

## Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system

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### SUMMARY

Cyclin-dependent kinase 5 (cdk5) was originally isolated on the basis of its close primary sequence homology to the human cdc2 serine/threonine kinase, the prototype of the cyclin-dependent kinases. While kinase activities of both cdc2 and cdk2 are detected in proliferating cells and are essential for cells to progress through the key transition points of the cell cycle, cdk5 kinase activity has been observed only in lysates of adult brain. In this study, we compared the activity and expression of cdk5 with that of cdc2 and cdk2 in the embryonic mouse forebrain. The expression and activity of cdk5 increased progressively as increasing numbers of cells exited the proliferative cycle. In contrast, the expression and activity of cdc2 and cdk2 were maximum at gestational day 11 (E11) when the majority of cells were proliferat-

ing and fell to barely detectable levels at E17 at the end of the cytogenetic period. Immunohistochemical studies showed that cdk5 is expressed in postmitotic neurons but not in glial cells or mitotically active cells. Expression of cdk5 was concentrated in fasciculated axons of postmitotic neurons. In contrast to other cell division cycle kinases to which it is closely related, cdk5 appears not to be expressed in dividing cells in the developing brain. These observations suggest that cdk5 may have a role in neuronal differentiation but not in the cell division cycle in the embryonic nervous system.

Key words: cyclin-dependent kinases, cell cycle, neuronal differentiation

### INTRODUCTION

The cell division cycle in eukaryotic cells is regulated by a series of coordinated events that are executed at defined transition points. Specific phosphorylations/dephosphorylations trigger progression from G<sub>1</sub> to S phase and from G<sub>2</sub> to M phase. Small protein serine/threonine kinases from the cdc2 family and their regulatory subunits, known as cyclins, appear to be the key regulators of the cell division cycle (see reviews (Hunt, 1989; Murray and Kirschner, 1989; Draetta, 1990; Nurse, 1990; Pines and Hunter, 1990)). Binding to the regulatory cyclin subunit (Draetta and Beach, 1988; Solomon et al., 1990) and post-translational phosphorylations/dephosphorylations of the kinase molecule are important regulatory steps in the activation of the kinase (Gould and Nurse, 1989; Solomon et al., 1990; Ducommun et al., 1991; Gould et al., 1991; Krek and Nigg, 1991a,b; Norbury et al., 1991).

Cyclins were first identified in marine invertebrates as proteins whose intracellular concentrations oscillate during the cell cycle (Evans et al., 1983). Multiple cyclins have subsequently been isolated from both yeast and mammalian cells. In mammalian cells, cyclins D1, D2, D3 and E are expressed during G<sub>1</sub> and are referred to as the 'G<sub>1</sub> cyclins';

cyclins A and B are preferentially expressed during S, G<sub>2</sub> and M phases and are referred to as the 'mitotic cyclins' (see reviews (Hunt, 1991; Hunter and Pines, 1991; Reed, 1991)).

In yeast, cdc2 (in *Schizosaccharomyces pombe*) or CDC28 (in *Saccharomyces cerevisiae*) appears to be the only kinase required for G<sub>1</sub>-to-S and G<sub>2</sub>-to-M progressions. When associated with the G<sub>1</sub> cyclins, cdc2/CDC28 controls the progression from G<sub>1</sub> into S phase. When associated with the mitotic cyclins, cdc2/CDC28 regulates the transition from G<sub>2</sub> to mitosis. In contrast to its master role in G<sub>1</sub>-to-S and G<sub>2</sub>-to-M progression in yeast, the role of cdc2 in vertebrate cells appears to be limited to the G<sub>2</sub>-to-M phase transition (Riabowol et al., 1989; Th'ng et al., 1990; Fang and Newport, 1991; Hamaguchi et al., 1992). In multicellular eukaryotes, a large family of cdc2-related kinases has been isolated (Elledge and Spottswood, 1991; Koff et al., 1991; Ninomiya-Tsuji et al., 1991; Paris et al., 1991; Tsai et al., 1991; Meyerson et al., 1992). These kinases are classified as the cyclin-dependent kinases (cdks) based on their requirement for cyclin association. Some of the cdks have been shown to rescue temperature-sensitive mutants of the cdc2 homologue in the budding yeast (Elledge and Spottswood, 1991; Koff et al., 1991; Ninomiya-Tsuji et al., 1991; Meyerson et al., 1992). cdk2 has been shown to be

necessary for initiation of DNA replication in vertebrate cells (Fang and Newport, 1991; Pagano et al., 1993; Tsai et al., 1993). Therefore, at least two cdk5s are required at different transition points for the progression of vertebrate cell division cycle. cdk2 is essential for G<sub>1</sub>-to-S transition and cdc2 for G<sub>2</sub>-to-M.

cdk5, formerly called PSSALRE, was isolated from our initial screening for cdc2-related kinases (Meyerson et al., 1992). It is 58% and 62% identical, respectively, with human cdc2 and cdk2. Despite its high degree of homology with kinases that are essential to progression of the cell division cycle, cdk5 does not complement the budding yeast strains harboring temperature-sensitive mutants of cdc2 (Meyerson et al., 1992). Recently, Xiong and colleagues reported the association of cdk5 and cyclin D1 in normal human fibroblast cell line WI38 (Xiong et al., 1992), hence the current nomenclature of cdk5. However, cdk5 kinase activity was not detected in their study.

cdk5 mRNA expression in different tissues, determined by northern blot analysis, is highest in brain, although low levels of expression are detected in many other tissues (Meyerson et al., 1992). In contrast, cdk5 mRNA is expressed at high levels in a wide variety of cell lines maintained in tissue culture (Meyerson et al., 1992). Cloning of a histone H1 kinase activity purified from bovine brain revealed it to be the bovine homologue of cdk5 (Lew et al., 1992b). Therefore cdk5, like cdk2 and cdc2, can phosphorylate similar substrates, at least in vitro. Homologues of cdk5 from human, bovine (Lew et al., 1992b), rat (Hellmich et al., 1992) and mouse (M. Meyerson and L.-H. T., unpublished results) share 99% identity with each other at the protein level suggesting that this protein is highly conserved within vertebrate species.

In this study, we have found that cdk5 has patterns of expression and kinase activity in the nervous system that are strikingly different from those of cdk2 and cdc2. These differences are unexpected because the three kinases are closely related in primary sequence, and share similar substrate specificities in vitro. Unlike the other two kinases, cdk5 kinase activity is found to be specific and limited in distribution to postmitotic neurons.

## MATERIALS AND METHODS

### Immunological reagents and cell lines

The cdk5 antibody (PA1) was raised against a seven amino acid residue peptide (CSDFCPP) corresponding to the carboxyl terminus of cdk5 with a cysteine residue added to the NH<sub>2</sub> terminus in order to facilitate coupling of the peptide to the carrier protein. PA1 was affinity purified with peptide immunogen cross-linked to SulfoLink Coupling Gel (Pierce). Anti-cdk2 antibody was raised and purified against a peptide corresponding to the carboxyl terminus of cdk2 as previously described (Tsai et al., 1993). Affinity-purified anti-cdc2 antibody was purchased from LTI/BRL-Gibco. GNS1 is a monoclonal antibody to human cyclin B kindly supplied by Dr S. Shiff. ML1 (myeloid leukemia), C33A (cervical cancer), T98G (glioblastoma), SKNSH (neuroblastoma), WI38 (diploid human fibroblast), SAOS2 (osteosarcoma), 293 (adenovirus E1A transformed human kidney epithelial cell), HepG2 (hepatocarcinoma) and BALB/c3T3 cell lines were grown in DMEM supplemented with 10% fetal calf serum. Nalm6 (pre-

B leukemia) cell line was grown in RPMI-1640 supplemented with 10% fetal calf serum and 0.1% 2-mercaptoethylamine. COS-1 (SV40 transformed monkey kidney cell) cell line was grown in DMEM supplemented with 5% calf serum. The plasmid containing the entire cdk5 open reading frame driven by the cytomegalovirus (CMV) early promoter was transfected into COS-1 cells using dextran sulfate method (Sambrook et al., 1989) and cell lysates were harvested 48 hours after transfection.

### Immunoprecipitation and kinase assay

For metabolic labelling, SKNSH cells or COS-1 cells 48 hours after transfection with the CMV-cdk5 construct were labeled with 200 µCi/ml of [<sup>35</sup>S]methionine in methionine-free medium for 4 hours. Cells were then lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, pH 8.0, 0.1% Nonidet P-40, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. Immunoprecipitation was carried out as previously described (Harlow et al., 1985). For denaturing immunoprecipitations, cell lysates were adjusted to contain 2% SDS, boiled for 10 minutes and diluted 20× before incubation with PA1 antibody. For making mouse tissue lysates, each tissue was quickly frozen in liquid nitrogen after it was removed from the animal. These tissues were ground into small pieces and subsequently *douce*-homogenized in the lysis buffer described above. For in vitro kinase assays, immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (50 mM Hepes, pH 7.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT and 1 µM cold ATP). The washed beads were then incubated with kinase buffer containing 2 µg of histone H1 and 5 µCi of [<sup>32</sup>P] ATP in a final volume of 50 µl at 30°C for 20 minutes. After incubation, 50 µl of 2× Laemmli sample buffer (Laemmli, 1970) was added to each sample and the samples were analyzed by SDS-PAGE. For quantitation, the protein bands corresponding to histone H1 were excised and radioactivity was measured by Cerenkov counting.

### Partial proteolytic mapping with V8 protease and western blotting

*S. aureus* V8 protease was used for partial proteolytic mapping as described (Cleveland et al., 1977). Briefly, [<sup>35</sup>S]methionine-labeled protein bands were excised, placed onto a stacking gel with 15% separation gel and digested with three different concentrations of the V8 protease during electrophoresis. For western blot, lysates containing 25 µg of total cellular protein were loaded in each lane. After electrophoresis proteins were transferred to Immobilon-P membrane (Milipore). All the affinity-purified antibodies were diluted 1 to 3000 for immunostaining. Proteins were detected with the ECL developing system (Amersham).

### Animals

CD1 mice, used for these studies, were maintained on a 12 hours (7:00 am-7:00 pm) light-dark schedule. Conception was ascertained by the presence of a vaginal plug with the day of conception considered to be embryonic day 0 (E0). Plug checks were conducted at 9:00 am. Embryos (E11-E17) were removed by hysterotomy from dams deeply anesthetized by an intraperitoneal injection of a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight). The E14 embryos were exposed cumulatively for 12.5 hours to bromodeoxyuridine (BrdU) (Takahashi et al., 1993) which was injected intraperitoneally (Sigma; 5 mg/ml in saline solution, 0.007 N for sodium hydroxide, Takahashi et al., 1992) into the pregnant dam.

### Immunocytochemistry

**PA1.** The embryos were decapitated and the whole heads (E11-E14) or the brains dissected from the skull (E15-E17) were fixed overnight by immersion in 70% ethanol and dehydrated in graded ethanol solutions, embedded in paraffin and sectioned at 4 µm in

the coronal plane. Adult mice anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) were perfused via the left ventricle with 70% ethanol for 5 minutes. Their brains were removed, postfixed in 70% ethanol overnight, dehydrated and embedded in paraffin as described above for embryos. Sections were cut at 4  $\mu$ m in the coronal plane. The sections were deparaffinized with xylene, rehydrated and incubated with PBS with 20% normal goat serum and 0.5% Tween-20 for 1 hour, then with PA1 antibody (25  $\mu$ g/ml in PBS with 20% normal goat serum and 0.5% Tween-20) for 24 hours, 0.45% biotinylated anti-rabbit IgG in phosphate buffer saline (PBS) for 45 minutes and then with 1.8% avidin-bound peroxidase complex (ABC Elite kit, Vector) for 60 minutes. The sections were reacted with 0.05% diaminobenzidine (DAB, Sigma) and hydrogen peroxide (0.01%) with cobalt chloride (0.025%) and nickel ammonium sulfate (0.02%) for 3 minutes.

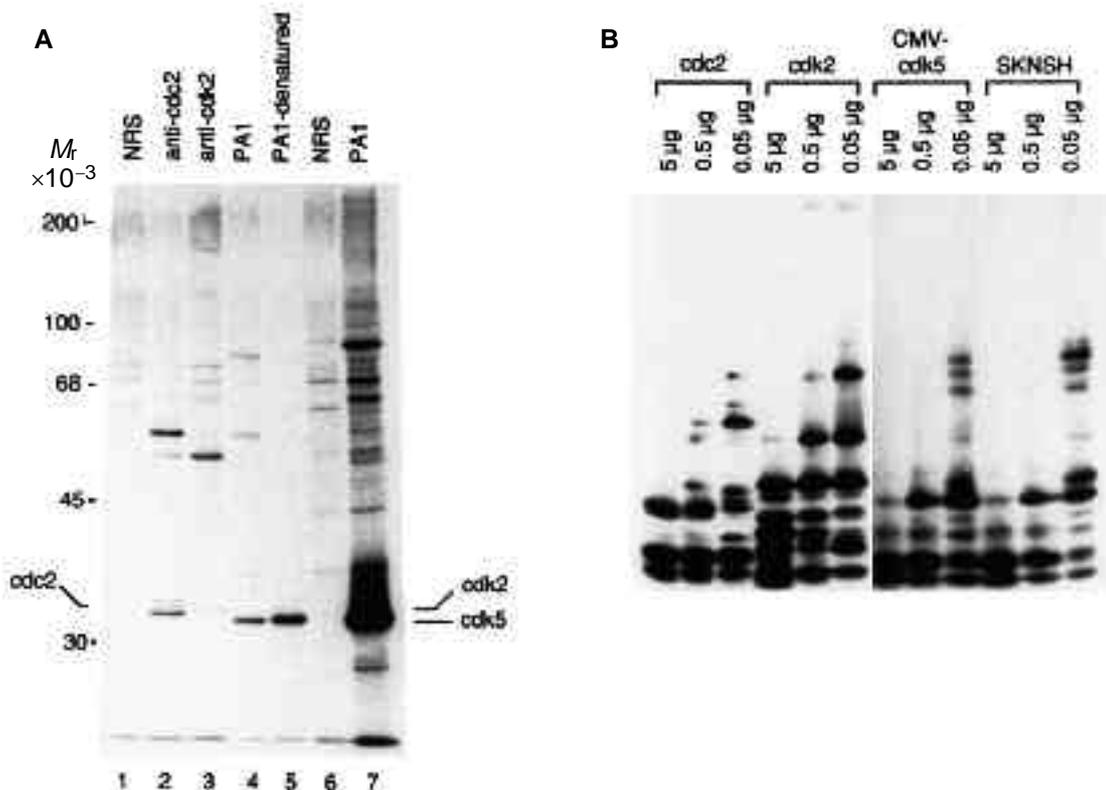
**GAP43.** Brains were fixed in 4% paraformaldehyde in 100 mM phosphate buffer for 48 hours, rinsed in PBS for 48 hours. 50  $\mu$ m vibratome sections were first incubated with 20% normal rabbit serum in 50 mM Tris, pH 7.4, and 0.1% Triton X-100 overnight. Sections were then incubated with anti-GAP43 antibody diluted 1:2000 in 50 mM Tris, pH 7.4, with 9% normal rabbit serum and 0.1% Triton X-100 for 3 days at 4°C. Sections were rinsed overnight and reacted with biotinylated anti-sheep rabbit IgG diluted 1:250 in 50 mM Tris, pH 7.4, containing 1% normal rabbit serum using ABC Elite kit (Vector). Sections were incubated with 0.05% DAB and hydrogen peroxide (0.01%) for 20 minutes.

BrdU (Takahashi et al., 1992) and RC2 (Misson et al., 1988) immunocytochemical procedures have been described previously.

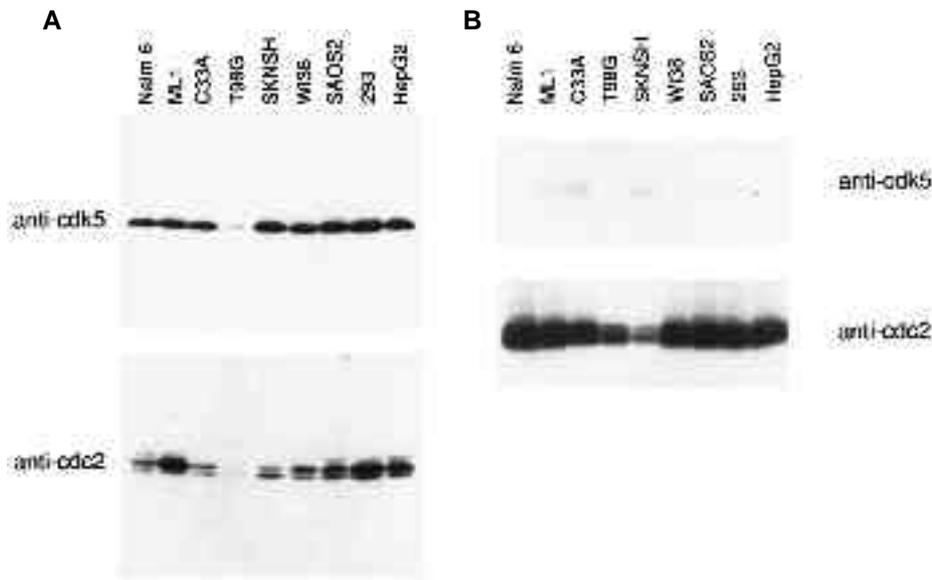
**RESULTS**

**Antibody specific for cdk5**

A polyclonal anti-peptide antibody (PA1) specific for the carboxy-terminal seven amino acids of human cdk5 was prepared by immunization of rabbits. After purification with peptide-affinity chromatography, this antibody recognized a  $32 \times 10^3 M_r$  protein species from cells transfected with a plasmid that expressed cdk5 from the CMV early promoter (Fig. 1A, lane 7). The  $32 \times 10^3 M_r$  species was also seen in immunoprecipitations of lysates from [<sup>35</sup>S]methionine-labeled neuroblastoma cell line SKNSH. This protein migrated faster than either cdc2 ( $34 \times 10^3 M_r$ , three forms) or cdk2 ( $33 \times 10^3 M_r$ , two forms) (Fig. 1A, compare lane 4 with lanes 2 and 3). *Staphylococcus aureus* V8 protease (cleaves after aspartic and glutamic acids) maps of the cdk5 protein expressed from CMV promoter were identical to the  $32 \times 10^3 M_r$  protein species made in the neuroblastoma cell line SKNSH (Fig. 1B). However, V8 maps of cdk5 are distinct from those of either cdc2 or cdk2 (Fig. 1B). PA1 did not



**Fig. 1.** Specificity of affinity-purified anti-cdk5 antibody PA1. (A) SKNSH cells and COS-1 cells transfected with CMV-cdk5 plasmid were labeled with [<sup>35</sup>S]methionine and the cell lysates were used for immunoprecipitations with various antibodies. Lanes 1 to 5 contain immunoprecipitates from SKNSH cells and lanes 6 and 7 contain immunoprecipitates from CMV-cdk5 transfected COS-1 cells. Immunoprecipitations were performed with: lane 1, non-immuned rabbit serum; lane 2, anti-cdc2; lane 3, anti-cdk2; lane 4, PA1; lane 5, denatured SKNSH lysates with PA1; lane 6, non-immuned rabbit serum; lane 7, PA1. (B) Partial V8 proteolytic maps of cdk5 made in vivo (SKNSH), recombinant cdk5 expressed from CMV promoter in COS-1 cells, cdc2 made in vitro and cdk2 made in vitro were compared as indicated.



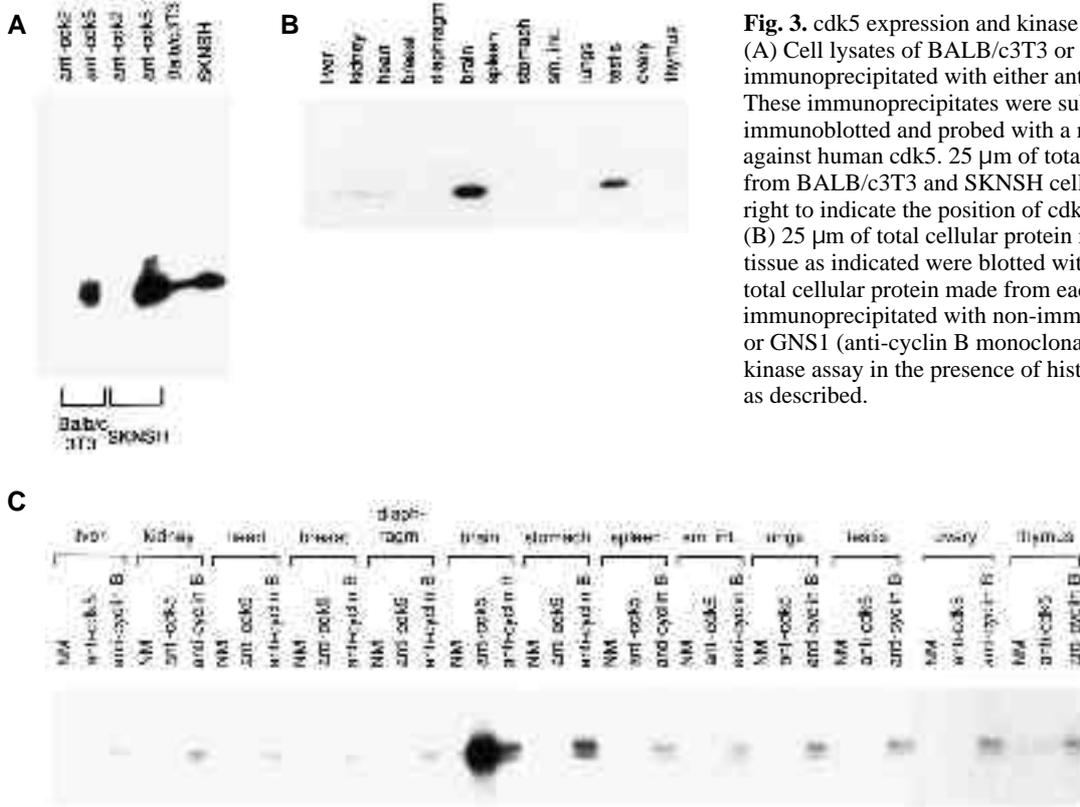
**Fig. 2.** Protein expression and kinase activity of cdk5 in cultured cell lines. (A) 25  $\mu$ m of total cellular protein from nine different cell lines were immunoblotted and probed with PA1 (higher panel), striped and probed with anti-cdc2 antibodies (lower panel). The blots were developed using the ECL detection system as described in Materials and Methods. (B) 200  $\mu$ m of total cellular protein were used for immunoprecipitation with PA1 (upper panel) and anti-cdc2 antibodies (lower panel). In vitro kinase assay was performed in the presence of histone H1 as described in Materials and Methods.

recognize either the cdc2 or cdk2 proteins synthesized in vitro (data not shown) or in vivo. When immunoprecipitation was carried out after 2% SDS denaturation of SKNSH cell lysates, PA1 only recognized the  $32 \times 10^3 M_r$  cdk5 protein (Fig. 1A, lane 5). In addition, when used for immunoblotting of cell lysates made from different sources, PA1 once again only recognized one  $32 \times 10^3 M_r$  protein species (see Fig. 2). Thus, the full set of evidence indicates that PA1 recognizes the cdk5 protein made in vivo and does

not cross-react with either the cdc2 or cdk2 proteins with which cdk5 shares extensive homology.

**cdk5 expression and kinase activity in cultured human cell lines**

PA1 was used to examine the distribution of cdk5 protein and its kinase activity in nine different cell lines: Nalm6 (pre-B leukemia), ML1 (myeloid leukemia), C33A (cervical cancer), T98G (glioblastoma), SKNSH (neuroblastoma),



**Fig. 3.** cdk5 expression and kinase activity in mouse tissues. (A) Cell lysates of BALB/c3T3 or SKNSH were immunoprecipitated with either anti-cdk2 antibody or PA1. These immunoprecipitates were subsequently immunoblotted and probed with a monoclonal antibody against human cdk5. 25  $\mu$ m of total cellular protein made from BALB/c3T3 and SKNSH cells were loaded on the right to indicate the position of cdk5 protein on the gel. (B) 25  $\mu$ m of total cellular protein made from each mouse tissue as indicated were blotted with PA1. (C) 200  $\mu$ m of total cellular protein made from each mouse tissue were immunoprecipitated with non-immuned rabbit serum, PA1 or GNS1 (anti-cyclin B monoclonal antibody). In vitro kinase assay in the presence of histone H1 was performed as described.

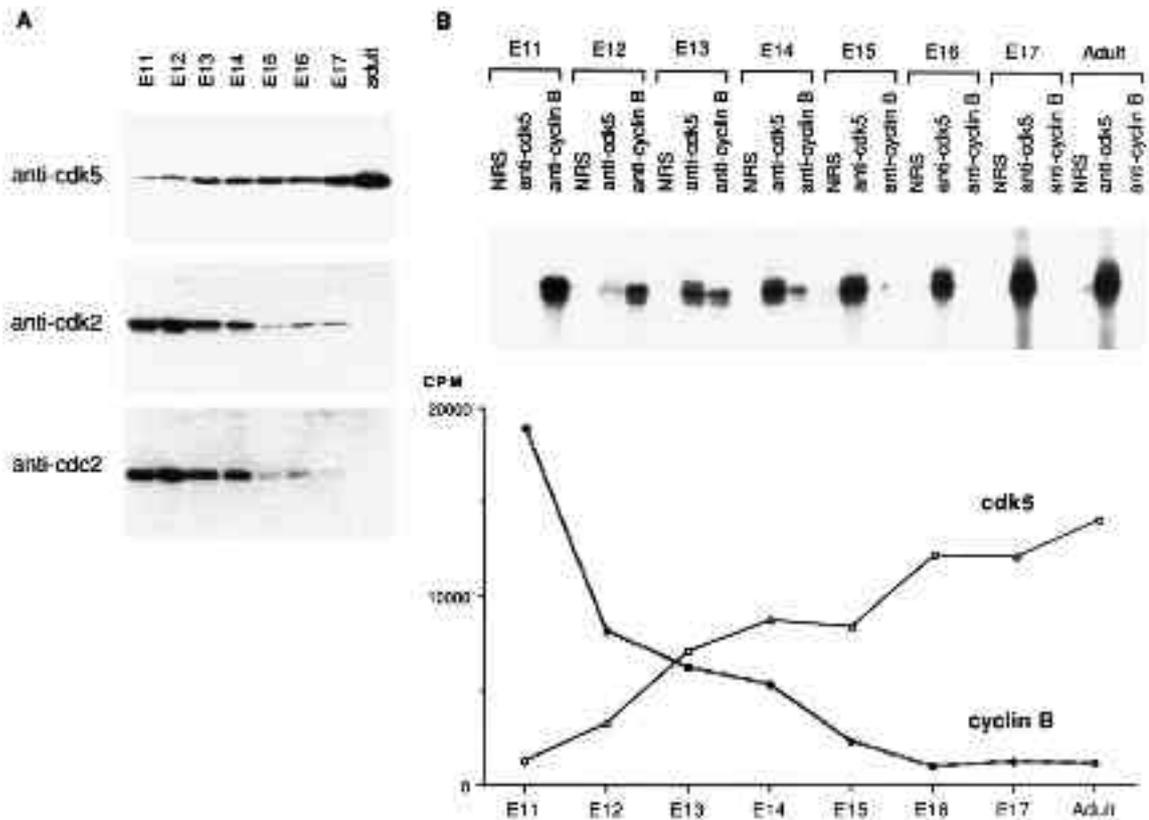
WI38 (diploid human fibroblast), SAOS2 (osteosarcoma), 293 (adenovirus transformed human kidney epithelial cell) and HepG2 (hepatocarcinoma). A single cdk5 protein band was detected in every cell line tested (Fig. 2A). This finding was expected because our previous study had identified high concentrations of cdk5 transcripts in a number of cell lines in tissue culture (Meyerson et al., 1992). The cell lines used in the current study were also shown to express cdc2 ubiquitously (Fig. 2A). Less protein was loaded in lane 4 containing T98G lysates and lower levels of both cdk5 and cdc2 were seen.

To investigate the distribution of cdk5 kinase activity, equal amounts of lysates from these cell lines were immunoprecipitated with PA1 or antibody specific to cdc2. In vitro kinase assays were then carried out on these immunoprecipitates in the presence of histone H1 as an exogenous substrate (see Materials and Methods). Fig. 2B shows that none of these lysates had detectable cdk5 kinase activity, but they do have abundant cdc2 kinase activity, phosphorylating histone H1 in vitro. Rabbit non-immune serum did not give rise to significant levels of histone H1 kinase activity (data not shown). The failure to detect cdk5 kinase activity in these preparations was not due to the inability of PA1 to recognize an active form of the cdk5 kinase, because these antibodies did immunoprecipitate active kinases in other experiments described below.

**Expression and activity of cdk5 in mice**

Since no cdk5-associated kinase activity was detected in any of the cell lines tested, we examined the distribution of the cdk5 protein and kinase activities in different tissues of intact animals. cdk5 cDNA clones were isolated from a neonatal mouse brain library (M. Meyerson, unpublished results), and sequence analyses of the mouse cdk5 revealed it to be 99% identical at the amino acid level to human cdk5 (L.-H. T., unpublished results). The carboxyl terminal sequence from human cdk5 sequence used to develop PA1 is identical to the corresponding sequence in the mouse form of cdk5 and, as expected, the antibody recognized cdk5 in the mouse fibroblast cell line BALB/c 3T3 (Fig. 3A). Therefore, we used this antibody to examine cdk5 in the mouse.

Previous work (Lew et al., 1992a) has shown that cdk5 purified from bovine brain can phosphorylate histone H1 in vitro. As expected, the PA1 antibody detected substantial cdk5 expression in the adult mouse brain (Fig. 3B). There was only moderate expression in testis and little or no expression of cdk5 in liver, kidney, heart, breast, diaphragm, spleen, stomach, small intestine, lungs, ovary or thymus (Fig. 3B). Lysates from all the 13 tissues screened here had detectable cdc2 kinase activity for histone H1 (assayed with cyclin B antibody immunoprecipitated cdc2/cyclin B complexes; Fig. 3C). cdk5 histone H1 kinase activity was detectable only in mouse brain (Fig. 3C). Higher levels of



**Fig. 4.** Expression and kinase activity of different cdk5 in embryonic mouse forebrain. (A) 25 µm of total cellular protein made from E11 to E17 forebrain were immunoblotted and probed with PA1, anti-cdk2, and anti-cdc2 antibodies as indicated. (B) 200 µm of total cellular protein made from E11 to E17 forebrain were immunoprecipitated with non-immuned rabbit serum, PA1 or GNS1 and in vitro kinase assay in the presence of histone H1 was performed as described. In the graph, these assays were quantitated by Cerenkov counting and plotted.

the cdk5 kinase were detected in the forebrain than the hindbrain (data not shown).

#### cdk5 expression and activity in the embryonic mouse brain

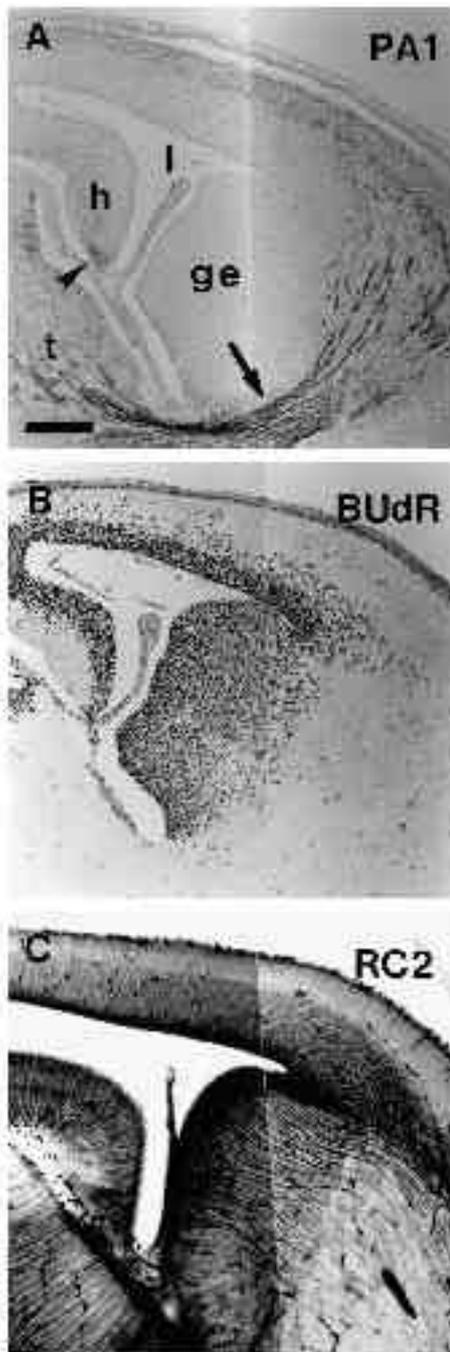
Given that cdk5 shares a high homology with cdc2 and cdk2 which play critical roles in cell cycle control, it was surprising that the adult mouse brain where most of the cells were terminally differentiated had high cdk5 kinase activity and that actively proliferating cell lines of neural origin, such as neuroblastomas and glioblastomas, did not. To gain further insight into the profile of cdk5 expression during

developmental stages when both actively dividing neural precursors and differentiating neurons are present simultaneously, we examined the expression of cdk5 in the forebrain of mouse embryos.

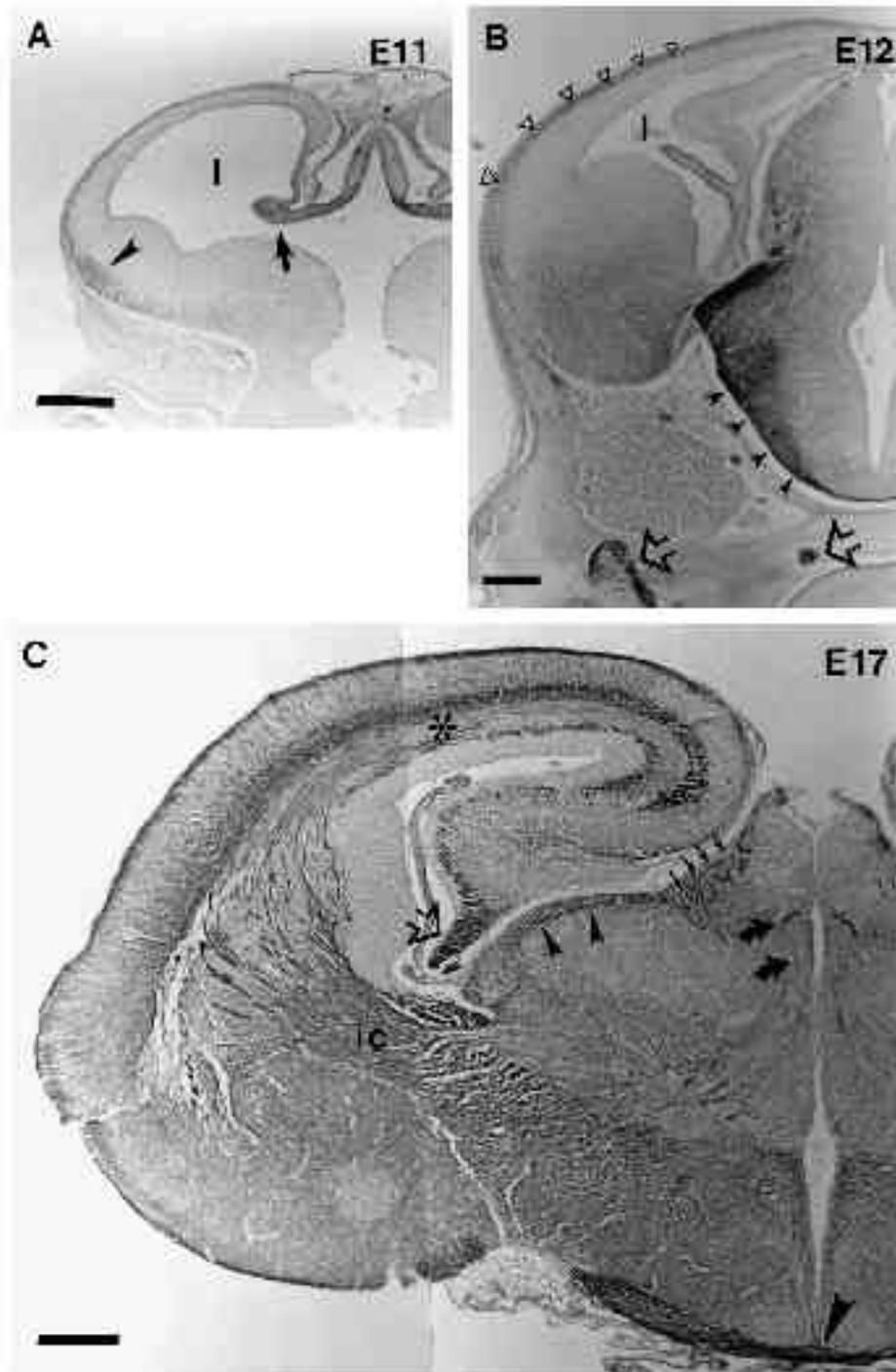
Expression and kinase activity of cdc2, cdk2 and cdk5 were investigated in mouse forebrain samples taken from E11-E17 (E0=day of coitus). The expression pattern of cdk5 from E11 to E17 appeared to have an inverse relationship to that of cdc2 and cdk2 (Fig. 4A,B). cdk5 expression was barely detectable on E11, the earliest gestational age studied. Its level increased gradually through E15 and then rapidly through E17 to maximum expression in the adult brain (Fig. 4A). By contrast, cdc2 and cdk2 expression was maximal at E11 and E12 and declined to barely detectable levels at E17 and remained at that level in the adult brain. It should be noted that the embryonic mouse forebrain is constituted of both terminally differentiated and actively proliferating cells, and the relative prominence of the former increases while that of the latter decreases as development proceeds.

The levels of histone H1 kinase activity closely matched the patterns of protein expression for these kinases (Fig. 4B). cdk5 kinase activity was not detectable at E11 and became maximal at E17 and in the adult brain, while cdc2 kinase activity was highest at E11 and declined to undetectable levels by E16 to E17 (Fig. 4B). cdc2 kinase activity was measured using anti-cyclin B monoclonal antibody in these experiments. In separate experiments, we have found that antibodies directed against the carboxy-terminal segment of human cdc2 do not efficiently recognize mouse cdc2-associated kinase activity, while immunoprecipitations prepared with the anti-cyclin B monoclonal antibody readily recognize the mouse cyclin B/cdc2 complex. The latter was thus used to measure the mouse kinase activity.

Given the close primary structural relationship between cdk5 and cdc2 or cdk2, we had expected cdk5 levels and kinase activity to resemble the general features of its cell cycle-regulating relatives. However, these results suggest that, at least in the developing mouse brain, cdk5 is expressed primarily after cell proliferation has subsided or terminated. Conversely, the expression and activity of cdc2



**Fig. 5.** Coronal view of the E14 cerebral wall at the level of the foramen of Monroe immunocytochemically stained with PA1 (A), anti-BrdU (B) and RC2 (C) antibodies. The plane of section passes through hippocampal formation, cerebral wall, ganglionic eminence, internal capsule and diencephalon. (A,B) 4  $\mu$ m-thick paraffin-embedded sections of 70% ethanol-fixed brains. (C) 50  $\mu$ m-thick Vibratome section of a 4% paraformaldehyde-fixed brain. (A) PA1 stains fasciculated axonal systems including the system of ascending fibers linking the developing neocortex and thalamus which spread tangentially through the neocortical subplate, continue through the internal capsule (arrow) and distribute within the thalamus (t). The fimbria (arrow head) of the hippocampus (h) is also stained. (B) The proliferative zones of the forebrain are delineated by nuclear labelling following a 12.5 hours period of cumulative S phase labelling with BrdU. There is no PA1 staining in the proliferative zones (compare A and B). (C) RC2 staining shows the distribution of radial glial fibers. The pattern of axonal fiber staining with PA1 contrasts in all details with that of radial glial fiber staining with RC2 (compare A and C). ge, ganglionic eminence. l, lateral ventricle. Bar in A, 100  $\mu$ m and also applies to B and C.



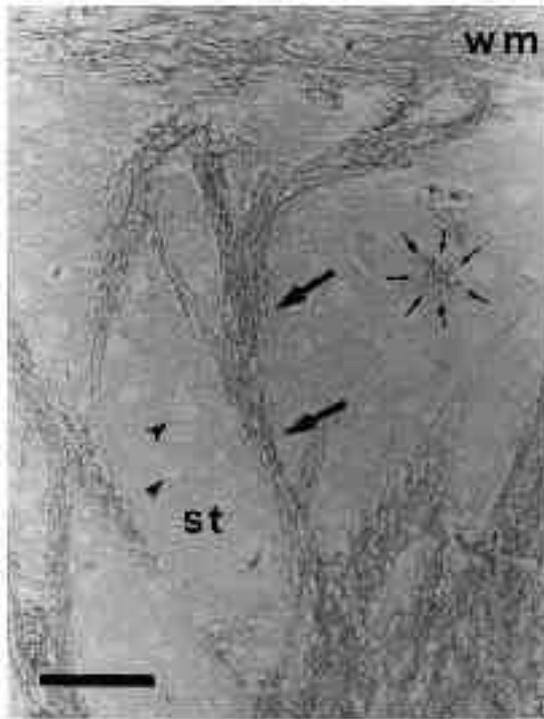
**Fig. 6.** PA1 staining of the forebrain on E11 (A), E12 (B) and E17 (C). The antibody appears to stain mainly fasciculated axonal systems. (A) Staining of the lateral olfactory tract (arrowhead) and the fimbria of the hippocampal system (arrow) is apparent as early as E11. l, lateral ventricle. (B) Cranial nerves (open arrows), optic tract (small arrowheads) and the tangential fiber systems of the primitive plexiform zone at the surface of the cerebral wall (open arrowheads) are stained on E12. Staining of cranial nerves is apparent on E11, the earliest age examined (data not illustrated). (C) The intensity of PA1 staining is robust by E17. In addition to the fiber systems shown in A and B, internal capsule (ic), axons in the intermediate zone (\*), stria medularis (small arrows), fasciculus retroflexus (curved arrows), external medullary lamina (small arrowhead), and the optic chiasm (large arrowhead) are stained. Open arrow, fimbria of the hippocampus. Bars, 200  $\mu\text{m}$ .

and cdk2 are biased toward that period of development when cell proliferation is active.

#### Immunohistochemistry for cdk5 in the mouse brain

To test the expression pattern of cdk5 in the nervous system, coronal sections of the brain and surrounding structures of the head from embryonic (E11-E17) and adult mice were stained with PA1. PA1 staining was confined to axonal pathways at all of the ages examined (Figs 5-8). PA1 did not stain any nuclei. On E14, PA1 staining pattern was

compared with the pattern of staining of proliferative cells (Fig. 5). The distribution of proliferative cells was detected immunohistochemically following a cumulative incorporation of the S phase marker BrdU (Takahashi et al., 1993). Proliferating cells identified by BrdU staining (Fig. 5B) were not stained by PA1 in any part of the nervous system. For example, the proliferative zones of the ganglionic eminence and the cerebral cortex were intensely labeled with BrdU (Fig. 5B) but were not labeled following PA1 immunocytochemistry (Fig. 5A). The PA1 staining pattern was also compared with that of RC2, an antibody selective



**Fig. 7.** A 4  $\mu\text{m}$ -thick, coronal, paraffin-embedded section through the striatum (st) and overlying white matter (wm) of an adult mouse processed for PA1 immunocytochemistry. PA1-labeled axon fascicles sectioned in the longitudinal (large arrows) and transverse planes (small arrows) delineate islands of unlabeled striatal neuropil which contains neuronal or glial somata (arrowheads) and dendrites. The overlying white matter also contains fascicles of PA1-labeled axons. Bar, 50  $\mu\text{m}$ .

for radial glial cells and other cells of astroglial lineage in the developing brain (Misson et al., 1988; Fig. 5C). The pattern of axonal staining by PA1 is strikingly different from the pattern of staining of radial glial fibers with RC2 (Fig. 5C). The radial glial fibers have a regularly spaced, transmural radial span throughout the developing nervous system, which bears no resemblance to the distribution of axonal fascicles. Thus, PA1 does not appear to stain the radial glial fiber system.

From as early as E11, PA1 staining was confined to zones of the nervous system where axonal pathways are known to form. Among the fiber systems well stained were the lateral olfactory tract, fimbria of the hippocampal formation (Fig. 6A) and peripheral nerve fascicles incidentally encountered within the extracranial tissues (Fig. 6B at E12). The optic tract was stained by E12 (Fig. 6B). By E14 (Fig. 5A) the full course of axonal systems linking neocortex and thalamus, and by E17 (Fig. 6C) the optic chiasm, the stria medullaris, the external medullary lamina and the fasciculus retroflexus were prominently stained. It appears that PA1 stains virtually all of the axonal systems in the embryonic mouse forebrain. It is possible that the onset of expression of *cdk5* is differentially regulated in different axonal systems during development but that possibility could not be examined in the present study. The pattern of PA1 staining in the adult nervous system was examined in

sections of the telencephalon of adult mice. PA1 staining in this part of the brain was comparable to that seen in the embryonic nervous system in that only axons appeared to be stained, neither cell bodies nor dendrites were labeled. An example of this staining pattern is shown for the striatum in Fig. 7. Thus, in our preparations, PA1 labelling appears to be restricted to axonal systems even in the mature brain.

In additional experiments, the pattern of PA1 staining was compared with that of an antibody against growth-associated protein-43 (GAP-43), a protein that is specific to developing axons (Jacobson et al., 1986; Benowitz and Routtenberg, 1987; Benowitz et al., 1988; Goslin et al., 1988; Meiri et al., 1988; Moya et al., 1989; Skene, 1989; Dani et al., 1991; Fig. 8). The distribution of PA1-stained structures was similar to that of structures stained with antibody against GAP-43 (Fig. 8A,B), an observation reinforcing the interpretation that PA1 stains axons. In the cerebral cortex, the intermediate zone and subplate showed strikingly similar patterns of staining with PA1 and anti-GAP-43 (compare Fig. 8C to D). However, there were some differences between the two patterns of staining. Anti-GAP-43 labeled varicosities along and growth cones at the tips of single axons whereas PA1 did not. This difference is illustrated in Fig. 8E and F for axons in the optic tract overlying lateral thalamus. In the optic tract, near the surface of the thalamus, prominent, anti-GAP-43-labeled growth cones at the tips of developing axons are visible (Fig. 8F). When sections of the same region of the brain are processed for immunocytochemistry with the PA1 antibody, none of the growth cones is labeled even though intensely labeled fascicles of optic tract axons are clearly visible (Fig. 8E). Thus, anti-GAP-43 appeared to stain developing axons throughout their trajectory, whereas PA1 appeared to stain only the established axonal shafts.

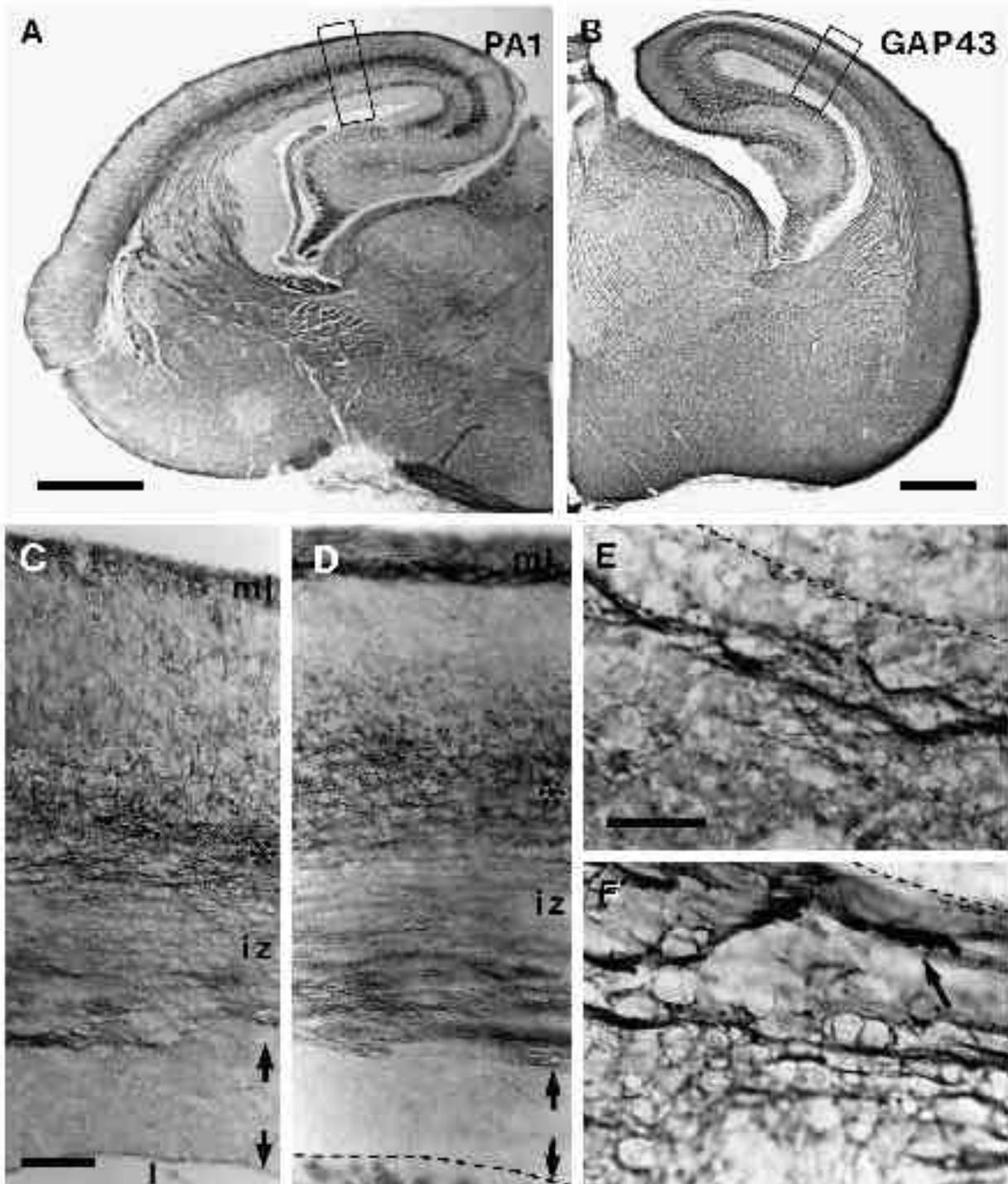
## DISCUSSION

*cdk5* is a protein serine/threonine kinase that bears approximately 60% structural identity to both *cdc2* and *cdk2*. Investigations of *cdk5* have highlighted a number of important differences with respect to the patterns of expression and activation of *cdc2* and *cdk2*. The essential basis for these differences has been partly resolved in the present analysis.

### *cdk5* versus *cdc2* and *cdk2*

The *cdc2* and *cdk2* kinases are indispensable to progression of the cell division cycle in all multicellular eukaryotes, and their kinase activities are ubiquitous in proliferative cell populations but not in cells that are terminally differentiated or in the quiescent ( $G_0$ ) state. Activation of *cdc2* and *cdk2* depends both on binding to regulatory subunits, known as cyclins, and on phosphorylations and dephosphorylations of the kinases themselves. Although *cdk5* shares similar physical structures and *in vitro* substrate specificity with both *cdc2* and *cdk2*, it displays unexpected unique properties in other respects.

Despite the fact that *cdk5* protein is expressed in virtually all proliferative cell lines *in vitro* and various tissues from the intact animal, with one exception, these populations of



**Fig. 8.** A comparison of the staining of axonal systems on E17 with PA1 (A,C,E) and GAP-43 (B,D,F). Both antibodies stain virtually the same elements in the forebrain (compare A and B). (C,D) High magnification views of the region of the cerebral wall enclosed by the rectangles in A and B, respectively. Neither antibody stains the ventricular proliferative zone (between two arrows). However, both antibodies intensely stain axons in the intermediate zone (iz), subplate-lower cortical plate level (\*) and in the molecular layer (ml). (E,F) High magnification views of coronal sections stained with PA1 and GAP-43, respectively, to show the optic tract overlying lateral thalamus. PA1 labels fascicles of optic tract axons in the thalamic neuropil but does not label growth cones at the tips of optic tract axons (E). In contrast, GAP-43 intensely labels growth cones of optic tract axons (arrow) and also axonal trunks coursing through the thalamus (F). In A and B, note differential tissue shrinkage due to different fixation techniques; the section in A (PA1 staining) was fixed with 70% ethanol and that in B (GAP-43 staining) with 4% paraformaldehyde (see Materials and Methods). Broken line in D indicates lateral ventricular surface and those in E and F indicate pial surface of the thalamus. Bars in A and B, 500  $\mu$ m; bar in C, 30  $\mu$ m and also applies to D; bar in E, 20  $\mu$ m and also applies to F.

cdk5 have no detectable associated kinase activity toward histone H1. The only exception has been adult brain which is composed virtually exclusively of terminally differentiated neurons and glial cells in the G<sub>0</sub> state, where the level of expression is matched by the level of kinase activity (Lew et al., 1992a, and this study). Proteins other than histone H1 (such as the retinoblastoma protein and the neurofilament-heavy (H) form) that served as good *in vitro* substrates for both cdc2 and cdk2 prepared from proliferating cell lines also failed to be phosphorylated by cdk5 prepared from the same cell lines, but were readily phosphorylated by cdk5 prepared from brains (L.-H. T., unpublished results). The significance of the expression of cdk5 in the proliferating cell lines is currently unclear. Nevertheless, we do not exclude the possibility that cdk5 is active as a kinase in the proliferating cell lines that phosphorylates protein substrates that we have not identified yet.

Several lines of evidence indicate that cdk5 may not be activated by conventional cyclin association in brain. First, cdk5/cyclin complexes with active kinase activity have not been found in vertebrate cell lines; although cdk5 has been found to complex with cyclin D1 in WI 38 cells (Xiong et al., 1992), this complex has not been demonstrated to have kinase activity. Second, an association between cdk5 and currently recognized cyclins has not been detected in brain tissue even though cdk5 is highly active as a histone H1 kinase in brain. Further, cdk5, unlike cdc2 and cdk2, does not complement the function of CDC28 when overexpressed in budding yeast (Meyerson et al., 1992) indicating that cdk5 cannot associate with the endogenous cyclins in yeast to form a cell cycle regulating kinase. A potential partner for cdk5 is a 25×10<sup>3</sup> M<sub>r</sub> protein co-purified with cdk5 from bovine brain (Lew et al., 1992a) and this protein is a potential candidate for a regulatory role of cdk5 kinase activity. It remains to be determined whether this cdk5 regulatory candidate is related to the current group of identified cyclins.

### Expression of cdk5 in the brain

In the forebrain, cell proliferation during development occurs deep within the cerebral wall. A pseudostratified ventricular epithelium that is immediately adjacent to the ventricular cavities of the forebrain is the principal source of neurons and glia (Sidman et al., 1959; Caviness and Sidman, 1973; Caviness, 1982; Bayer and Altman, 1991; Takahashi et al., 1993). After neurons undergo their terminal mitoses, they migrate outward to their destinations (Rakic, 1972, 1988; Misson et al., 1991). Neocortical neurons become postmitotic largely between E11 and E17 in mice (Caviness, 1982), while glial formation is most intense in the early postnatal period (Misson et al., 1991; Takahashi et al., 1991).

The principal conclusions that have emerged from this study are: (1) cdk5 is not expressed in proliferating cells of the nervous system, (2) it is expressed and active in terminally differentiated neurons; and (3) it is not expressed in neuroglial cells. When the levels of expression and activation of cdc2 and cdk5 were compared during development, it was found that on E11 cdk5 kinase was barely detectable but the levels of cdc2 were the highest. As development proceeds, the levels of expression and activation of cdk5

increase while those of cdc2 show a corresponding decrease. These gradients in the levels of expression and activity of the two kinases correspond to the maturational gradients in the cerebrum.

The immunohistochemical distribution of cdk5 at days E11-E17 is consistent with the analysis of tissue lysates in that there is no histological detection of cdk5 in forebrain proliferative populations. The distribution is specific for neuronal populations and not neuroglial populations. Although the focus of the analysis was the forebrain, we were able to determine that cdk5 was expressed in the post-mitotic neurons of the peripheral as well as the central nervous system.

PA1 stained axons representing virtually all the major systems. In particular, the fasciculated axonal systems with relatively advanced maturity were readily stained with PA1. This staining pattern was preserved throughout development and into maturity: PA1 staining was restricted to axons at all of the embryonic stages examined and in the adult telen-cephalon. Conspicuously absent from the histological profile were immature axons in the course of their primary elaboration. Specifically, neither axonal varicosities nor growth cones at the tips of axons were observed in the immunohistological preparations at any age, although axonal staining was robust at all ages after E12.

Varicosities and growth cones are characteristic of axons during their primordial stages of development, appearing in advance of the elaboration of the rigid neurofilament structure with H-form cross bridging between the longitudinal members. Neurofilament H- and medium (M)-forms appear to contain consensus phosphorylation sites for the cdk5 and can be phosphorylated by cdc2 kinase *in vitro* (Hisanaga et al., 1991). The purified cdk5 kinase from bovine brain was also reported to phosphorylate neurofilament H-form *in vitro* (Lew et al., 1992b). Therefore, it is possible that cdk5 kinase activity, by phosphorylating the H-form, may be critical to the formation and maintenance of this more rigid neurofilament axonal skeleton (Nixon and Sihag, 1991). Other candidate substrates of cdk5 in brain include the microtubule-associated protein *tau* which also appears to contain phosphorylation consensus sequences for the cdk5 (Drewes et al., 1992). *Tau* has been shown to be involved in axonal morphogenesis and aberrant phosphorylation of *tau* has been found in paired helical filaments of Alzheimer's brain (see review by Kosik, 1993).

cdk5 isolated from human, cow, rat and mouse share greater than 99% homology with each other. Homologous kinases with a high degree of structural identity with the human kinase have also been isolated from the nematode *Caenorhabditis elegans* (S. v. d. Heuvel and L.-H. T., unpublished results) and the fruit fly *Drosophila melanogaster* (L.-H. T. unpublished results). Thus, cdk5 appears to be an evolutionarily highly conserved protein kinase. To date, efforts to isolate cdk5 from yeast have been unsuccessful (L.-H. T., unpublished results) indicating that the evolved functions of cdk5 are limited to multicellular organisms, where a role in neuronal differentiation is most conspicuous.

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