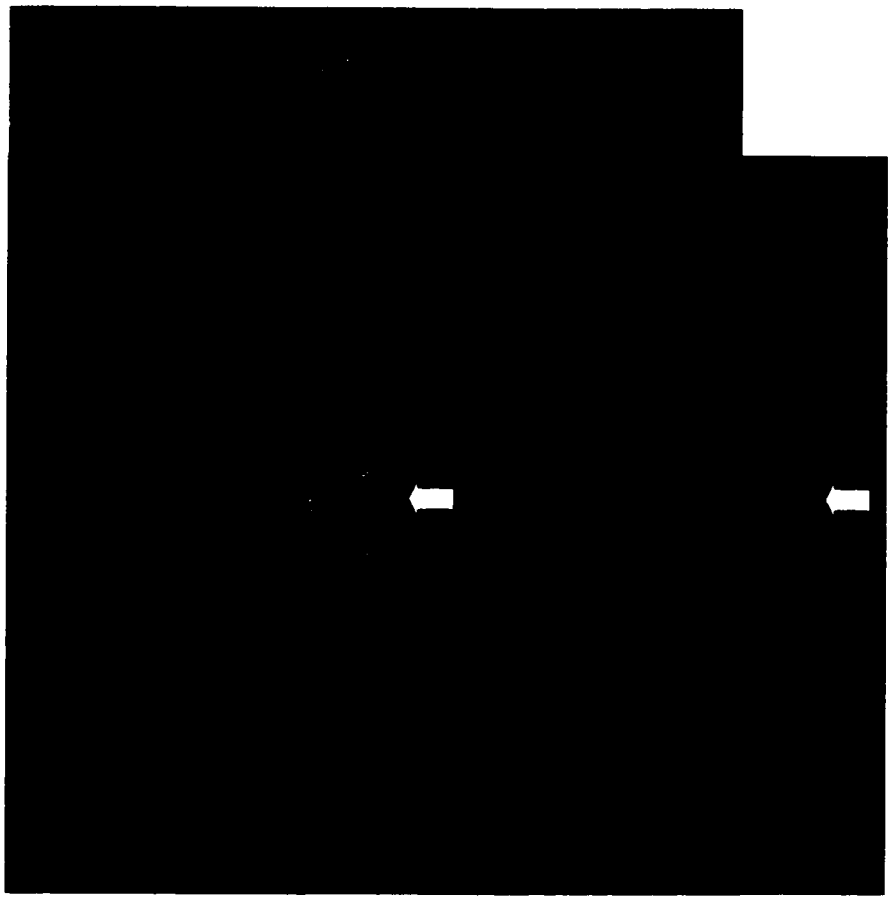

Fig. 12. Cdk5 nuclear immunostaining of M059J glioma cells. This figure depicts the cross-sectional view through the cell. Cdk5 staining (red) is present within the nuclear structure (green) indicated by the blue line arrow. Note the absence of Cdk5 stain within the nucleoli (pink block arrow).

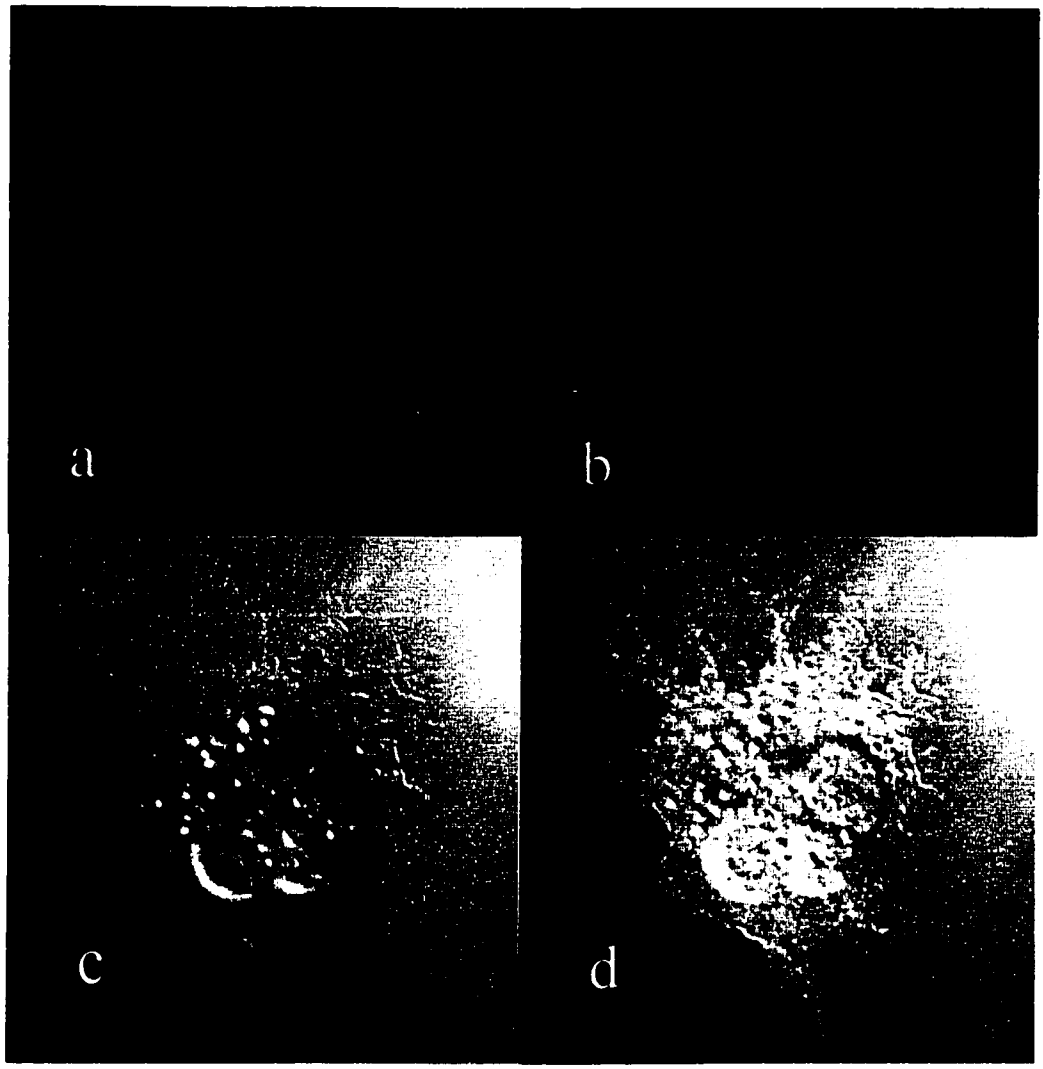


Fig. 13. Confocal analysis of the subcellular distribution of Cdk5 protein in a M059J glioma cell. Cells were double immunolabeled using (a) anti-Cdk5 (C-8) antibodies (red) and (b) Hoechst 33258 DNA stain (blue). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

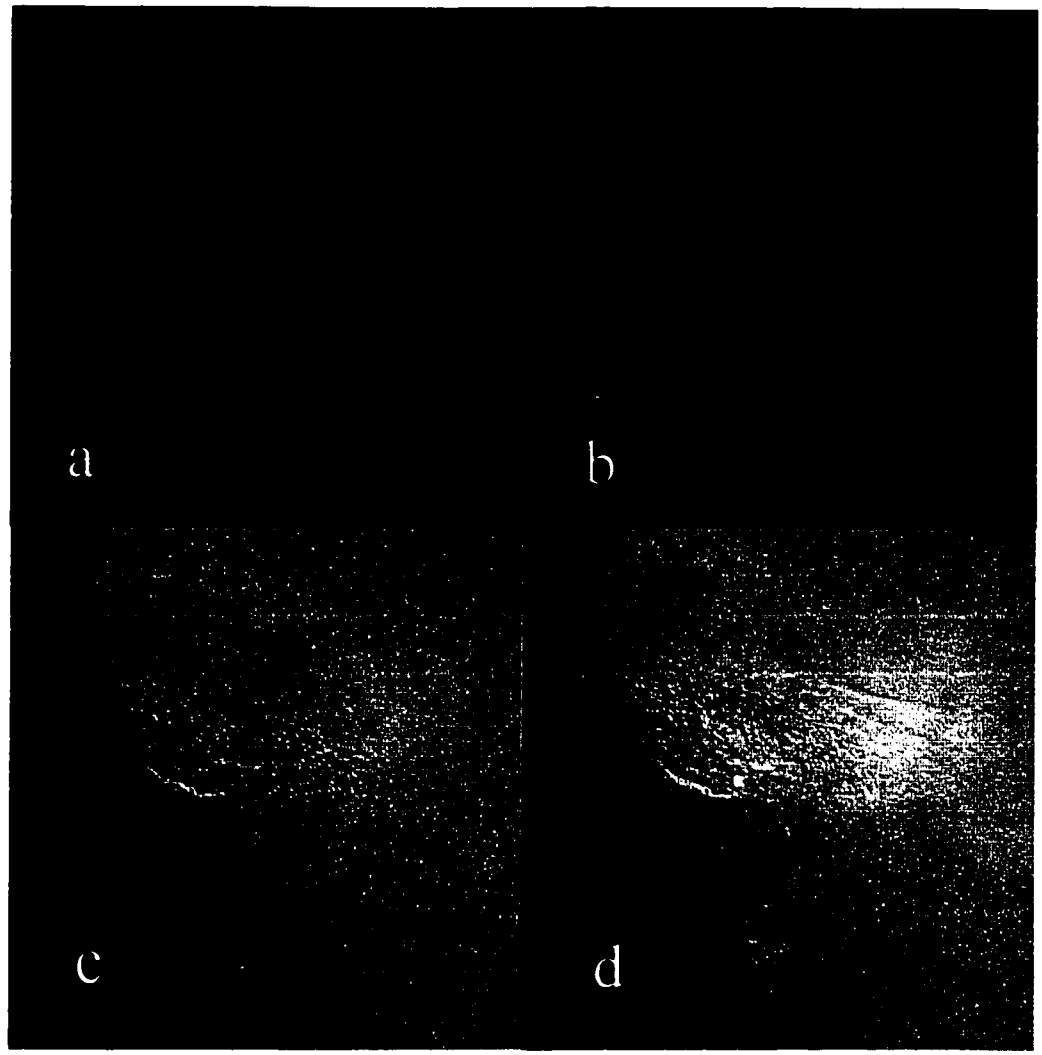


Fig. 14. Effect of control peptide on Cdk5 (C-8) immunostaining in M059J glioma cells. Following preincubation of antibodies with control peptide, cells were double immunolabeled using (a) anti-Cdk5 (C-8) antibodies (red) and (b) Hoechst 33258 DNA stain (blue) . The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

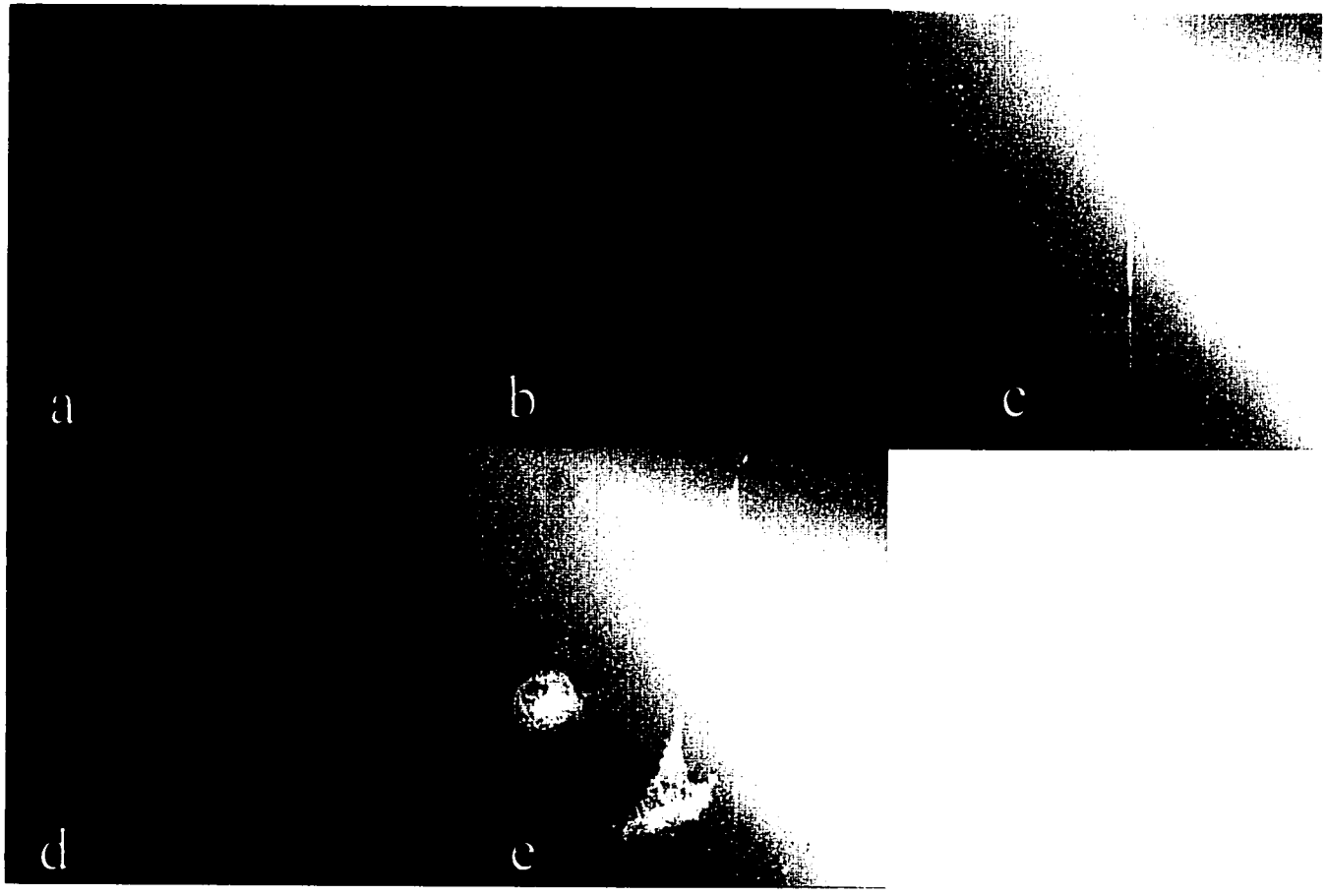


Fig. 15. Colocalization of p35 and Cdk5 in mature M059J glioma cells. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-Cdk5 (C-8) antibodies (red) and (d) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all four images (a,b,c and d) is shown in (e).

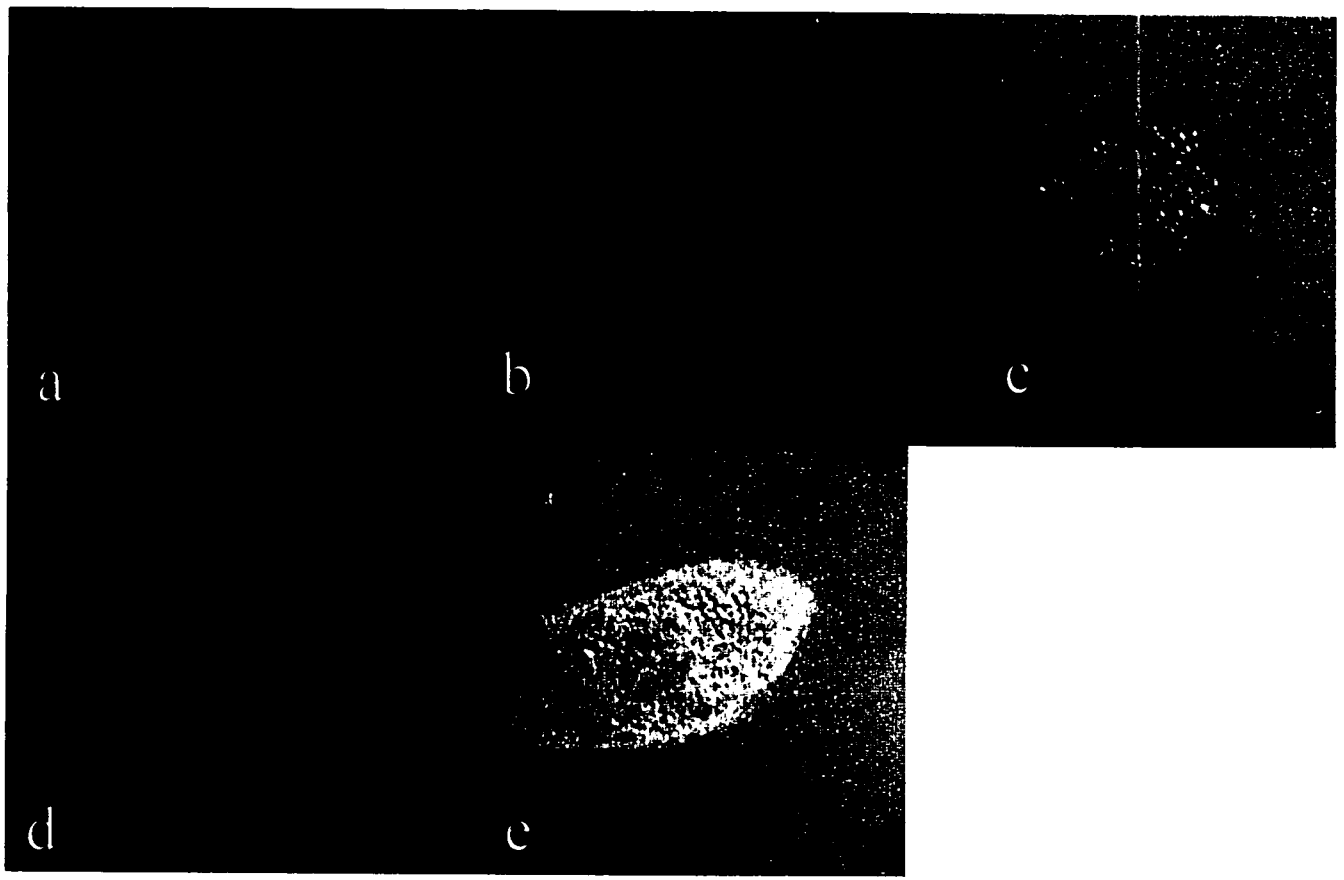


Fig. 16. Colocalization of p35 and Cdk5 in mitotic M059J glioma cells. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-Cdk5 (C-8) antibodies (red) and (d) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c ) the brightfield image. An overlay of all four images (a,b,c and d) is shown in (e).

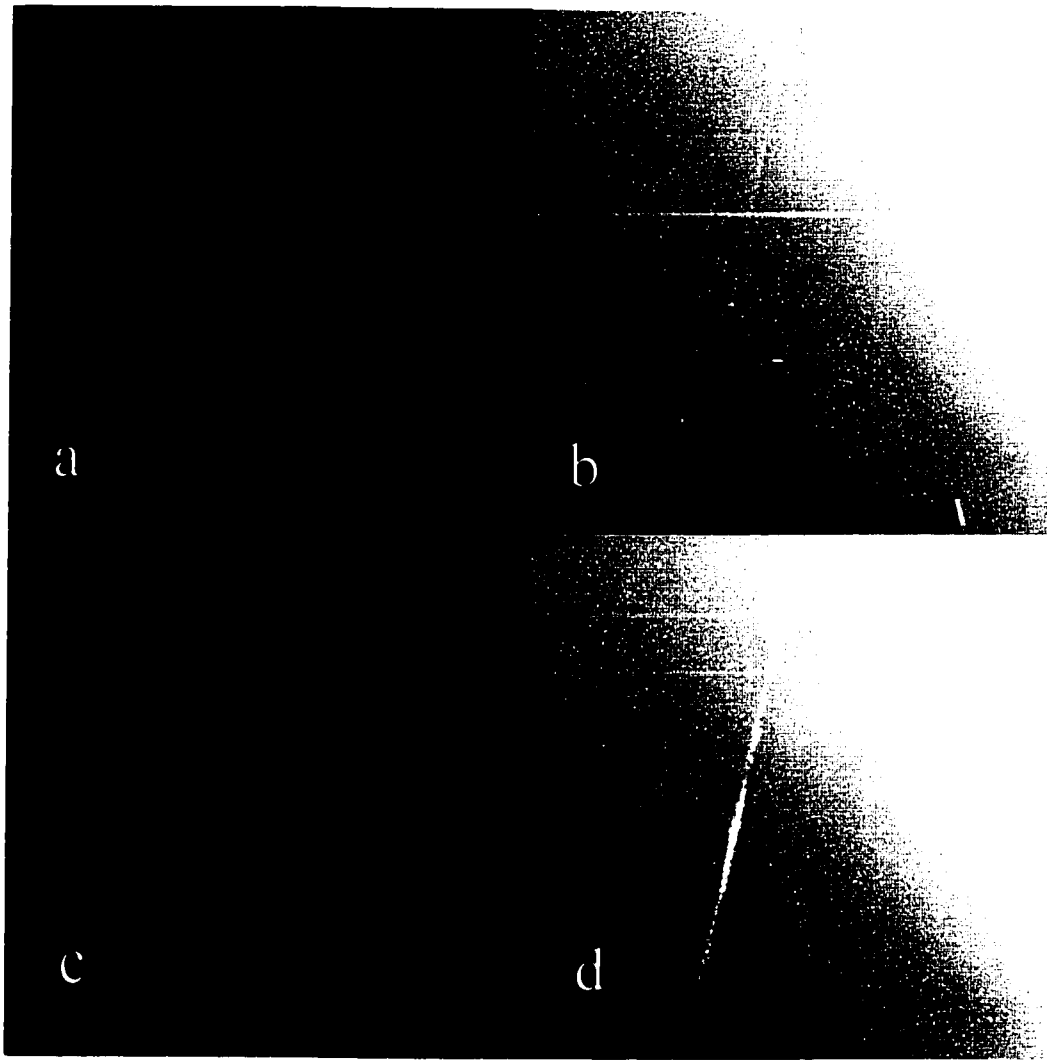


Fig. 17. Colocalization of Cdk5 and p35 in glioma cell process extensions. M059J glioma cells were double immunolabeled using (a) anti-Cdk5 (C-8) (red) and (c) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (b) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

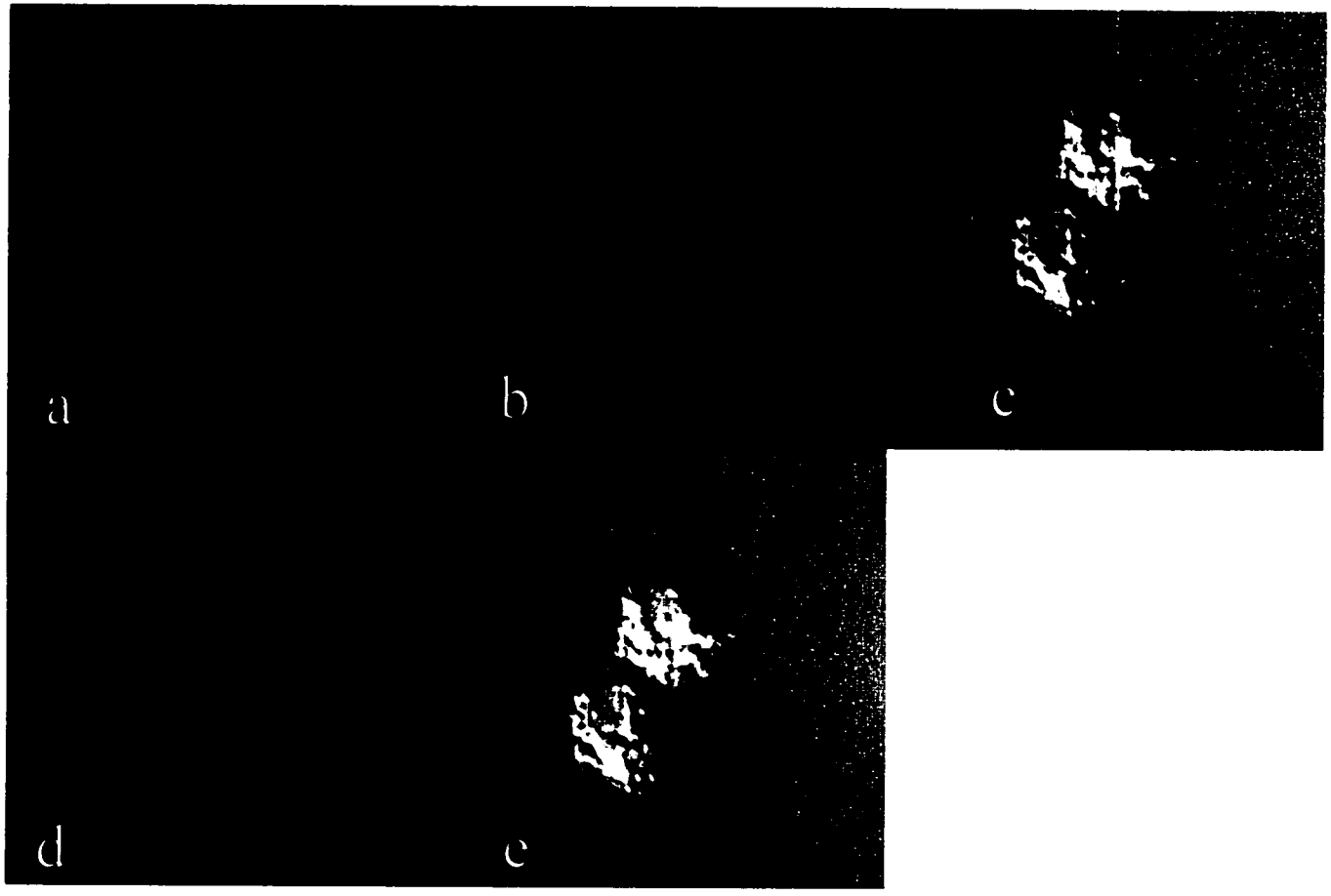


Fig. 18. Colocalization of p35 and Cdk5 in M059J glioma cells during cytokinesis. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-Cdk5 (C-8) antibodies (red) and (d) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c ) the brightfield image. An overlay of all four images (a,b,c and d) is shown in (e).

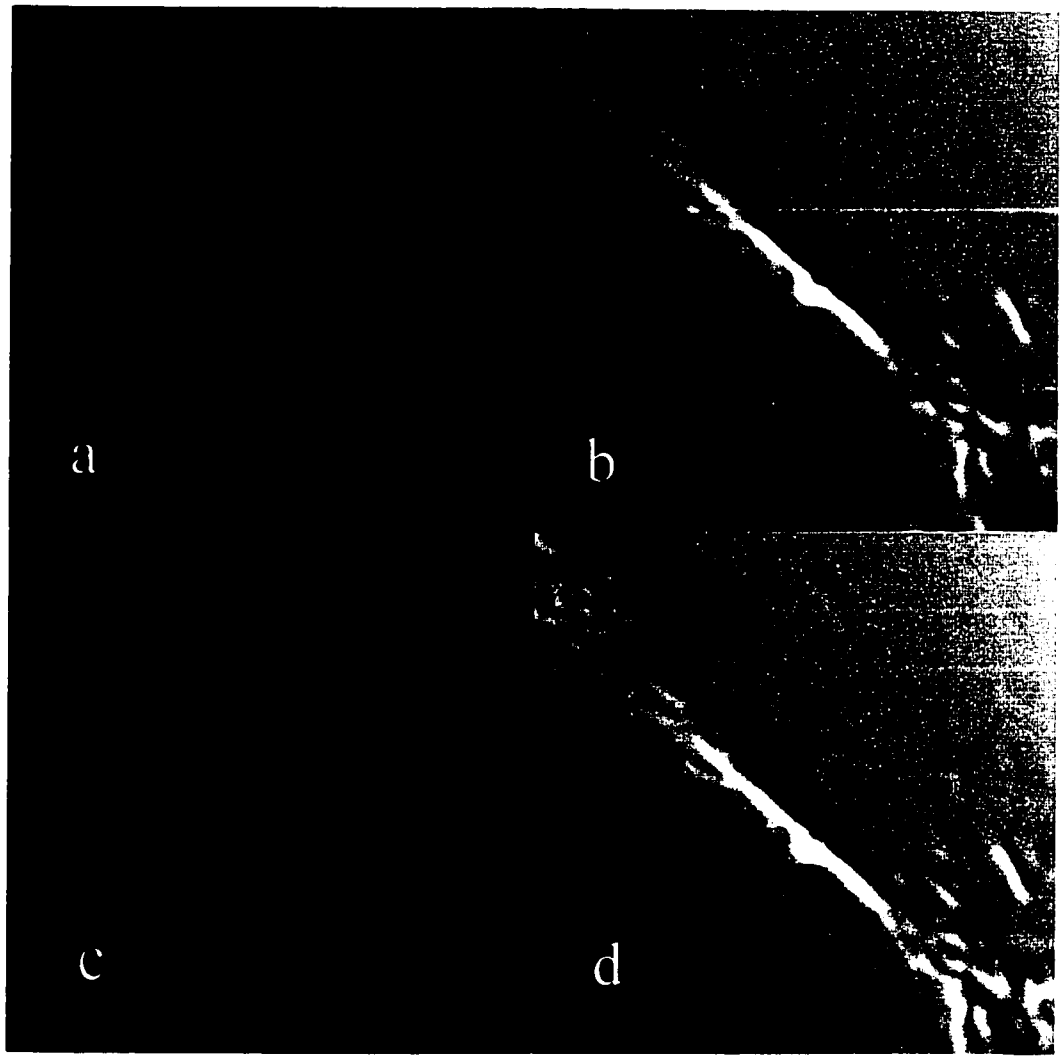


Fig. 19. Colocalization of Cdk5 and p35 in the midbody of M059J glioma cells during cytokinesis. Cells were double immunolabeled using (a) anti-Cdk5 (C-8) (red) and (c) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (b) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

### 4.1.4 Double Immunofluorescent Staining of p35 and α-Tubulin in Untreated and Microtubule-Disrupted Glioma Cells

To assess the possible association of p35 with the microtubule cytoskeleton in M059J cultured glioma cells, cells were double-labeled with p35 (C-19) and anti-α-tubulin antibodies followed by anti-rabbit-Alexa 488 (green) and anti-mouse-Cy3 (red) secondary antibodies, respectively. The results indicate that p35 immunolabeling colocalizes with the microtubule structure as seen in Figs. 20e, 21e and 22e.

To further examine the relationship between p35 and microtubules, nocodazole, an inhibitor of microtubule assembly, was used to treat M059J cells prior to double immunofluorescent labeling of p35 and tubulin. Nocodazole (2.5 μg/ml) treatment resulted in the expected disassembly of the microtubule network as exhibited by a non-specific, diffuse cytoplasmic tubulin staining pattern (Fig. 23a). The normal, lattice-like staining pattern of p35 was also disrupted by nocodazole and displayed a speckled appearance within both the cytoplasm and the nucleus (Fig. 23b).

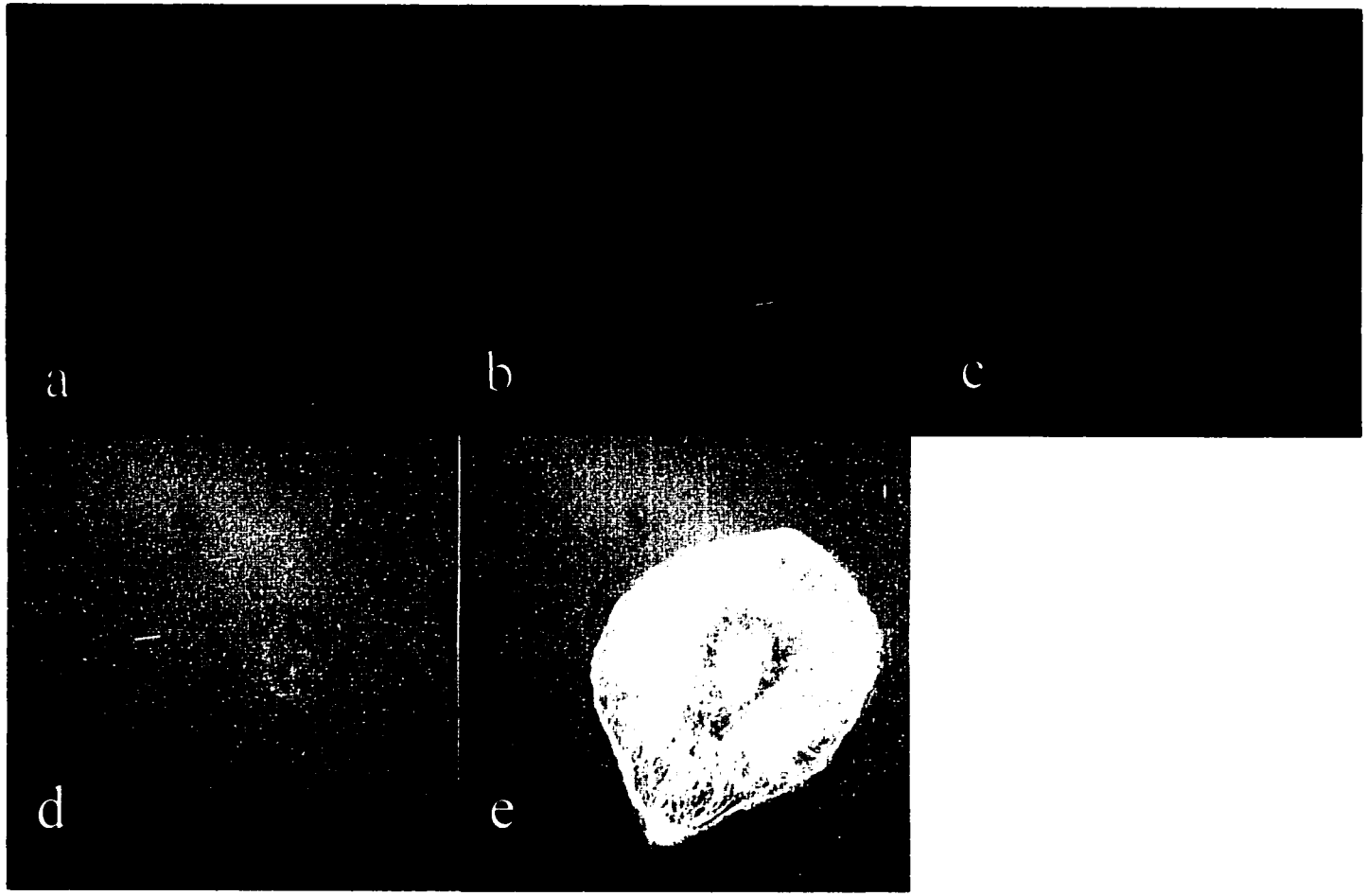


Fig. 20. Colocalization of p35 and α-tubulin in M059J glioma cells. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-α-tubulin antibodies (red) and (c) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (d) the brightfield image. An overlay of all four images (a,b,c and d) is shown in (e) where colocalization of p35 and α-tubulin is depicted by a yellow color.

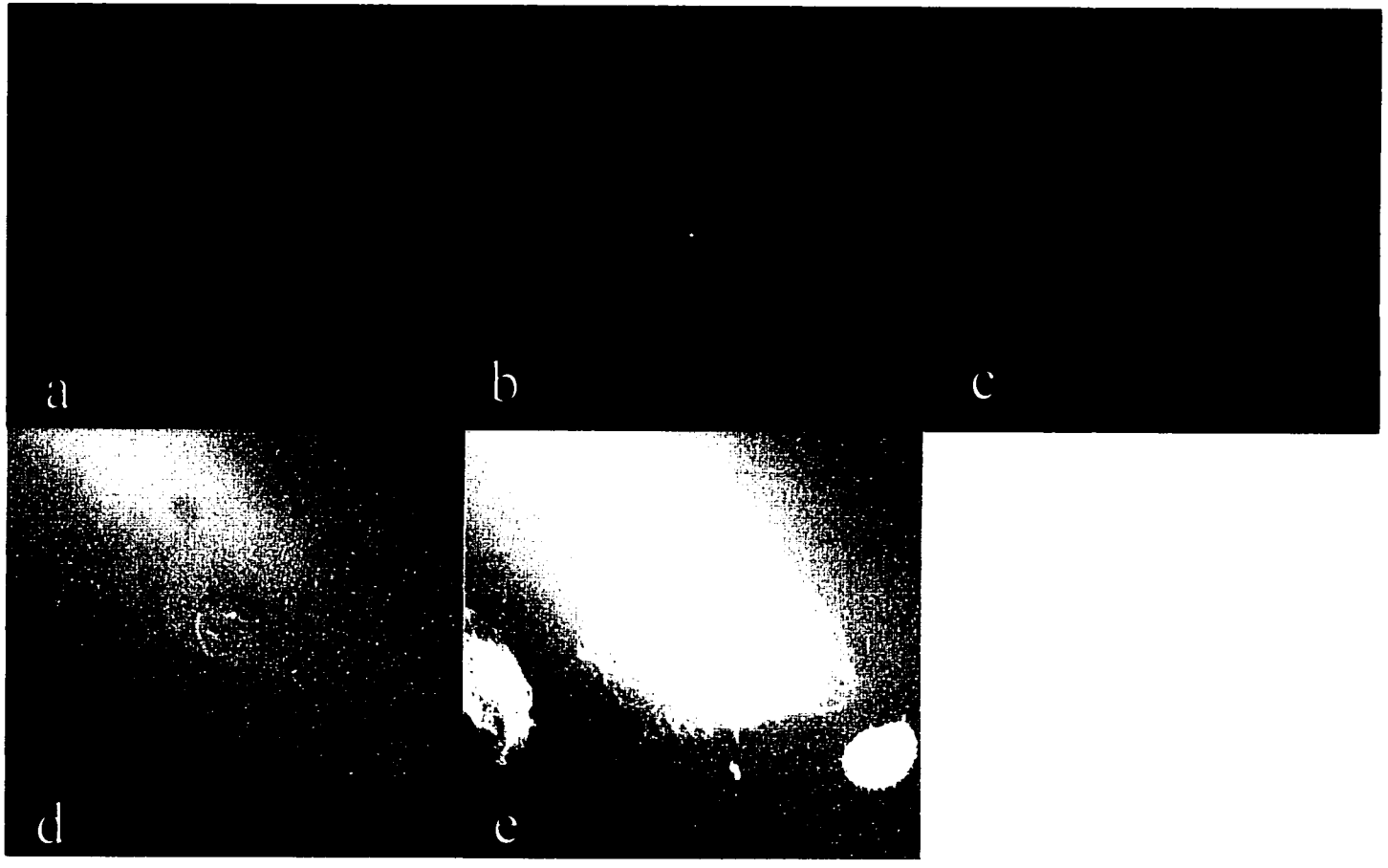


Fig. 21. Colocalization of p35 and α-tubulin in M059J glioma cells. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-α-tubulin antibodies (red) and (c) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (d) the brightfield image. An overlay of all four images (a,b,c and d) is shown in (e) where colocalization of p35 and α-tubulin is depicted by a yellow color.

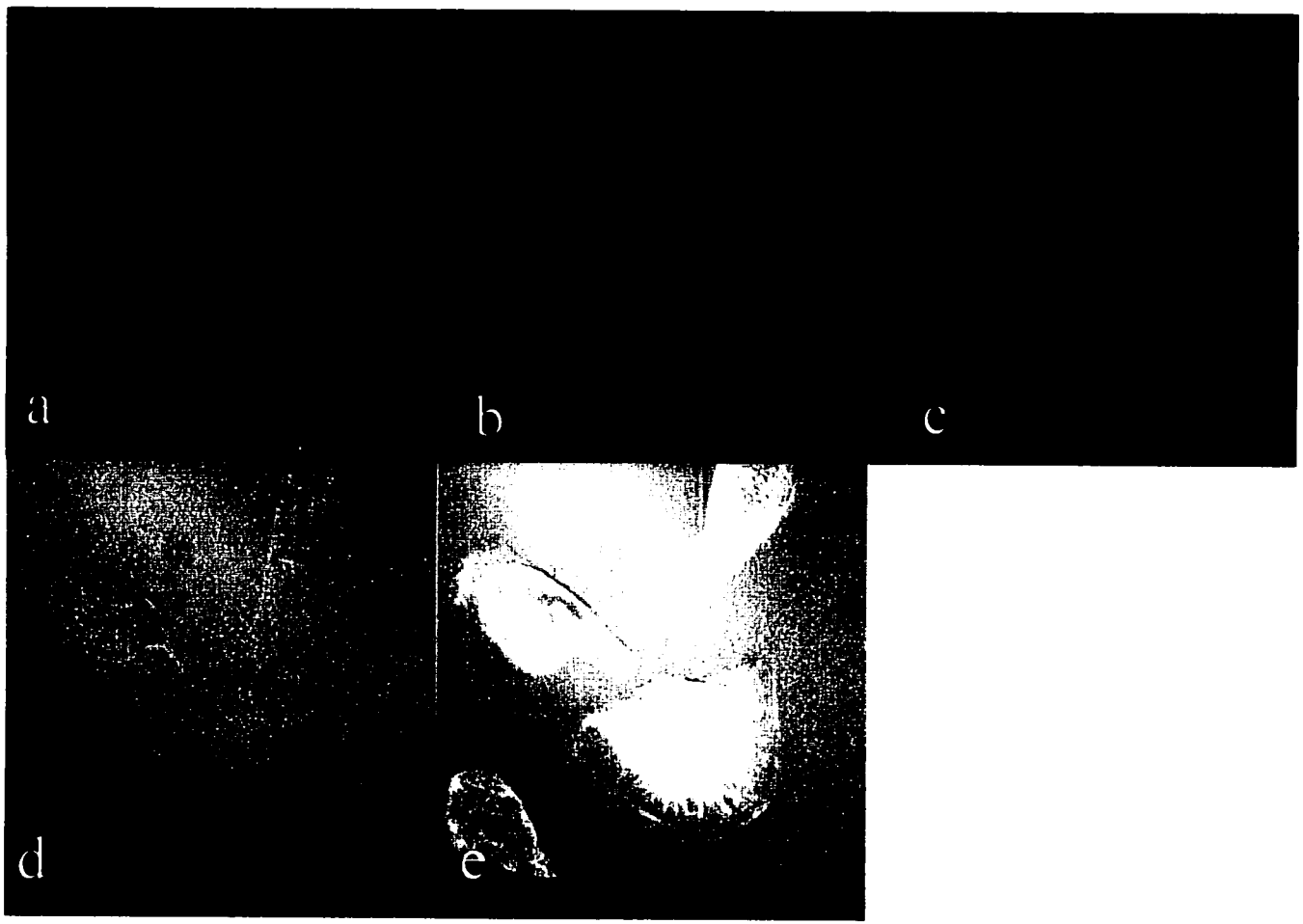


Fig 22. Colocalization of p35 and α-tubulin in M059J glioma cells. Cells were triple labeled using (a) Hoechst 33258 DNA stain (blue), (b) anti-α-tubulin antibodies (red) and (c) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (d) the brightfield image. An overlay of all four images (a, b, c and d) is shown (e) where colocalization of p35 and α-tubulin is depicted by a yellow color.

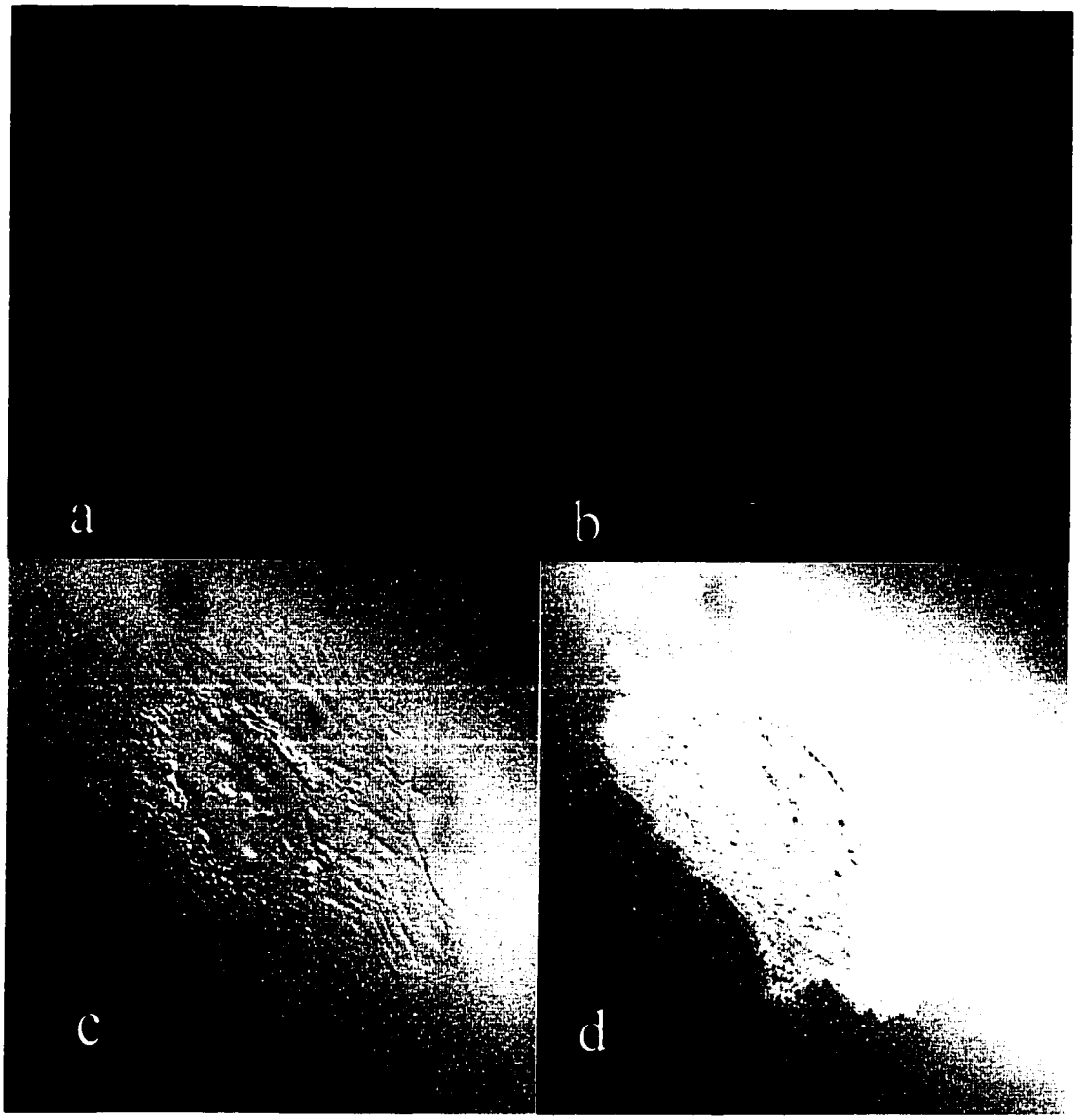


Fig. 23. p35 and α-tubulin immunostaining in nocodazole-treated M059J glioma cells. Cells were treated with nocodazole for 2 h, fixed and double immunolabeled using (a) anti-α-tubulin antibodies (red) and (b) anti-p35 (C-19) antibodies (green). The overall cell morphology is depicted in (c) the brightfield image. An overlay of all three images (a,b and c) is shown in (d).

## 4.2 Cdk5/p35 Kinase Involvement in Apoptotic Cell Death in Gliomas

### 4.2.1 Correlation of Cdk5 and p35 Protein Expression with Apoptosis in Human Glioma Biopsy Specimens

As noted in Section 3.1.2, a small subset of cells in high grade glioblastoma multiforme biopsy tissue specimens displayed morphological characteristics consistent with apoptosis as well as intense Cdk5 immunostaining. To confirm the apoptotic nature of the Cdk5-expressing cells, serial sections of the same glioblastoma multiforme biopsy specimens were analyzed *in situ* for evidence of DNA fragmentation. The DNA fragmentation assay revealed that a subset of cells exhibiting p35 protein expression (Fig. 24b) and intense focal distribution of Cdk5 protein (Fig. 24c) also showed evidence of chromatin fragmentation characteristic of apoptotic cells (Fig. 24d).

### 4.2.2 Confocal Analysis of CDK5/p35 Protein Distribution and Apoptosis

A fluorescent double-labeling technique for *in situ* DNA fragmentation and p35 protein detection was used to confirm the association between p35 protein and apoptosis in individual cells of the same human glioblastoma multiforme tumor biopsy specimen. This technique used FITC-labeled terminal d-UTP nick end labeling (TUNEL) to detect DNA fragmentation (green fluorescence) and Cy3-conjugated secondary antibody to detect p35 protein (red fluorescence). Although the TUNEL assay produced diffuse low background staining, individual apoptotic cells were identified by combining positive TUNEL labeling with overall morphological

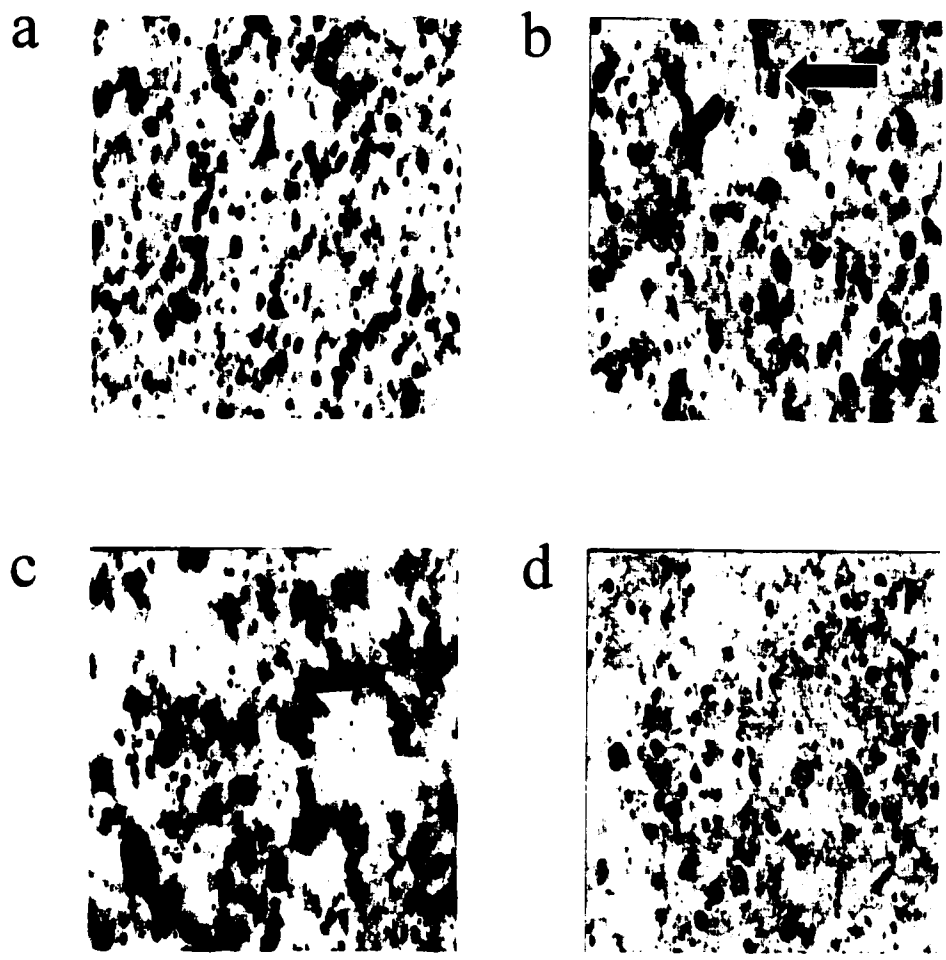


Fig. 24. Association of Cdk5 and p35 proteins with apoptosis in a human glioblastoma multiforme biopsy specimen. In (a), (b) and (c), tissue sections were stained using Fast Red substrate and counterstained with hematoxylin for nuclear identification (blue). Pink staining (green block arrow) or red staining (yellow line arrow) indicates positive (b) p35 or (c) Cdk5 staining. In (d), sections were treated with FragEl DNA fragmentation assay (brown is positive) and counterstained with methyl green for nuclear identification (green). (a) IgG control stain indicates background levels of staining. A similar subset of cells displaying (b) p35 and (c) Cdk5 staining are positive for (d) DNA fragmentation assay.

appearance of the nuclei visualized by Hoechst DNA staining. Individual cells were considered apoptotic when exhibiting both TUNEL-positive staining and condensed nuclei or compact, round Hoechst-positive material indicative of apoptotic bodies. Fig. 25 displays an individual apoptotic cell surrounded by viable cells within a tumor tissue. This same apoptotic cell, seen in the absence of the brightfield image in Fig. 26, exhibited positive TUNEL-staining (~ 20-fold greater intensity than viable cells) and met the morphological criteria as exemplified by the bead-like structures within the nucleus.

When biopsy tissue sections were triple-labeled with the TUNEL, p35 and Hoechst stains, individual apoptotic cells displaying positive TUNEL staining and an apoptotic morphology also exhibited intense p35 immunoreactivity. A typical apoptotic cell surrounded by viable malignant cells in tumor biopsy tissue is seen in Fig. 27 and displays the colocalization of p35 (red) and TUNEL (green) staining as indicated by the yellow color. Fig. 28 displays the same cell seen in Fig. 27 with removal of the brightfield and red images and confirms the apoptotic nature of the cell through independent TUNEL staining (~ 20-fold increase above background) and nuclear morphology. This same apoptotic cell also displayed p35 protein up-regulation with ~ 8-fold increase in p35 immunoreactivity as compared to background staining (Fig. 29).

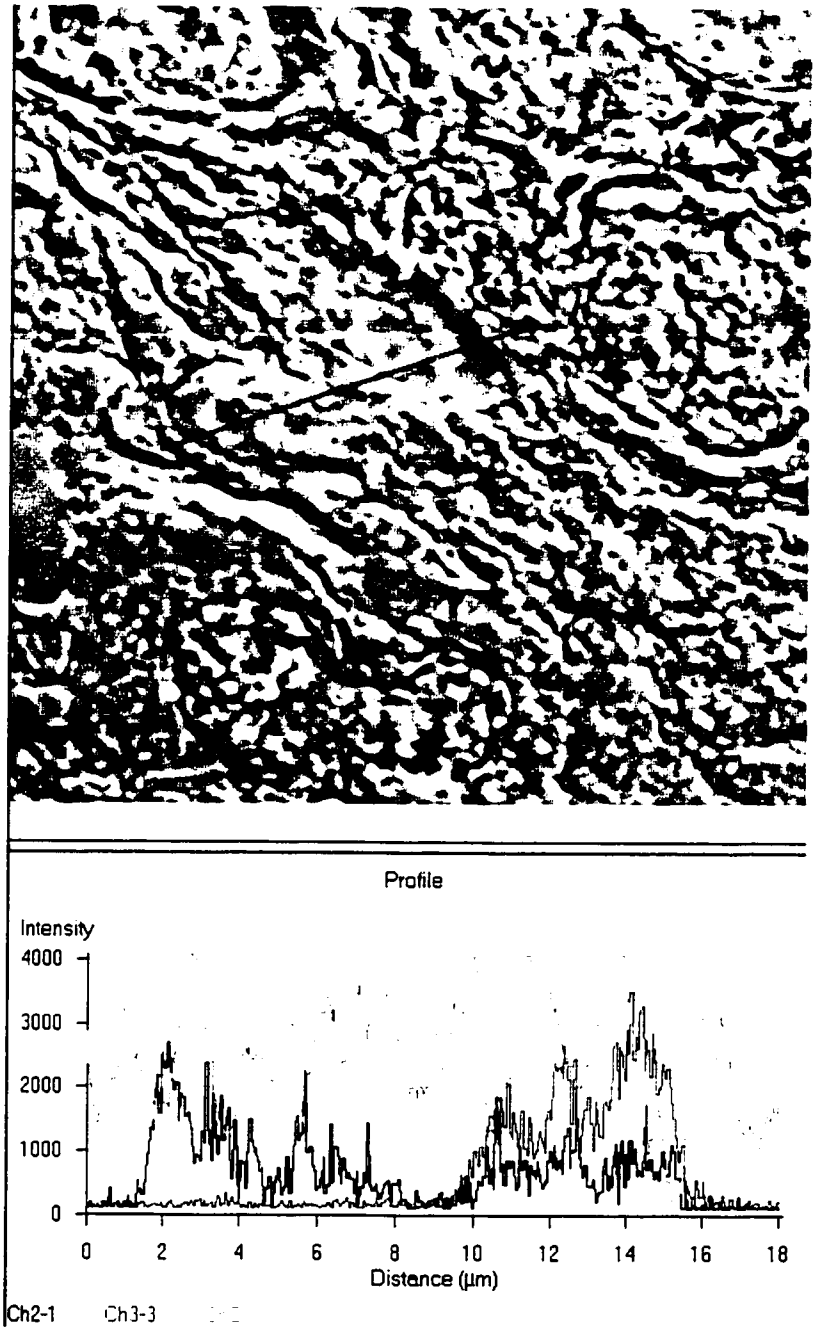


Fig. 25. DNA fragmentation assay of human glioblastoma multiforme tissue. Paraffin-embedded glioblastoma multiforme tissue sections were subjected to TUNEL DNA fragmentation assay (positive is green) followed by Hoechst 33258 DNA stain (positive is blue). This picture includes the brightfield image of the tissue. The chart below depicts the relative fluorescent intensities of the two stains detected along the length of the red arrow.

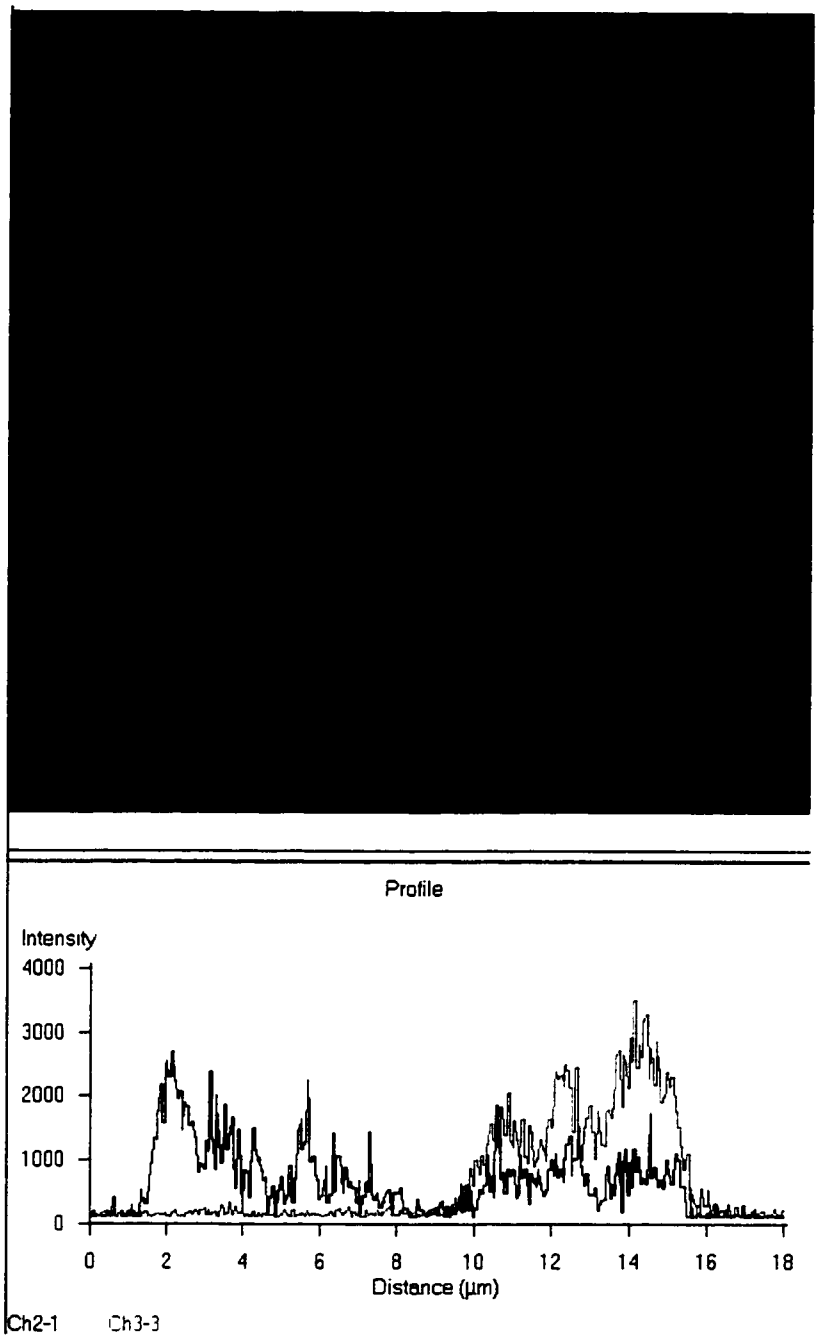


Fig. 26. DNA-fragmentation assay of human glioblastoma multiforme tissue. Paraffin-embedded glioblastoma multiforme tissue sections were subjected to TUNEL DNA fragmentation assay (positive is green) followed by Hoechst 33258 DNA stain (positive is blue). This figure illustrates a TUNEL-positive apoptotic cell surrounded by viable tumor cells. The chart below depicts the relative fluorescent intensities of the two stains detected along the length of the red arrow.

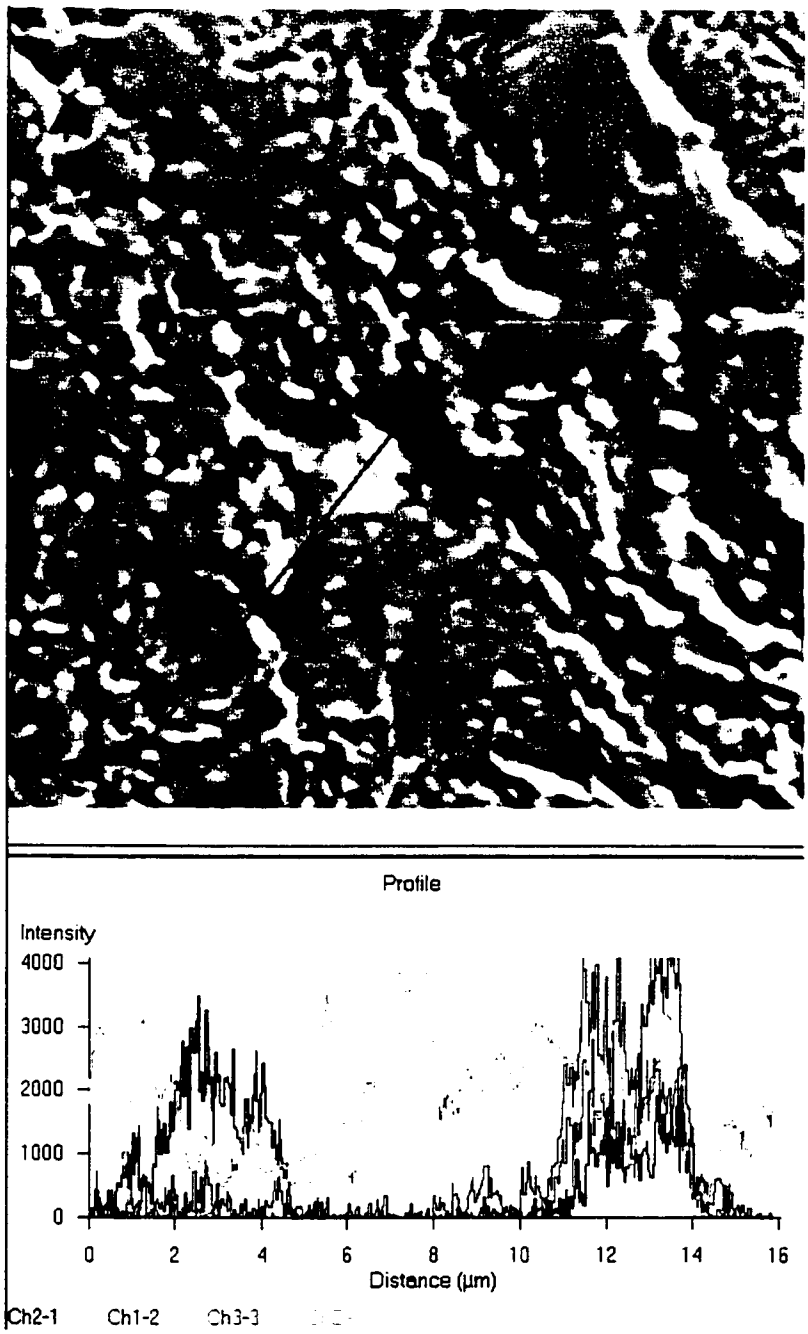


Fig. 27. Colocalization of p35 and DNA fragmentation assay in human glioblastoma multiforme tissue. Paraffin-embedded glioblastoma biopsy tissue sections were subjected to TUNEL DNA fragmentation assay (positive is green) followed by p35 immunostaining (positive is red) and Hoechst 33258 DNA stain (positive is blue). Colocalization of p35 and TUNEL is observed as a yellow color. This picture includes the brightfield image of the tissue. The chart below depicts the profiles of the relative fluorescent intensities of all three stains detected along the length of the red arrow.

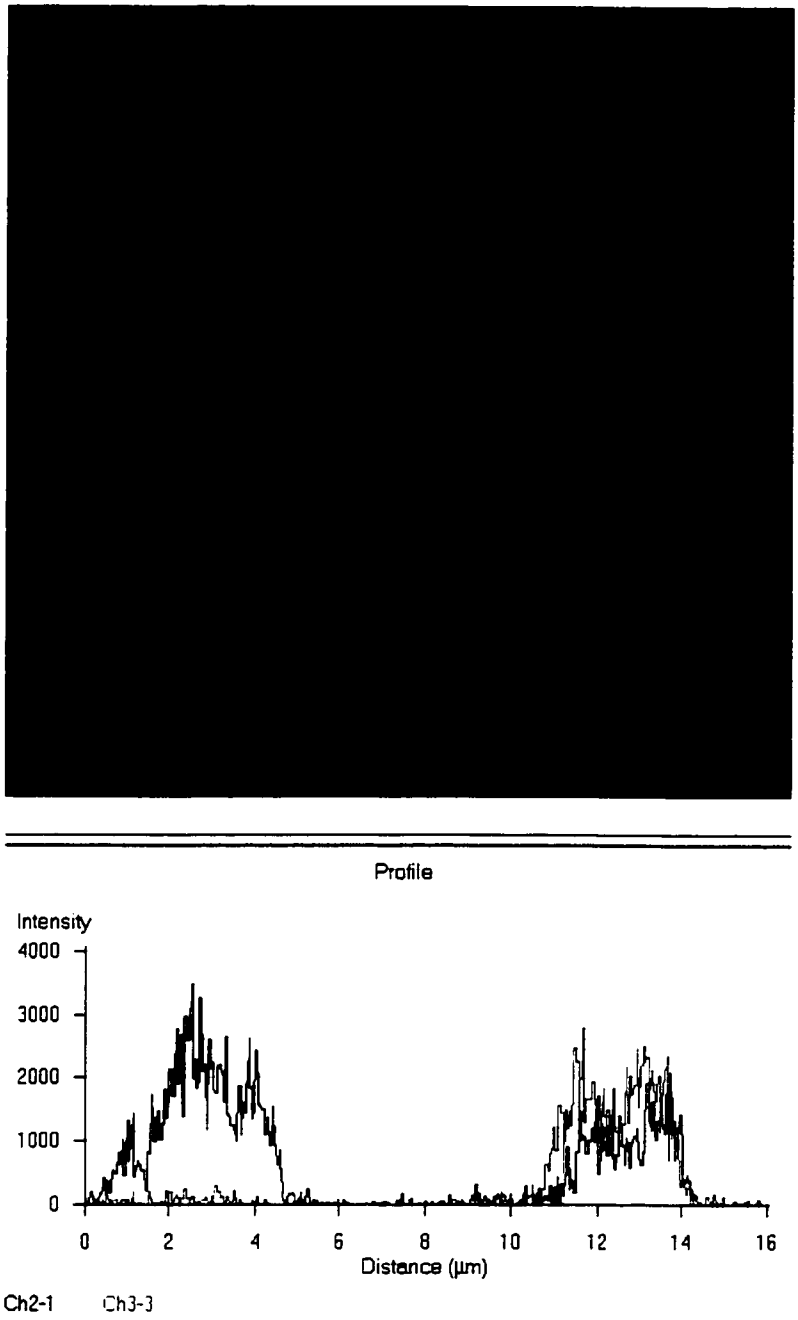


Fig. 28. DNA fragmentation assay of human glioblastoma multiforme tissue. Paraffin-embedded glioblastoma biopsy tissue sections were subjected to TUNEL DNA fragmentation assay (positive is green) followed by p35 immunostaining and Hoechst 33258 DNA stain (positive is blue). This figure illustrates a TUNEL-positive cell with subtraction of the red image (p35 staining). The chart below depicts the profiles of the relative fluorescent intensities of the green and blue stains along the length of the red arrow.

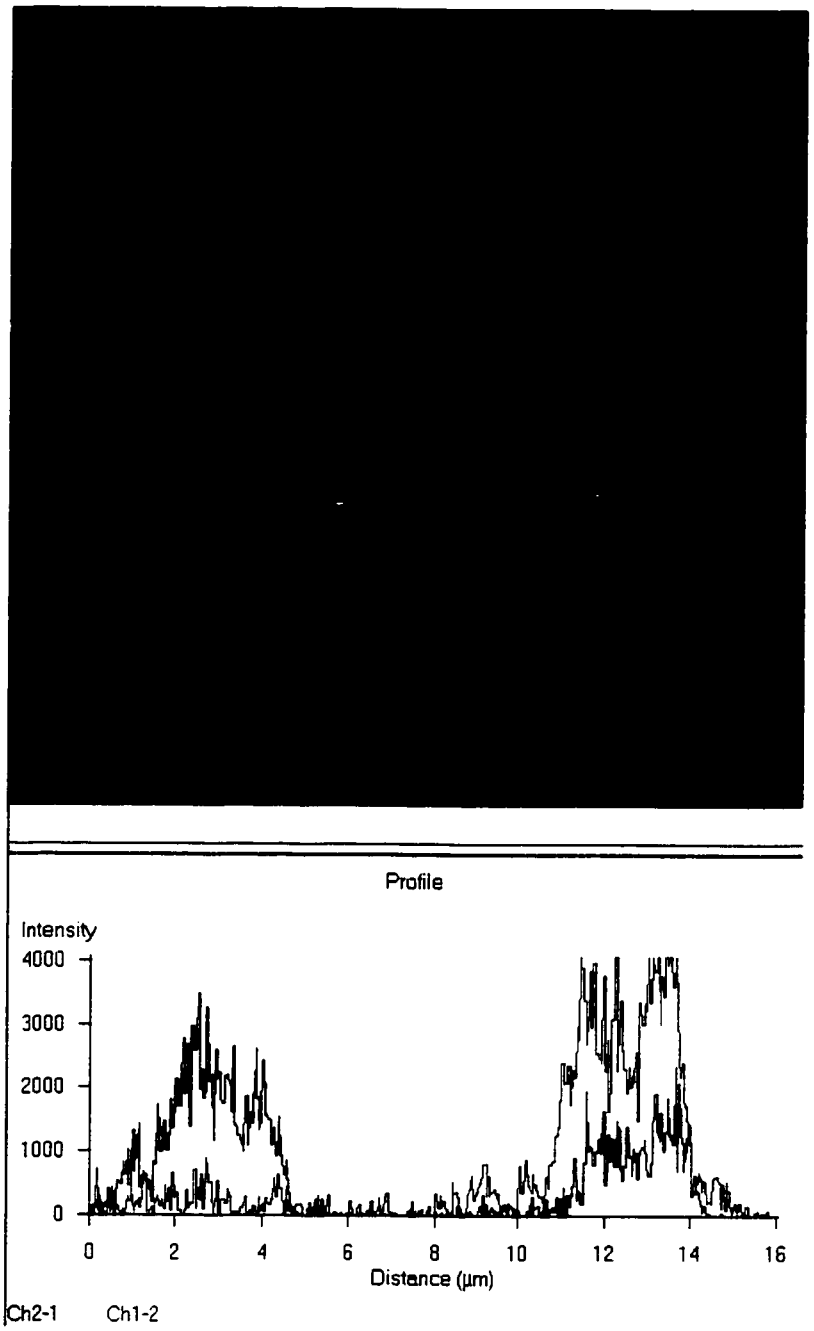


Fig. 29. p35 immunostaining of apoptotic cells in human glioblastoma multiforme tissue. Paraffin-embedded glioblastoma biopsy tissue sections were subjected to TUNEL DNA fragmentation assay followed by p35 immunostaining (positive is red) and Hoechst 33258 DNA stain (positive is blue). This figure illustrates the same p35-positive apoptotic cell seen in Fig. 29 with subtraction of the green image (TUNEL stain). The chart below depicts the profiles of the relative fluorescent intensities of red and blue stains detected along the length of the red arrow.

### 4.2.3 Immunohistochemical Detection of Cdk5 and p35 Proteins in an Irradiated Glioma Cell Line

To assess the association of p35 with apoptotic cell death in gliomas, p35 immunoreactivity was examined in cytospin preparations made from control and irradiated (36 h post-irradiation) M059J glioma cells. The time point chosen is consistent with peak incidence of radiation-induced apoptosis in this cell line. Unirradiated M059J control preparations indicated a low level of p35 protein expression with relatively diffuse, weak, pink staining accompanied by several aggregations of darker stained cells scattered thoughout the slide (Fig. 30a). After 36 h post-irradiation, p35 protein expression increased and all cells exhibited intense p35 immunoreactivity (Fig. 30b).

### 4.2.4 Western Blot Analysis of p35 and Cdk5 Protein Expression in Irradiated Glioma Cell Lines

To further examine p35 and Cdk5 protein levels during radiation-induced apoptosis, cell lysates were prepared from control and irradiated M059J and M059K cells. Early (15 min, 30 min, 1h and 3h) and later time points (24 h, 36 h and 48 h) post-irradiation were analyzed by western blotting techniques. Cdk5 was present in both cell lines as a single band of 33 kDa (Fig. 31a & b), consistent with the reported size of Cdk5. Relatively high basal levels of Cdk5 protein were detected in control cells. Densitometric analysis indicated that neither cell line showed any significant change in Cdk5 protein levels after radiation (Fig. 32). Although Cdk5 protein levels remained relatively constant in the radioresistant M059K cells at all time points after

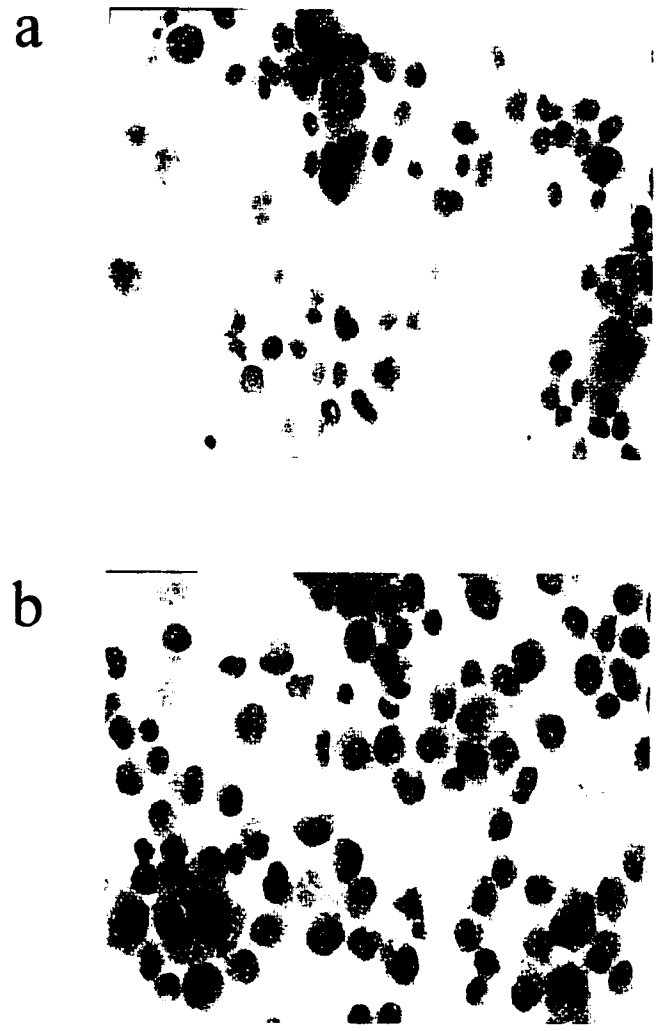


Fig. 30. p35 expression in M059J glioma cells. (a) Unirradiated, control cells display a diffuse, low level of p35 staining with multiple aggregations of darker stained cells. (b) p35 immunoreactivity increases in M059J cells 36 h post-irradiation where all cells exhibit intense p35 staining.

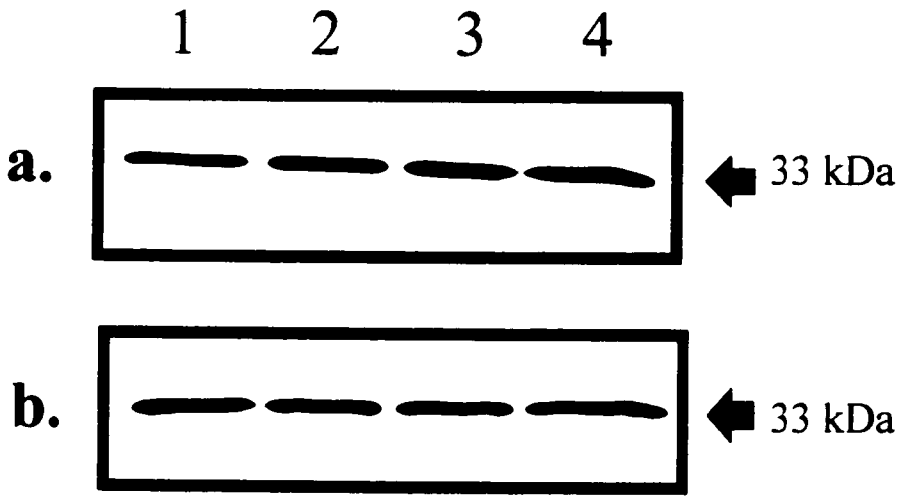


Fig. 31. Western blot analysis of Cdk5 protein expression in post-irradiated M059J and M059K glioma cells. (a) M059J and (b) M059K cultured glioma cells after no exposure to ionizing radiation (lane 1) or 24 h (lane 2), 36 h (lane 3), or 48 h (lane 4) after 10 Gy $\gamma$ -radiation.

exposure to ionizing radiation (Fig. 32, lanes 4-6), a slight increase in the level of Cdk5 was detected in post-irradiated M059J cells at later time points with maximum expression at 36 h (Fig. 31, lanes 1-3).

Assuming that Cdk5 kinase activity is regulated by the presence of an activator protein, p35 protein levels were also examined in control and irradiated M059J and M059K cells at similar time points post-irradiation. p35 was detected as a 25 kDa band in both cell lines suggesting the presence of the active proteolytic fragment of p35 (Fig. 33). In contrast to Cdk5, basal p35 protein levels were much lower in both cell lines. p35 protein expression was increased at 24 hours post-irradiation, with highest levels of expression reached after 36 h in both M059K cells (Fig. 32, lanes 7-9) and M059J cells (Fig. 32, lanes 10-12). p35 levels were slightly reduced by 48 h (60% and 67% of maximal level as determined by densitometric analysis of ECL blots for M059K and M059J cells, respectively), although they remained at higher levels than controls (Fig. 32, lanes 6 & 12, respectively). Although p35 induction was detected in both cell lines, a more dramatic increase relative to unirradiated controls was apparent in the radiosensitive M059J cells as shown in Fig. 32 (lane 8). Sample blots of p35 protein expression shown in Fig. 33 illustrate the up-regulation of p35 protein levels 24 h after radiation-induced apoptosis with maximal expression at 36 h in both M059K (a) and M059J (b) cell lines.

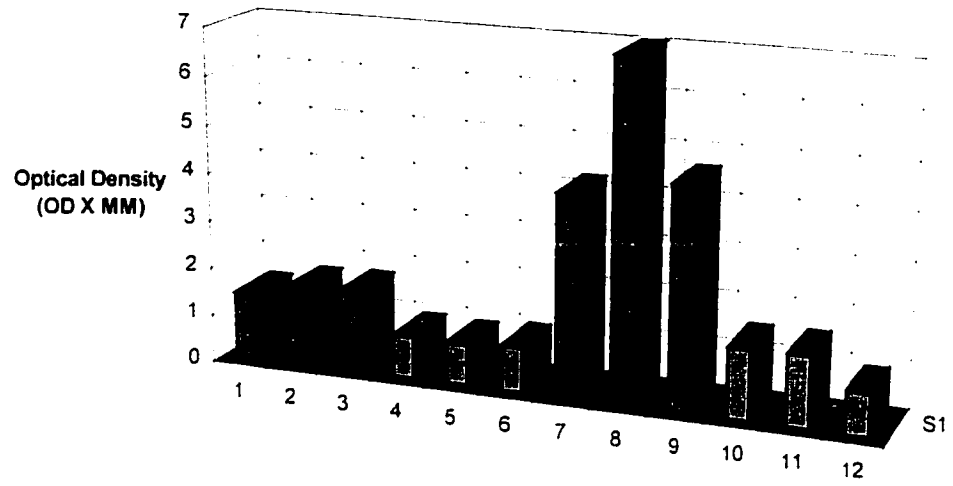


Fig. 32. Densitometric analysis of western blots examining p35 and Cdk5 levels in M059J and M059K glioma cells at 24-48 h post-irradiation. These values represent the optical density (OD) of each band relative to the unirradiated control OD values for each blot. Each group of three lanes represents a single experiment. Lanes 1-3 represent Cdk5 levels in M059J cells 24 h, 36 h, and 48 h post-irradiation. Lanes 4-6 represent Cdk5 level in M059K cells 24 h, 36 h and 48 h post-irradiation. Lanes 7-9 represent p35 levels in M059J cells 24 h, 36 h and 48 h post-irradiation. Lanes 10-12 represent p35 levels in M059K cells 24 h, 36 h, and 48 h post-irradiation.

### 4.2.5 Cdk5 Histone H1 Kinase Assay

Experiments were designed to assess whether increased Cdk5 and p35 protein levels observed after radiation treatment are associated with induction of Cdk5 kinase activity. Cdk5 protein was immunoprecipitated from control and irradiated M059J and M059K cells at 5 min and 36 h post-irradiation and examined for H1 histone kinase activity. Normalized values were obtained by densitometric analysis of four

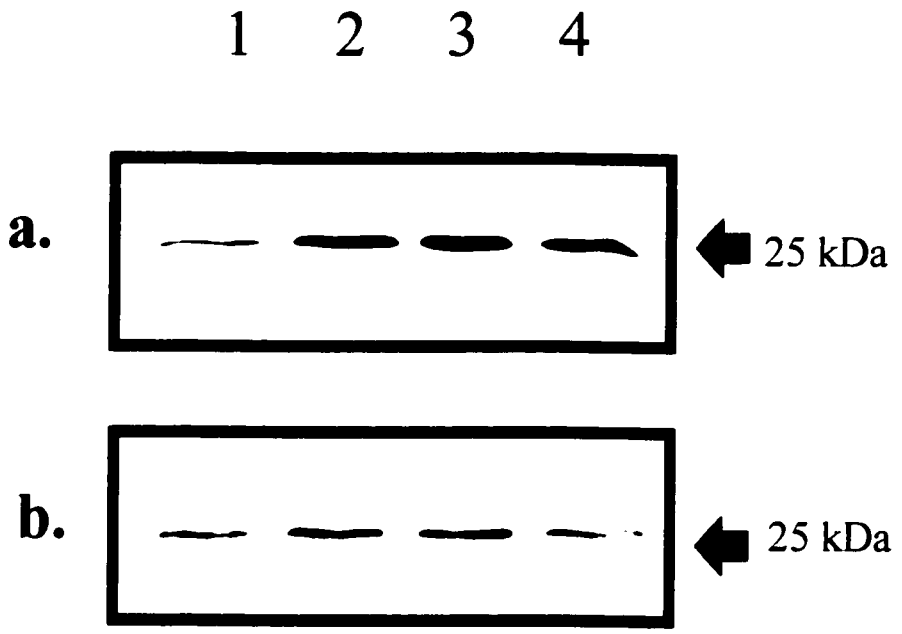


Fig. 33. Western blot analysis of p35 protein expression in post-irradiated M059J and M059K glioma cells. (a) M059J and (b) M059K cultured glioma cells after either no exposure to ionizing radiation (lane 1) or 24 h (lane 2), 36 h (lane 3), or 48 h (lane 4) after exposure to 10 Gy γ-radiation.

separate determinations of Cdk5 kinase activity in M059J cells and are displayed in Table 3. The average level of Cdk5 kinase activity increased ~3.6 fold in M059J cells 36 h post radiation-induced apoptosis as compared to unirradiated controls (Table 3). This was shown to be a statistically significant increase (Student t-test, $p < 0.01$). Cdk5 kinase activity also increased in M059K cells 36 h after exposure to ionizing radiation as indicated by densitometric analysis of autoradiographs (Table 3). However, there was greater inter-sample variation for M059K cells. A representative kinase assay of M059J and M059K cells in Fig. 34 illustrates the induction of Cdk5 kinase activity in M059J cells 36 h following exposure to ionizing radiation. A less pronounced increase in M059K cells 36 h post-irradiation is noted. Preincubation of the Cdk5 C-8 antibody with the control peptide prior to use in immunoprecipitation decreased histone H1 kinase activity in M059J cells 36 h post-irradiation by 23% based on densitometric analysis.

| | | | |
|---|---|---|---|
| M059J | 1.0 | - | 4.1 |
| | 1.0 | 3.7 | 3.0 |
| | 1.0 | 0.5 | 5.3 |
| | 1.0 | 1.3 | 2.1 |
| AVG | 1.0 | 1.8 ± 1.7 | *3.6 ± 1.4 |
| | | | |
| M059K | 1.0 | - | 16.6 |
| | 1.0 | 2.3 | 4.3 |
| AVG | 1.0 | 2.3 | 10.5 |

Table 3. Densitometric analysis of Cdk5 histone H1 kinase activity in M059J and M059K glioma cells. All values have been normalized relative to unirradiated controls. (* statistically significantly different from (0); $p < 0.01$)

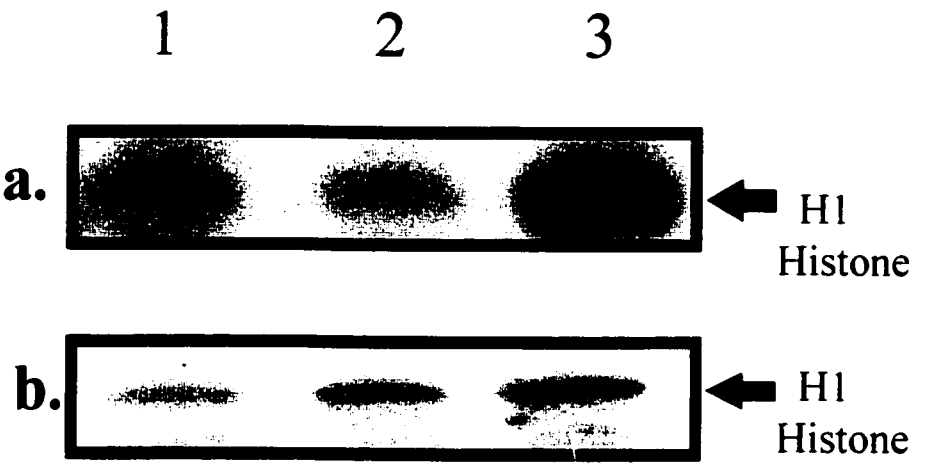


Fig. 34. Cdk5 histone H1 kinase assay of post-irradiated M059J and M059K glioma cells. (a) M059J and (b) M059K cells after either no exposure to radiation (lane 1), 5 min after exposure to 10 Gy γ -radiation (lane 2) or 36 h post-irradiation (lane 3).

## 3.3 p35 Protein Stability in Glioma Cells

Previous studies have demonstrated p35 to be subject to rapid turnover in cultured neuronal cells with a half-life of approximately 20-30 mins (Patrick *et al.*, 1998; Saito *et al.*, 1998). To examine p25/p35 protein stability in cultured glioma cells, M059J cells were treated with cycloheximide (75 μg/ml) for 4 h, 8 h, 12 h, 24 h and 36 h and protein levels examined by western blot techniques. p35 was detected as a 25 kDa protein and was still present at appreciable levels 36 h after - cycloheximide treatment (Fig. 35) indicating it to be a stable protein in glioma cells.

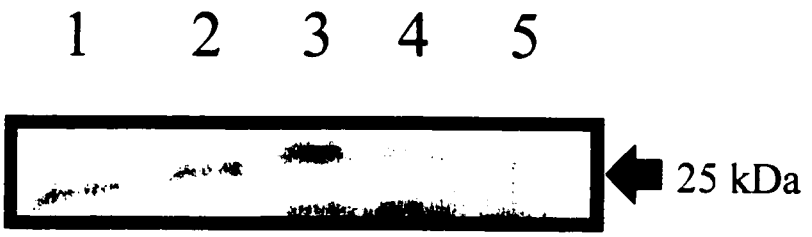


Fig. 35. p35 protein stability in M059J glioma cells. Cells were untreated (lane 1) or exposed to cycloheximide for 4 h (lane 2), 8 h (lane 3), 24 h (lane 4) or 36 h (lane 5).

# V. DISCUSSION AND CONCLUSION

## 5.1 Introduction

Cdk5 is a member of the cyclin-dependent kinase family and is expressed at basal levels in all tissues with highest levels in neurons of the forebrain (Lew and Wang, 1995). Like other Cdk's, the Cdk5 monomer is inactive and requires binding of a regulatory subunit, p35. p35 also exists as a 25 kDa proteolytic derivative (p25) and shows complete specificity in binding and activating Cdk5 (Tsai *et al.*, 1994). p35 shows no sequence homology to any known protein (Lew and Wang, 1995; Lew *et al.*, 1994). Although it is well-established that normal astrocytes are devoid of Cdk5 expression (Tsai *et al.*, 1993; Ino *et al.*, 1994; Nakamura *et al.*, 1998; Hayashi *et al.*, 1999), previous studies in our lab, using western blot techniques, indicated that Cdk5 and p35 are aberrantly expressed in all glioma cell lines examined. Also, p35 is unexpectedly present in the 'active' proteolytic form (25 kDa) in all glioma cell lines tested. p35 was further detected by immunohistochemistry in 7 of 7 astrocytoma biopsies suggesting that its expression in glioma cells is not an artifact of *in vitro* tissue culture. The aim of this study is to characterize the expression patterns of Cdk5 and p35 in human malignant glioma cells and to examine the functional role of Cdk5/p35 kinase in these cells.

## 5.2. Expression and Localization of Cdk5 and p35

Human non-malignant neocortical and hippocampal brain tissue of varying pathologies was examined for Cdk5 protein expression using immunohistochemistry. Cdk5 immunoreactivity is detected in neurons of all six cortical layers and the pyramidal cell layer and granular layer of the hippocampus. Within these neurons, Cdk5 is expressed in both dendrites and the cell body, including the cytoplasm and, to a lesser extent, the nucleus, but is absent from axons of the same neurons. Axonal pathways within the white matter and glial cells, including both astrocytes and oligodendrocytes, are devoid of Cdk5 staining.

Immunohistochemical analysis of Cdk5 expression in murine paraffin-embedded brain sections reveals similar Cdk5 staining patterns with both cytoplasmic and nuclear protein expression in neurons of the CNS and PNS, and absence of staining in glia and nucleoli (Ino and Chiba, 1996). Our methodology is similar to that of Ino and Chiba (1996) as the same source of antibodies is used and all brain sections are heat-treated prior to staining. In contrast, Tsai *et al.* (1993) and Tomiwaza *et al.* (1997) have reported an exclusively axonal Cdk5 expression pattern in neurons of mouse and rat brain. This discrepancy may be due to differences in the source of antibodies used and differences in preparation of tissue sections. Ino and Chiba (1996) suggested that heat-treatment may be essential for unmasking nuclear Cdk5. Neither Tsai *et al.* (1993) nor Tomiwaza *et al.* (1996) utilized this technique. *In situ* hybridization studies in mouse (Ino *et al.*, 1994) and rat (Zheng *et al.*, 1998)

further support our immunohistochemical analyses. In these studies, Cdk5 transcript is observed in pyramidal and granular cells of the hippocampus and all layers of the cerebral cortex except lamina I. It is absent from axonal pathways within the white matter and from glial cells of the hippocampus and cortex.

After confirming that human astrocytes are devoid of Cdk5 protein expression, we wished to determine if the aberrant expression of Cdk5 and p35 proteins in astrocytoma cells is associated with histological grade or any pathological features of astrocytic tumors. In these studies, surgical biopsy specimens of human astrocytic tumors of varying grades were examined for Cdk5 and p35 protein expression. Cdk5 and p35 protein expression weakly correlates with malignant grade. To our knowledge, there is no previous systematic study of Cdk5/p35 protein expression in human tumor tissues of varying histological grades. Because primary and secondary glioblastomas are know to develop from a cascade of multiple genetic alterations (Peraud *et al.*, 1999), it is not expected that changes in the expression of a protein kinase would be a singular feature of tumor progression.

Interestingly, our study of human brain tumor biopsy specimens shows that Cdk5 and p35 immunoreactivity is strongly associated with cells in perinecrotic areas. These regions within a tumor are known to contain populations of cells undergoing apoptosis (Tachibana *et al.*, 1996; Kordek *et al.*, 1996). This association with apoptotic cell death will be discussed further when examining the functional role of Cdk5/p35 kinase in gliomas.

Our analyses of Cdk5 and p35 protein expression in human tumor specimens provides strong evidence that the expression of these proteins occurs *in vivo*. We used human glioma cells maintained in tissue culture to further examine the intracellular distribution of Cdk5 and p35 proteins. Immunohistochemical staining for Cdk5 protein using two different antibodies reveals two different patterns of distribution. Although both antibodies detect Cdk5 protein within the nucleus and the cytoplasm, the relative fluorescence intensity of the cytoplasmic *vs.* nuclear staining is different. We believe these results reflect the fact that the two Cdk5 antibodies recognize different epitopes on the protein. The Upstate antibody is raised against a full-length recombinant protein and the specific epitope recognized by this antibody is unknown. The Santa Cruz (C-8) antibody is directed towards an epitope in the C-terminus of the Cdk5 protein. At present, there is no crystal structure and/or molecular model describing the three dimensional conformation adopted by Cdk5 when it is bound to its putative substrates. It is possible that the C-terminal epitope cannot be recognized by the Santa Cruz (C-8) antibody when the Cdk5 protein is engaged in specific protein-protein interactions within the cytoplasm. If this is the case, it provides at least a partial explanation for the somewhat different staining patterns observed using two different antibodies.

In general, our results are consistent with previous reports which describe both nuclear and cytoplasmic Cdk5 subcellular localization in both primary neuronal cultures (Nikolic *et al.*, 1996) and cultured proliferative rat myoblasts (Lazaro *et al.*, 1997). Interestingly, a redistribution of Cdk5 protein from cytoplasm to nucleus has

been demonstrated in differentiating proliferative myoblasts (Lazaro *et al.*, 1997). Considering the nuclear localization of its activator protein, p35, Cdk5 kinase may have a functionally distinct role in the cell nucleus which has yet to be determined.

Imunohistochemical staining for p35 protein reveals a cytoskeletal-like distribution pattern with a minute amount of labeling detected in the nucleus of some cells. The pattern of staining suggests an association of p35 with some component of the microtubule infrastructure. Confocal microscopy and double labeling techniques were used to further investigate this colocalization where we observe staining patterns consistent with a colocalization of p35 and α-tubulin. When α-tubulin polymerization is disrupted by nocodazole treatment, both the co-localization and the lattice-like staining pattern of p35 and α-tubulin is lost. These results suggest that the p35/Cdk5 kinase may be associated with tubulin subunits.

Support for this hypothesis is provided by the work of several investigators. Pigino *et al.* (1997) have shown that Cdk5 and p35 associate with the microtubule cytoskeleton in the distal end of axonal processes and within the central region of growth cones of cerebellar neurons. Furthermore, it has been demonstrated that Cdk5, as well as other kinases and phosphatases, can bind to microtubules *in vitro* (Ishiguro *et al.*, 1992; Pigino *et al.*, 1997). Cdk5 also phosphorylates various microtubule-associated proteins including tau (Lew and Wang, 1995), MAP2 (Nikolic *et al.* 1996), and MAP1B (Pigino *et Al.*, 1998) *in vitro*. The tumor suppressor protein, adenomatous polyposis coli (APC), which binds microtubules in neurons, is believed to regulate axonal transport through its interaction with Cdk5 (Ratner *et al.*,

1998). Furthermore, suppression of both Cdk5 and p35 expression by antisense oligonucleotide treatment reduces MAP1B phosphorylation during laminin-enhanced axonal growth (Pigino *et al.*, 1997; Paglini *et al.*, 1998).

Cdk5 and p35 may associate with a number of cytoskeletal components. Extensive *in vitro* analyses suggest the possibility that the p35/Cdk5 kinase phosphorylates a repertoire of cytoskeletal proteins. For example, the intermediate filament proteins, vimentin and desmin both contain a Cdk5 consensus KSP.YK phosphorylation motif (Inagaki *et al.*, 1996) and are both expressed in human malignant gliomas (Osborn *et al.*, 1981; Hirato *et al.*, 1994). Neurofilament proteins are also possible Cdk5 substrates. In normal brain, neurofilament proteins are neuron-specific intermediate filaments. However, NF-L, NF-M, and NF-H proteins (Bodey *et al.*, 1991, Abaza *et al.*, 1998), as well as the neuronal microtubule-associated proteins MAP1B (Tohyama *et al.*, 1993) and tau (Lopes *et al.*, 1992; Miyazono *et al.*, 1994) have been detected in primary human glioma specimens and are known *in vitro* substrates of Cdk5 kinase (Lew and Wang, 1995; Pigino *et al.*, 1998). Although GFAP is another intermediate filament which contains a Cdk2/Cdk5 phosphorylation motif (Tsujimura *et al.*, 1994) and is an obvious candidate substrate for Cdk5 in glioma cells, previous studies have shown that the M059J glioma cells used in this study express low levels of GFAP transcript (Godbout *et al.*, 1998) and protein (Allalunis-Turner *et al.*, 1993). We have ruled out the possibility of a direct association between p35 and microfilaments as staining patterns using p35 and actin antibodies showed no evidence of protein colocalization.

## 5.3 Cdk5/p35 Involvement in Apoptotic Cell Death

In our analysis of Cdk5/p35 protein expression in human primary brain tumor specimens, we observe that positively stained cells are distributed in the perinecrotic regions of the tumor. These regions are generally associated with apoptotic tissue. To confirm the association between Cdk5 and p35 positivity and apoptosis, two DNA fragmentation assays were used to detect apoptotic cell death and Cdk5/p35 immunoreactivity in perinecrotic areas of adjacent tissue sections and within individual dying cells of human glioma biopsy sections.

The association between Cdk5/p35 immunostaining and apoptotic cell death has been reported in a variety of physiological and experimental apoptosis model systems using both adult and embryonic tissue. For example, p35 andCdk5 immunostaining has been found to correlate with apoptosis in atretic ovarian follicular cells, regions of the developing retina and nervous system, androgen-withdrawn regressing prostate and interdigital zones of developing mouse hindlimbs (Zhang *et al.*, 1997; Henchcliffe and Burke, 1997; Ahuja *et al.*, 1997). To our knowledge, there has been no study investigating a possible association between p35/Cdk5 and apoptosis in a human tumor model. The results of our experiments show that, within the panel of human glioma biopsy tissue sections examined, cells positive for both Cdk5/p35 protein expression and DNA fragments characteristic of apoptosis are observed almost exclusively in tumor cells surrounding foci of necrosis. In accordance with these observations, apoptotic cell death has been shown to correlate with tumor grade in astrocytic tumors. The proportion of cells undergoing

apoptosis is greater in higher-grade tumors such as anaplastic astrocytomas and GBM's than in lower-grade astrocytomas (Kordek *et al.*, 1996). Also consistent with our findings is the observation that apoptosis occurs more frequently in glioma cells within perinecrotic areas of GBM tumors (Tachibana *et al.*, 1996; Kordek *et al.*, 1996).

Our analyses of Cdk5/p35 protein expression and apoptosis in human primary tumor specimens provide strong evidence for the association of this kinase in the process of apoptosis occuring *in vivo*. We used human glioma cell lines to further examine the role of Cdk5/p35 in apoptosis induced following exposure to the DNA damaging agent, ionizing radiation. Two glioma cell lines with differing sensitivities to radiation-induced apoptosis are used for these studies. Consistent with the results of our *in vivo* experiments, up-regulation of the p35 proteolytic fragment, p25, is detected in apoptosis-sensitive cells by western blot analysis and immunohistochemistry. p25 protein expression is maximally expressed 36 h after radiation treatment and is more pronounced in the radiosensitive M059J cells. In addition to increased protein expression, a concomitant increase in histone H1 Cdk5 kinase activity was observed 36 h post-irradiation in both M059J and M059K glioma cells. Previous work from this laboratory shows that the peak incidence of radiation-induced apoptosis in these cell lines occurs between 24 h - 48 h post-irradiation (Leithoff, 1995). Our finding of increased p25 protein expression and Cdk5 kinase activity at 36 h post-irradiation is consistent with this work. In addition, 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).

Association of Cdk5/p35 protein up-regulation and induction of Cdk5 kinase activity with apoptotic cell death has been demonstrated in several normal tissue systems. For example, Ahuja *et al.* (1997) use western blotting to detect an increase in Cdk5 and p35 protein expression that is associated with increased interdigital cell death in developing mouse limbs. In chick sympathetic neurons, the pattern of Cdk5 protein expression in oxidative stress-induced apoptosis is reported to change as a function of the apoptotic process and to coincide with the commitment of these cells to die (Shirvan *et al.*, 1998). Finally, an association of Cdk5 kinase activity with apoptotic cell death is shown in the mouse embryonic hindlimb where apoptosis increases between E12.5 and E14.5 along with a concomitant increase in Cdk5 kinase activity (Zhang *et al.*, 1997).

Although the specific role of Cdk5/p35 kinase in apoptotic cell death remains to be elucidated, our *in vivo* and *in vitro* results suggest that Cdk5 may be involved in the apoptosis signaling cascade in glioma cells. Cdk5 may mediate its functional role through its association with the microtubule system. Rearrangement of the cytoskeleton is a prominent feature of apoptosis frequently resulting in membrane blebbing (Levee *et al.*, 1996; Van Engeland *et al.*, 1997). Various agents disrupt the microtubule cytoskeleton during apoptosis. Many chemotherapeutic drugs that disrupt microtubule turnover such as taxol, vinblastine and vincristine also promote cell death through phosphorylation of various signaling proteins (Blagosklonny *et al.*, 1997; Haldar *et al.*, 1997). Glutamate-induced apoptosis in neurons results in degradation of tubulin sub-units during early stages of apoptosis (Ankarcrona *et al.*, 1996). By confocal microscopy, we have demonstrated the possible association of

p35, a Cdk5 activator, with the microtubule structure in glioma cells. Furthermore, it is well established that Cdk5 is an *in vivo* kinase for various microtubule-associated proteins, including tau (Lew and Wang, 1995), MAP2 (Nikolic *et al.*, 1996), and MAP1B (Pigino *et al.*, 1997; Paglini *et al.*, 1998). Given its possible role in regulation of phosphorylation of MAPs, it is reasonable to postulate that Cdk5-mediated phosphorylation regulates rearrangement of the microtubule structure during apoptotic cell death.

Interestingly, in these studies, p35 is detected in western blots as its 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). Tau protein kinase II, originally purified from bovine brain microtubules, was also found to be composed of Cdk5 and p25 (Ishiguro *et al.*, 1992). Although p35 is the predominant form found in brain extracts and primary neuronal cultures (Tsai *et al.*, 1994), both p35 (Tsai *et al.*, 1994) and p25 are fully competent for Cdk5 activation (Lew *et al.*, 1994, Lee and Johnston, 1997). Their differential expression may be the result tissue- or species- specific post-translational regulatory processes.

## 5.4 p35 Protein Stability in Glioma Cells

Recent studies using cultured rat cortical neurons indicate that p35 is a short-lived protein with a half-life of approximately 20-30 mins (Patrick *et al.*, 1998; Saito *et al.*, 1998). At present, there is no information about the stability of this protein in non-neuronal tissue. We therefore undertook to determine whether the half-life of p35

protein in human glioma cells is similar to that determined for neuronal p35. Our results indicate that in glioma cells, the proteolytic p35 fragment, p25, displays appreciably greater protein stability than that of the full-length p35 protein detected in neurons. In accordance with our observations, it has recently been demonstrated that p25 lacks one of the four Cdk5 phosphorylation motifs within the amino-terminus which mediate protein degradation. Absence of this motif would result in decreased ubiquitination and ATP-dependent degradation, making the p25 activator a more stable protein (Patrick *et al.*, 1998).

## 5.5 Conclusion

This study is the first to demonstrate, *in vitro* and *in vivo*, the aberrant expression of Cdk5/p35 kinase in glioma cells. In addition, it is the first to describe the association of Cdk5/p35 protein expression and kinase activity with apoptosis in human cells, in general, and cells of glial origin, in particular. Although we suggest Cdk5/p35 may function in glioma cells as a signaling factor during apoptotic cell death, we have shown that both Cdk5 and p35 are ubiquitously expressed at basal levels in all glioma cells examined. This suggests that in addition, Cdk5 may mediate normal functions within these cells.

One possible function is Cdk5-mediated phosphorylation of cytoskeletal proteins involved in the migratory and invasive behaviour of glioma cells. Local invasion is a hallmark of the progression of malignant gliomas and one of the most important determinants of the poor prognosis associated with these tumors (Halperin *et al.*, 1998). Single migratory tumor cells can disseminate out of the primary tumor

mass into adjacent normal brain parenchyma and can be found at distances greater than 2 cm beyond the tumor margin (Halperin *et al.*, 1988). The physical interaction between cell surface integrins and extracellular matrix (ECM) proteins is believed to mediate alterations in internal protein-scaffolding necessary for cell shape changes and cell motility (Tysnes *et al.*, 1996; Haugland *et al.*, 1997; Giese *et al.*, 1997; Knott *et al.*, 1998; Uhm *et al.*, 1999).

The distal growth cones in the axonal tip of cultured cerebellar neurons also interact with ECM molecules to promote axonal extension and cell motility (Reichard and Tomaselli, 1991). Neuronal cytoskeletal proteins are rearranged throughout this process to maintain cell shape and promote axonal outgrowth (Julien and Mushynski, 1998). It has been suggested that Cdk5/p35 may serve as an important regulatory linker between ECM molecules and constituents of the intracellular cytoskeletal machinery (Paglini *et al.*, 1998; Pigino *et al.*, 1997). In neurons, it has been shown that: (1) Cdk5 phosphorylates MAP1b, a neuronal microtubule-associated protein involved in axon elongation (Pigino *et al.*, 1998); (2) laminin, an extracellular matrix molecule known to promote neurite outgrowth (Lein 1992), accelerates the redistribution of Cdk5 to the axonal tip and increase its kinase activity; (3) inhibition of Cdk5 kinase activity reduces laminin-mediated axonal elongation, and (4) antibodies directed against integrin β1, a laminin receptor, significantly reduce Cdk5 kinase activity (Pigino *et al.*, 1997; Paglini *et al.*, 1998). These data suggest that Cdk5/p35 kinase plays an important role during laminin-mediated axonal elongation.

Several aspects of axonal growth are shared by the ECM-mediated adhesive and migratory behaviour of human glioma cells. These include: (1) glioma tumor cells adhere and migrate on ECM proteins including laminin, a particularly potent promoter of glioma cell adhesion and migration (Fukushima *et al.*, 1998); (2) antibodies against integrin β1, a laminin receptor, significantly reduces laminin-mediated glioma cell migration (Tysnes *et al.*, 1996); and (3) GBM primary tumors have been shown to express neuronal cytoskeletal proteins such as MAP1B (Tohyama *et al.*, 1993), MAP2 (Lopes *et al.*, 1992), tau (Miyazono *et al.*, 1993), NF-L, NF-M, and NF-H proteins (Tohyama *et al.*, 1993; Bodey *et al.*, 1992). Both NF proteins and microtubule-associated proteins are known substrates of Cdk5/p35 kinase in neurons (Lew and Wang, 1995; Nikolic *et al.*, 1996; Lee *et al.*, 1997b). As in neurons, Cdk5/p35 kinase may regulate the phosphorylation of these neuronal proteins in glioma cells, and through its modulation of cytoskeletal elements, contribute to the development of a motile and invasive phenotype of glioma cells. If this is the case, Cdk5/p35 kinase would be an important target for anti-glioma therapies. Interference with Cdk5/p35 activity would eliminate an important link between cytoskeletal proteins and the extracellular environment required for glioma cell motility.

The lineage relationship between astrocytes and neurons is still somewhat controversial. Multipotential progenitors may give rise to both astrocytes and other CNS cells (Turner and Cepko, 1987; Gray and Sanes, 1992; Levison and Goldman, 1993). Alternatively, astrocytes and neurons may be derived from two separate populations of stem cells (Levison and Goldman, 1993). As described above, glioma

cells share a number properties with neurons including similar components of the cell motility machinery and the expression a number of neuronal cytoskeletal proteins. In addition to the neuronal cytoskeletal-associated proteins mentioned above, glioma cells have been shown to express other non-glial (neuronal) proteins such as a number of glutamate transporters (Boado *et al.*, 1994), neuron-specific enolase (Vinores *et al.*, 1984) and neuron-specific protein gene product 9.5 (PGP 9.5) (Giambanco *et al.*, 1991). These data raise an intriguing question regarding the cell lineage of gliomas: Is malignant glioma the transformation of a mature, differentiated astrocyte or is it the result of the malignant conversion of an earlier, multipotential progenitor stem cell? Further insight into the origin and mechanisms of progression of malignant gliomas will provide a greater understanding of the biology of human malignant gliomas and aid in the development of more effective glioma-directed therapies.

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