THE UNIVERSITY OF CALGARY

Regulatory Properties of Neuronal Cdc2-like Kinase

by

Zhong Qi

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

MAY, 1996

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a dissertation entitled "Regulatory Properties of Neuronal Cdc2-like Kinase" submitted by Zhong Qi in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Supervisor: Dr. Jerry H. Wang, Department of Medical Biochemistry

Dr. Donald J. Fujita, Department of Medical Biochemistry

Dr. H. Joseph Goren, Department of Medical Biochemistry

Dr. David L. Severson, Department of Pharmacology and Therapeutics

Dr. Michael P. Walsh, Department of Medical Biochemistry

External Examiner: Dr. Patrick W.K. Lee
Department of Microbiology and Infectious Diseases

External Examiner: Dr. Gerald M. Carlson
Department of Biochemistry, University of Tennessee

June 17, 1996
Date

ABSTRACT

Neuronal Cdc2-like kinase (NCLK) is a heterodimer of Cdk5 and a neuron-specific protein, p35. Three p35-derived fragments, p25, p23 and p21, were expressed in and purified from *E. coli* as glutathione *S*-transferase recombinant proteins. p21 was used to precipitate an active Cdk5 from bovine brain extract. When recombinant p35 fragments were incubated with bacterially expressed Cdk5, all three p35 fragments activated Cdk5 with equal efficacy. NCLK has been reconstituted from bacterially expressed Cdk5 and the shortest form of p35, p21. After purification, reconstituted NCLK displays similar specific enzyme activity and substrate specificity to NCLK isolated from bovine brain.

Activation of Cdk5 by p35-derivatives has been characterized in detail with p21. Several observations suggest that, unlike other well characterized Cdc2-like kinases whose activities depend on phosphorylation of the catalytic subunits by a distinct kinase, activation of Cdk5 by p35-derivatives is independent of Cdk5 phosphorylation. Cdk5 is highly activated by p35-derivatives without requiring any other protein in the reconstitution reaction. The rate and extent of Cdk5 activation by reconstitution were not significantly affected by inclusion of ATP-Mg^{2+} in the reaction. Potential autophosphorylation of Cdk5 has been ruled out, as no phosphorylation occurred on Cdk5 during Cdk5-catalyzed phosphorylation. Protein phosphatase 2A, which dephosphorylates and inactivates Cdk2/cyclin A, has no discernible effect on reconstituted NCLK activity.

Cdk5/p35 may exist in a complex with several proteins in brain. Seven different sequences encoding p35-interacting proteins were isolated by the yeast two-hybrid system from a human brain cDNA library. They are fragments of glial fibrillary acidic

protein (GFAP), $n(\alpha_2)$-chimaerin, clusterin, and four novel peptides displaying no significant homology to any clearly defined protein in the current database. $n(\alpha_2)$-chimaerin and clusterin are expressed in neurons. GFAP is a glial-specific protein. However, neurofilaments are neuronal homologues of GFAP, and contain a region of homology to the isolated p35-interacting region in GFAP. Further study is required to verify interactions between p35 and these isolated binding proteins and to determine the nature of these interactions.

ACKNOWLEDGMENTS

I would like to thank the members of my supervisory committee, Drs. Donald J. Fujita, H. Joseph Goren, David L. Severson, Michael P. Walsh, and Jerry H. Wang, for all their guidance and support. In particular, my deepest appreciation goes to Dr. J.H. Wang who was an excellent mentor throughout my studies.

I wish to acknowledge the members of the J.H. Wang laboratory, past and present, who have given me invaluable help, support and advice. I also owe enormous thanks to Dr. C.J. Brandl (University of Western Ontario) for all his advice and help on the yeast two-hybrid system.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
ATP	adenosine 5'-triphosphate
BCR	product of the breakpoint-cluster-region gene
CAK	Cdk-activating kinase
CDK	cyclin-dependent kinase
CSF-1	colony-stimulating factor-1
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
GAP	GTPase-activating protein
GDP	guanosine 5'-diphosphate
GFAP	glial fibrillary acidic protein
GSH	glutathione
GST	glutathione *S*-transferase
GTP	guanosine 5'-triphosphate
IL-2	interleukin-2
IPTG	isopropylthio-β-D-galactoside
JNK	c-Jun N-terminal kinase
LB medium	Luria-Broth medium
MOPS	4-morpholinepropane-sulfonic acid
NCLK	neuronal Cdc2-like kinase
NF-H	high molecular weight subunit of neurofilaments
NF-M	medium molecular weight subunit of neurofilaments
NFT	neurofibrillary tangle

ONPG	*o*-nitrophenyl-β-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PEG-4000	polyethylene glycol of average molecular weight of 3350
PHF	paired helical filament
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A
pol δ	DNA polymerase δ
SAPK	stress-activated protein kinase
SD medium	synthetic dropout medium
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
TBS	Tris buffered saline
TGFβ	transforming growth factor β
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD medium	yeast peptone dextrose medium

INTRODUCTION

1. Cyclin-dependent kinases

Cell proliferation is controlled by an intricate network including protein-protein interaction and protein modification events. This network integrates external and internal signals to cell functions. The dividing cells pass through a regular sequence of cell growth and division, known as the cell cycle (Norbury and Nurse, 1992). The cycle consists of four major phases: G_1, S, G_2 and M. The timing of cell cycle events is normally under stringent control. The information is integrated during one event in order to determine the readiness of a cell to enter the next one. If cycle progression is halted at any stage, and the subsequent step is consequently prevented, the cell cycle is arrested (Hartwell and Weinert, 1989).

It is believed that cell cycle progression is governed by a biochemical cycle in which a family of Ser/Thr kinases is activated and inactivated in a precisely timed program. To date there are eight recognized members in this family: Cdc2 (Cdk1), Cdk2, Cdk3, Cdk4, Cdk5, Cdk6, Cdk7, and Cdk8; Cdc2 is the founding member of this family (Lees, 1995; Morgan, 1995; Tassan *et al.*, 1995). *cdc2* in fission yeast was selected from a collection of cell division cycle (*cdc*) mutants as it controls two critical steps in the cell division cycle, G_1/S and G_2/M transitions (Murray and Kirschner, 1991). *cdc2* mutations in *S. pombe* cause either cell cycle arrest or cells to enter mitosis prematurely before they have reached their normal size.

Studies using *Xenopus* oocytes provided evidence for biochemical mechanisms that control cell division (Murray and Kirschner, 1989). Microinjection of cytoplasm from mature oocytes into immature oocytes, which are naturally arrested in meiotic

prophase, promotes their full maturation without requiring further protein synthesis (Masui and Markert, 1971; Reynhout and Smith, 1974). It was proposed that a maturation-promoting factor (MPF) existed in mitotic cells. Such a MPF was detected in metaphase-arrested eggs and in extracts of somatic cells from distantly related eukaryotes (Kishimoto and Kanatani, 1976; Murray and Kirschner, 1991). Purification of MPF revealed that this factor was a complex composed of two subunits of 34 kDa and 45 kDa (Lohka *et al.*, 1988). The 34 kDa polypeptide displays high sequence homology to the *cdc2* gene product found in budding yeast (Arion *et al.*, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988). The 45 kDa subunit, which was shown to cross react with an antibody against cyclins in clams, is a homologue of the yeast *cdc13* gene product (Gautier *et al.*, 1989).

The *cdc2* gene product in fission yeast, whose functional counterpart in budding yeast is *cdc28*, is a protein Ser/Thr kinase. In animal cells, while the regulation by Cdc2 of entry into and exit from mitosis is conserved, the distinct Cdc2 homologues, Cdk2, Cdk4 and Cdk6, are primarily involved in control of G_1 progression and subsequent DNA synthesis (Fang and Newport, 1991; Koff *et al.*, 1992; Rosenblatt *et al.*, 1992; Tsai *et al.*, 1993a). In addition, there is an extended family of Cdc2 homologous proteins, which are highly conserved and share 40-75% sequence identities (Meyerson *et al.*, 1992).

The monomeric Cdc2-like proteins do not display any kinase activity. Association with the primary regulatory subunit, which is cyclin, is essential for their catalytic activities (Solomon *et al.*, 1990). Therefore, they are designated as cyclin-dependent kinases (Cdks). Cdk-cyclin are subjected to an integrated regulatory mechanism of

phosphorylation and dephosphorylation, as well as association with additional regulatory subunits.

2. Cdk regulation by cyclin binding

Cyclins were initially discovered in clam embryos as proteins periodically synthesized during early embryonic development (Evans *et al.*, 1983). In surf clams, cyclin levels were seen to peak at each M phase, with two types, A and B, being distinguished by their gel mobilities and the slightly earlier appearance and disappearance of the A type. Moreover, the *cdc13* gene product of fission yeast was identified as a homologue of the 45 kDa subunit of *Xenopus* MPF, which is now known as cyclin B (Booher and Beach, 1988; Gautier *et al.*, 1990).

Cyclins compose a rapidly growing family, based on sequence homology and functional complementation. In mammalian cells, there are now cyclins A-H with subspecies (Lees, 1995; Sherr, 1993). The conserved similarity in the cyclin family is restricted to an internal region of approximately 100 amino acid residues, which is termed the cyclin box. It is thought that the cyclin box is the region responsible for Cdk binding and activation (Kobayashi *et al.*, 1992; Lees and Harlow, 1993). Deletion or mutation in this region abolishes association with Cdks and their Cdk-activating activity. In addition to supporting Cdk activity, cyclin contributes to the substrate selection of the Cdk-cyclin complex. The interaction between cyclin and substrate facilitates phosphorylation of particular substrates (Dynlacht *et al.*, 1994; Hoffmann *et al.*, 1993; Peeper *et al.*, 1993).

The crystal structures of monomeric Cdk2 and the Cdk2 complex with an active fragment of cyclin A provided the structural basis for Cdk2 activation by cyclin A (De Bondt *et al.*, 1993; Jeffrey *et al.*, 1995). Cdk2 has a bilobal structure resembling that of

the catalytic subunit of protein kinase A (PKA). There are variations between the structures of free Cdk2 and PKA. Firstly, the core of the protein substrate binding cleft between the two lobes in Cdk2 is blocked by a large loop, which includes residues 152-170 and is called the T-loop. Secondly, the residues in the catalytic core required to coordinate ATP orientation are positioned quite differently in Cdk2 from those equivalent residues in PKA. With the misalignment of the catalytic residues, it is impossible for Cdk2 to catalyze the phosphate-transfer reaction. The binding of cyclin A induces dramatic changes of Cdk2 structure including removal of the T-loop from the substrate binding cleft and rearrangement of the catalytic residues in the ATP-binding site, which restores the active conformation of Cdk2. Since sequences in the catalytic domain of Cdk members are well conserved and display extensive similarity to other protein Ser/Thr kinases, the structural features of the Cdk2/cyclin A complex are most likely shared by other Cdk-cyclin complexes.

Cyclin function is primarily controlled by its protein level, which is regulated by protein synthesis and turnover. The cyclin levels peak at particular stages of the cell cycle corresponding to their functions. Indeed, they are categorized by the stages in which they function, such as mitotic cyclins, which control the progression from G_2 to M phase, and G_1 cyclins, which control the G_1/S transition (Sherr, 1993). Cdc2/cyclin B is the well characterized complex that controls crossing of the G_2/M boundary. The quantity of cyclin B gradually accumulates during interphase until its maximal level is reached in late G_2 phase. At that time, the Cdc2/cyclin B complex is ready for activation. After induction of M phase and cytokinesis, cyclin B is rapidly proteolyzed via a ubiquitination pathway; cyclin degradation is essential for cell exit from mitosis (Glotzer *et al.*, 1991; Murray, 1995).

The CLN genes were identified in budding yeast to encode a class of G_1 cyclins. Human cyclins C, D1 and E were discovered in screens for cDNAs that could rescue CLN-deficient yeast cells (Lew *et al.*, 1991). Three mouse homologues of human cyclin D1 were uncovered as the genes induced by colony-stimulating factor-1 (CSF-1) in macrophage cells, indicating that the expression of D type cyclins is inducible by growth factors (Matsushime *et al.*, 1991; Motokura *et al.*, 1992). D type cyclins are normally expressed in G_1 and activate Cdk4 and Cdk6. Cyclin E appears later than cyclin D in G_1 and peaks at the G_1/S transition. Like cyclin A, cyclin E associates with and activates Cdk2, but not Cdk4 or Cdk6. Each Cdk may bind a subset of cyclins and *vice versa.* A number of kinase complexes are derived from combinations of Cdk and cyclin and function in particular stages of the cell cycle (Fig. 1).

3. Cdk regulation by phosphorylation/dephosphorylation

Accumulation of cyclin during S and G_2 is a gradual process, and the activity of the Cdc2/cyclin complex is regulated by phosphorylation and dephosphorylation events at least at residues of Thr^{14}, Tyr^{15} and Thr^{161} in human Cdc2, or the equivalent residues in Cdc2 homologues from other organisms (Gould *et al.*, 1991; Gould and Nurse, 1989). It is thought that phosphorylation of Thr^{161} is required for activation of the enzyme, whereas phosphorylation of Thr^{14} and Tyr^{15} inhibits the enzyme activity (Norbury *et al.*, 1991).

Phosphorylation of Cdc2 at Thr^{14} and Tyr^{15} occurs after Cdc2 association with cyclin (Solomon *et al.*, 1990). When exogenous Cdc2 was added to *Xenopus* extracts, it was induced to undergo phosphorylation at threonine and tyrosine residues only after cyclin binding (Solomon *et al.*, 1990). Phosphorylation of Cdc2 at Thr^{161} has been suggested to facilitate cyclin binding. The Ala^{161} mutant of Cdc2 exhibited much reduced

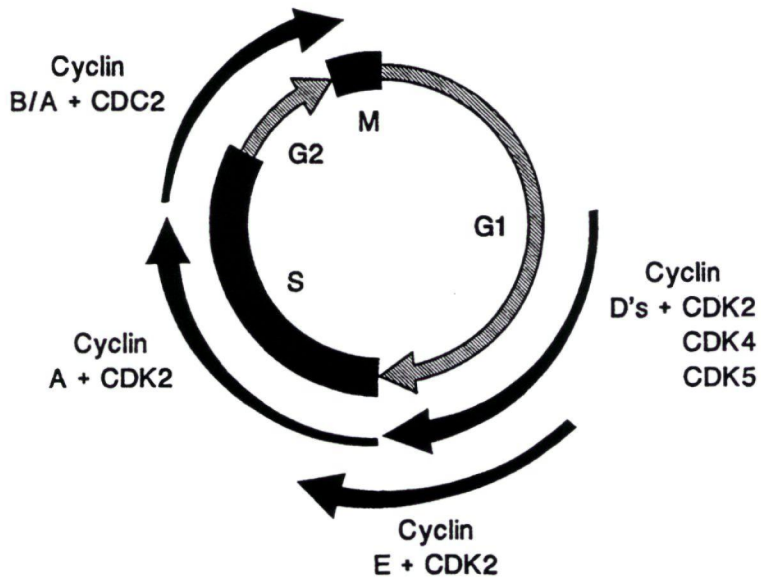


FIG. 1. Combinatorial interactions of cyclins and Cdks during the cell cycle (revised from Sherr, C.J., 1993).

cyclin binding, and was not phosphorylated at Thr^{14} and Tyr^{15}, while the double mutation of Ala^{14} and Phe^{15} could still bind cyclin B (Norbury *et al.*, 1991).

Cyclin accumulates in cells in interphase and binds Cdc2. The cyclin binding induces immediate phosphorylation of Thr^{14}, Tyr^{15} and Thr^{161}, among which Thr^{14} and Tyr^{15} phosphorylation prevents premature activation of Cdc2/cyclin. When cells approach the G_2/M transition, both Thr^{14} and Tyr^{15} are rapidly dephosphorylated to produce an active Cdc2/cyclin complex. After completion of cell division, cyclin is proteolyzed and then phospho-Thr^{161} is dephosphorylated. Cells exit from mitosis.

3.1. Activation by phosphorylation

In addition to cyclin association, Cdc2 activation shows absolute dependence on phosphorylation of Thr^{161} (Desai *et al.*, 1992; Solomon *et al.*, 1990). The binding of cyclin A to Cdk2 establishes a basal activity of Cdk2; full activity requires phosphorylation of Thr^{160}, which is the residue corresponding to Thr^{161} of Cdc2 (Connell-Crowley *et al.*, 1993). In the Cdk2 structure, Thr^{160} is at the apex of the T-loop (De Bondt *et al.*, 1993). Phosphorylation of Thr^{160} introduces ionic interactions between Phospho-Thr^{160} and a cationic pocket formed by the basic residues Arg^{50}, Arg^{126} and Arg^{150} (Jeffrey *et al.*, 1995). These interactions are suggested to stabilize the active conformation of the T-loop introduced by association with cyclin A. Thus, the requirement of both cyclin association and Thr^{160}-phosphorylation for the kinase activity is due to the unique features of the Cdk structure.

Cdk-activating kinase (CAK) was initially identified from *Xenopus* egg extracts by virtue of its ability to phosphorylate and activate Cdc2 (Solomon *et al.*, 1990). Peptide sequencing information of CAK, which was isolated from *Xenopus* eggs and starfish

oocytes, as well as immunochemical data, revealed that $p40^{MO15}$ was a major component and the catalytic subunit of CAK (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). $p40^{MO15}$ was previously cloned in a screen of Cdc2 homologues from *Xenopus* eggs (Shuttleworth *et al.*, 1990). A second subunit of CAK was identified independently by cloning the protein that co-purified with MO15 from HeLa cell extracts, and by a yeast two-hybrid screen for MO15-binding proteins (Fisher and Morgan, 1994; Makela *et al.*, 1994). It is a homologue of cyclin C, and therefore termed cyclin H. Cyclin H is a major component in MO15 immunoprecipitates, and is required for MO15 activity in the *in vitro* reconstitution experiments. Due to the sequence relatedness of MO15 to Cdc2 and Cdk2, as well as the requirement of cyclin H for its activity, MO15 was renamed Cdk7 (Fisher and Morgan, 1994).

CAK does not display any phosphorylating activity towards histone H1, the conventional substrate of Cdc2 and Cdk2 (Fisher and Morgan, 1994). Phosphorylation of Cdc2- and Cdk2-cyclin complexes by CAK occurs exclusively at Thr^{161} and Thr^{160}, respectively. Later, it was shown that CAK could activate Cdk4/cyclin D and Cdk6/cyclin D by phosphorylating Cdk4 and Cdk6 (Kato *et al.*, 1994a; Matsuoka *et al.*, 1994; Meyerson and Harlow, 1994). The substrate selection of CAK seems not to be restricted to the Cdk family. CAK associates with the general transcription factor TFIIH and phosphorylates RNA polymerase II, indicating that it potentially functions in other than activation of Cdk (Feaver *et al.*, 1994; Liao *et al.*, 1995; Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995).

Relatively little is known about the regulation of CAK. Its activity was initially found to associate with a macromolecular complex, suggesting the existence of regulatory proteins other than cyclin H in cells (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et*

al., 1992). Cdk7 contains a Cdc2 Thr^{161}-equivalent residue, which is Thr^{176} in Cdk7 (Shuttleworth *et al.*, 1990). The putative inhibitory phosphorylation sites, which correspond to Thr^{14} and Tyr^{15} of Cdc2, do not exist in Cdk7. The sequence surrounding Thr^{176} shows significant homology to that of Cdc2. Mutation of Thr^{176} to Ala dramatically reduced CAK activity, indicating that phosphorylation of Thr^{176} is required for CAK activity (Fisher and Morgan, 1994). However, it is presently not known whether Cdk7/cyclin H undergoes autophosphorylation at Thr^{176} or phosphorylation via a distinct kinase. An understanding of CAK regulation will increase our knowledge of the Cdk/cyclin network, and the fine tuning of cell cycle events.

3.2. Inhibition by phosphorylation

While phosphorylation of Thr^{14} in yeast has not been observed, phospho-Tyr^{15} was found to be abruptly dephosphorylated when yeast cells entered mitosis (Gould and Nurse, 1989). Expression of a Cdc2 mutant in which Tyr^{15} was replaced by phenylalanine resulted in yeast cells that entered mitosis prematurely (Gould and Nurse, 1989). In mammalian cells, both Thr^{14} and Tyr^{15} are phosphorylated in parallel with the expression of cyclin B when cells approach the G_2/M boundary. Presumably, phosphorylation of Tyr^{15} and Thr^{14} normally delays the entry of mitosis by preventing Cdc2 activation.

From genetic analysis in fission yeast, the *wee1* and *mik1* gene products are the potential kinases that catalyze Tyr^{15} phosphorylation (Featherstone and Russell, 1991; Parker *et al.*, 1991). The yeast *wee1* product, $p107^{wee1}$, has the dual function protein Ser/Thr and Tyr kinase activities towards peptide substrates, and was therefore expected to be a kinase of dual-specificity, phosphorylating both Thr^{14} and Tyr^{15}. However, it catalyzes phosphorylation of Cdc2 solely at Tyr^{15}. Moreover, the human Wee1

homologue, Wee1Hu, has been identified to encode a tyrosine-specific kinase, and phosphorylates Cdc2 at Tyr^{15} but not Thr^{14}. In the Cdk family, Cdk2 is another well documented substrate of mammalian Wee1. Cdk4 has been found to undergo a tyrosine phosphorylation-dephosphorylation circuit in G_1 progression and entry into S phase (Terada *et al.*, 1995). Cells transfected with the Phe^{17} mutant of Cdk4, in which the Cdc2 Tyr^{15}-equivalent residue, Tyr^{17}, was mutated to unphosphorylatable phenylalanine, failed to arrest in G_1 in response to DNA damage. However, the tyrosine-phosphorylating kinase of Cdk4 has not yet been identified.

In fission yeast, Nim1 kinase potently inactivates Wee1 activity by phosphorylating its C-terminal region (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). The *pyp* family of protein tyrosine phosphatases has also been found to negatively regulate Wee1 activity (Millar *et al.*, 1992). However, their homologues have not been identified in mammalian cells. In mitotic *Xenopus* egg extracts, a kinase activity was found to specifically phosphorylate the N-terminal region of Wee1, resulting in Wee1 inactivation. This inhibitory phosphorylation could be reversed by a type 2A-like activity in the extracts, indicating the existence of an upstream regulatory network of Wee1 consisting of kinases and phosphatases.

Recent studies have shown the existence of at least two kinases capable of phosphorylating Thr^{14}. Kinase activities have been found in the membrane fraction of *Xenopus* eggs and HeLa cells that promote phosphorylation of Cdc2 at both Thr^{14} and Tyr^{15} (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994). A distinct Wee1 homologue, Myt1, has been cloned from *Xenopus*, and shown to be responsible for such a dual specific phosphorylation event (Mueller *et al.*, 1995). Myt1 is located exclusively in the plasma membrane of *Xenopus* eggs. Moreover, from the cytosolic fraction of

bovine thymus, a Thr^{14}-specific kinase has been isolated and shown to potently inactivate Cdc2, Cdk2 and Cdk5 by phosphorylation exclusively on Thr^{14} (Matsuura and Wang, 1996). However, the molecular identity of this enzyme has not yet been established.

3.3. Regulation by dephosphorylation

Dephosphorylation of phospho-Tyr^{15} and activation of Cdc2 in fission yeast appear to depend on the activity of the *cdc25* gene product, $p80^{cdc25}$, which is a phosphatase directed toward phospho-Tyr^{15} (Millar *et al.*, 1991). The functional homologues of Cdc25 were cloned from animal cells either by complementation of a fission yeast *cdc25* mutant or on the basis of the conserved amino acid sequences (Galaktionov and Beach, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991). The mammalian Cdc25 homologues display dual specificity of phosphatase activity, catalyzing dephosphorylation of Cdc2 at both Thr^{14} and Tyr^{15}.

There are three defined members of the Cdc25 family in mammalian cells, Cdc25A, Cdc25B and Cdc25C (Galaktionov and Beach, 1991; Nagata *et al.*, 1991; Sadhu *et al.*, 1990). Cdc25 proteins are fully active and function at the G_2/M boundary (Galaktionov and Beach, 1991; Millar *et al.*, 1991). Particularly, Cdc25A is required for the G_1/S transition (Jinno *et al.*, 1994). It has been shown that Cdc25 activity is subject to regulation by phosphorylation and dephosphorylation (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Kumagai and Dunphy, 1992). During the G_2/M transition, Cdc25 is extensively phosphorylated in its N-terminal region and fully activated. In interphase, a phosphatase 2A-like activity was found to act on Cdc25 and efficiently inhibit its activity. When cells are passing through the G_2/M border, the Cdc25-stimulating kinase activity increases and the Cdc25-inhibiting phosphatase activity decreases in coordinating Cdc25 activation. Interestingly, Cdc25 can be activated *in vitro* by Cdc2 and Cdk2 through

phosphorylation of its N-terminal domain, suggesting a positive-feedback mechanism to rapidly amplify Cdk activity during the G_2/M transition (Hoffmann *et al.*, 1993; Izumi and Maller, 1993).

It was shown by Galaktionov and coworkers that Cdc25A, and possibly Cdc25B, interacted with Raf1, which is a protein Ser/Thr kinase, and Cdc25A could be phosphorylated by Raf1 immunoprecipitated from insect cell extracts to result in Cdc25 activation (Galaktionov *et al.*, 1995a). This finding provides the possibility for cross-talk between cell cycle machinery and multiple signal pathways through Ras and Raf1 proto-oncogene products. Furthermore, Cdc25A and Cdc25B were found to cooperate with oncogenic Ras or deletion of pRb protein in mitogenesis, leading to the proposal of *cdc25* as a human oncogene (Galaktionov *et al.*, 1995b).

During mitosis, cyclin B is degraded and Cdc2 undergoes dephosphorylation at phospho-Thr^{161} and recycles. Inactivation of Cdc2 can be achieved *in vitro* by treatment with protein phosphatase 2A, presumably dephosphorylating phospho-Thr^{161} (Lee *et al.*, 1991). Performing two-hybrid screens for Cdk2-interacting proteins, two research groups independently cloned a novel protein phosphatase, designated differently Cdi1 and KAP (Gyuris *et al.*, 1993; Hannon *et al.*, 1994b). Cdi1/KAP binds Cdc2 and Cdk3 as well as Cdk2, but not with Cdk4. Later, it was documented by Poon and Hunter (1995) that Cdi1/KAP was a phosphatase acting on phospho-Thr^{160} of Cdk2. Cdi1/KAP catalyzed dephosphorylation of monomeric Cdk2 at phospho-Thr^{160}, but not phospho-Tyr^{15}, although it had been previously shown to have dual specificity of phosphatase activity (Gyuris *et al.*, 1993; Hannon *et al.*, 1994b). Dephosphorylation of Cdk2 by Cdi/KAP prevented subsequent Cdk2 activation by cyclin *in vitro*. Furthermore, binding of cyclin to Cdk2 abolished the dephosphorylating activity of Cdi1/KAP at phospho-

Thr^{160}, indicating that dephosphorylation of Cdk2 by Cdi1/KAP occurs only after cyclin degradation (Poon and Hunter, 1995).

4. Cdk inhibitors

It is thought that there are "checkpoints" in the cell cycle, at which completion of the cell event is monitored (Hartwell and Weinert, 1989). The negative regulation of cycle progression at checkpoints prevents premature entry into the next stage. A number of Cdk inhibitors have been found to set brakes on the cycle machinery (Hunter, 1993; Sherr and Roberts, 1995). These inhibitors may function in response to external signals or as programmed steps in the cell cycle. They bind Cdk, cyclin or the Cdk/cyclin complexes, and suppress Cdk activities without covalent modification of Cdk.

In *S. cerevisiae*, p40 was originally identified independently by two research groups and called SIC1 and SDB25 (Donovan *et al.*, 1994; Mendenhall, 1993; Nugroho and Mendenhall, 1994). During the cycle p40 appears only in G_1 and binds the Cdc28/Clb5 and Cdc28/Clb6 complexes. Following passage through the START point in late G_1, p40 is proteolyzed in a ubiquitin pathway, and DNA replication consequently starts (Schwob *et al.*, 1994). It seems that p40 is programmed in cells to ensure initiation of DNA replication at proper time.

Far1 is another inhibitor in budding yeast, and shows response to the mating factor, α factor (Peter *et al.*, 1993; Peter and Herskowitz, 1994). Regulation of Far1 has been well-studied at both transcriptional and post-transcriptional levels. α factor induces Far1 transcription as well as phosphorylation of Far1 by Fus3, and the phosphorylation of Far1 is required for its activity to bind and inhibit the Cdc28/Cln complexes. The third inhibitor in budding yeast is Pho81. It inhibits the activity of the Cdk/cyclin complex

Pho85/Pho80, which regulates the expression of the acid phosphatase gene Pho5 (Schneider *et al.*, 1994). In fission yeast, the *rum 1* gene, which controls G_1 progression, has been found to encode an inhibitor of Cdc2/cyclin (Moreno and Nurse, 1994).

There are three categories of Cdk inhibitors demonstrated in mammalian cells, p21, $p27^{Kip1}$ and its homologue $p57^{Kip2}$, and the INK4 family inhibitors. These inhibitors function in G_1/S progression instead of G_2/M transition, and provide important control mechanisms for initiation of DNA synthesis.

4.1. p21

p21 is one of the first identified Cdk inhibitors in mammalian systems. It was cloned by several research groups and named differently: p21 (Xiong *et al.*, 1993), Cip1 for the Cdk-interacting protein (Harper *et al.*, 1993), WAF1 for the wild-type p53-activated fragment (el-Deiry *et al.*, 1994), and Sdi1 for the senescent cell-derived inhibitor (Noda *et al.*, 1994). CAP20 (Cdk2-associated protein 20) is a mouse homologue of human p21 (Gu *et al.*, 1993). p21 is known as a universal Cdk inhibitor, displaying inhibitory activity on multiple Cdk/cyclin complexes (Xiong *et al.*, 1993).

The regulation of p21 occurs primarily in the transcriptional stage. The expression of p21 is cell cycle-dependent. p21 mRNA is present in quiescent fibroblasts and T lymphocytes, and reaches its highest level following serum stimulation (Firpo *et al.*, 1994; Li *et al.*, 1994; Nourse *et al.*, 1994). When the G_1/S boundary nears, the level of p21 mRNA begins to decrease and reaches its lowest level during S phase. As the cells exit S phase, p21 mRNA accumulates again (Li *et al.*, 1994). The reduced p21 mRNA during crossing of the G_1/S boundary and its lowest level in S phase suggest a negative effect of p21 in entering and progressing through S phase of the cell cycle.

The recognition that p21 is transcriptionally activated by the tumor suppressor gene product p53 provides insight in understanding of how p53 affects cell cycle progression. The level of p21 mRNA in early passage Li-Fraumeni cells that are heterozygous for p53 and its mutant remains similar to that in normal fibroblasts, but is undetectable in immortalized Li-Fraumeni cells that are homozygous for p53 mutant, suggesting the effect of wild type p53 on p21 expression (Li *et al.*, 1994). In searching for cDNAs induced by wild type but not p53 mutants, the WAF1 gene was identified as producing a major p53-inducible transcript in mammalian cells, which is p21 (el-Deiry *et al.*, 1994). Moreover, the promoter region of the p21 gene contains a p53-binding site 2.4 kb upstream of the coding region, indicating the inducibility of the p21 gene by p53 (el-Deiry *et al.*, 1994). Taken together, p21 is a p53-inducible Cdk inhibitor. Additionally, the p21 gene was independently identified in a search for cDNAs corresponding to mRNAs preferentially expressed in senescent human cells, pointing out its function in cell senescence (Noda *et al.*, 1994).

Over-expression of p21 prevents growing cells from entering S phase and DNA synthesis in human diploid fibroblasts and mouse NIH 3T3 cells, indicating the inhibitory role of p21 in cell cycle progression. This inhibition can be reversed by co-expression of SV40 T-antigen, which binds the tumor suppressing gene products pRb and p53 and blocks their negative effects on cell cycle progression (Harper *et al.*, 1993).

In normal cells, Cdks exist predominantly in quaternary complexes containing Cdk, cyclin, proliferating cell nuclear antigen (PCNA) and p21 (Xiong *et al.*, 1993; Zhang *et al.*, 1993). It has been shown by immunoprecipitation that p21 exists in multiple Cdk/cyclin complexes from human diploid fibroblasts involving Cdc2, Cdk2, Cdk4, Cdk5, cyclins A, B, D, and E. p21 displays potent inhibitory activity towards

Cdk2/cyclins A and E, and Cdk4/cyclin D, which are essential for G_1 and S progression (Harper *et al.*, 1993). However, cyclin B-containing complexes are affected much less efficiently by p21. p21 seems to be specified to control the progression through G_1 and S by inhibition of the G_1/S-related Cdk/cyclin complexes.

Cdk inhibition by p21 does not require any additional cellular proteins. The inhibitory mechanism does not involve disruption of Cdk/cyclin complexes, dephosphorylation of Cdk2 at phospho-Thr^{160} or the equivalent residues in other Cdks, or phosphorylation at the negative regulatory sites (Harper *et al.*, 1993; Xiong *et al.*, 1993). It has been reported that most Cdk/cyclin complexes from proliferating cells contain p21 and still display kinase activities (Zhang *et al.*, 1994). Addition of excess p21 to the active Cdk complexes containing endogenous p21 effectively inactivates the kinases, indicating that binding of multiple p21 to the Cdk/cyclin complexes is required to turn off the kinase activities (Harper *et al.*, 1995; Zhang *et al.*, 1994). Moreover, it has been shown that the p21 binding to Cdk/cyclin prevents phosphorylation of Cdk by CAK, providing a second mode of Cdk inhibition by p21.

In addition to binding to Cdk/cyclin, p21 binds directly to PCNA in the complex of Cdk-cyclin-p21-PCNA. PCNA is a cofactor of DNA polymerase δ (pol δ), which enhances processivity of pol δ. PCNA is also involved in nucleotide excision repair of DNA. Association of p21 with PCNA blocked SV40 DNA replication with pol δ *in vitro*, but not the excision repair function of PCNA (Flores-Rozas *et al.*, 1994; Li *et al.*, 1994; Shivji *et al.*, 1994; Waga *et al.*, 1994). Two separate domains were mapped in p21 to bind Cdk/cyclin and PCNA (Chen *et al.*, 1995; Luo *et al.*, 1995; Nakanishi *et al.*, 1995). The N-terminal domain consisting of amino acid residues 1-71 interacts with and inhibits Cdk/cyclin, and has no effect on pol δ-dependent SV40 DNA replication. The C-terminal

domain is sufficient for PCNA binding, and efficiently inhibits DNA synthesis in the experiments of pol δ-dependent SV40 DNA replication. Moreover, the p21 mutant with deletion of the Cdk/cyclin-binding domain still retained the ability to inhibit DNA synthesis, suggesting that the PCNA-binding domain in p21 may also contribute to the growth inhibitory effect (Nakanishi *et al.*, 1995).

4.2. $p27^{Kip1}$ and $p57^{Kip2}$

In studies of cell contact inhibition and cell cycle arrest in G_1 by treatment with transforming growth factor β (TGFβ), Polyak *et al.* (1994a, b) isolated and cloned a Cdk inhibitor from the inactive Cdk2/cyclin E complex , which is a 27 kDa thermostable protein, and designated it Kip1 for Cdk inhibitory protein 1. Independently, Toyoshima and Hunter (1994) identified $p27^{Kip1}$ in a yeast two-hybrid screen for Cdk4/cyclin D-binding proteins.

Kip1 is a p21-related protein sharing significant sequence homology in the Cdk/cyclin-binding domain, but lacking the PCNA-binding domain (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). An N-terminal peptide of 60 amino acids from Kip1 displays 44% identity to the region in p21 that represents the Cdk/cyclin binding and inhibiting activity (Polyak *et al.*, 1994b). Like p21, the binding of Kip1 prevents Cdk phosphorylation by CAK. Kip1 has a preference for the Cdk/cyclin complex over monomeric Cdk. It effectively inhibits many Cdk complexes in which cyclins D, E, A and B are involved. Cdk4/cyclin D1 has been detected in Kip1 immunoprecipitates from proliferating cells, whereas Cdc2 and Cdk2, as well as cyclins E, A and B1, are barely detected (Toyoshima and Hunter, 1994).

Studies have been carried out to understand the regulation of Kip1. Kip1 expression in fibroblast cells appears constant throughout the cell cycle, and is not affected by TGFβ treatment (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). Instead, Cdk4 expression is reduced in response to TGFβ (Ewen *et al.*, 1995; Ewen *et al.*, 1993). As Cdk4/cyclin D is known to sequester the majority of Kip1 in cells, the decreased cell level of Cdk4/cyclin D results in more free Kip1, which consequently acts on Cdk2/cyclin E (Toyoshima and Hunter, 1994).

Macrophages requires growth factor CSF-1 (colony-stimulating factor-1) in the culture for G_1 progression (Kato *et al.*, 1994b). G_1 arrest of macrophages induced by cAMP treatment or deprivation of CSF-1 increases the overall cell level of Kip1. Consistently, the Kip1 level is decreased by CSF-1 stimulation. Similar cell responses to mitogen treatment were observed in T cells as well (Firpo *et al.*, 1994; Nourse *et al.*, 1994). Upon treatment with the antigen receptor, high levels of Kip1 accumulated in T cells at quiescent and G_1 stages. Subsequent stimulation with interleukin-2 (IL-2) dramatically reduced the Kip1 levels, and allowed the cells to pass through the G_1/S boundary (Firpo *et al.*, 1994; Nourse *et al.*, 1994). Moreover, the IL-2-mediated diminution of Kip1 can be prevented by the immunosuppressant rapamycin, which blocks the G_1 progression. These phenomena have also been observed with human fibroblasts and with HeLa cells in which Kip1 had accumulated when the cells were arrested in G_1 by lovastatin treatment (Hengst *et al.*, 1994; Nourse *et al.*, 1994). Thus, Kip1 expression is high in quiescent cells and is negatively regulated by mitogenic stimulation, displaying an inverse pattern to p21 transcription in response to mitogenic signals.

$p57^{Kip2}$ was identified as containing an N-terminal Cdk inhibitory domain homologous to those in Kip1 and p21, and a C-terminal region homologous to Kip1 (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). Kip2 binds the cyclin complexes of Cdk2, Cdk3, Cdk4 and Cdk6, and blocks their activities. The chromosomal location of Kip2 was mapped to 11p15.5 on the human chromosome, which is a region with frequent abnormality in a number of human cancers, promoting the proposal of Kip2 as a tumor suppressor gene (Matsuoka *et al.*, 1995).

4.3. INK4 family inhibitors

INK4 is a family of Cdk4- and Cdk6-specific inhibitors. The first described member in this family, $p16^{INK4A}$, was originally uncovered as an inhibitor of Cdk4 (Serrano *et al.*, 1993). The INK4 family members are homologous 15-19 kDa proteins with four repeated ankyrin motifs, and display no sequence relatedness to either p21 or $p27^{Kip1}$ (Guan *et al.*, 1994; Hirai *et al.*, 1995; Serrano *et al.*, 1993). They form complexes with Cdk4 or Cdk6 by displacing cyclin D, but have no effect on other Cdks. It is thought that association of the INK4 inhibitors with Cdk4 or Cdk6 *in vivo* interferes with cyclin binding and the subsequent activation of Cdk, resulting in G_1 arrest (Guan *et al.*, 1994; Lukas *et al.*, 1995; Serrano *et al.*, 1995).

The expression of $p16^{INK4A}$ is significantly elevated in tumor cells lacking functional pRb, such as SV40 T-antigen transformed cells, suggesting that pRb suppresses the expression of $p16^{INK4A}$ (Okamoto *et al.*, 1994; Parry *et al.*, 1995; Tam *et al.*, 1994). Since pRb might be the sole critical substrate for cyclin D-dependent Cdk4 and Cdk6, these kinases appear redundant and are sequestered by $p16^{INK4A}$ when functional pRb is deprived in cells (Serrano *et al.*, 1993).

The expression of $p15^{INK4B}$, but not $p16^{INK4A}$, is greatly induced (30-fold) in human keratinocytes (HaCaT cells) treated with TGFβ, but this treatment does not affect Cdk4 expression, suggesting that $p15^{INK4B}$ is an effector of TGFβ (Hannon and Beach, 1994a). During TGFβ treatment, complex formation of Cdk4 with $p15^{INK4B}$ instead of cyclin D would release $p27^{Kip1}$ from the Cdk4/cyclin D complex, subsequently blocking Cdk2 activity.

$p16^{INK4A}$ and $p15^{INK4B}$ have been located in tandem on human chromosome 9p21, and designated as MTS1 and MTS2, respectively (Kamb *et al.*, 1994; Nobori *et al.*, 1994). This region is frequently deleted or mutated in many human tumors, suggesting that they are potential tumor suppressor gene products. Indeed, alterations at the $p16^{INK4A}$ locus have been detected frequently in melanomas and gliomas (Kamb *et al.*, 1994; Nobori *et al.*, 1994). The loci of $p18^{INK4C}$ and $p19^{INK4D}$ have been mapped to chromosomes 1p32 and 19p13, respectively (Chan *et al.*, 1995; Guan *et al.*, 1994; Hirai *et al.*, 1995). Neither of them has been shown to act as a tumor suppressor gene product.

5. Yeast two-hybrid system

Protein interactions are essential for most biological events. In order to understand function and regulation of a protein of interest, intense research efforts are made to investigate its interacting proteins. Various approaches are used, such as protein affinity chromatography, affinity blotting, co-immunoprecipitation, cross-linking, protein probing of expression libraries, yeast two-hybrid screening, and phage display libraries. The yeast two-hybrid system is a powerful approach to study *in vivo* protein-protein interactions (Fields and Song, 1989). It has been applied in testing interactions between two cloned proteins, mapping domains or amino acid residues critical for interactions, and searching DNA libraries for interacting proteins.

The two-hybrid system is based on the structural features of many eukaryotic transcription activators, which contain two distinct domains for binding to specific DNA sequences and transcriptional activation (Brent and Ptashne, 1985; Hope and Struhl, 1986; Keegan *et al.*, 1986). A DNA binding domain binds DNA to recruit a transcriptional activation domain to the upstream activation sequences of particular genes, where the activation domain contacts other components of the transcriptional machinery to initiate transcription. The covalent linkage between these two domains is not necessarily required for transcriptional activation, and it is still functional if an activation domain is brought to a DNA binding domain by interaction between two proteins (Ma and Ptashne, 1988). The feasibility of the two-hybrid strategy was shown by Fields and Song (1989) with two known interacting proteins, SNF1 and SNF4. They were separately fused to the DNA binding domain and the transcriptional activation domain of yeast GAL4. The association of SNF1 with SNF4 successfully reconstituted the transcription activation activity. As an extension, Chien *et al.* (1991) carried out a library screen with the yeast SIR4 protein fused to the GAL4 DNA binding domain and a cDNA library fused to the GAL4 activation domain.

The two-hybrid system has been developed in a few research groups with slight modifications. It is composed of a plasmid for expression of a DNA binding domain hybrid, a plasmid to express a transcriptional activation domain hybrid, and yeast strains containing reporter genes with the binding sites for the DNA binding domains. The DNA binding domain is derived from yeast GAL4 or *E. coli LexA* (Chien *et al.*, 1991; Durfee *et al.*, 1993; Vojtek *et al.*, 1993; Zervos *et al.*, 1993). The transcriptional activation domain is derived from yeast GAL4 or Herpes simplex virus VP16 (Chien *et al.*, 1991; Dalton and Treisman, 1992; Durfee *et al.*, 1993). The reporter genes are *lacZ* from *E. coli*

and nutrient selectable genes from yeast such as HIS3 and LEU2 (Durfee *et al.*, 1993; Fields and Song, 1989; Zervos *et al.*, 1993).

Application of the two-hybrid system requires construction of hybrid genes for expression of two chimeric proteins, a DNA binding domain fused to the target protein X, and a transcriptional activation domain fused to the protein Y or a DNA library (Fig. 2). These two hybrids are co-expressed in yeast cells, which contain the reporter genes. If protein X interacts with protein Y, a transcriptional activator is reconstituted via the specific protein-protein interaction between X and Y. Consequently, the reporter activities are readily detected, such as β-galactosidase activity (Fields and Song, 1989). This system is highly sensitive and allows detection of low-affinity interactions or transient interactions, which may not be revealed by other methods. For example, the interaction of mammalian Ras and Raf was demonstrated by the two-hybrid approach, but had not been detected by co-immunoprecipitation (Van Aelst *et al.*, 1993). In the two-hybrid assay, protein-protein interaction takes place in the native cell environment of yeast, in which the expressed recombinant proteins are more likely to be in their native conformations, and interacting conditions are theoretically similar to those required for presumptive *in vivo* interactions.

Not all protein-protein interactions can be detected by the two-hybrid system. If the target protein alone activates transcription when fused to a DNA binding domain, the intrinsic transcription activation activity in the target must be removed by deletion or mutation of certain regions before the two-hybrid assay is performed. If the domains responsible for protein-protein interactions are in close proximity to the fusion part in the chimeric proteins, the interaction may be excluded by steric hindrance. In some cases, certain post-translational modifications are required for protein-protein interactions, such

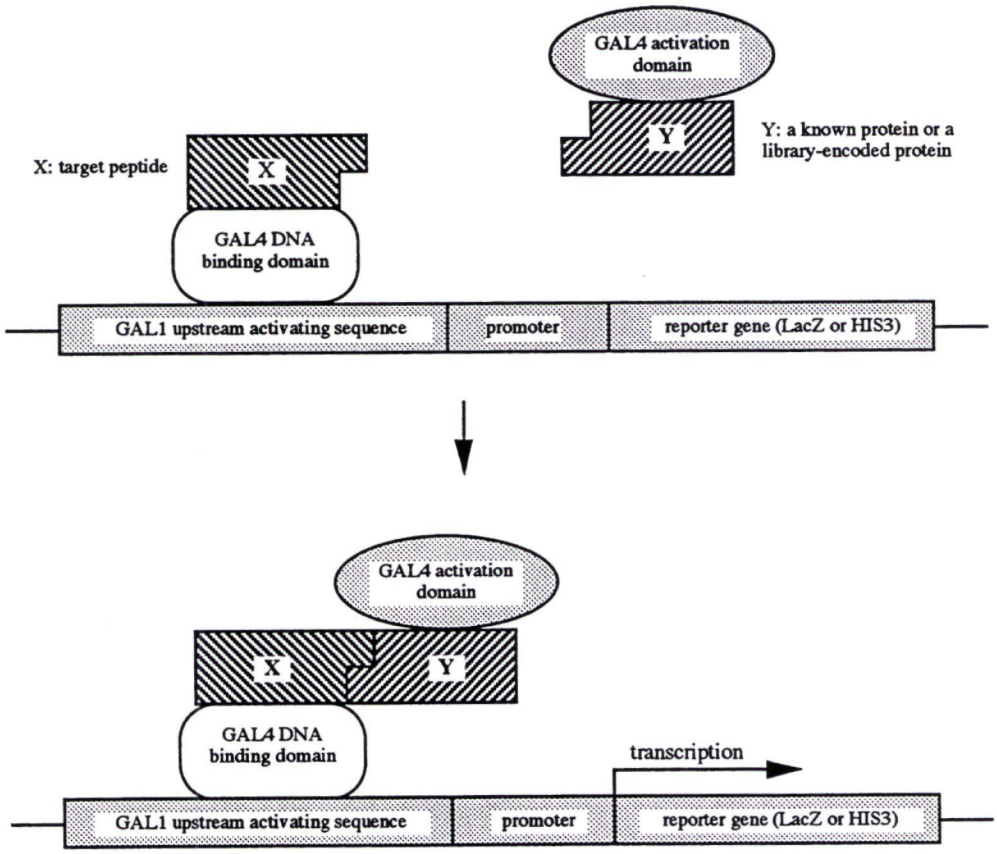


FIG. 2. A diagram of the yeast two-hybrid assay.

as tyrosine phosphorylation being required for binding of SH2 domains, and such a modification system may not exist in yeast cells.

False positives occur in the two-hybrid assays. Some library-derived proteins activate transcription of reporters, but may not interact with the target (Bartel *et al.*, 1993). These false positives alone may cause reporter gene transcription if the encoded proteins are involved in the transcriptional machinery. In some cases, reporter transcription caused by the false positives requires the presence of a DNA binding domain hybrid containing an unspecific protein moiety. To identify false positives, library-isolated clones are introduced into yeast alone and together with the following plasmids individually: the bait used in the original screen, the DNA binding domain vector, and the DNA binding domain hybrid containing an unrelated protein. If reporter activities are detected only when the specific bait is co-transformed, the library-isolated plasmids are taken as true positive clones that encode interacting proteins with the target protein.

The interactions identified in the two-hybrid assays need to be verified by an independent approach, such as co-purification on an affinity column or co-precipitation of the two proteins. A positive result from the two-hybrid system does not definitely mean direct interaction between a target and its isolated interacting proteins. It is possible that a transcriptional activator is reconstituted by interactions of both two hybrid proteins to a third party that comes from the yeast host (Hannon *et al.*, 1993). Moreover, if the target and the isolated interacting proteins are not expressed in the same cells, in the same stage of the cell cycle, or co-localized in cell compartments, the interaction does not occur in cells.

The two-hybrid system is widely used to detect protein-protein interactions. Cell cycle control study is one of the fields with successful application of this approach. A pool of Cdk inhibitors has been demonstrated in the two-hybrid screens for Cdk- and cyclin-interacting proteins: $p21^{Cip1}$ in the screen for Cdk2-binding proteins (Harper *et al.*, 1993), $p27^{Kip1}$ in the screen using Cdk4/cyclin D as a bait (Toyoshima and Hunter, 1994), $p57^{Kip2}$ in the cyclin D screen (Matsuoka *et al.*, 1995), and the INK4 family inhibitors, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$, in the Cdk4 screens (Hirai *et al.*, 1995; Serrano *et al.*, 1993). There are some other protein components of the cell cycle machinery identified by the two-hybrid system, including: Cdi1/KAP, which is a Cdk2 Thr^{160}-phosphatase (Gyuris *et al.*, 1993; Hannon *et al.*, 1994b), cyclin H, which binds Cdk7 (Makela *et al.*, 1994), and $p130^{Rbr-2}$, which is a pRb-related protein that binds cyclins (Hannon *et al.*, 1993). Identification of these proteins greatly facilitated the understanding of regulation and function of Cdks.

6. Neuronal Cdc2-like kinase

While Cdks are predominantly involved in the control of cell proliferation, Northern blot analysis revealed the existence of Cdc2 homologues in specifically differentiated cells and tissues (Meyerson *et al.*, 1992), suggesting that the physiological role of the Cdc2 family members may not be restricted to the cell cycle control. In brain, neurofilaments and tau are phosphorylated on residues in the consensus Cdc2 substrate motif, which is the so-called proline-directed Ser/Thr phosphorylation motif (Xu *et al.*, 1992). It was shown that Cdc2 phosphorylated the dephosphorylated medium and high molecular weight subunits of neurofilaments (NF-M and NF-H, respectively) to restore their characteristics, such as electrophoretic mobility and dissociation from microtubules, to the state of the *in vivo* phosphorylated forms of NF-M and NF-H (Hisanaga *et al.*, 1991).

Tau is a family of low molecular weight microtubule-associated proteins, which are involved in maintenance of neuronal polarity. Tau from Alzheimer's neurons differs from normal tau in its low solubility in aqueous solution, its inability to promote microtubule assembly, and its retarded mobility in SDS-PAGE. Abnormal hyperphosphorylation of tau appears to be a contributing factor to these anomalous behaviors (Hasegawa *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992). The proline-directed kinase activities are suggested to be responsible for phosphorylating most of the abnormally phosphorylated sites in Alzheimer's tau.

A Cdc2-like kinase was isolated from bovine brain by its phosphorylating activity towards tau or a synthetic peptide containing the proline-directed Ser/Thr phosphorylation site (Ishiguro *et al.*, 1994; Lew *et al.*, 1992a). This kinase is designated as neuronal Cdc2-like kinase (NCLK). Close-to-homogeneous NCLK purified from bovine brain displays a high histone kinase activity of 5-10 μmol PO4/min/mg measured with a histone H1-derived peptide containing the Cdc2 phosphorylation site (Lew *et al.*, 1992a). NCLK appears to represent the predominant proline-directed kinase activity in bovine brain extracts. It catalyzes *in vitro* phosphorylation of NF-M, NF-H and tau at the sites identical to those phosphorylated by Cdc2 (Lew *et al.*, 1992a). The NCLK-phosphorylated sites in tau are abnormally phosphorylated in Alzheimer's tau (Paudel *et al.*, 1993). Therefore, NCLK is a potential kinase to phosphorylate NF-M, NF-H and Alzheimer's tau in brain.

NCLK is a heterodimer of 33 kDa and 25 kDa subunits. Molecular cloning revealed that the 33 kDa protein is Cdk5, which was formerly called "PSSALRE" due to its "PSSALRE" sequence corresponding to the conserved "PSTAIRE" motif in Cdc2 (Lew *et al.*, 1992b; Meyerson *et al.*, 1992). The 25 kDa protein (p25) is a fragment of a

novel 35 kDa protein (p35) with the N-terminal 98 amino acids missing in p25 (Lew *et al.*, 1994; Tsai *et al.*, 1994). p35 is predominant in bovine brain extract; p25 is a proteolytic derivative of p35 and appears in extensively purified NCLK (Lew *et al.*, 1994).

Transcription of *cdk5* is detected in all cultured cell lines and human tissues examined (Lew *et al.*, 1994; Meyerson *et al.*, 1992). Brain contains a high level of *cdk5* mRNA, which is 3- to 5-fold above that in other human tissues. The expression of *cdk5* in brain is enhanced during neuronal differentiation. In contrast, *cdc2* and *cdk2* mRNAs, although present at high levels in proliferating neuronal precursor cells, are not detected in terminally differentiated neurons (Tsai *et al.*, 1993b). When expression of p35 was examined by Northern blot analysis and *in situ* hybridization, p35 signals were detectable only in neurons of the central nervous system (Lew *et al.*, 1994; Tsai *et al.*, 1994). Like *cdk5*, p35 appears to be highly expressed in post-mitotic neurons (Tsai *et al.*, 1994). Thus, NCLK is a proline-directed kinase in differentiated neurons.

Association with cyclin is essential for Cdk activity. Although the name of Cdk5 implies that the $p33^{cdk5}$ protein possesses the potential to be activated by cyclin, cyclin activation of Cdk5 has not yet been demonstrated. The observation that highly active NCLK contains Cdk5 and p25 as its sole protein components suggests that p25 has a cyclin-like function to support Cdk5 activity (Lew *et al.*, 1992a). However, the sequence of p35 displays little homology to cyclins (Lew *et al.*, 1994; Tsai *et al.*, 1994). In the present thesis, activation of Cdk5 has been demonstrated with bacterially-expressed fragments of p35, including that corresponding to the 25 kDa subunit in NCLK purified from bovine brain. A simple procedure is described to purify the highly active enzyme reconstituted from recombinant proteins. Characterization of the kinase activation has

been carried out in detail. It reveals a major difference between the activation mechanisms of Cdk5 and Cdc2 or Cdk2.

Multiple protein bands have been observed in Cdk5 immunoprecipitates from a brain extract (Tsai *et al.*, 1994). Moreover, Cdk5 has been found to exist in bovine brain homogenates in three forms: monomeric Cdk5, a heterodimer of Cdk5 and p25, and a heterodimer of Cdk5 and p35 (Lee *et al.*, 1996). After gel filtration chromatography of Cdk5-containing samples isolated from bovine brain extracts, monomeric Cdk5 and the Cdk5/p25 complex was detected in the eluate corresponding to molecular masses of 30 kDa and 60 kDa, respectively (Lee *et al.*, 1996). However, Cdk5/p35 behaved as a macromolecular complex of 670 kDa on gel filtration chromatography, suggesting the presence of proteins other than Cdk5 and p35 in the Cdk5/p35 complex. In the present thesis, the yeast two-hybrid system has been employed to investigate p35-interacting proteins. Seven different sequences have been isolated that code for putative p35-binding proteins in human brain, suggesting the existence of multiple effectors or regulators of NCLK.

MATERIALS AND METHODS

1. Materials

[γ-^{32}P]ATP (4500 Ci/mmol) was purchased from ICN. [α-^{32}P]ATP and [α-^{35}S]dATP (3000 Ci/mmol) were obtained from Amersham. Restriction enzymes, *Taq* polymerase, IPTG, and X-gal were obtained from Life Technologies, Inc. *Vent* DNA polymerase was purchased from New England Biolabs. P81 phosphocellulose paper and Whatman No.1 filter paper circles were purchased from Whatman. The materials for bacterial and yeast cultures were from Difco. Chemicals were purchased from Sigma. Oligonucleotide primers were synthesized at the DNA Synthesis Facility of the University of Calgary. The synthetic peptides were produced by the MRC Peptide Synthesis Facility at the University of Calgary.

2. Protein concentration determination

Protein concentration was measured either by absorbance at 280 nm (A_{280}) or by a modified Bradford method (Bradford, 1976). The samples were mixed with 150 μl of Bradford reagent in a microtiter plate, and A_{570} read using a Dynatec MR 600 microtiter plate reader. The Coomassie Blue-stained gel with the purified enzyme was scanned using a Pharmacia LKB 2202 Ultroscan laser densitometer. Protein in the stained bands was estimated by densitometry and compared with that of the protein standard carbonic anhydrase.

3. SDS-PAGE and immunoblot

SDS-PAGE was performed by the method of Laemmli in 10% vertical slab gels, and gels were stained with silver or 0.2% Coomassie Brilliant Blue (Laemmli, 1970; Wray *et al.*, 1981). For immunoblotting, proteins were transferred to Immobilon PVDF

membranes (Millipore). The membranes were blocked in TBS-milk (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5% skimmed milk powder), and then incubated with indicated primary antibodies. The secondary antibodies were coupled with alkaline phosphatase for visualization by *p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, or with horseradish peroxidase and visualized by the Enhanced Chemiluminescence Detection Kit (ECL, Amersham).

Antibodies were rabbit polyclonal antibody preparations raised against synthetic peptides coupled to keyhole limpet hemocyanin. The peptides were synthesized with an additional cysteine at the N-terminus for coupling to the carrier protein. The antibodies were prepared with the following peptides: α-Cdc2N is against a peptide from an N-terminal region of human Cdc2 (EKIGEGTYGVVYK), which is conserved in Cdc2, Cdk2, Cdk3, and relatively conserved in Cdk5 with variation of two residues; α-Cdc2C is a Cdc2-specific antibody against a peptide from the C-terminus of human Cdc2 (NDLDNQIKKM); α-Cdk2 is against a peptide corresponding to the C-terminus of human Cdk2 (QDVTKPVPHLRL); α-Cdk5C and α-Cdk5I are against peptides corresponding to a C-terminal sequence (FSDFCPPGC) and an internal sequence (YPMYPATTSLVNVVPKC), respectively, of bovine Cdk5; and α-p25N is against an N-terminal peptide of the 25 kDa subunit (p25) in NCLK purified from bovine brain (PPASQLSGSQTG). Antisera were purified by affinity chromatography with protein A or the immobilized peptide antigen columns.

4. DNA sequencing

DNA was sequenced by dideoxynucleotide termination. Sequencing reactions using [α-^{35}S]dATP were performed with the dGTP analogue of 7-deaza-dGTP (USB) to avoid problems caused by secondary structures of GC-rich sequences. Reaction mixtures

were resolved on a 6% polyacrylamide denaturing gel. The gels were then dried and exposed to X-OMAT AR scientific imaging films (Eastman Kodak). Cycle sequencing reactions were performed with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The polymerase chain reactions (PCRs) were as follows: 25 cycles of 30 sec at 96°C, 15 sec at 50°C, and 4 min at 60°C. After the reaction, free nucleotides were removed through a Centri-Sep spin column (Princeton Separations, Inc.). The amplified fragments were analyzed on an automated DNA Sequencer at the DNA Sequencing Facility of the University of Calgary.

5. Protein kinase activity measurement

The protein kinase activity of NCLK was determined by measuring the incorporation of ^{32}P-phosphate into the histone H1 derived peptide, HS(9-18): PKTPKKAKKL (Beaudette *et al.*, 1993). Kinase reactions were performed at 30°C with 20 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (~300 dpm/pmol), the peptide substrate as indicated, and protein kinase samples. In the assay of highly purified enzymes, 0.5 mg/ml bovine serum albumin was included for enzyme stability. The kinase reactions were terminated by addition of 10% acetic acid. Aliquots of the mixture were spotted on P81 paper squares. The papers were then extensively washed with 0.3% H_3PO_4, once with acetone, and dried. Incorporation of ^{32}P-phosphate was quantified by counting in a liquid scintillation counter (Pharmacia LKB).

6. Reconstitution of NCLK

6.1. Bacterial strains, plasmids and enzymes

E. coli DH5 (F^-, *rec*A1, *end*A1, *hsd*R17, *sup*E44, *thi*1, *gyr*A, *rel*A1) was used as the transformation recipient in plasmid constructions. *E. coli* B strain BL21 was employed for expression of glutathione *S*-transferase (GST) fusion proteins (Studier *et*

al., 1990). The plasmids for expression of GST-cyclin D1 and $(His)_6$-tagged cyclin A were kindly provided by Dr. Tim Hunt (ICRF Clare Hall Laboratories, England). The *cdk5*/pGEX2T plasmid was a gift from Drs. Li-Huei Tsai and Ed Harlow (Massachusetts General Hospital). Protein phosphatase 2A (PP2A) catalytic subunit was a gift from Dr. Anna A. DePaoli-Roach (Indiana University School of Medicine).

6.2. Construction of recombinant plasmids

A PCR approach was employed to construct recombinant plasmids for expression of GST fusion proteins. Four sets of primers were designed according to the open reading frame sequence of p35 (Fig. 3). Primer 1 (5'-CCCGGGGATCCATGGGCACGGTGCTG-3') and primer 3 (5'-CCCGGGAATTCTCACCGGTCCAGCCC-3') are for the full length protein of 307 amino acids, p35. Primer 2 (5'-CCGGGGATCCGCCCAGCCCCCGCCGG-3') and primer 3 are for the 25 kDa protein (p25) found in NCLK purified from bovine brain. Primer 4 (5'-CCCGGGGATCCAAGTCGCTGTCGTGC-3') and primer 6 (5'-CCCGGGAATTCTAGTTCTTCAGGTCGGAGA-3') are for the 23 kDa fragment (p23) of p35, which encodes amino acids 88-291 of p35. Primer 5 (5'-CCCGGATCCTGTCCCCCTGCCAGC-3') and primer 6 are for the 21 kDa fragment (p21) of p35, which encodes amino acids 108-291 of p35. These fragments, as well as the full length p35, are shown schematically in Figure 3. The PCR reactions were performed with *Vent* DNA polymerase under cycling conditions of 30 cycles of 30 sec at 96°C, 1 min at 55°C, and 1 min at 72°C. The PCR products were cloned in pGEX-2T vector at *BamH*I and *EcoR*I sites. The constructs were verified by DNA sequencing.

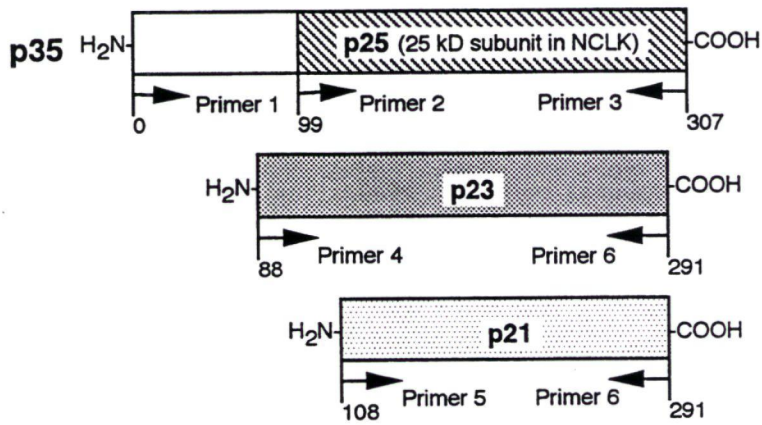


FIG. 3. Schematic representation of p35 and p35-derived fragments.

6.3. Expression and purification of GST fusion proteins

The recombinant pGEX constructs encoding the GST fusion proteins, GST-p21, GST-p23, GST-p25, GST-Cdk5 and GST-cyclin D1, were individually expressed in *E. coli* strain BL21 (DE3). The host cells were cultured in 1 litre of LB medium containing 100 μg/ml ampicillin to A_{600} of 0.5-0.8 at 37°C. The synthesis of a GST recombinant protein was induced by 0.4 mM IPTG and the culture was continued for 2-3 h. The cells were then harvested by centrifugation at 2000 g. The cell pellets were washed with PBS (1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4, 140 mM NaCl, and 2.7 mM KCl) and collected for subsequent purification of the recombinant proteins.

Purification of GST fusion proteins was carried out essentially as described by Smith and Johnson (1988). Cell pellets were suspended in 20 ml of 50 mM Tris-HCl (pH 7.4) buffer containing 2 mM EDTA, 1% Tween-20, 1 mM DTT, 0.25 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin and pepstatin. The purification procedures were performed at 0-5°C. The cells were lysed using a French press at 1000 psi. The lysate was then clarified at 18,000 g for 15 min. The supernatant was applied to a 2 ml column of glutathione-Agarose (GSH-Agarose) which had been equilibrated with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, and the peptide protease inhibitors described above. The column was sealed and allowed to sit on an end-over-end shaker for 1 h. After loading, the column was washed with 20 bed volumes of the same buffer, followed by 5 bed volumes of PBS supplemented with 1 mM DTT. The GST fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and 1 mM DTT. The collected sample was dialyzed against PBS containing 1 mM EDTA and 1 mM DTT to remove glutathione, and then concentrated to a small volume by dialysis against Aquacide II (Calbiochem).

6.4. Expression and purification of $(His)_6$-tagged cyclin A

The plasmid for expression of $(His)_6$-tagged cyclin A was introduced into *E. coli* strain BL21 (DE3). Overexpression and cell lysis followed the procedure described for GST fusion proteins. After clarification of the cell lysate by centrifugation, the supernatant was applied to a Ni-NTA-Agarose column (Qiagen). After sample loading, the column was washed sequentially with 5 bed vol. of 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 5 mM imidazole; 5 bed vol. of 20 mM Tris-HCl, pH 8.0, 1.0 M NaCl, 25 mM imidazole, 0.5% Triton X-100, and 0.5% Tween 20 ; and 5 bed vol. of 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 50 mM imidazole. $(His)_6$-tagged cyclin A was eluted with 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 150 mM imidazole. The collected sample was dialyzed against PBS containing 1 mM EDTA and 1 mM DTT, and then concentrated to a small volume by dialysis against Aquacide II.

6.5. Gel filtration chromatography of GST fusion proteins

The affinity purified GST-p21 and GST-Cdk5 samples were resolved on FPLC Superose 12 columns (Pharmacia LKB). 3 ml of the concentrated GST-p21 sample was loaded on a 100 ml column equilibrated in PBS supplemented with 1 mM EDTA and 1 mM DTT, and eluted at a flow rate of 1 ml/min. Chromatography of the GST-Cdk5 sample was performed with an analytical column (25 ml bed volume) at a flow rate of 0.5 ml/min in the same buffer as that in the GST-p21 chromatography. The collected fractions were examined by SDS-PAGE and immunoblots.

6.6. Reconstitution and purification of NCLK

2 mg of GST-Cdk5 was mixed with 4 mg of GST-p21 in 5 ml of PBS buffer containing 1 mM EDTA and 1 mM DTT. The mixture was incubated at 30°C for 1 h for enzyme reconstitution, and subsequently treated with 10 U human thrombin (Sigma) per

mg recombinant protein at room temperature for 1 h. After thrombin excision of GST, the sample was dialyzed against 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.25 mM PMSF and 100 μg/ml benzamidine at 4°C for 2 h. The sample was then clarified by centrifugation and loaded on an FPLC Mono S column (HR5/5, Pharmacia LKB) equilibrated in the same buffer. The flow rate was 0.5 ml/min. After loading, the column was washed with 10 bed vol. of the column equilibration buffer. The bound kinase activity was eluted with a linear salt gradient (0-0.3 M NaCl/10 ml). Protein kinase activity was monitored by phosphorylation of histone H1 peptide HS(9-18).

6.7. Determination of substrate specificity

The phosphorylation specificity of NCLK was examined with a set of peptides derived from histone H1 peptide HS(9-18), in which each residue was individually substituted to determine its contribution to the peptide phosphorylation. Phosphorylation was performed with either reconstituted Cdk5/p21 or the enzyme isolated from bovine brain under the conditions indicated above. The relative phosphorylation rates of the peptide analogues were measured with 5 mM of each individual peptide in the phosphorylation reaction and expressed as a percentage of the parent peptide. The K_m of the reconstituted enzyme for each peptide was determined with four substrate concentrations and calculated from Lineweaver-Burk plots using linear regression analysis.

6.8. Autophosphorylation of reconstituted NCLK

Aliquots of reconstituted NCLK purified by Mono S chromatography were incubated at 30°C with 100 μM [γ-^{32}P]ATP (~5000 dpm/pmol) in 20 mM MOPS, pH 7.4, and 5 mM $MgCl_2$. Reactions were stopped by addition of SDS-PAGE sample buffer and boiling at 95°C for 5 min. The samples were resolved on SDS-PAGE (10%

acrylamide). Phosphorylated proteins were visualized by autoradiography with exposure at -70°C for 3 h with intensifying screens.

7. Searching p35-interacting proteins by the yeast two-hybrid system

7.1. Strains, plasmids and cDNA library

In the yeast two-hybrid assays, the yeast strains used were CG-1945 [MATa, *ura*3-52, *his*3-200, *lys*2-801, *trp1*-901, *ade2*-101, *leu2*-3, 112, *gal*4-542, *gal*80-538, LYS2::GAL1-HIS3, *cyh*^{r}2, URA3::(GAL4 17-mers)3-CYC1-*lacZ*] and Y190 (MATa, *leu2*-3, 112, *ura3*-52, *trp1*-901, *his3*-Δ200, *ade2*-101, *gal*4Δ*gal*80Δ URA3 GAL-*lacZ*, LYS GAL-HIS3, cyh^r). The DNA binding domain vector pAS2, and the cDNA library in the transcription activation domain vector pACT2 were purchased from Clontech Laboratories, Inc. The control plasmids, SNF1 or human Lamin C fragment in the DNA binding domain plasmid pAS1 and SNF4 in the transcription activation domain plasmid pACT, were generous gifts from Dr. C.J. Brandl (University of Western Ontario). *E. coli* HB101 [F$^-$, *sup*E44, *lac*Y1, *ara*-14, *gal*K2, *xyl*-5, *mtl*-1, *leu*B6, *pro*A2, *delta*(mcrC-mrr), *rec*A13, *rps*L20, *thi*-1] was employed to selectively recover the pACT2-based plasmids from a mixture of the pAS2 and pACT2 plasmids.

Yeast cells were propagated in YPD or SD medium. YPD medium contains 2% peptone, 1% yeast extract, and 2% dextrose. SD is a minimal medium containing 0.67% Difco yeast nitrogen base and an amino acid dropout mix of 30 μg/ml L-isoleucine, 150 μg/ml L-valine, 20 μg/ml L-adenine hemisulfate salt, 20 μg/ml L-arginine HCl, 20 μg/ml L-histidine HCl monohydrate, 100 μg/ml L-leucine, 30 μg/ml L-lysine HCl, 20 μg/ml L-methionine, 50 μg/ml L-phenylalanine, 200 μg/ml L-threonine, 20 μg/ml L-tryptophan, 30 μg/ml L-tyrosine, and 20 μg/ml L-uracil. To select particular phenotypes, a certain amino acid(s) is omitted from the dropout to make a selective SD medium.

Constructs of pAS2 and pACT2 are shown in Figure 4. The recombinant protein expressed from pAS2 consists of a GAL4 DNA binding domain, a hemagglutinin (HA) epitope tag, and a peptide encoded by the inserted sequence. The DNA binding domain protein is targeted to yeast nuclei by the GAL4 nuclear localization sequence. The cDNA library of a human adult brain is constructed in pACT2 at *EcoR*I and *Xho*I sites with cDNA inserts of 0.5-4.5 kb. There is an HA tag and an engineered nuclear localization sequence from SV40 T-antigen in the encoded transcription activation domain hybrids. The library was provided by Clontech Laboratories, Inc. as transformants of *E. coli* BNN132. Library amplification was performed on 100 plates (150 mm diameter) of LB/ampicillin agar. The plasmids were isolated by alkaline lysis and column purification using the QIAprep Plasmid Kit (Qiagen).

7.2. Construction of the bait plasmid

The entire open reading frame of p35 was amplified by PCR with the following oligonucleotide primers: 5'-CCAGCCATGGGCACGGTGCTGTCC-3', and 5'-GGCTGGGATCCTCACCGGTCCAGCCCGAG-3', which contain the *NcoI* and *BamHI* restriction sites, respectively. The PCR fragment was inserted in frame at the *NcoI* and *BamHI* sites in pAS2. The construct was verified by DNA sequencing with the primer 5'-TCATCGGAAGAGAGTAG-3', which is derived from the GAL4 DNA binding domain sequence and reads toward the junction of the GAL4 DNA binding domain and the cloned sequence. The resulting construct, p35pAS2, encodes a fusion protein of the GAL4 DNA binding domain and p35.

7.3. Transformation of shuttle plasmids to yeast cells

Shuttle plasmids were introduced into yeast cells by the Li^+ and heat shock procedure (Schiestl and Gietz, 1989). The following protocol can be used for

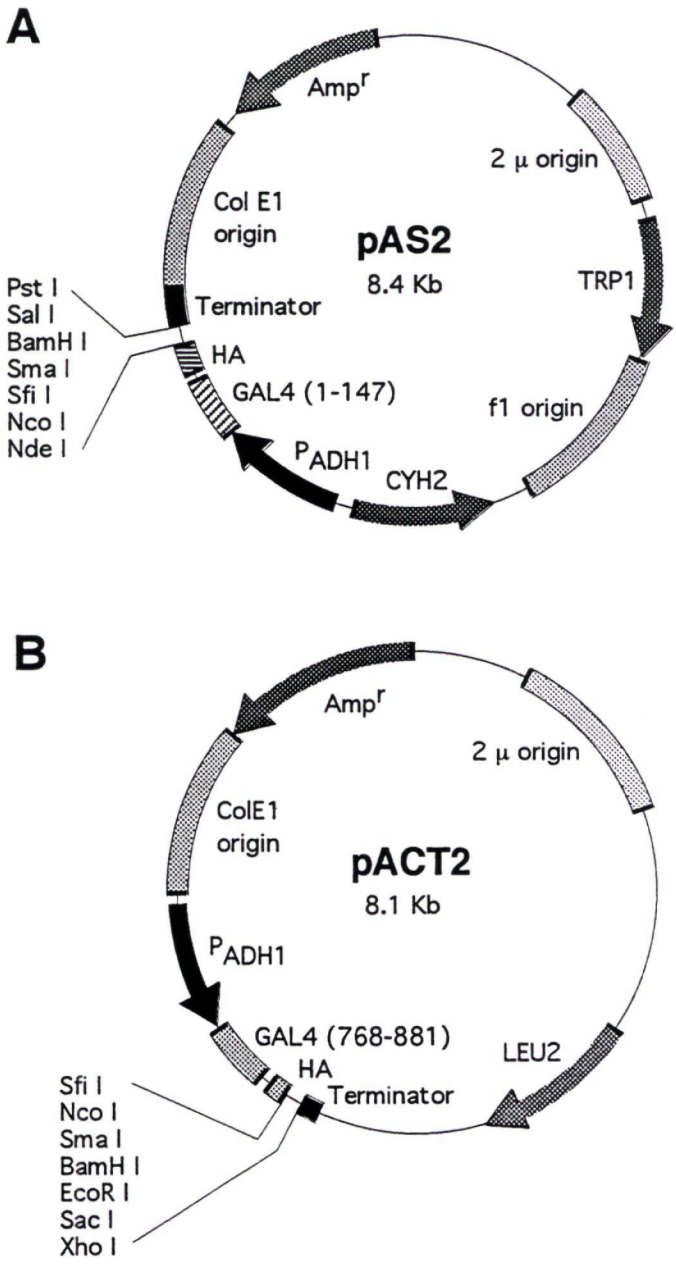


FIG. 4. Maps of the DNA binding domain plasmid pAS2 (A) and the transcripional activation domain plasmid pACT2 (B). Amp^r: ampicillin resistance gene, TRP1 and LEU2: nutritional selection markers, P_{ADH1}: ADH1 promoter, HA: hemagglutinin epitope tag, CYH2: cycloheximide sensitivity gene.

transformation of single plasmid or cotransformation of two plasmids. 20 ml of YPD medium was inoculated with a single yeast colony and cultured at 30°C overnight with shaking. This culture was then diluted into 300 ml of YPD medium to an A_{600} of 0.1-0.2 (~3-6 x 10^6 cells/ml), and the culture was continued for 3-4 h. The cells were harvested by centrifugation at 1000 g for 5 min at room temperature. After being washed with 50 ml of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), the cell pellet was resuspended in 1.5 ml of TE/LiAc (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 M LiAc), which was freshly made from 10 x TE and 10 x LiAc (1 M LiAc, pH 7.5). The cells were kept on ice until the next step.

In a microcentrifuge tube, 0.1 μg of transforming plasmid(s) and 100 μg of carrier DNA (sheared and denatured salmon sperm DNA) were prepared in a volume up to 10 μl. The DNA mixture was subsequently mixed with 100 μl of the prepared yeast cells, and 0.6 ml of PEG/LiAc solution (40% PEG-4000, 1 x TE and 1 x LiAc, freshly made from 50% PEG stock, 10 x TE and 10 x LiAc). The mixture was then incubated at 30°C with agitation for 30 min. After incubation, dimethyl sulfoxide (DMSO) was gently mixed with the cell mixtures to 10% final concentration. The cells were incubated at 42°C for 15 min, and then cooled to room temperature on ice. The cells were pelleted in a microcentrifuge at 12,000 rpm for 10 sec, washed with TE buffer, and resuspended in TE buffer. The cells were then plated onto appropriate selective media. The plates were incubated at 30°C until the transformant colonies appeared.

7.4. Library screening by the two-hybrid system

To perform a two-hybrid screen, the bait and prey plasmids were sequentially transformed into yeast cells. p35pAS2 was introduced into the reporter strain CG-1945, and the p35pAS2 transformant was used as the host in the prey transformation. To

prepare cells for the library transformation, 5 ml of overnight culture of the p35pAS2 transformant in SD-Trp medium was diluted into 100 ml of the same medium, and the culture was continued overnight. The saturated culture was used to inoculate 1 litre of YPD medium to an A_{600} of 0.2-0.3. After growth for 4 h in YPD medium, the cells were harvested by centrifugation at 1000 g for 10 min at room temperature. The pellet was washed with 500 ml of TE buffer. The cells were resuspended in 8 ml of freshly prepared TE/LiAc and kept on ice.

100 μg of the brain cDNA library plasmids and 2 ml of 12 mg/ml carrier DNA (sheared and denatured salmon sperm DNA) were added to the cells in a flask. The cells were then mixed with 60 ml of PEG/LiAc solution, and incubated at 30°C for 30 min with agitation. After incubation, 7 ml of DMSO was added. The mixture was heated in a 42°C water bath for 15 min, and then rapidly cooled to room temperature in a cold water bath. The cells were pelleted by centrifugation at 1000 g for 10 min, washed with 500 ml of TE buffer, resuspended in 1 litre of YPD medium, and then incubated at 30°C for 1 h with shaking. The cells were then collected by centrifugation, washed twice with TE buffer, and resuspended in a small volume of TE buffer. The cells were then plated onto SD media lacking Trp, Leu, and His, but containing 5 mM 3-amino-1,2,4-triazole (3-AT) to select the cotransformants harboring p35pAS2 and p35-interacting proteins.

7.5. Filter lift assay of β-galactosidase activity

β-galactosidase activity produced in yeast colonies was examined on Whatman No.1 filter paper circles with the chromogenic substrate X-gal (Breeden and Nasmyth, 1985). After colony lift, cells on the filter paper circle were permeabilized by freezing in liquid nitrogen and thawing at room temperature. In a petri dish, the filter was laid, with colonies facing up, on a second filter paper circle pre-saturated with Z buffer (60 mM

Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol) containing 0.334 mg/ml X-gal (diluted from 20 mg/ml stock solution in N,N-dimethylformamide). The plate was kept in a 30°C incubator until blue color appeared, which required from approximately 30 min to overnight.

7.6. Quantification of β-galactosidase activity

β-galactosidase activity was quantitatively measured with the chromogenic substrate *o*-nitrophenyl-β-D-galactoside (ONPG). Cells from a single colony were cultured in 10 ml of SD medium lacking Trp and Leu to maintain the fusion plasmids of both pAS2 and pACT2. The cells were harvested at mid- to late-log phase (OD_{600} of 0.5-1.0) and washed with Z buffer. The cell pellet was resuspended in 100 μl of Z buffer in a microcentrifuge tube, and then mixed with 0.3 g of acid-washed glass beads (425-600 μm). After vigorous vortexing for 2 min, the supernatant was collected by centrifugation in a microcentrifuge at 12,000 rpm for 5 min at 4°C.

25 μl of the lysate was mixed with 975 μl of Z buffer in a capped tube. The blank tube was set up with 1 ml of Z buffer without any cell sample. The tubes were prewarmed in a water bath at 30°C for 5 min. The reaction was initiated by addition of 200 μl of 4 mg/ml ONPG in Z buffer, and continued in the water bath until a pale-yellow color developed. The reaction was then stopped by addition of 300 μl of 1 M Na_2CO_3. The sample absorption was read at 420 nm. The protein in the cell lysate was determined by the Bradford method (Bradford, 1976). β-galactosidase activity was calculated and expressed in Miller unit (Miller, 1972).

7.7. Plasmid preparation from yeast cells

Total yeast DNA was prepared as previously described (Hoffman and Winston,

1987; Kaiser and Auer, 1993). Briefly, the yeast cells were collected from 5 ml of the overnight culture in SD-Leu medium to maintain selection of the pACT2-based fusion plasmids. The cells were suspended in 0.2 ml of the yeast lysis buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 100 mM NaCl, 2% Triton X-100, and 1% SDS). After addition of 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads (425-600 μm), the tube was vortexed vigorously for 2 min. The cell lysate was clarified by centrifugation in a microcentrifuge at 12,000 rpm for 5 min at room temperature. The aqueous solution containing the total yeast DNA was collected.

7.8. Recovery of pACT2 fusion plasmids from *E. coli*

Electrocompetent cells of *E. coli* HB101 were prepared from bacteria grown in LB medium at 37°C and harvested at early- to mid-log phase (A_{600} of 0.5-0.7). The cells were sequentially washed with ice-cold water, 10% glycerol, and then suspended in 10% glycerol at 1/500th the original culture volume. The cell suspension were stored at -70°C in aliquots.

Electroporation was performed with 1 μl of the prepared yeast DNA and 40 μl of the electrocompetent cells. The parameters of a Gene Pulsar (Bio Rad) were set at 25 μF capacitor, 200 Ω and 2.5 kV using 0.2 cm cuvettes. After pulsing, the cells were immediately transferred to 1 ml of LB medium in a microcentrifuge tube and incubated at 37°C for 1 h with shaking. The cells were then collected by centrifugation in a microcentrifuge at 12,000 rpm for 5 sec. After two washes with 1 ml of M9 medium (52.0 mM Na_2HPO_4, 22.0 mM K_2HPO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, and 0.4% glucose), the cells were plated on M9 agar supplemented with 50 μg/ml ampicillin, 40 μg/ml proline, and 1 mM thiamine-HCl. During the incubation at 37°C, only colonies harboring the pACT2-based plasmids grew.

7.9. Dot blot analysis of isolated clones

To prepare a probe from a library-isolated clone, the cDNA insert was excised by *EcoRI*/*Xho*I digestion, and isolated from agarose electrophoresis resolved bands. The *cdk5* probe was a PCR fragment of the entire open reading frame of human *cdk5*. The DNA fragments were radiolabelled by the method of random priming with the Megaprime DNA Labelling System (Amersham). The unincorporated ^{32}P-dATP was removed from the labeled DNA fragments by gel filtration using a Centri-Sep spin column.

75 ng of plasmid DNA from each library-isolated clone was spotted individually on a nitrocellulose filter (Schleicher & Schuell). The DNA was denatured on the membrane for 5 min in a solution containing 0.5 M NaOH and 1.5 M NaCl, and then neutralized for 5 min in a solution containing 0.5 M Tris-HCl, pH 7.4 and 1.5 M NaCl. After the filter was dried, the DNA was fixed by UV cross-linking at 120 mJoules/cm^2 in a UV cross linker (Stratagene Stratalinker).

The blot was washed in 5 x concentration of SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, and pH 7.0) supplemented with 0.5% SDS and 1 mM EDTA at 37°C for 30 min. Prehybridization was performed in 6 x concentration of SSC, 0.5% SDS, 5 x concentration of Denhardt (50 x concentration of Denhardt is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin) and 100 μg/ml carrier DNA (sheared and denatured salmon sperm DNA) at 37°C for 1 h. Hybridization was carried out overnight at 37°C in the same solution supplemented with the radiolabelled DNA probe. The blot was then washed with solutions of increasing stringency (2 x concentration of SSC and 0.1% SDS at room temperature, and SSC and 0.1% SDS at 37°C) with a few changes. The filter was then exposed to Kodak X-OMAT film.

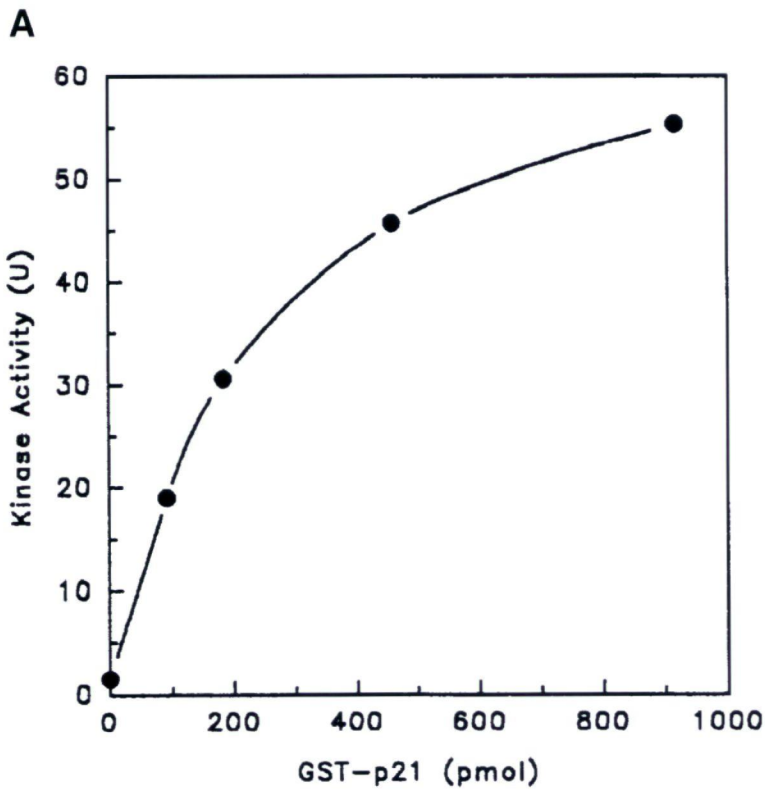
RESULTS

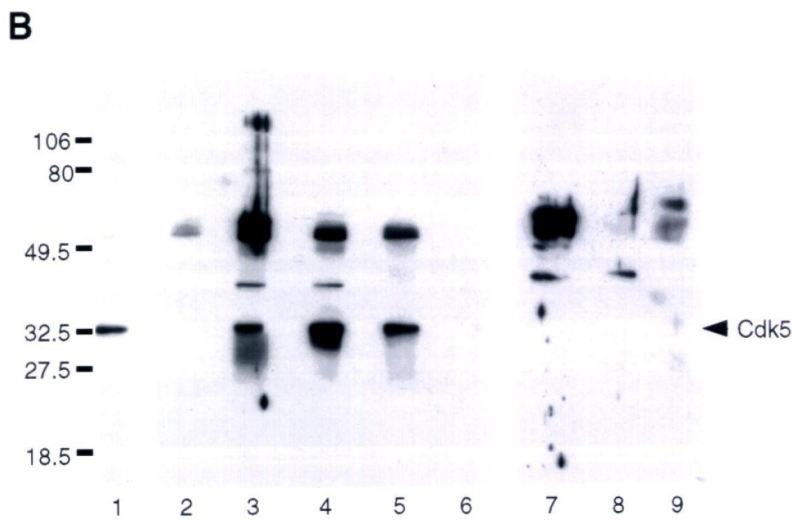
1. Activation of Cdk5 by expressed p35 fragments

Expression of the GST-fusion proteins of Cdk5, p35, as well as p35-derived p25, p23 and p21 (Fig. 3), was attempted in *E. coli* strain BL21 (DE3). The bacterial lysates were centrifuged to separate soluble and particulate fractions, and expression of the recombinant proteins was examined in both fractions by SDS-PAGE and immunoblotting with antibodies recognizing Cdk5 and p35 individually. Expression of full-length p35 was not successful; essentially no fusion protein was detectable in the bacterial lysate. An attempt to express full-length p35 using hexahistidine-tagged vector, PQE30 (Qiagen), were also unsuccessful. Most of the expressed p35-derivatives, GST-p21, GST-p23 and GST-p25, were found in the insoluble fraction. In all cases, the soluble recombinant proteins appeared to have undergone considerable proteolytic degradation. Lowering the induction temperature from 37°C to room temperature or decreasing the induction time to 1 h did not significantly affect the extent of protein degradation.

The expressed proteins were purified from the soluble fraction of bacterial lysates. To investigate functional properties, the shortest p35-derivative, GST-p21, was preincubated with a 120,000 g supernatant fraction of bovine brain extract, and subsequently affinity-precipitated with GSH-Agarose beads. Fig. 5A shows that the addition of GST-p21 resulted in high histone H1 kinase activity associated with the GSH-Agarose beads and this kinase activity was dose-dependent on GST-p21 bound on GSH-Agarose. Proteins bound on the beads were examined by Western blots with antibodies recognizing Cdk5, Cdc2 and Cdk2 individually. A protein band of 33 kDa was visualized in the precipitate with GST-p21, but not in the control sample, by three antibodies

FIG. 5. Activation of Cdk5 in crude brain extract by GST-p21. **(A)** 0.5 ml of the 120,000 g supernatant fraction of bovine brain extract was preincubated with the indicated amounts of GST-p21 for 40 min at 30°C. 50 μl of packed GSH-Agarose beads were then added, and incubation was continued at 4°C for 1 h. After centrifugation, the beads were extensively washed with the brain homogenization buffer. The histone kinase activity associated with the beads was assayed in 50 ul of a reaction mixture containing 5 mM $MgCl_2$, 30 mM MOPS, pH 7.2, 100 μM of [γ-^{32}P]ATP, and 100 μM of histone peptide HS(9-18) for 20 min at 30°C. **(B)** Western blot analysis of the proteins bound to the GSH-Agarose beads. Lane 1: NCLK standard purified from bovine brain was probed with α-Cdc2N. Lanes 2-6: Brain extract was preincubated with GST-p21 and precipitated with GSH-Agarose. Blots were probed with α-Cdc2C (lane 2), α -Cdk5C (lane 3), α-Cdk5I (lane 4), α-Cdc2N (lane 5), and α-Cdk2 (lane 6). Lanes 7-9: Brain extract was preincubated without GST-p21 and precipitated with GSH-Agarose. Blots were probed with α-Cdk5C (lane 7), α-Cdk5I (lane 8), and α-Cdc2N (lane 9).


A
60
50
40
30
20
10
0
Kinase Activity (U)
0
200
400
600
800
1000
GST−p21 (pmol)


B
106
80
49.5
32.5
27.5
18.5
Cdk5
1
2
3
4
5
6
7
8
9

reacting with Cdk5, which are α-Cdc2N, α-Cdk5I and α-Cdk5C. There is no such band stained by the Cdc2- or Cdk2-specific antibodies (Fig. 5B). The results indicate that Cdk5, but not Cdc2 nor Cdk2, from the brain extract bound to immobilized GST-p21, resulting in an active kinase complex.

To further test the function of the p35-derivatives in Cdk5 activation, GST-Cdk5 was mixed with the p35-derived GST fusion proteins. After incubation for 30 min at 30°C, the protein mixtures were analyzed for histone H1 kinase activity. As shown in Figure 6, GST-Cdk5 did not exhibit any kinase activity by itself, nor did any of the p35-related fusion proteins. Protein kinase activity, however, could be readily detected upon mixing GST-Cdk5 with the p35-derived fragments, GST-p21, GST-p23 and GST-p25. In contrast, bacterially expressed cyclin A or cyclin D1 did not give rise to kinase activity upon mixing with GST-Cdk5 (Fig. 6), suggesting that activation of Cdk5 by the p35-fragments is specific. Since GST-p21, GST-p23 and GST-p25 showed similar activity in Cdk5 activation, only Cdk5 activation by p21 was characterized further in this study.

In order to achieve maximal activity of Cdk5 in the complex with GST-p21, appropriate reconstitution conditions were explored. Dose-dependent activation of GST-Cdk5 by GST-p21 indicated that maximal activation was approached at an approximate mass ratio of Cdk5/p21 of 1:1.5 (Fig. 7). Cdk5 activation by p21 displayed pronounced time-dependence. The time course of the kinase reaction with a mixture of GST-Cdk5 and GST-p21 displayed low kinase activity and upward curvature, consistent with a slow increase of the kinase activity during the reaction (Fig. 8). When the two GST-proteins were pre-incubated for 1 h at 30°C before the kinase assay was initiated, a linear initial time course with greatly increased kinase activity was observed (Fig. 8). Consequently, the kinase activity was examined as a function of preincubation time. Fig. 9 shows that

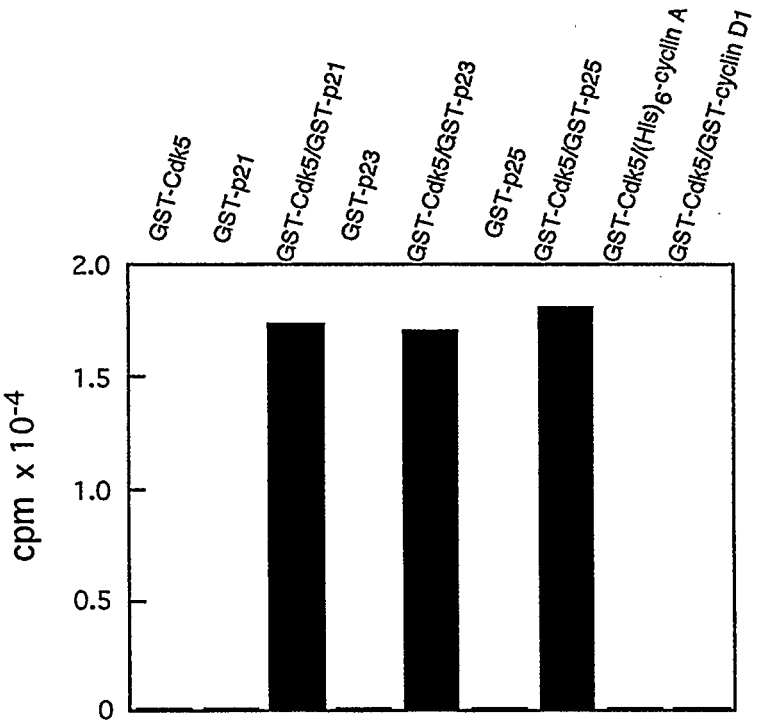


FIG. 6. Activation of bacterially expressed Cdk5. 0.5 μg GST-Cdk5 and 5 μg each of GST-p21, GST-p23, GST-p25, $(His)_6$-cyclin A and GST-cyclin D1 were preincubated alone or together at 30°C for 30 min in 15 μl of PBS buffer containing 1 mM EDTA and 1 mM DTT. The protein kinase assay was then started by addition of 100 μM substrate peptide HS(9-18), 5 mM Mg^{2+} and 100 μM [γ-^{32}P]ATP.

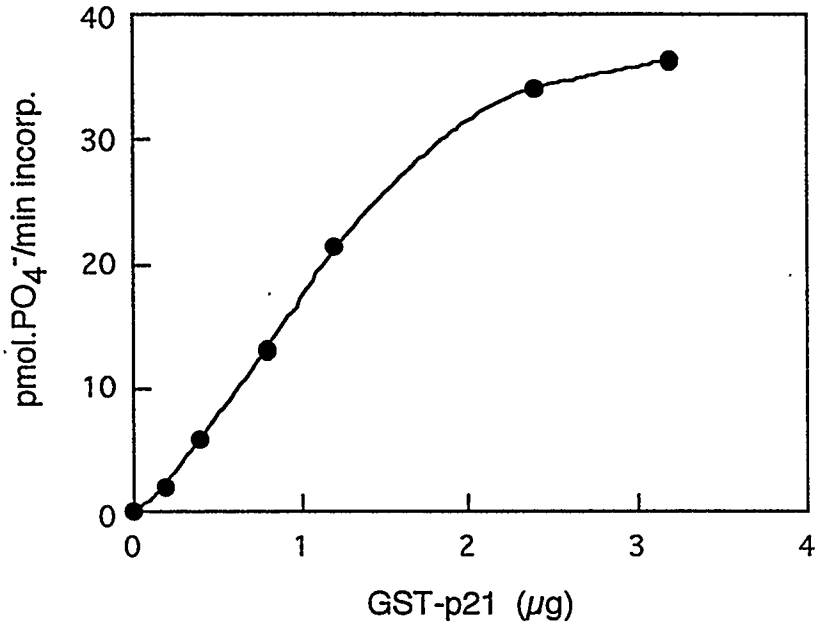


FIG. 7. Dose-dependent activation of Cdk5 by p21. 2 μg GST-Cdk5 was reconstituted with various amount of GST-p21 at 30°C for 1 h. The kinase reaction was performed with 100 μM substrate peptide HS(9-18), 5 mM Mg^{2+} and 100 μM [γ-^{32}P]ATP at 30°C for 20 min.

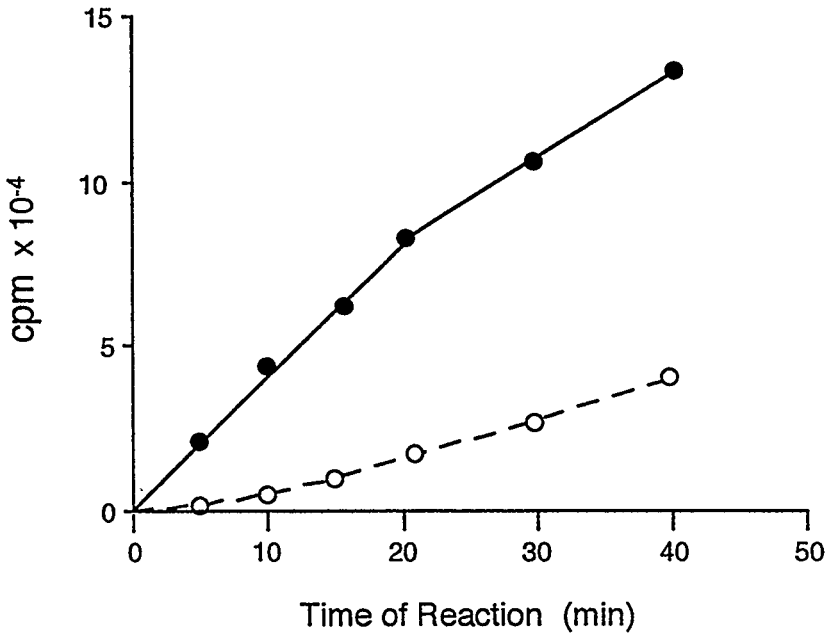


FIG. 8. Effect of preincubation on the reconstituted kinase activity. GST-Cdk5 (10 μg) and GST-p21 (20 μg) were mixed in 150 μl PBS buffer containing 1 mM EDTA and 1 mM DTT. The kinase reaction was initiated by addition of [γ-^{32}P]ATP, Mg^{2+} and peptide HS(9-18) immediately (o- - -o) or after incubation of the mixture at 30°C for 1 h (•—•). At indicated times after initiation of the kinase reaction, aliquots (15 μl) were withdrawn for analysis of phosphate incorporation into the histone peptide.

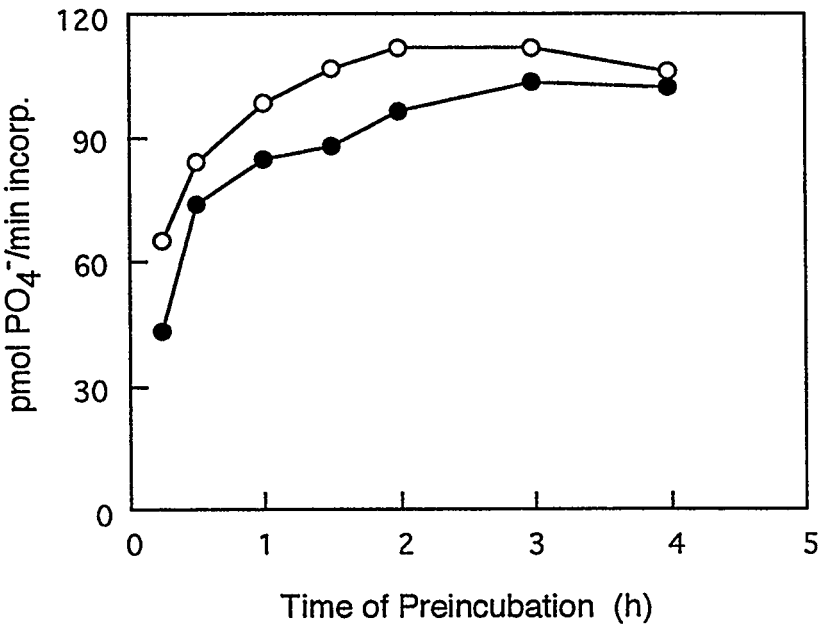


FIG. 9. Time-dependence of Cdk5/p21 kinase reconstitution. GST-Cdk5 (20 μg) and GST-p21 (40 μg) were mixed in 300 μl of PBS buffer containing 1 mM EDTA, 1 mM DTT and 0.5 mg/ml bovine serum albumin. The preincubation was performed at 30°C with (•—•) or without (o—o) 100 μM ATP-Mg^{2+}. Aliquots (30 μl) were removed at the indicated intervals for measurement of the protein kinase activity at a fixed time of 5 min.

the kinase activity increased rapidly during the first hour of pre-incubation, and reached its maximal activity at about 2 h. The time-dependence of Cdk5 activation was not significantly affected with inclusion of ATP and Mg^{2+} in the incubation mixture, suggesting that an autophosphorylation is not involved in the enzyme activation. This aspect of the enzyme activation is probed further in a later section.

2. Purification of reconstituted Cdk5/p21

The specific kinase activity of nearly homogeneous NCLK from bovine brain is approximate 4-10 μmoles PO_4 transferred per min. per mg. enzyme using the histone H1 derived peptide as a substrate (Lew *et al.*, 1992a). This value is similar to that of the purified Cdc2/cyclin B complex. The maximal kinase activity obtained by the mixture of GST-Cdk5 and GST-p21, however, was only several tens of nanomoles PO_4 per min. per mg. protein. A number of factors may contribute to the low activity displayed by reconstituted GST-Cdk5 and GST-p21. As indicated above, considerable proteolysis of the isolated recombinant proteins has occurred, and the partially degraded proteins may not be functional. Moreover, some of the recombinant proteins are likely to be folded incorrectly and, therefore, inactive. Experiments have been carried out to test the factors responsible for the low activity of the reconstituted enzyme.

Figure 10, lane *os*, shows that at least 50% of the GST-p21 sample purified by GSH-Agarose existed as proteolyzed fragments. The sample could be separated into two major protein peaks with gel filtration chromatography. The second peak contained most of the proteolytic derivatives and exhibited little Cdk5-activating activity. Although the first protein peak was enriched with intact GST-p21, only the trailing edge of the protein peak showed Cdk5-activating activity, suggesting that only a portion of the expressed intact protein was folded correctly (Fig. 10).

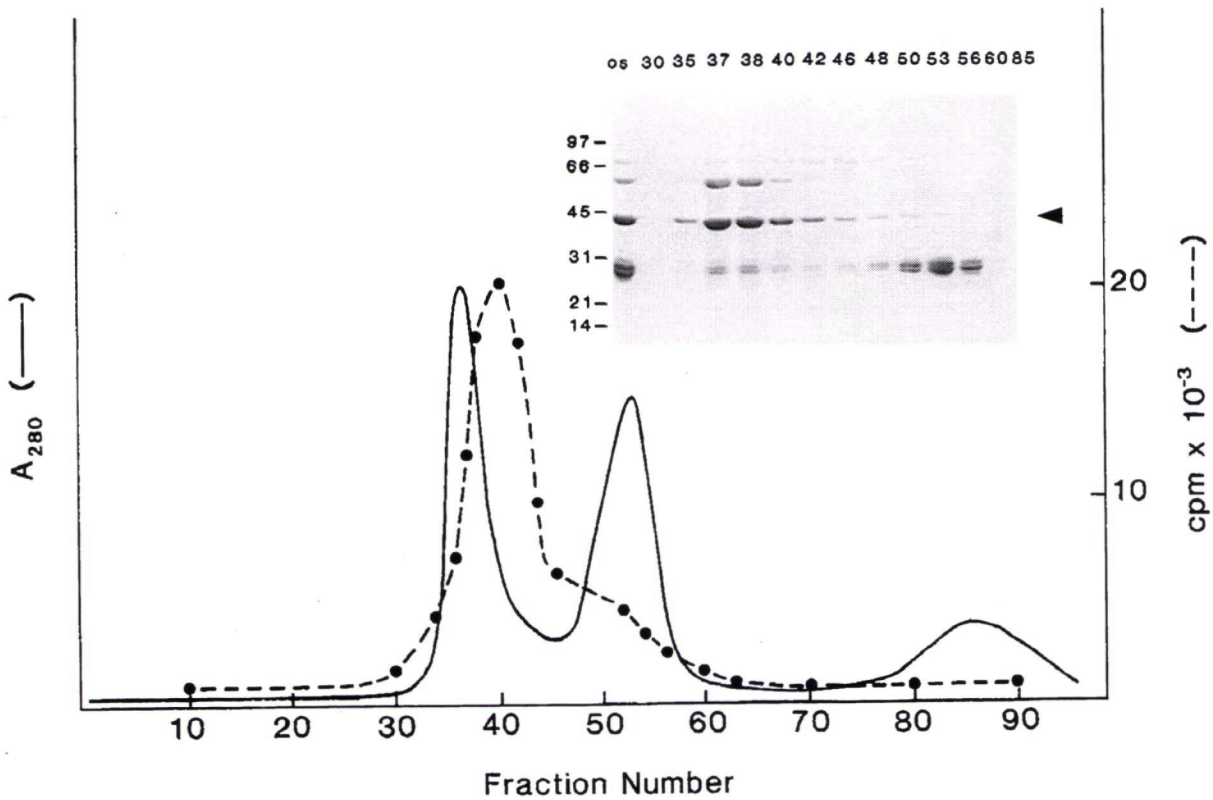


FIG. 10. Gel filtration chromatography of GST-p21. The concentrated sample (3 ml) of GSH-agarose purified GST-p21 was applied to an FPLC Superose 12 column (100 ml bed volume) equilibrated in PBS suplemented with 1 mM each EDTA and DTT, and the column was run at 1 ml/min. Samples (3 μl) were taken from the indicated fractions and incubated with 3 μg GST-Cdk5 for 1 h at 30°C. The kinase activity was then measured by phosphorylation of histone peptide HS(9-18). Inset is the SDS-PAGE of indicated fractions. The arrow indicates GST-p21 bands. The gel was stained with Coomassie Blue. *os* is the sample loaded on the column.

Like GST-p21, the GST-Cdk5 sample purified by GSH-Agarose contained large amounts of proteolytic derivatives of GST-Cdk5 (Fig. 11, lane *os*). The sample could be resolved into three major protein peaks after gel filtration chromatography (Fig. 11). While the intact fusion protein was enriched in both the first and second peaks, high kinase activity could be activated by GST-p21 only with the fractions at the trailing edge of the second peak (Fig. 11). Fractions of the last major peak, which contained mostly degraded fusion proteins, also failed to show kinase activity in the presence of GST-p21 (Fig. 11). Thus, the majority of GST-Cdk5 is present in functionless conformations or proteolytic derivatives.

Since the low specific activity of the reconstituted enzyme appeared to be due, at least in part, to the existence of large amounts of degraded and incorrectly folded recombinant proteins, attempts were made to purify a active kinase from the reconstituted enzyme sample. The enzyme was reconstituted from GST-Cdk5 and GTS-p21, and GST was then excised by thrombin. After dialysis, the sample was subjected to chromatography on an ion exchange Mono S column. A large amount of protein, mostly GST, was found in the flow-through, whereas about 85% of the kinase activity bound to the column. Almost 80% of this activity was eluted by a salt gradient in a sharp peak along with a small amount of protein (Fig. 12A).

Analysis by SDS-PAGE revealed that proteins of 33 kDa and 21 kDa were abundant in the histone kinase active fractions (Fig. 12B), and they were identified as Cdk5 and p21, respectively, by immunoblots with Cdk5 and p25 specific antibodies. Densitometric scanning of the Coomassie Blue-stained gels indicated that Cdk5 and p21 constituted about 50% of the total protein, and the molar ratio of the 21 kDa protein to the 33 kDa protein was about 1.3 (Fig. 13). However, the purified sample was still

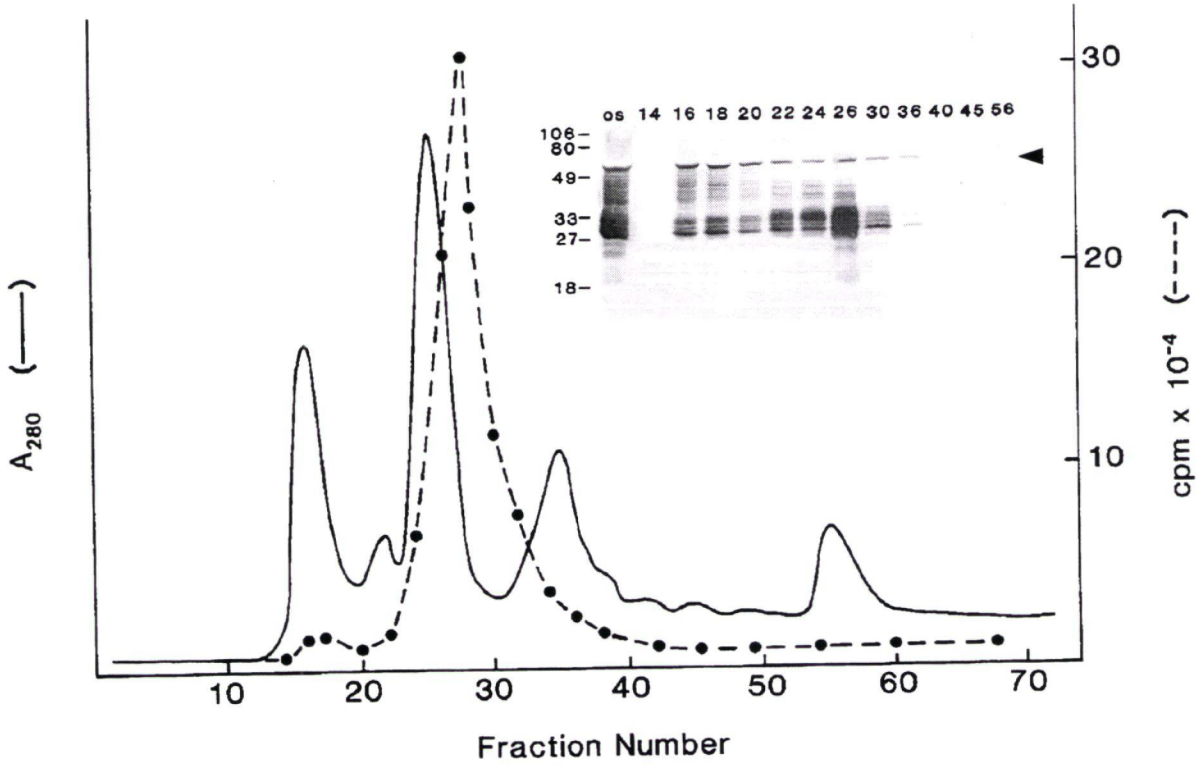


FIG. 11. Gel filtration analysis of GST-Cdk5. 200 μl of GST-Cdk5 purified by GSH-Agarose was applied to an FPLC Superose 12 analytical column (24 ml bed volume) equilibrated in PBS supplemented with 1 mM each of EDTA and DTT. The column was run at 0.5 ml/min and 0.5 ml was collected in each fraction. Samples (15 μl) from the indicated fractions were incubated with 2 μg GST-p21 for 1 h at 30°C. The histone kinase activity of the reconstituted samples was then measured. Inset is the Western blot with anti-Cdk5C. The arrow indicates GST-Cdk5 bands. *os* is the sample loaded on the column.

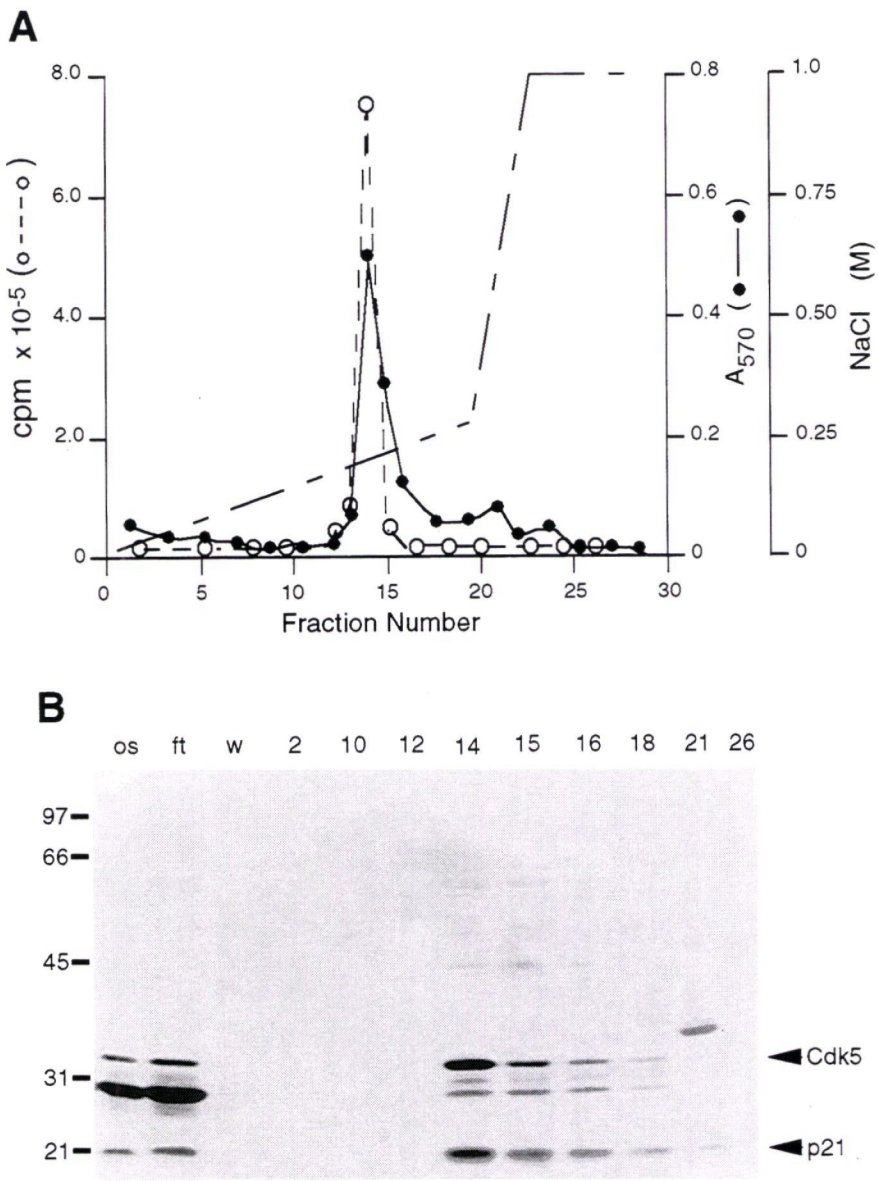


FIG. 12. FPLC Mono S chromatography of reconstituted Cdk5/p21. The reconstitution and purification of the Cdk5/p21 complex were carried out as described in detail in *Materials and Methods*. After sample loading and column washing, the collection was started by fractions of 0.5 ml. **(A)** Column elution profiles of protein and histone peptide kinase activity. Protein was determined by the Bradford method performed in a microtiter plate. **(B)** SDS-PAGE of indicated fractions from (A). Gel was stained with silver. *os* is the original sample loaded on the column. *ft* is the flow-through fraction of the sample loading. *w* is the column washing fraction after the sample loading.

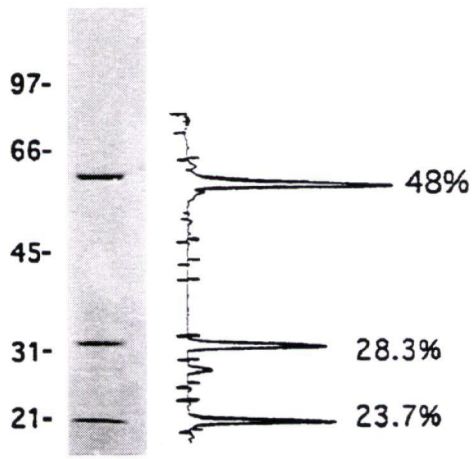


FIG. 13. Composition of the purified reconstituted kinase sample. A sample from Mono S profile Fraction 14 was resolved by SDS-PAGE. The gel was stained with Coomassie Blue. Beside the gel is the densitometry scanning profile of the stained bands.

contaminated with a bacterial protein having a molecular weight about 60 kDa, which was visualized by Coomassie Blue stain but not silver stain, and a trace amount of free GST. Purification data for a typical preparation are summarized in Table I. Protein concentration of the most active fraction (Fr. 14) was determined by both densitometric scanning of the Coomassie Blue-stained gels and Bradford assays performed in a microtiter plate using carbonic anhydrase and bovine serum albumin as protein standards, respectively.

The purified, reconstituted Cdk5/p21 complex has a specific kinase activity of 3.8 μmoles PO_4/min/mg protein towards histone H1 peptide HS(9-18). This activity is 65-fold higher than that of GST-Cdk5 and GST-p21 mixture. Since about 50% of the protein in the purified sample was Cdk5 and p21 (Fig. 13), a homogeneous reconstituted enzyme is expected to have a specific kinase activity of at least 7 μmol PO_4/min/mg protein. Thus, the active kinase reconstituted from bacterially expressed Cdk5 and a fragment of p35 has a specific activity as high as that of NCLK purified from bovine brain, which is 4-10 μmoles PO_4/min/mg enzyme.

3. Substrate specificity of reconstituted Cdk5/p21

Previously, a set of peptide analogs was employed to elucidate substrate determinants for NCLK (Beaudette *et al.*, 1993). These peptides were designed by systematic substitution of each residue in the parent peptide HS(9-18), PKTPKKAKKL. HS(9-18) contains the consensus proline-directed Ser/Thr phosphorylation motif (Fig. 14). To characterize the substrate specificity, the reconstituted enzyme catalyzed phosphorylation of the HS(9-18) analogues was compared to that catalyzed by bovine brain NCLK. Figure 14A shows the relative phosphorylation rates of the different peptide analogues. The reconstituted and native enzymes display very similar phosphorylation rates of the substrate analogues. Substrate Km values were determined for reconstituted

TABLE I. Purification of reconstituted Cdk5/p21 by Mono S chromatography

	Protein mg	Total activity μmol PO_4/min	Specific activity μmol PO_4/min/mg	Purification	Yield
Crude reconstituted enzyme	3.43	0.20	0.058		
Mono S purified sample (Fr.14)	0.04	0.15	3.8	65-fold	75%

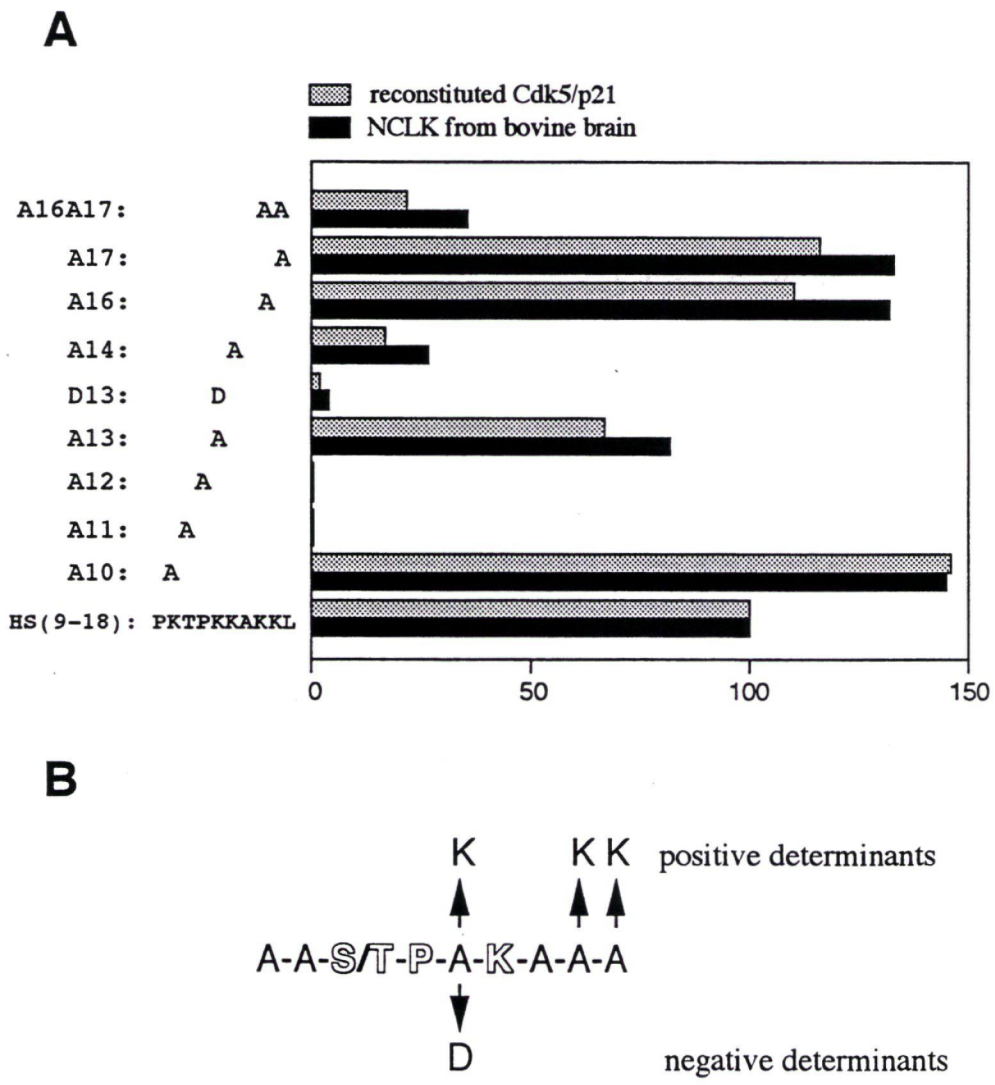


FIG. 14. Substrate specificities of NCLK from bovine brain and reconstituted Cdk5/p21. (**A**) Relative phosphorylation rates of the histone peptide analogues by NCLK from bovine brain and reconstituted Cdk5/p21. The phosphorylation reaction was performed with 5 mM of each individual synthetic peptide analogue and the indicated enzymes. The phosphorylation rates were expressed as a percentage of the parent peptide, HS(9-18). (**B**) Summary of the positive and negative determinants of the proline-directed Ser/Thr phosphorylation motif.

Cdk5/p21. They are highly similar, if not identical, to those for NCLK from bovine brain (Table II).

4. Phosphorylation-independent activation of Cdk5 by p21

Cdc2 activation by cyclin B is absolutely dependent on Cdc2 phosphorylation at Thr^{161} by CAK. Although Cdk2 exhibits some activity upon reconstitution with cyclin A, the activity of Cdk2/cyclin A is greatly increased by the CAK-catalyzed phosphorylation of Cdk2 on Thr^{160}. Sequence alignment of Cdk5 with Cdc2 and Cdk2 has shown that the residue corresponding to Thr^{161}/Thr^{160} of Cdc2/Cdk2 is conservatively substituted in Cdk5 by a serine, Ser^{159} (Table III). Since the sequence surrounding Ser^{159} is highly conserved in Cdk5, Cdc2 and Cdk2, Cdk5 might also be regulated by CAK (Lew *et al.*, 1992b). Reconstitution and isolation of the highly active Cdk5/p21 complex in the absence of any other protein factor, however, argues against such a suggestion.

CAK activity has been detected in our laboratory in bovine brain, which phosphorylates and activates the Cdk2/cyclin A complex (Z. Zhu and J.H. Wang, unpublished data). To test the involvement of CAK activity, which is isolated from bovine brain, in the regulation of Cdk5/p21 activity, the purified, reconstituted kinase was subjected to a phosphorylation reaction in the presence of brain CAK. The kinase activity of Cdk5/p21 was then measured. The result showed that the treatment had little or no effect on Cdk5/p21 activity. As a control, a mixture of bacterially expressed Cdk2 and cyclin A was treated with brain CAK under the same conditions; CAK treatment greatly enhanced Cdk2 kinase activity. Furthermore, the treatment of a Cdk5 and cyclin A mixture with brain CAK did not enhance the kinase activity. This observation further supports the notion that Cdk5 is specifically activated by the p35 derivatives.

TABLE II. K_m values of NCLK purified from bovine brain and the reconstituted kinase

The kinetic parameters were determined as detailed under *Materials and Methods* with the purified enzymes. These K_m values for NCLK purified from bovine brain were determined by Beaudette *et al.* (1993).

Peptide	Sequence	Brain Purified Kinase	Reconstituted Kinase
		K_m (μM)	
HS(9-18)	PKTPKKAKKL	6	7
HS(9-18)A14	PKTPKAAKKL	376	260
HS(9-18)A13	PKTPAKAKKL	50	57
HS(9-18)D13	PKTPDKAKKL	649	333
HS(9-18)A16	PKTPKKAAKL	26	33
HS(9-18)A17	PKTPKKAKAL	27	27
HS(9-18)A16,A17	PKTPKKAAAL	155	152

TABLE III. Sequence alignments of the potential regulatory phosphorylation sites in Cdks

	inhibiting sites	activating sites
Cdc2 (Cdk1)	EKIGEGT[14]Y[15]GVVYKG	AFGIPIRVYT[161]HEVVTLWYR
Cdk2	EKIGEGT[14]Y[15]GVVYKA	AFGVPVRTYT[160]HEVVTLWYR
Cdk3	EKIGEGT[14]Y[15]GVVYKA	AFGVPLRTYT[160]HEVVTLWYR
Cdk4	AEIGVGA[16]Y[17]GTVYKA	RIYSYQMALT[172]PVVVTLWYR
Cdk5	EKIGEGT[14]Y[15]GTVFKA	AFGIPVRCYS[159]AEVVTLWYR
Cdk6	AEIGEGA[23]Y[24]GKVFKA	RIYSFQMALT[177]SVVVTLWYR

While reconstitution of a highly active kinase from bacterially expressed Cdk5 and p35-derived fragments may rule out the involvement of an activating kinase in enzyme activation, the possibility that Ser^{159} phosphorylation is required in Cdk5 activation was not excluded. The enzyme may undergo autophosphorylation at this serine residue during reconstitution, resulting in increased kinase activity. However, the assumption of autophosphorylation and activation was weakened by the results from the time dependence of Cdk5 activation by p21 (Fig. 9). The time course of Cdk5 activation by p21 was not significantly affected by inclusion of ATP-Mg^{2+} (Fig. 9) in the preincubation mixture, suggesting that activation may not be a function of phosphorylation.

To probe further whether the reconstituted kinase underwent autophosphorylation on Cdk5, a sample of the purified, reconstituted kinase was incubated with [γ-^{32}P]ATP-Mg^{2+} at 30°C. Aliquots of the sample were withdrawn at various intervals and analyzed by SDS-PAGE and autoradiography. Figure 15A shows that while p21 is rapidly phosphorylated, no radioactivity is seen to associate with Cdk5 even after 40 min incubation. Figure 15B shows that samples taken from the kinase reactions with the substrate histone H1 peptide were phosphorylated on the 21 kDa subunit but not on Cdk5. The capability of reconstituted Cdk5/p21 to catalyze phosphorylation of p21 is compatible with the results of a previous study showing that the regulatory subunit of bovine brain NCLK is phosphorylated under phosphorylation conditions (Lew *et al.*, 1992a). Phosphorylation of p21 does not appear to affect the enzyme activity. As shown in Figure 15B, phosphorylation of p21 in the kinase reaction was significantly suppressed in the presence of the substrate peptide. While the extent of p21 phosphorylation diminished with elevated peptide substrate concentrations, there was no indication of substrate-dependent activation or inactivation of this enzyme.

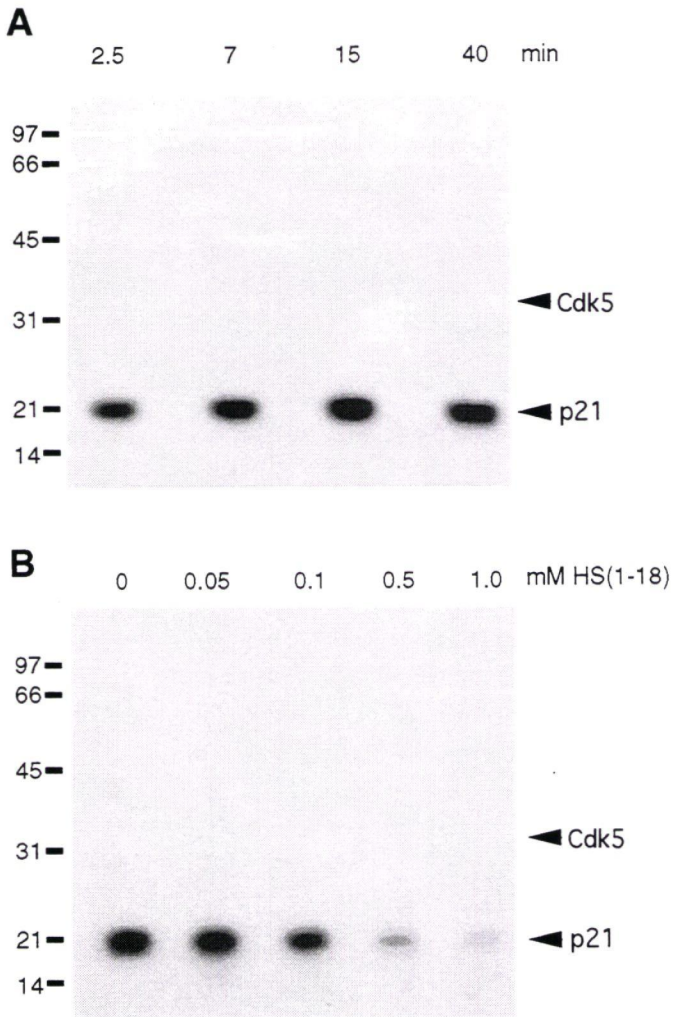


FIG. 15. Autophosphorylation of Cdk5/p21. **(A)** Time course of autophosphorylation reaction. Aliquots (1 μg) of reconstituted and purified Cdk5/p21 were incubated at 30°C with 100 μM [γ-^{32}P]ATP (~5000 dpm/pmol) in 20 mM MOPS, pH7.4, 5 mM $MgCl_2$ for different intervals as indicated. **(B)** Substrate effect on autophosphorylation. Aliquots (0.4 μg) of the reconstituted enzyme were incubated at 30°C for 5 min under the same condition as above with substrate peptide HS(1-18) at the indicated concentrations. Reactions were stopped by addition of SDS-PAGE sample buffer and boiling at 95°C for 5 min. The samples were analyzed by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

To substantiate further the suggestion that Cdk5 activation by p21 does not depend on phosphorylation of Cdk5, the possibility, albeit remote, that the bacterially expressed Cdk5 used for enzyme reconstitution had already been phosphorylated was tested by examining the effect of a protein phosphatase on the reconstituted kinase. Protein phosphatase 2A (PP2A) catalytic subunit, which was previously shown to dephosphorylate and inactivate the isolated Cdc2/cyclin complex, was applied in such experiments (Lee *et al.*, 1991). The enzyme reconstitution was performed with GST-Cdk5 and GST-p25 at 30°C for 1 h in the presence of 0.5 mg/ml bovine serum albumin. 20 μl aliquots containing 1 μg GST-Cdk5 and 1.5 μg GST-p25 were then treated with 20 ng PP2A catalytic subunit at 30°C for 30 min. The phosphatase activity was stopped by addition of 100 nM okadaic acid before the kinase activity was measured. The kinase reaction was carried out for a short interval (6 min at 30°C) to minimize the possibility of autophosphorylation during the reaction. It was found that the kinase activity of the phosphatase-treated sample was not discernibly different from that of the control samples, which had been treated in the same manner except that okadaic acid was added before addition of the phosphatase.

5. Cloning of p35-interacting proteins in human brain

NCLK has been suggested to represent the predominant activity of proline-directed kinases in adult brain (Lew *et al.*, 1992a). In contrast to the widely expressed Cdk5, the regulatory subunit in NCLK, p35, is expressed strictly in neurons of the central nervous system, establishing the neuronal specificity of NCLK (Tsai *et al.*, 1994). Moreover, p35 is a specific activator of Cdk5. This observation prompted us to investigate p35-binding proteins in brain. The yeast two-hybrid system, which is a genetic tool to study *in vivo* protein-protein interactions, was employed in this study.

5.1. cDNA library screening by the two-hybrid system

The entire open reading frame of bovine p35 was inserted in frame into pAS2 to make a bait construct, which expresses a fusion protein of the GAL4 DNA binding domain and p35 in yeast cells. A functional transcriptional activator can be reconstituted by the interaction of p35 in the bait protein with its binding protein, which is fused to the GAL4 transcriptional activation domain. Reconstitution of the transcriptional activator can be readily monitored by enhanced transcription of the reporter genes HIS3 and *lacZ*. However, there is a basal level of HIS3 expression in the reporter strains CG-1945 and Y190 in the presence of pAS2 to allow slow growth of the cells in the medium without histidine. The addition of a minimal amount of 3-AT, which is a competitive inhibitor of imidazole glycerol phosphate dehydratase (HIS3 gene product), to the selective medium requires a higher level of HIS3 expression to allow cell growth (Durfee *et al.*, 1993). p35pAS2 alone has little transcriptional activity in CG-1945 cells, and 5 mM 3-AT in the selective medium was sufficient to suppress cell growth due to the residual HIS3 expression.

The prey is a human adult brain cDNA library constructed in pACT2. The library contains 5×10^6 independent clones with >85% inserts. The bait p35pAS2 and the prey were introduced into CG-1945 cells by sequential transformation. 100 μg of the library plasmids were used to transform the p35pAS2-containing cells. A total of 3.5×10^5 co-transformants were plated on SD media lacking Trp, Leu and His but containing 5 mM 3-AT. About 100 colonies appeared after incubation at 30°C for 7 days, of which 39 were positive in the filter assay of β-galactosidase activity. These 39 colonies were considered as positives in the screen. Cells from the positive colonies were streaked on SD-Trp-Leu plates and grown up for a retest of β-galactosidase activity. The plasmids were then isolated from colonies displaying positive results in the β-galactosidase assays.

Separation of the pACT2-based plasmids from p35pAS2 and recovery of the pACT2-based plasmids were achieved through transformation of a *leu*B$^-$ strain of *E. coli*, HB101, which allows only the transformants harboring the pACT2-based plasmids to grow on minimal media lacking leucine.

5.2. Elimination of false positives

The library-isolated plasmids were reintroduced into CG-1945 cells by co-transformation with p35pAS2 or SNF1. Co-transformants of p35pAS2 and the library-isolated clones should yield positive results in the reporter assays. The yeast protein SNF1 should not interact with the isolated p35-interacting proteins, and therefore, a negative result in the reporter assay was expected for the co-transformants of SNF1 and the isolated clones. Expression of HIS3 and *lac*Z in the co-transformants was tested by growth on plates of SD-Trp-Leu-His but including 5 mM 3-AT and by the filter assay of β-galactosidase activity, respectively. The p35pAS2-dependent reporter activities were detected with 14 library-isolated clones. The interaction of p35pAS2 and these 14 clones was verified in another reporter strain, Y190. In this case, a fragment of human lamin C in the pAS1 vector was used as a control to test the specificity of the interaction assays (Bartel *et al.*, 1993). All 14 library-isolated clones displayed specific interaction with p35pAS2 in the reporter assays.

5.3. Classification of isolated clones

To analyze the isolated cDNAs, PCR amplification using *Taq* polymerase was performed with the primers flanking the insert region in pACT2. The primer sequences are 5'-TACCACTACAATGGATG-3' and 5'-GTTGAAGTGAACTTGCGGGG-3'. The cycling conditions were as follows: 30 cycles of 30 sec at 96°C, 1 min at 55°C, and 1.5 min at 72°C. The products were subsequently analyzed by agarose electrophoresis. As

shown in Figure 16A, the cDNA inserts were amplified from 9 clones, clones C7, C35, C42, C48, C51, C53, C61, C73, and C105. The sizes of the PCR fragments are 0.7-2.1 kb. However, the PCR amplification failed with the remaining five clones, clones C1, C11, C28, C33 and C37. Attempts to PCR amplify these five clones remained unsuccessful even after changing denaturing and annealing temperatures.

In attempting to sort the positive clones by sequence similarities, restriction digestion and dot blot hybridization were applied in combination. The amplified cDNA inserts were treated with the restriction enzymes *BamH*I, *Cla*I, *Hinf*I and *EcoR*V, and the digested fragments were resolved by agarose electrophoresis. Figure 16B shows that clones C7, C35 and C51 have identical restriction fragment patterns. In addition, the sizes of their cDNA inserts are the same (Fig. 16A), suggesting that they are identical clones. Likewise, clone C48 displays the same restriction pattern and clone size as clone C105. The restriction patterns appear to be unique for clones C42, C53, C61 and C73, indicating different sequences in these clones (Fig. 16B).

Clones C1, C11, C28, C33 and C37 harbor 1.4 kb cDNA inserts. In order to find similar sequences, the cDNA insert of clone C1 was used as a probe to hybridize to all 14 positive clones dot-blotted on a nitrocellulose filter. Specific hybridization was seen with clones C11, C28, C33 and C37, indicating that they contain the same sequence as C1. Taken together, the 14 positive clones from the screen for p35-interacting proteins are sorted into 7 groups by sequence similarities (Table IV), which represent seven putative p35-interacting proteins in human brain.

To further test the interaction of p35 with the library-isolated clones, β-galactosidase activity was quantified using substrate ONPG with the yeast cells co-

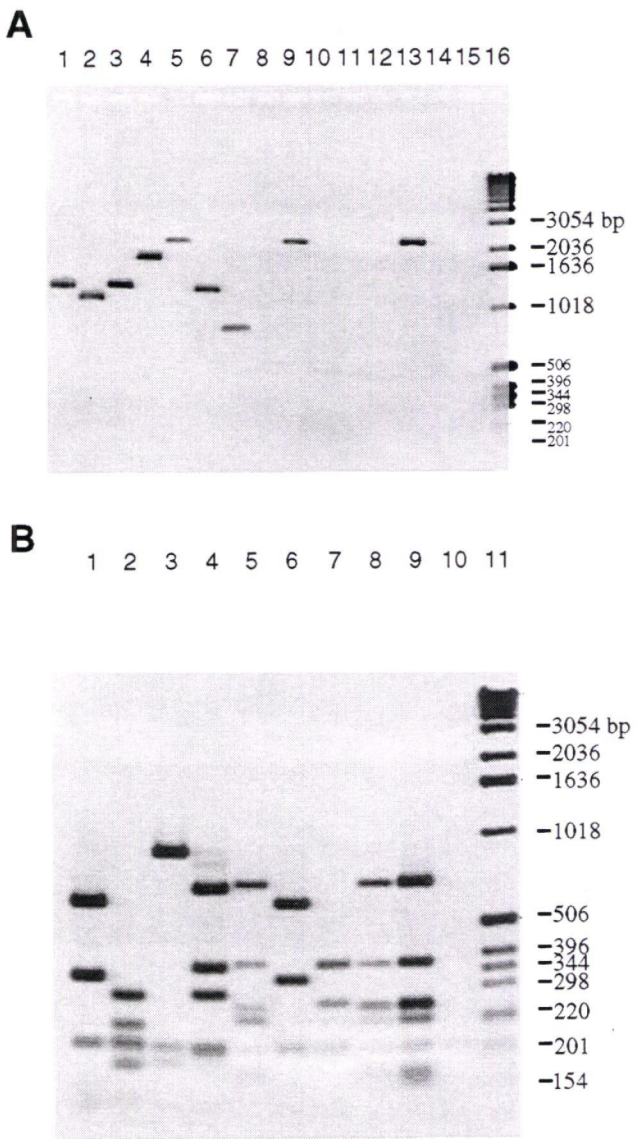


FIG. 16. Restriction analysis of the isolated cDNA clones. (A) cDNA inserts of the isolated clones. The PCR products were resolved by electrophoresis on 1.5% agarose gel in the following order: lane 1, C105; lane 2, C73; lane 3, C61; lane 4, C53; lane 5, C51; lane 6, C48; lane 7, C42; lane 8, C37; lane 9, C35; lane 10, C33; lane 11, C28; lane 12, C11; lane 13, C7; lane 14, C1; lane 15, vector pACT2; lane 16, 1 kb DNA ladder. **(B)** Restriction patterns of the isolated cDNA fragments. The PCR fragments of the isolated cDNAs were digested with *BamHI*, *ClaI*, *EcoRV* and *HinfI*, and the products were analyzed by electrophoresis with 1.5% agarose gel as follows: lane 1, C105; lane 2, C73; lane 3, C61; lane 4, C53; lane 5, C51; lane 6, C48; lane 7, C42; lane 8, C35; lane 9, C7; lane 10, vector pACT2; lane 11, 1 kb DNA ladder.

TABLE IV. Classification of isolated clones

Groups	Isolated clones	cDNA insert size (kb)
A	C1, C11, C28, C33, C37	1.4
B	C7, C35, C51	2.1
C	C42	0.7
D	C48, C105	1.2
E	C53	1.6
F	C61	1.1
G	C73	0.9

transformed with p35pAS2 and the selected clones from each group. In controls, a human lamin C fragment in pAS1 was used instead of p35pAS2 to measure the background activities in the cells. All seven assayed clones displayed low background activities in the presence of lamin C (Table V). The interaction between p35 and the library-isolated proteins enhanced expression of β-galactosidase to higher levels, which are 3.4- to 40-fold above their background activities (Table V), suggesting that the proteins coded for by the library-isolated sequences bind p35 with different affinities.

5.4. Identification of library-isolated clones

As NCLK was identified as a complex of Cdk5 and p25/p35, it was expected that some of the library-isolated clones might contain *cdk5*-derived sequences. These could be readily identified by hybridization with a ^{32}P-labeled *cdk5* probe. However, no clone was found containing the *cdk5* sequence in dot blot analysis.

In order to identify the proteins coded for by the 7 groups of the positive clones, a cDNA clone from each group was subjected to DNA sequencing. The nucleotide and deduced amino acid sequences were used to search the Genbank database. Clone C35 encodes a 122-amino acid peptide with a termination codon. A Genbank homology search showed that the deduced amino acid sequence of C35 was identical to the carboxyl terminal region of human glial fibrillary acidic protein (GFAP, Fig. 17). Likewise, clone C61 was found to encode the last 98 amino acids of human *n*-chimaerin (α_1-chimaerin) and α_2-chimaerin, which are alternative splice products (Fig. 18). When the sequence of C73 was put on query, the result suggested that it was the C-terminal region of human clusterin precursor (Fig. 19). Therefore, the C-terminal regions of GFAP, *n*(α_2)-chimaerin, and clusterin were isolated in the screen for p35-interacting proteins.

TABLE V. Interactions of two hybrids in yeast cells

Library cDNA/Activation domain hybrid	Protein moiety in DNA binding domain	β-galactosidase activity (Miller unit)
C33	Lamin C	0.8
	p35	29.3
C35	Lamin C	1.0
	p35	16.0
C42	Lamin C	1.7
	p35	20.1
C48	Lamin C	1.0
	p35	40.2
C53	Lamin C	1.0
	p35	13.4
C61	Lamin C	1.6
	p35	5.5
C73	Lamin C	2.4
	p35	10.7

A

```
CGCGAGCAGGAGGAGCGGCACGTGCGGGAGGCGGCCAGTTATCAGGAGGCGCTG    54
 R  E  Q  E  E  R  H  V  R  E  A  A  S  Y  Q  E  A  L     18

GCGCGGCTGGAGGAAGAGGGGCAGAGCCTCAAGGACGAGATGGCCCGCCACTTG   108
 A  R  L  E  E  E  G  Q  S  L  K  D  E  M  A  R  H  L     36

CAGGAGTACCAGGACCTGCTCAATGTCAAGCTGGCCCTGGACATCGAGATCGCC   162
 Q  E  Y  Q  D  L  L  N  V  K  L  A  L  D  I  E  I  A     54

ACCTACAGGAAGCTGCTAGAGGGCGAGGAGAACCGGATCACCATTCCCGTGCAG   216
 T  Y  R  K  L  L  E  G  E  E  N  R  I  T  I  P  V  Q     72

ACCTTCTCCAACCTGCAGATTCGAGAAACCAGCCTGGACACCAAGTCTGTGTCA   270
 T  F  S  N  L  Q  I  R  E  T  S  L  D  T  K  S  V  S     90

GAAGGCCACCTCAAGAGGAACATCGTGGTGAAGACCGTGGAGATGCGGGATGGA   324
 E  G  H  L  K  R  N  I  V  V  K  T  V  E  M  R  D  G    108

GAGGTCATTAAGGAGTCCAAGCAGGAGCACAAGGATGTGATGTGAGGCAGGACC   378
 E  V  I  K  E  S  K  Q  E  H  K  D  V  M  *              122

CACCTGGTGGCCTCTGCCCCGTCTCATGAGGG                          410
```

B

```
GFAP MERRRITSAARRSYVSSGEMMVGGLAPGRRLGPGTRLSLARMPPPLPTRV   50
GFAP DFSLAGALNAGFKETRASERAEMMELNDRFASYIEKVRFLEQQNKALAAE  100
GFAP LNQLRAKEPTKLADVYQAELRELRLRLDQLTANSARLEVERDNLAQDLAT  150
GFAP VRQKLQDETNLRLEAENNLAAYRQEADEATLARLDLERKIESLEEEIRFL  200
GFAP RKIHEEEVRELQEQLARQQVHVELDVAKPDLTAALKEIRTQYEAMASSNM  250
GFAP HEAEEWYRSKFADLTDAAARNAELLRQAKHEANDYRRQLQSLTCDLESLR  300
GFAP GTNESLERQMREQEERHVREAASYQEALARLEEEGQSLKDEMARHLQEYQ  350
GFAP DLLNVKLALDIEIATYRKLLEGEENRITIPVQTFSNLQIRETSLDTKSVS  400
GFAP EGHLKRNIVVKTVEMRDGEVIKESKQEHKDVM*                    432
```

FIG. 17. Sequences of C35 and GFAP. **(A)** Partial nucleotide and deduced amino acid sequences of C35. **(B)** Amino acid sequence of GFAP. The underlined part is the sequence of C35.

A

```
AAGTTTATAGAATCTGCCAAAATTATGGATCCGGATGAGCAATTGGAAACCCTT    54
 K  F  I  E  S  A  K  I  M  D  P  D  E  Q  L  E  T  L     18

CATGAAGCACTGAAACTACTGCCACCTGCTCACTGCGAAACCCTCCGGTACCTC   108
 H  E  A  L  K  L  L  P  P  A  H  C  E  T  L  R  Y  L     36

ATGGCACATCTAAAGAGAGTGACCCTCCACGAAAAGGAGAATCTTATGAATGCA   162
 M  A  H  L  K  R  V  T  L  H  E  K  E  N  L  M  N  A     54

GAGAACCTTGGAATCGTCTTTGGACCCACCCTTATGAGATCTCCAGAACTAGAC   216
 E  N  L  G  I  V  F  G  P  T  L  M  R  S  P  E  L  D     72

GCCATGGCTGCATTGAATGATATACGGTATCAGAGACTGGTGGTGGAGCTGCTT   270
 A  M  A  A  L  N  D  I  R  Y  Q  R  L  V  V  E  L  L     90

ATCAAAAACGAAGACATTTTATTTTAAATTTTTAATTTGAGGGGAAAAGAAATG   324
 I  K  N  E  D  I  L  F  *                                98

TTTTACAGATGAAGGAATGTTTTATAGTAATTTAATTTGCTCCTGTAGCTGCAT   378
TATTTCTTGATTAGAGGTTTGGGCATATAACCAGATTAAAGTGAAGGAACTTTC   432
TGTTGTTTTTGTAGCACCGCTCAGCTGTCTTGTAAAACAGTGAACACACGCTTT   486
CTGGTTCTAGTAATCCTGG                                      505
```

B

α_2-chim	MALTLFDTDEYRPPVWKSYLYQLQQEAPHPRRITCTCEVENRPKY	45
α_2-chim	YGREFHGMISREAADQLLIVAEGSYLIRESQRQPGTYTLALRFGS	90
α_2-chim	QTRNFRLYYDGKHFVGEKRFESIHDLVTDGLITLYIETKAAEYIA	135
n-chim	MPSKESWSGR	10
α_2-chim	KMTINPIYEHVGYTTLNREPAYKKHMPVLKETHDERDSTGQDGVS	180
n-chim	KTNRAAVHKSKQEGRQQDLLIAALGMKLGSPKSSVTIWQPLKLFA	55
α_2-chim	EKRLTSLVRRATLKENEQIPKYEKIHNFKVHTFRGPHWCEYCANF	225
n-chim	YSQLTSLVRRATLKENEQIPKYEKIHNFKVHTFRGPHWCEYCANF	100
α_2-chim	MWGLIAQGVKCADCGLNVHKQCSKMVPNDCKPDLKHVKKVYSCDL	270
n-chim	MWGLIAQGVKCADCGLNVHKQCSKMVPNDCKPDLKHVKKVYSCDL	145
α_2-chim	TTLVKAHTTKRPMVVDMCIREIESRGLNSEGLYRVSGFSDLIEDV	315
n-chim	TTLVKAHTTKRPMVVDMCIREIESRGLNSEGLYRVSGFSDLIEDV	190
α_2-chim	KMAFDRDGEKADISVNMYEDINIITGALKLYFRDLPIPLITYDAY	360
n-chim	KMAFDRDGEKADISVNMYEDINIITGALKLYFRDLPIPLITYDAY	235
α_2-chim	PKFIESAKIMDPDEQLETLHEALKLLPPAHCETLRYLMAHLKRVT	405
n-chim	PKFIESAKIMDPDEQLETLHEALKLLPPAHCETLRYLMAHLKRVT	280
α_2-chim	LHEKENLMNAENLGIVFGPTLMRSPELDAMAALNDIRYQRLVVEL	460
n-chim	LHEKENLMNAENLGIVFGPTLMRSPELDAMAALNDIRYQRLVVEL	325
α_2-chim	LIKNEDILF*	469
n-chim	LIKNEDILF*	334

FIG. 18. Sequences of C61, *n*-chimaerin and α_2-chimaerin. **(A)** Nucleotide and deduced amino acid sequences of C61. **(B)** Amino acid sequences of *n*-chimaerin and α_2-chimaerin. The underlined parts are the sequence of C61.

A

```
CTCAGCAGGCCATGGACATCCACTTCCATAGCCCGGCCTTCCAGCACCCGCCAACA  56
  Q  Q  A  M  D  I  H  F  H  S  P  A  F  Q  H  P  P  T   18

 GAATTCATACGAGAAGGCGACGATGACCGGACTGTGTGCCGGGAGATCCGCCAC 110
  E  F  I  R  E  G  D  D  D  R  T  V  C  R  E  I  R  H   36

 AACTCCACGGGCTGCCTGCGGATGAAGGACCAGTGTGACAAGTGCCGGGAGATC 164
  N  S  T  G  C  L  R  M  K  D  Q  C  D  K  C  R  E  I   54

 TTGTCTGTGGACTGTTCCACCAACAACCCCTCCCAGGCTAAGCTGCGGCGGGAG 218
  L  S  V  D  C  S  T  N  N  P  S  Q  A  K  L  R  R  E   72

 CTCGACGAATCCCTCCAGGTCGCTGAGAGGTTGACCAGGAAATACAACGAGCTG 272
  L  D  E  S  L  Q  V  A  E  R  L  T  R  K  Y  N  E  L   90

 CTAAAGTCCTACCAGTGGAAGATGCTCAACACCTCCTCCTTGCTGGAGCAGCTG 326
  L  K  S  Y  Q  W  K  M  L  N  T  S  S  L  L  E  Q  L  108

 AACGAGCAGTTTAACTGGGTGTCCCGGCTGGCAAACCTCACGCAAGGCGAAGAC 380
  N  E  Q  F  N  W  V  S  R  L  A  N  L  T  Q  G  E  D  126

 CAGTACTATCTGCGGGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTT 434
  Q  Y  Y  L  R  V  T  T  V  A  S  H  T  S  D  S  D  V  144

 CCTTCCGGTGTCACTGAGGTGGTCGTGAAGCTCTTTGACTCTGATCCCATCACT 488
  P  S  G  V  T  E  V  V  V  K  L  F  D  S  D  P  I  T  162

 GTGACGGTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTG 542
  V  T  V  P  V  E  V  S  R  K  N  P  K  F  M  E  T  V  180

 GCGGAGAAAGCGCTGCAGGAATACCGCAAAAAGCACCGGGAGGAGTGAGATGTG 596
  A  E  K  A  L  Q  E  Y  R  K  K  H  R  E  E  *         195

 GATGTTGCTTTTGCACCTACGGGGGCATCTGAGTCCAGCTCCCCCCAAGATGAG 650
 CTGCAGCCCCCCAGAGAGAGCTCTGCACGTCACCAAGTAACCAGGCCCCAGCCT 704
 CCAGGCCCCCAACTCCGCCCAGCCTCTCCCCGCTCTGGATCCTGCACTCTAACA 758
 CTCGACTCTGCTGCTCATGGGAAGAACAGAATTGCTCCTGCATGCAACTAATTC 812
 AATAAAACTGTCTTGTGAGCTGATAAAAAAAAAAAAAAAAAAA            856
```

B

```
Clus  MMKTLLLFVGLLLTWESGQVLGDQTVSDNELQEMSNQGSKYVNKEIQNAV   50
Clus  NGVKQIKTLIEKTNEERKTLLSNLEEAKKKKEDALNETRESETKLKELPG  100
Clus  VCNETMMALWEECKPCLKQTCMKFYARVCRSGSGLVGRQLEEFLNQSSPF  150
Clus  YFWMNGDRIDSLLENDRQQTHMLDVMQDHFSRASSIIDELFQDRFFTREP  200
Clus  QDTYHYLPFSLPHRRPHFFFPKSRIVRSLMPFSPYEPLNFHAMFQPFLEM  250
Clus  IHEAQQAMDIHFHSPAFQHPPTEFIREGDDDRTVCREIRHNSTGCLRMKD  300
Clus  QCDKCREILSVDCSTNNPSQAKLRRELDESLQVAERLTRKYNELLKSYQW  350
Clus  KMLNTSSLLEQLNEQFNWVSRLANLTQGEDQYYLRVTTVASHTSDSDVPS  400
Clus  GVTEVVVKLFDSDPITVTVPVEVSRKNPKFMETVAEKALQEYRKKHREE*  449
```

FIG. 19. Sequences of C73 and clusterin precursor. **(A)** Nucleotide and deduced amino acid sequences of C73. **(B)** Amino acid sequence of clusterin precursor. The underlined part is the sequence of C73.

During searches of the Genbank database, the predicted peptide sequences of C33, C42, C48 and C53 were found to have no significant homology to any known protein sequence (Figs. 20, 21, 22 and 23). The nucleotide sequence of C33 displayed no obvious homology to any sequence in the data base. The nucleotide sequences of C42, C48 and C53, however, aligned with three human cDNA sequences in the data base with minor discrepancies. These are designated as EST05911 Homo sapiens cDNA clone HIBAA65 5' end, yu73b11.r1 Homo sapiens cDNA clone 239421 5', and ym49a06.r1 Homo sapiens cDNA clone 51364 5', respectively (Figs. 21, 22 and 23). These three sequences are among those so-called "unknown genes" obtained by sequencing random human brain cDNA clones to generate the "expressed sequence tags" (ESTs) in the human genome project (Adams *et al.*, 1992; Adams *et al.*, 1991).

```
GGACGGCCGAGGGCCAGGCCGCTCCGGCCGGGCTCGTGGTGGGGGCAGCCCCAG    54
 G  R  P  R  A  R  P  L  R  P  G  S  W  W  G  Q  P  Q     18

CGGCGGCGGCGGCGGCGTGGGCTGGCGAGGCCGCGCGGACGGCGCCCGACAGCA   108
 R  R  R  R  R  R  R  G  L  A  R  P  R  G  R  R  P  T  A  36

GCTGGAGGAGCGGTTTGCGGACCTGGCGGCGAGCCACTTGGAGGCCATCCGTGC   162
 A  G  G  A  V  C  G  P  G  G  E  P  L  G  G  H  P  C     54

GCGGGACGAGTGGGACCGGCAGAACGCGCGGCTGCGTCAGGAGAACGCCCGGCT   216
 A  G  R  V  G  P  A  E  R  A  A  A  S  G  E  R  P  A     72

GCGGCTCGAGAACCGGCGGCTGAAGCGCGAGAACCGCAGCCTCTTCCGTCAGGC   270
 A  A  R  E  P  A  A  E  A  R  E  P  Q  P  L  P  S  G     90

TTTGCGGCTCCCCGGCGAAGGCGGCGACGGGACGCCCGCGGAGGCGCGCCGGGT   324
 F  A  A  P  R  R  R  R  R  R  D  A  R  G  G  A  P  G     108

CCCTGAAGAGGCCAGCACGAACCGGAGGGCTAGAGACAGCGGTCGAGAGGACGA   378
 P  *                                                      109

GCCGGGGAGTCCCAGGGCCCTGAGGGCCCGACTTGAGAAGCTGGAAGCCATGTA   432
CCGCCGGGTCCTGTTGTAGCTGCACCTCGAGCAGCGGGGACCACGTCCGAGCGG   486
GGACAAGGAGGAGCAGTCTCTACAGGAACCCGACTCCGGTCTCCGTTCCCGGGA   540
CTCGGAGCCCGTTGG                                          555
```

FIG. 20. Nucleotide and deduced amino acid sequences of C33.

A

```
CGGAAGAGGTAAAATTAAGGCGTTTGGAGGAACTCATCACTATCTTCCGAGAA     53
  E  E  V  K  L  R  R  L  E  E  L  I  T  I  F  R  E      17

 GAAGCAACAAAAGCCAATCAGACCTCTGTGGGCTGTACCCAGTTGGTGCTA     104
  E  A  T  K  A  N  Q  T  S  V  G  C  T  Q  L  V  L      34

 GTGGAAGGGCTCAGTAAACGCTCTGCCACTGACCTGTGTGGCAGGAATGAT     155
  V  E  G  L  S  K  R  S  A  T  D  L  C  G  R  N  D      51

 GGAAACCTTAAGGTGATCTTCCCTGATGCAGAGATGGAGGATGTCAATAAC     206
  G  N  L  K  V  I  F  P  D  A  E  M  E  D  V  N  N      68

 CCTGGGCTCAGGGTCAGAGCCCAGCCTGGGGACTATGTGCTGGTGAAGATC     257
  P  G  L  R  V  R  A  Q  P  G  D  Y  V  L  V  K  I      85

 ACCTCAGCCAGTTCTCAGACACTTAGGGGACATGTTCTCTGCAGGACCACT     308
  T  S  A  S  S  Q  T  L  R  G  H  V  L  C  R  T  T     102

 CTGAGGGACTCTTCTGCATATTGCTGACCTGAGAGGATGGCCTCAGAGCTG     359
  L  R  D  S  S  A  Y  C  *                              110

 ACTTGGGCAATCCTCCCCAACAGGAAGGGGAGACATTGCCTGCCACTGAGG     410
 AAACAGGTCATGAAGGTGGAGATAAGCTGCAAGGGGCGAAGCAACTTTATG     461
 TCAGTGGAAAACGTGTCTCTTTAAAGCTGCTATGTGAACAGCTTTTACAGT     512
 CATTAAATTTACCTAAACTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA        558
```

B

```
C42   CGGAAGAGGTAAAATTAAGGCGTTTGGAGGAACTCATCACTATCTTCCGA   50
HIB                              GAGGAACTCATCACTATCTTCCGA   24

C42   GAAGAAGCAACAAAAGCCAATCAGACCTCTGTGGGCTGTACCCAGTTGGT  100
HIB   GAAGAAGCAACAAAAGCCAATCAGACCTCTNTGGGCTGTACCCAGTTGGT   74

C42   GCTAGTGGAAGGGCTCAGTAAACGCTCTGCCACTGACCTGTGTGGCAGGA  150
HIB   GCTAGTGGAAGGGCTCAGTAAACGCNCTGCCACTANCCTGTGTGGCAGGA  124

C42   ATGATGGAAACCTTAAGGTGATCTTCCCTGATGCAGAGATGGAGGATGTC  200
HIB   ATNATGGAAACCTTAAGGTGATCTTCCCTGATGCAGAGATGGAGGATGTC  174

C42   AATAACCCTGGGCTCAGGGTCAGAGCCCAGCCTGGGGACTATGTGCTGGT  250
HIB   AATAACCCTGGGCTCAGGGTCAGAGCCCAGCCTGGG                210

C42   GAAGATCACCTCAGCCAGTTCTCAGACACTTAGGGGACATGTTCTCTGCA  300
C42   GGACCACTCTGAGGGACTCTTCTGCATATTGCTGACCTGAGAGGATGGCC  350
C42   TCAGAGCTGACTTGGGCAATCCTCCCCAACAGGAAGGGGAGACATTGCCT  400
C42   GCCACTGAGGAAACAGGTCATGAAGGTGGAGATAAGCTGCAAGGGGCGAA  450
C42   GCAACTTTATGTCAGTGGAAAACGTGTCTCTTTAAAGCTGCTATGTGAAC  500
C42   AGCTTTTACAGTCATTAAATTTACCTAAACTAAAAAAAAAAAAAAAAAAA  550
C42   AAAAAAAA                                            558
```

FIG. 21. Sequence of C42. **(A)** Nucleotide and deduced amino acid sequences of C42. **(B)** Sequence alignment of C42 with a cDNA clone in the Genbank database. HIB: EST05911 Homo sapiens cDNA clone HIBAA65 5' end.

A

```
GTTTCAGAGACTCCTCCACTCTCTGGGAATGACACGGACTCCCTCTCCTGCGAC    54
 V  S  E  T  P  P  L  S  G  N  D  T  D  S  L  S  C  D     18

AGTGGCAGTTCGGCAACTAGCACTCCGTGTGTGTCCCGCCTGGTCACTGGCCAC   108
 S  G  S  S  A  T  S  T  P  C  V  S  R  L  V  T  G  H     36

CACCTGTGGGCCAGCAAGAATGGCCGCCATGTCCTGGGCCTGATTGAGGACTAT   162
 H  L  W  A  S  K  N  G  R  H  V  L  G  L  I  E  D  Y     54

GAGGCCCTGCTCAAACAGATCAGCCAGGGACAGAGGCTCCTTGCTGAAATGGAC   216
 E  A  L  L  K  Q  I  S  Q  G  Q  R  L  L  A  E  M  D     72

ATTCAAACCCAAGAGGCTCCCAGCTCCACAAGTCAAGAGCTGGGAACAAAGGGT   270
 I  Q  T  Q  E  A  P  S  S  T  S  Q  E  L  G  T  K  G     90

CCACACCCAGCACCACTGAGCAAGTTTGTGAGCAGTGTGAGCACGGCCAAGCTG   324
 P  H  P  A  P  L  S  K  F  V  S  S  V  S  T  A  K  L    108

ACCCTGGAAGAGGCCTACAGGCGGCTGAAGCTTCTCTGGAGAGTCTCACTCCCC   378
 T  L  E  E  A  Y  R  R  L  K  L  L  W  R  V  S  L  P    126

GAGGATGGCCAGTGCCCCTTCACTGTGAGCAGATTGGAGAATGAAGGCAGAGGT   432
 E  D  G  Q  C  P  F  T  V  S  R  L  E  N  E  G  R  G    144

CACCAA                                                    438
 H  Q                                                     146
```

B

```
C48   GTTTCAGAGACTCCTCCACTCTCTGGGAATGACACGGACTCCCTCTCCTG   50

C48   CGACAGTGGCAGTTCGGCAACTAGCACTCCGTGTGTGTCCCGCCTGGTCA  100
yu73         GGCAGTTCGGCAACTAGCACTCCGTGTGTGTCCCGCCTGGTCA   43

C48   CTGGCCACCACCTGTGGGCCAGCAAGAATGGCCGCCATGTCCTGGGCCTG  150
yu73  CTGGCCACCACCTGTGGGCCAGCAAGAATGGCCGCCATGTCCTGGGCCTG   93

C48   ATTGAGGACTATGAGGCCCTGCTCAAACAGATCAGCCAGGGACAGAGGCT  200
yu73  ATTGAGGACTATGAGGCCCTGCTCAAACAGATCAGCCAGGGACAGAGGCT  143

C48   CCTTGCTGAAATGGACATTCAAACCCAAGAGGCTCCCAGCTCCACAAGTC  250
yu73  CCTTGCTGAAATGGACATTCAAACCCAAGAGGCTCCCAGCTCCACAAGTC  193

C48   AAGAGCTGGG-AACAAAGGGTCCACACCCAGCACCACTGAGCAAGTTTGT  300
yu73  AAGAGCTGGGGAACAAAGGGTNCACACCCAGCACCACTGAGCAAGTTTGT  243

C48   GAGCAGTGTGAGCACGGCCAAGCTGACCCTGG-AAGAGGCCTACAGG-CG  350
yu73  GAGCAGTGTGAGCACGGCCAAGNTGACC-TGGGAAGAGGCNTACAGGG-G  291

C48   GCT-GAAGCTTCTC-TGGAGAGTCTCACTCCCCGAGGATGGCC-AGTGCC  400
yu73  GCTTGAAGCTTCTTTTGGAGAGTCTCANTCCCCGAGGATGGCCCANTGCC  341

C48   CCTT-CACTGTGAGCAGATT-GGAGAAT-GAAGGCAGAGGTCACCAA     447
yu73  CCTTTCATTTGAAGCAGATTTGGAGAATTGAAGGCAGAGGTTACCAAATT  391

yu73  ACTTAAAAATTGTTTNAAACAAGAAAAGAAGTTTGCAAAAACACCTTGAA  441
yu73  GGTTTTTCAGGTTGATTG                                 459
```

FIG. 22. Partial sequence of C48. **(A)** Partial nucleotide and deduced amino acid sequences of C48. **(B)** Sequence alignment of C48 with a cDNA clone in the Genbank database. yu73: yu73b11.r1 homo sapiens cDNA clone 239421 5'.

A

```
CTCAAAGGCACAGAGGCCTCCACGAAGAATATTTTTGGCCGATACTCTTCACAG    54
 L  K  G  T  E  A  S  T  K  N  I  F  G  R  Y  S  S  Q     18

CGGATGAAGGATTGGCAGGAGATTATAGCTCTGTATGAGAAGGACAACACCTAC   108
 R  M  K  D  W  Q  E  I  I  A  L  Y  E  K  D  N  T  Y     36

TTAGTGGAACTCTCTAGCCTCCTGGTTCGGAATGTCAACTATGAGATCCCCTCA   162
 L  V  E  L  S  S  L  L  V  R  N  V  N  Y  E  I  P  S     54

CTGAAGAAGCAGATTGCCAAGTGCCAGCAGCTGCAGCAAGAATACAGCCGCAAG   216
 L  K  K  Q  I  A  K  C  Q  Q  L  Q  Q  E  Y  S  R  K     72

GAGGAGGAGTGCCAGGCAGGGGCTGCCGAGATGCGGGAGCAGTTCTACCACTCC   270
 E  E  E  C  Q  A  G  A  A  E  M  R  E  Q  F  Y  H  S     90

TGCAAGCAGTATGGCATCACGGGCGAAAATGTCCGAGGAGAACTGCTGGCCCTG   324
 C  K  Q  Y  G  I  T  G  E  N  V  R  G  E  L  L  A  L    108

GTGAAGGACCTGCCGAGTCAGCTGGCTGAGATTGGGGCAGCGGCTCAGCAGTCC   378
 V  K  D  L  P  S  Q  L  A  E  I  G  A  A  A  Q  Q  S    126

CTGGGGGAAGCCATTGACGTGTACCAGGCGTCTGTGGGGTTTGTGTGTGAGAGC   432
 L  G  E  A  I  D  V  Y  Q  A  S  V  G  F  V  C  E  S    144

CCCACAGAGCAGGTGTTGCCAATGCTGCGGTTCGTGCAGAAGCGGGGAAACTCA   486
 P  T  E  Q  V  L  P  M  L  R  F  V  Q  K  R  G  N  S    162

ACGGTGTACGAGTGGAGGACAGGGACAGAGCCCTCTGTGGTGGAACGACCCCAC   540
 T  V  Y  E  W  R  T  G  T  E  P  S  V  V  E  R  P  H    180

CTCGAGGAGCTTCCTGAGCAGGTGGCAGAAGATGCGATTGACTGGGGGCGACTT   594
 L  E  E  L  P  E  Q  V  A  E  D  A  I  D  W  G  R  L    198

TGGGGTAGAGGCAGTGTCTGAGGGGACTGACTCTGGCATCTCTgCCGAGGCTGC   648
 W  G  R  G  S  V  *                                     204

TGGAATCGACTGGGGCATCTTCCC                                  672
```

B

```
ym49                                                TGGCTGGTGGACAGAAGG   18
ym49  CACTGCAGCCTGAAATGGCAGAGTCTGGTGCTGACGATCCGCGAGAAGAT   68
ym49  CAATNCTGCCATCCAGGACATGCCAGAGAGCGAAGAGATCGCCCAGCTGC  118
ym49  TGTCTGGGTCCTACATTCACTACTTTCACTGCCTAAGAATCCTGGACCTT  168

ym49  CTCAAAGGCACAGAGGCCTCCACGAAGAATATTTTTGGCCGATACTCTTC   218
C53   CTCAAAGGCACAGAGGCCTCCACGAAGAATATTTTTGGCCGATACTCTTC    50

ym49  ACAGCGGATGAAGGATTNGCAGGAGATTATAGCTCTGTATGAGAAGGACA  268
C53   ACAGCGGATGAAGGATTGGCAGGAGATTATAGCTCTGTATGAGAAGGACA  100

ym49  ACACCTACTTAGTGGAACTCTCTAGCCTCCTGGTTTCGGAATGTCAACTA  318
C53   ACACCTACTTAGTGGAACTCTCTAGCCTCCTGGTT-CGGAATGTCAACTA  149

ym49  TGAAGATC                                           326
C53   TGA-GATCCCCTCACTGAAGAAGCAGATTGCCAAGTGCCAGCAGCTGCAG  198

C53   CAAGAATACAGCCGCAAGGAGGAGGAGTGCCAGGCAGGGGCTGCCGAGAT  248
C53   GCGGGAGCAGTTCTACCACTCCTGCAAGCAGTATGGCATCACGGGCGAAA  298
C53   ATGTCCGAGGAGAACTGCTGGCCCTGGTGAAGGACCTGCCGAGTCAGCTG  348
C53   GCTGAGATTGGGGCAGCGGCTCAGCAGTCCCTGGGGGAAGCCATTGACGT  398
C53   GTACCAGGCGTCTGTGGGGTTTGTGTGTGAGAGCCCCACAGAGCAGGTGT  448
C53   TGCCAATGCTGCGGTTCGTGCAGAAGCGGGGAAACTCAACGGTGTACGAG  498
C53   TGGAGGACAGGGACAGAGCCCTCTGTGGTGGAACGACCCCACCTCGAGGA  548
C53   GCTTCCTGAGCAGGTGGCAGAAGATGCGATTGACTGGGGGCGACTTTGGG  598
C53   GTAGAGGCAGTGTCTGAGGGGACTGACTCTGGCATCTCTgCCGAGGCTGC  648
C53   TGGAATCGACTGGGGCATCTTCCC                           672
```

FIG. 23. Sequence of C53. **(A)** Nucleotide and deduced amino acid sequences of C53. **(B)** Sequence alignment of C53 with a cDNA clone in the Genbank database. ym49: ym49a06.r1 Homo Sapiens cDNA clone 51364 5'.

DISCUSSION

When NCLK was first purified to near homogeneity, it was shown to be a heterodimer of 33 kDa and 25 kDa subunits (Lew *et al.*, 1992a). Molecular cloning revealed that the 33 kDa subunit was a Cdc2-related protein, Cdk5; the 25 kDa subunit turned out to be a proteolyzed product of a neuron-specific 35 kDa protein, p35 (Lew *et al.*, 1994; Tsai *et al.*, 1994). From the analogy of cyclin activation of Cdc2 and Cdk2, it was speculated that the 25 kDa protein possessed a cyclin-like function, i.e. is capable of supporting the kinase activity of Cdk5 in NCLK. This assumption was highlighted by the observation of co-precipitation of active Cdk5 with the recombinant 21 kDa fragment of p35 from bovine brain extract. Furthermore, the proposal that the 25 kDa subunit is a kinase-activating protein for Cdk5 is unequivocally established by the successful reconstitution of active histone H1 kinase from p35-derived recombinant proteins and bacterially expressed Cdk5. Consistently, in an independent study, monomeric Cdk5 isolated from bovine brain could also be activated by truncated forms of p35 (Qi *et al.*, 1995). Thus, the interaction between Cdk5 and p35 or p25 plays significant regulatory functions.

The reconstituted kinase activity from the bacterially-expressed GST-Cdk5 and p35-fragments displayed very low specific activity: almost three orders of magnitude lower than that of NCLK purified from bovine brain. The lower-than-expected reconstituted kinase activity may be attributed to the fact that most bacterially-expressed proteins were present in degraded and/or incorrectly folded forms. After removal of inactive proteins by ion-exchange chromatography using a Mono S column, the reconstituted enzyme displays specific histone H1 peptide phosphorylating activity similar to that of highly purified NCLK from bovine brain. The reconstituted enzyme and the

brain purified enzyme show similar Km values and substrate specificities. The amount of the reconstituted kinase obtained from a typical preparation with a liter of each bacterial culture of the protein components is equivalent to 2 preparations (500 gm tissue each) of the brain enzyme. In other words, reconstitution from bacterially-expressed proteins is a convenient source to provide functional NCLK.

Three truncated forms of p35 (p21, p23 and p25) have been expressed as GST fusion proteins in *E. coli* and tested as Cdk5 activators. The expressed p25 sequence is identical to the 25 kDa subunit found in NCLK from bovine brain. Given that all these p35-derivatives showed similar Cdk5-activating activities, and that NCLK from brain containing the 25 kDa subunit is highly active, it is likely that all three derivatives contain the complete Cdk5-activating domain. Although purified reconstituted NCLK used for most of the detailed characterizations was the Cdk5/p21 heterodimer, other reconstituted kinases (Cdk5/p23 and Cdk5/p25) are expected to have similar general characteristics.

Although Cdk5 shows a high degree of sequence identity with other Cdk family members, it has not been found to be activated by any known cyclin. A previous study showed the association of cyclin D with Cdk5, but the protein complex appeared to be devoid of kinase activity (Xiong *et al.*, 1992). Thus, p35 and its truncated fragments are the only activators found for Cdk5 to date. It has been reported that Cdk5 immunoprecipitated from brain, but not from the extract of any other tissue, displays histone H1 kinase activity (Tsai *et al.*, 1993b), suggesting that the histone H1 kinase activity of Cdk5 is specifically activated by p35. Like other Cdc2-like proteins, whose kinase activities depend on association with particular cyclins, Cdk5 appears to depend absolutely on association with p35 or its derivatives for its kinase activity.

In addition to association with cyclins, a few well-characterized members of the Cdk family have shown absolute dependence to achieve full activity on phosphorylation of a particular threonine residue by CAK (Morgan, 1995). Cdk5 displays 58% and 59% sequence identities to Cdc2 and Cdk2, respectively (Lew *et al.*, 1992b; Meyerson *et al.*, 1992). Thus, the highly conserved domains in well characterized Cdks are expected to be conserved in Cdk5 as well. In addition, Cdk5 contains a phosphorylatable residue, Ser^{159}, at the position corresponding to Thr^{161}/Cdc2 and Thr^{160}/Cdk2. The peptide sequence around Ser^{159} in Cdk5 is homologous to the corresponding Cdc2 and Cdk2 sequences (Table III), which form the T-loop revealed in the Cdk2 crystal structure. However, the mechanism of Cdk5 activation by p25 differs significantly from that of Cdk activation by cyclin. First of all, a highly active kinase could be isolated from reconstitution of Cdk5 and p21 without requiring any additional protein factor. The purified reconstituted kinase could not be further activated by a CAK-like kinase isolated from brain. Second, the enzyme reconstitution and Cdk5 activation were not affected by ATP-Mg^{2+}. There was no detectable phosphorylation of Cdk5 when the highly active, reconstituted enzyme was incubated under phosphorylation conditions, ruling out the possibility that Cdk5 is phosphorylated at Ser^{159} by an autocatalytic reaction. Lastly, the activity of the reconstituted enzyme was not affected by treatment with the protein phosphatase 2A catalytic subunit, which has been shown to effectively inactivate Cdc2 and Cdk2 by dephosphorylation (Lee *et al.*, 1991). These observations strongly support the view that Cdk5 activation by p25 is independent of Cdk5 phosphorylation. The demonstration of a phosphorylation-independent mechanism of Cdk5 activation suggests that the phosphorylation-dependent activation mechanism observed for the Cdc2- and Cdk2-cyclin complexes may not be extrapolated to all Cdk family members.

The crystal structure of cyclin A-bound Cdk2 shows that cyclin A binds to one side of the catalytic cleft involving both the N- and C-terminal lobes of Cdk2, and has extensive interaction with the PSTAIRE and T-loop regions (Jeffrey *et al.*, 1995). The binding of cyclin A induces large conformational and positional changes of the T-loop, and consequently removes the steric hindrance of substrate access to the catalytic core and exposes Thr^{160} for better access by CAK. In addition, the binding of cyclin A realigns the residues in the catalytic core region including Lys^{33}, Glu^{51} and Asp^{145}, which are involved in ATP phosphate orientation and magnesium coordination. Misalignment of the residues in the catalytic site in monomeric Cdk2 is thought to make it unable to catalyze phosphoryl group transfer reactions (De Bondt *et al.*, 1993). The reformed catalytic core of Cdk2 upon cyclin A binding adopts a structural conformation close to that of the catalytically active protein kinase A (Jeffrey *et al.*, 1995). Therefore, cyclin A plays a critical role in Cdk2 activation.

As p35 shows little overall sequence homology to the cyclin family members, it is tempting to suggest that the unique, phosphorylation-independent activation of Cdk5 is due to unique structural properties of p35. This suggestion, unfortunately, is difficult to test, since there is no evidence for Cdk5 activation by any cyclin as yet. Furthermore, the phosphorylation-dependent and phosphorylation-independent mechanisms may not be mutually exclusive in activation of a particular Cdk. Thus, although high Cdk5 kinase activity is reconstituted in the absence of Cdk5 phosphorylation, the possibility that Cdk5 can be activated by a phosphorylation-dependent mechanism is not excluded. Cdk5 may be activated by a distinct protein in a phosphorylation-dependent manner. Similarly, Cdc2 or Cdk2 may be activated by a p25-like protein in a phosphorylation-independent manner.

In contrast to widely expressed Cdk5, p35 expression is restricted to neurons of the central nervous system (Lew *et al.*, 1994; Tsai *et al.*, 1994). Moreover, Cdk5-associated histone H1 kinase activity has been found only in brain extracts, suggesting that the kinase specificity of NCLK is dictated by p35 (Tsai *et al.*, 1993b). Thus, p35-interacting proteins may play important roles in Cdk5/p35 regulation and function in brain. The yeast two-hybrid strategy was employed to screen a human brain cDNA library for p35-binding proteins, which interacted with p35 and consequently reconstituted a functional transcriptional activator in the reporter yeast.

A variety of false positive clones have been reported in the literature to activate either or both reporter genes irrespective of the presence of a specific bait (Bartel *et al.*, 1993). The employment of both HIS3 and *lacZ* in strain CG-1945 under the control of different promoters that share only the GAL4 responsive element automatically eliminates a number of clones that activate either HIS3 or *lacZ*. However, some clones caused elevated expression of both HIS3 and *lacZ*, i.e. growth of the yeast cells in the selective medium without histidine and enhanced β-galactosidase activity. These library-derived plasmids may encode proteins with transcriptional activation potential against the reporter genes. In some cases, they appear to require an nonspecific hybrid of the DNA binding domain for their activities. Therefore, the specificity of the detected interactions in the two-hybrid screen should be tested in yeast cells with the following combinations: the library-isolated clones and the original bait, and the library-isolated clones and a hybrid construct of the DNA binding domain with an unrelated protein, such as SNF1 or human lamin C. The isolated clones that cause enhanced reporter activities when co-transformed with the original bait, but not with the construct of an unrelated protein, are considered as true positive clones.

It is somewhat unexpected that the *cdk5* sequence was not found in the isolated clones of p35-interacting proteins. Cdk5 is activated by association with p35, or its derivatives, to display high histone H1 kinase activity, suggesting that, like Cdc2/cyclin, overexpression of the Cdk5/p35 complex could be quite toxic to host yeast and might not be tolerable. On the other hand, 3.5×10^5 co-transformants were assayed for the reporter activities in this screen. Presumably, one library-derived plasmid was in each co-transformant with p35pAS2. Approximate 1.2×10^5 cDNAs could be covered in three reading frames. Thus, low-abundance mRNAs could be missed in this screen. In addition, some p35-interacting proteins might not be detectable in the two-hybrid assay due to their poor expression in yeast or to their inability to be localized into the nuclei where they are supposed to bind p35 and reconstitute a functional transcriptional activator. Therefore, the *bona fide* p35-interacting proteins coded for in the cDNA library could be more than the seven isolated sequences.

Protein-protein interactions detected in the two-hybrid assays do not necessarily mean that these proteins form a complex *in vivo*. A C-terminal fragment of GFAP was isolated in the screen by its interaction with p35. GFAP is an intermediate filament protein specifically expressed in astroglial cells (Steinert and Roop, 1988). However, the expression of p35 is restricted to neurons of the central nervous system (Tsai *et al.*, 1994). The different cell-type expressions make their association impossible in brain even if they could form a complex *in vitro* as suggested by the two-hybrid result.

Members of the intermediate filament family contain a conserved central rod domain flanked by end domains (Steinert and Roop, 1988). The sequence of C35 is composed of the partial central rod domain and the entire C-terminal domain of GFAP. The C-terminal domain of GFAP displays conserved structural characteristics and

moderate sequence conservation with a region C-terminal to the central rod domain in neurofilaments (Reeves *et al.*, 1989). Thus, the C35 fragment of GFAP is relatively conserved in sequence and structure in neurofilaments. The sequence homology between C35 and a corresponding region in NF-M is shown in Fig. 24. p35 is likely to bind neurofilaments in neurons via the region homologous to the C-terminal stretch of GFAP. Furthermore, it has been shown that NCLK is a potential kinase to carry out the proline-directed Ser/Thr phosphorylation of NF-H and NF-M in brain (Lew *et al.*, 1992a). Conceivably, the binding of p35 to neurofilaments would facilitate the phosphorylation by Cdk5, which is reminiscent of the cyclin effect on the substrate preference of Cdc2 and Cdk2 (Dynlacht *et al.*, 1994; Hoffmann *et al.*, 1993; Peeper *et al.*, 1993).

The isolation of multiple p35-interacting proteins suggests the existence of multiple effectors or regulators of Cdk5/p35 in brain. The C-terminal peptide of *n*- or α_2-chimaerin was isolated by virtue of its relatively weak interaction with p35 in the two-hybrid assay (Table V). α_2-chimaerin is an alternatively spliced transcript of the *n*-chimaerin gene (Hall *et al.*, 1993). Both *n*-chimaerin and α_2-chimaerin are expressed at relatively high levels in post-mitotic neurons, and *n*-chimaerin is a neuron-specific protein (Hall *et al.*, 1993; Lim *et al.*, 1992). The expression of *n*-chimaerin in rat brain appears to increase in parallel with neuronal development, which correlates with the developmental pattern of p35 (Lim *et al.*, 1992).

$n(\alpha_2)$-chimaerin is a GTPase-activating protein (GAP) selectively acting on Rac and Cdc42Hs, which are members of the Rho family in the Ras superfamily of GTP-binding proteins (Diekmann *et al.*, 1991). $n(\alpha_2)$-chimaerin contains two separate domains related to two protein families. The N-terminal cysteine-rich domain has high sequence identity to the C1 regulatory region of protein kinase C, displaying diacylglycerol and

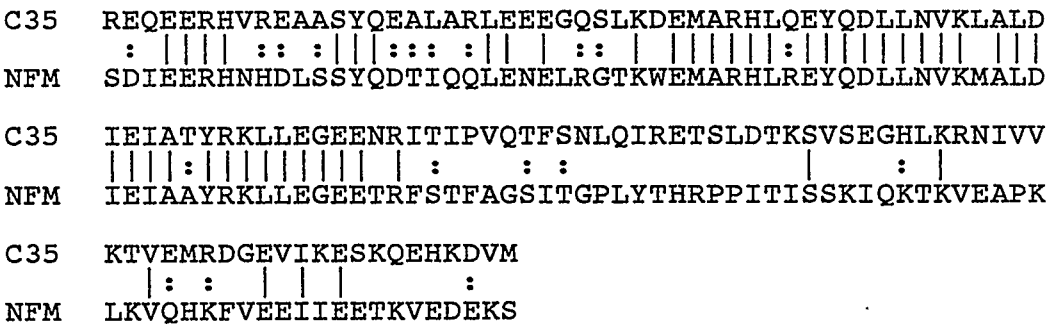


FIG. 24. Homology between the last 98 amino acids of GFAP and a region in NF-M.

phorbol ester binding activity (Hall *et al.*, 1990; Hall *et al.*, 1993). The GAP activity resides in its C-terminal region, which is homologous to the C-terminal region of the BCR protein (product of the breakpoint-cluster-region gene). It has been shown that bacterially-expressed *n*-chimaerin and the related C-terminal domain of BCR act as GAPs towards Rac (Diekmann *et al.*, 1991). In addition, α_2-chimaerin has an SH2 domain at its amino terminus, which is lacking in *n*-chimaerin, with the capability to respond to phosphotyrosine-linked signals (Hall *et al.*, 1993).

The Rho family members are thought to regulate cytoskeletal organization. Rac is involved in membrane ruffling in response to growth factors, and Cdc42 stimulates formation of actin-containing microspikes (Nobes and Hall, 1995; Ridley *et al.*, 1992). The function of Rho, Rac and Cdc42 are required for cell cycle progression through G_1 and subsequent DNA synthesis (Olson *et al.*, 1995). Furthermore, recent studies have shown that Rac and Cdc42 are particularly required in the MAP kinase pathway leading from Ras to c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (Coso *et al.*, 1995; Minden *et al.*, 1995). Activation of protein Ser/Thr kinase PAK65, which is a specific effector of Rac and Cdc42Hs, by GTP- but not GDP-loaded forms of Rac or Cdc42Hs, results in a kinase cascade through MEKK1 and SEK/MKK4 to JNK/SAPK. Consistent with overexpression of rhoGAP, which acts on all members of the Rho family, diminishes JNK activation. .

The association of p35 and $n(\alpha_2)$-chimaerin implies p35 in neuronal signaling mediated by Rac and Cdc42Hs. The putative p35-binding region in $n(\alpha_2)$-chimaerin resides in the domain responsible for GAP activity. It is possible that the association of p35 with this region blocks the GAP activity of $n(\alpha_2)$-chimaerin. As a result, Rac and Cdc42Hs could potentially accumulate in their GTP-bound active forms to mediate down

stream signaling. Alternatively, the regulatory effect could be exerted through phosphorylation of $n(\alpha_2)$-chimaerin by Cdk5/p35. Moreover, the binding of p35 to $n(\alpha_2)$-chimaerin could have an effect on regulation of Cdk5/p35 activity as well.

p35 was found to interact with a C-terminal fragment of human clusterin in the two-hybrid screen. Clusterin is a glycoprotein with various names and potential functions (Table VI). The mature protein is a heterodimer of disulfide-linked α and β subunits, which are generated by proteolytic cleavage of a precursor peptide. The mRNA and protein of clusterin have been detected in neurons by *in situ* hybridization and immunohistochemistry, respectively (O'Bryan *et al.*, 1993). Enhanced clusterin expression has been observed in correlation with brain maturation. Moreover, the synthesis of clusterin is dramatically induced under neurodegenerative conditions. Clusterin has been visualized by immunostaining in dystrophic neurites, neuropil threads, amyloid deposits in senile plaques, pyramidal neurons, and intracellular neurofibrillary tangles (NFTs) in Alzheimer brain, suggesting its involvement in neuropathology and neuronal cell death (Duguid *et al.*, 1989; May *et al.*, 1990; McGeer *et al.*, 1992).

NFTs are one of the two neuropathological hallmarks of Alzheimer's disease. The predominant fibrous structures in NFTs are paired helical filaments (PHFs) in which altered forms of tau are the major protein components. The PHF tau proteins are abnormally hyperphosphorylated on a number of Ser/Thr sites followed by proline, and NCLK has been proposed as a potential kinase to phosphorylate PHF tau in brain (Paudel *et al.*, 1993). A loss of control of NCLK activity might be associated with Alzheimer pathology. The association of clusterin and p35 might have a role in the abnormal neuronal processes in Alzheimer brain.

TABLE VI. Species homologues of clusterin

Name	Species	Putative Functions	References
Clusterin	Sheep	A protein from testis fluid with cell aggregating activity	Blaschuk *et al.*, 1983
SGP-2	Rat	A major secretory product of Sertoli cells	Collard and Griswold, 1987
CLI SP40, 40	Human	An inhibitor of the terminal complement complex	Jenne and Tschopp, 1989; Kirszbaum *et al.*, 1989
T64	Quail	mRNA induced in neuroretinal cells by Rous sarcoma virus	Michel *et al.*, 1989
TRPM-2	Rat	mRNA induced during involution of the prostate after androgen withdrawal; highy expressed after cell injury and during apoptosis	Buttyan *et al.*, 1989
GpIII	Bovine	A component of chromaffin granules	Palmer and Christie, 1990
Apo-J NA1/NA2	Human	Apolipoprotein	de Silva *et al.*, 1990
Gp80	Dog	Glycoprotein secreted apically from kidney epithelial cells	Hartmann *et al.*, 1991
pADHC-9	Human	A cDNA clone isolated from Alzheimer hippocampus; mRNA highly expressed during neurodegenerative diseases	May *et al.*, 1990
pTB16	Human	A cDNA clone isolated from glioma library; mRNA highly expressed in epileptic foci	Danik *et al.*, 1991
HISL-19	Human	Immunocytochemical marker of neuroendocrine cells	Krisch *et al.*, 1988

Four novel peptides, which are coded for by C33, C42, C48 and C53, have been identified through interaction with p35 in the two-hybrid screen. These peptide sequences show no clear homology to any known protein. In the two-hybrid assays, these four peptides displayed relatively strong interaction with p35 (Table V). For example, the interaction of the C48-encoded peptide with p35 resulted in a 40-fold increase of β-galactosidase activity, suggesting high binding affinity of the C48-encoded peptide for p35. However, their interactions with p35 must be verified by an independent method, such as co-precipitation or affinity purification. The expression and localization patterns in brain of these novel gene products must still be characterized.

In summary, NCLK is specified in neurons of the central nervous system. Cdk5 is homologous to other Cdks, and contains potential regulatory phosphorylation sites that are conserved in members of the Cdk family. The regulatory subunit, p35, which has little homology to cyclins, is a cell-specific activator of Cdk5. The active domain in p35 has been narrowed to a 21 kDa fragment. Activation of Cdk5 by the active fragments of p35 proceeds by a distinct phosphorylation-independent mechanism. The possibility of phosphorylation-dependent activation of Cdk5 has not been excluded; however, the upstream factors imparting such regulation remain unidentified.

Like Cdc2- or Cdk2-cyclin, Cdk5/p35 may exist in macromolecular complexes. The yeast two-hybrid system has been applied to investigate p35-interacting proteins. Seven sequences have been isolated in the screen with p35, including C-terminal fragments of GAP, $n(\alpha_2)$-chimaerin, and clusterin, as well as four novel peptides. Studies of p35-interacting proteins will contribute to delineation of regulation and function of Cdk5/p35 in neurons.

REFERENCES

Adams, M.D., Dubnick, M., Kerlavage, A.R., Moreno, R., Kelley, J.M., Utterback, T.R., Nagle, J.W., Fields, C. and Venter, J.C. (1992). Sequence identification of 2,375 human brain genes. *Nature* **355**, 632-4.

Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., Moreno, R.F. *et al.* (1991). Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**, 1651-6.

Arion, D., Meijer, L., Brizuela, L. and Beach, D. (1988). cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. *Cell* **55**, 371-8.

Atherton-Fessler, S., Liu, F., Gabrielli, B., Lee, M.S., Peng, C.Y. and Piwnica-Worms, H. (1994). Cell cycle regulation of the p34cdc2 inhibitory kinases. *Mol Biol Cell* **5**, 989-1001.

Bartel, P., Chien, C.T., Sternglanz, R. and Fields, S. (1993). Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**, 920-4.

Beaudette, K.N., Lew, J. and Wang, J.H. (1993). Substrate specificity characterization of a cdc2-like protein kinase purified from bovine brain. *J Biol Chem* **268**, 20825-30.

Blaschuk, O., Burdzy, K. and Fritz, I.B. (1983). Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J Biol Chem* **258**, 7714-20.

Booher, R. and Beach, D. (1988). Involvement of cdc13+ in mitotic control in Schizosaccharomyces pombe: possible interaction of the gene product with microtubules. *Embo J* **7**, 2321-7.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Breeden, L. and Nasmyth, K. (1985). Regulation of the yeast HO gene. *Cold Spring Harb Symp Quant Biol* **50**, 643-50.

Brent, R. and Ptashne, M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**, 729-36.

Buttyan, R., Olsson, C.A., Pintar, J., Chang, C., Bandyk, M., Ng, P.Y. and Sawczuk, I.S. (1989). Induction of the TRPM-2 gene in cells undergoing programmed death. *Mol Cell Biol* **9**, 3473-81.

Chan, F.K., Zhang, J., Cheng, L., Shapiro, D.N. and Winoto, A. (1995). Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol Cell Biol* **15**, 2682-8.

Chen, J., Jackson, P.K., Kirschner, M.W. and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374**, 386-8.

Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* **88**, 9578-82.

Coleman, T.R., Tang, Z. and Dunphy, W.G. (1993). Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. *Cell* **72**, 919-29.

Collard, M.W. and Griswold, M.D. (1987). Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochemistry* **26**, 3297-303.

Connell-Crowley, L., Solomon, M.J., Wei, N. and Harper, J.W. (1993). Phosphorylation independent activation of human cyclin-dependent kinase 2 by cyclin A in vitro. *Mol Biol Cell* **4**, 79-92.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-46.

Dalton, S. and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell* **68**, 597-612.

Danik, M., Chabot, J.G., Mercier, C., Benabid, A.L., Chauvin, C., Quirion, R. and Suh, M. (1991). Human gliomas and epileptic foci express high levels of a mRNA related to rat testicular sulfated glycoprotein 2, a purported marker of cell death. *Proc Natl Acad Sci U S A* **88**, 8577-81.

De Bondt, H.L., Rosenblatt, J., Jancarik, J., Jones, H.D., Morgan, D.O. and Kim, S.H. (1993). Crystal structure of cyclin-dependent kinase 2. *Nature* **363**, 595-602.

de Silva, H.V., Stuart, W.D., Duvic, C.R., Wetterau, J.R., Ray, M.J., Ferguson, D.G., Albers, H.W., Smith, W.R. and Harmony, J.A. (1990). A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J Biol Chem* **265**, 13240-7.

Desai, D., Gu, Y. and Morgan, D.O. (1992). Activation of human cyclin-dependent kinases in vitro. *Mol Biol Cell* **3**, 571-82.

Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21rac. *Nature* **351**, 400-2.

Donovan, J.D., Toyn, J.H., Johnson, A.L. and Johnston, L.H. (1994). P40SDB25, a putative CDK inhibitor, has a role in the M/G1 transition in Saccharomyces cerevisiae. *Genes Dev* **8**, 1640-53.

Duguid, J.R., Bohmont, C.W., Liu, N.G. and Tourtellotte, W.W. (1989). Changes in brain gene expression shared by scrapie and Alzheimer disease. *Proc Natl Acad Sci U S A* **86**, 7260-4.

Dunphy, W.G., Brizuela, L., Beach, D. and Newport, J. (1988). The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54**, 423-31.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev* **7**, 555-69.

Dynlacht, B.D., Flores, O., Lees, J.A. and Harlow, E. (1994). Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes Dev* **8**, 1772-86.

el-Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y. *et al.* (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* **54**, 1169-74.

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389-96.

Ewen, M.E., Oliver, C.J., Sluss, H.K., Miller, S.J. and Peeper, D.S. (1995). p53-dependent repression of CDK4 translation in TGF-beta-induced G1 cell-cycle arrest. *Genes Dev* **9**, 204-17.

Ewen, M.E., Sluss, H.K., Whitehouse, L.L. and Livingston, D.M. (1993). TGF beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. *Cell* **74**, 1009-20.

Fang, F. and Newport, J.W. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66**, 731-42.

Featherstone, C. and Russell, P. (1991). Fission yeast p107wee1 mitotic inhibitor is a tyrosine/serine kinase. *Nature* **349**, 808-11.

Feaver, W.J., Svejstrup, J.Q., Henry, N.L. and Kornberg, R.D. (1994). Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. *Cell* **79**, 1103-9.

Fesquet, D., Labbe, J.C., Derancourt, J., Capony, J.P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M. and Cavadore, J.C. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *Embo J* **12**, 3111-21.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245-6.

Firpo, E.J., Koff, A., Solomon, M.J. and Roberts, J.M. (1994). Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol* **14**, 4889-901.

Fisher, R.P. and Morgan, D.O. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78**, 713-24.

Flores-Rozas, H., Kelman, Z., Dean, F.B., Pan, Z.Q., Harper, J.W., Elledge, S.J., O'Donnell, M. and Hurwitz, J. (1994). Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc Natl Acad Sci U S A* **91**, 8655-9.

Galaktionov, K. and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell* **67**, 1181-94.

Galaktionov, K., Jessus, C. and Beach, D. (1995a). Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. *Genes Dev* **9**, 1046-58.

Galaktionov, K., Lee, A.K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. and Beach, D. (1995b). CDC25 phosphatases as potential human oncogenes. *Science* **269**, 1575-7.

Gautier, J., Matsukawa, T., Nurse, P. and Maller, J. (1989). Dephosphorylation and activation of Xenopus p34cdc2 protein kinase during the cell cycle. *Nature* **339**, 626-9.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T. and Maller, J.L. (1990). Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* **60**, 487-94.

Gautier, J., Norbury, C., Lohka, M., Nurse, P. and Maller, J. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+. *Cell* **54**, 433-9.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F. and Kirschner, M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* **67**, 197-211.

Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132-8.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S. and Nurse, P. (1991). Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. *Embo J* **10**, 3297-309.

Gould, K.L. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature* **342**, 39-45.

Gu, Y., Turck, C.W. and Morgan, D.O. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* **366**, 707-10.

Guan, K.L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X., O'Keefe, C.L., Matera, A.G. and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev* **8**, 2939-52.

Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791-803.

Hall, C., Monfries, C., Smith, P., Lim, H.H., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T. and Lim, L. (1990). Novel human brain cDNA encoding a 34,000 Mr protein n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. *J Mol Biol* **211**, 11-6.

Hall, C., Sin, W.C., Teo, M., Michael, G.J., Smith, P., Dong, J.M., Lim, H.H., Manser, E., Spurr, N.K., Jones, T.A. *et al.* (1993). Alpha 2-chimerin, an SH2-containing GTPase-activating protein for the ras-related protein p21rac derived by alternate splicing of the human n-chimerin gene, is selectively expressed in brain regions and testes. *Mol Cell Biol* **13**, 4986-98.

Hannon, G.J. and Beach, D. (1994a). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257-61.

Hannon, G.J., Casso, D. and Beach, D. (1994b). KAP: a dual specificity phosphatase that interacts with cyclin-dependent kinases. *Proc Natl Acad Sci U S A* **91**, 1731-5.

Hannon, G.J., Demetrick, D. and Beach, D. (1993). Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes Dev* **7**, 2378-91.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805-16.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E. *et al.* (1995). Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell* **6**, 387-400.

Hartmann, K., Rauch, J., Urban, J., Parczyk, K., Diel, P., Pilarsky, C., Appel, D., Haase, W., Mann, K., Weller, A. *et al.* (1991). Molecular cloning of gp 80, a glycoprotein complex secreted by kidney cells in vitro and in vivo. A link to the reproductive system and to the complement cascade. *J Biol Chem* **266**, 9924-31.

Hartwell, L.H. and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. [Review]. *Science* **246**, 629-34.

Hasegawa, M., Morishima-Kawashima, M., Takio, K., Suzuki, M., Titani, K. and Ihara, Y. (1992). Protein sequence and mass spectrometric analyses of tau in the Alzheimer's disease brain. *J Biol Chem* **267**, 17047-54.

Hengst, L., Dulic, V., Slingerland, J.M., Lees, E. and Reed, S.I. (1994). A cell cycle-regulated inhibitor of cyclin-dependent kinases. *Proc Natl Acad Sci U S A* **91**, 5291-5.

Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A. and Sherr, C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* **15**, 2672-81.

Hisanaga, S., Kusubata, M., Okumura, E. and Kishimoto, T. (1991). Phosphorylation of neurofilament H subunit at the tail domain by CDC2 kinase dissociates the association to microtubules. *J Biol Chem* **266**, 21798-803.

Hoffman, C.S. and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. *Gene* **57**, 267-272.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E. and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. *Embo J* **12**, 53-63.

Hope, I.A. and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46**, 885-94.

Hunter, T. (1993). Braking the cycle. [Review]. *Cell* **75**, 839-41.

Ishiguro, K., Kobayashi, S., Omori, A., Takamatsu, M., Yonekura, S., Anzai, K., Imahori, K. and Uchida, T. (1994). Identification of the 23 kDa subunit of tau protein kinase II as a putative activator of cdk5 in bovine brain. *FEBS Lett* **342**, 203-8.

Izumi, T. and Maller, J.L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. *Mol Biol Cell* **4**, 1337-50.

Jeffrey, P.D., Russo, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N.P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* **376**, 313-20.

Jenne, D.E. and Tschopp, J. (1989). Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci U S A* **86**, 7123-7.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H. and Okayama, H. (1994). Cdc25A is a novel phosphatase functioning early in the cell cycle. *Embo J* **13**, 1549-56.

Kaiser, P. and Auer, B. (1993). Rapid shuttle plasmid preparation from yeast cells by transfer to E. coli. *Biotechniques* **14**, 552.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S.3rd, Johnson, B.E. and Skolnick, M.H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264**, 436-40.

Kato, J.Y., Matsuoka, M., Polyak, K., Massague, J. and Sherr, C.J. (1994b). Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell* **79**, 487-96.

Kato, J.Y., Matsuoka, M., Strom, D.K. and Sherr, C.J. (1994a). Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol Cell Biol* **14**, 2713-21.

Keegan, L., Gill, G. and Ptashne, M. (1986). Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science* **231**, 699-704.

Kirszbaum, L., Sharpe, J.A., Murphy, B., d'Apice, A.J., Classon, B., Hudson, P. and Walker, I.D. (1989). Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: a link between the complement and reproductive systems. *Embo J* **8**, 711-8.

Kishimoto, T. and Kanatani, H. (1976). Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocyte. *Nature* **260**, 321-2.

Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J.P., Gannon, J. and Hunt, T. (1992). Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. *Mol Biol Cell* **3**, 1279-94.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R. and Roberts, J.M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257**, 1689-94.

Kornbluth, S., Sebastian, B., Hunter, T. and Newport, J. (1994). Membrane localization of the kinase which phosphorylates p34cdc2 on threonine 14. *Mol Biol Cell* **5**, 273-82.

Krisch, K., Horvat, G., Krisch, I., Wengler, G., Alibeik, H., Neuhold, N., Ulrich, W., Braun, O. and Hochmeister, M. (1988). Immunochemical characterization of a novel secretory protein (defined by monoclonal antibody HISL-19) of peptide hormone producing cells which is distinct from chromogranin A, B, and C. *Lab Invest* **58**, 411-20.

Kumagai, A. and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. *Cell* **70**, 139-51.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Lee, K.-Y., Rosales, J.L., Tang, D. and Wang, J.H. (1996). Interaction of cyclin-dependent kinase 5 (Cdk5) and neuronal Cdk5 activator in bovine brain. *J Biol Chem* **271**, 1538-1543.

Lee, M.H., Reynisdottir, I. and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* **9**, 639-49.

Lee, T.H., Solomon, M.J., Mumby, M.C. and Kirschner, M.W. (1991). INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell* **64**, 415-23.

Lees, E. (1995). Cyclin dependent kinase regulation. *Curr Opin Cell Biol* **7**, 773-780.

Lees, E.M. and Harlow, E. (1993). Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Mol Cell Biol* **13**, 1194-201.

Lew, D.J., Dulic, V. and Reed, S.I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* **66**, 1197-206.

Lew, J., Beaudette, K., Litwin, C.M. and Wang, J.H. (1992a). Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J Biol Chem* **267**, 13383-90.

Lew, J., Huang, Q.Q., Qi, Z., Winkfein, R.J., Aebersold, R., Hunt, T. and Wang, J.H. (1994). A brain-specific activator of cyclin-dependent kinase 5. *Nature* **371**, 423-6.

Lew, J., Winkfein, R.J., Paudel, H.K. and Wang, J.H. (1992b). Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. *J Biol Chem* **267**, 25922-6.

Li, R., Waga, S., Hannon, G.J., Beach, D. and Stillman, B. (1994). Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* **371**, 534-7.

Li, Y., Jenkins, C.W., Nichols, M.A. and Xiong, Y. (1994). Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* **9**, 2261-8.

Liao, S.M., Zhang, J., Jeffery, D.A., Koleske, A.J., Thompson, C.M., Chao, D.M., Viljoen, M., van Vuuren, H.J. and Young, R.A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. *Nature* **374**, 193-6.

Lichtenberg-Kraag, B., Mandelkow, E.M., Biernat, J., Steiner, B., Schroter, C., Gustke, N., Meyer, H.E. and Mandelkow, E. (1992). Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer tau. *Proc Natl Acad Sci U S A* **89**, 5384-8.

Lim, H.H., Michael, G.J., Smith, P., Lim, L. and Hall, C. (1992). Developmental regulation and neuronal expression of the mRNA of rat n-chimaerin, a p21rac GAP:cDNA sequence. *Biochem J* **287**, 415-22.

Lohka, M.J., Hayes, M.K. and Maller, J.L. (1988). Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. *Proc Natl Acad Sci U S A* **85**, 3009-13.

Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J., Strauss, M., Peters, G. and Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**, 503-6.

Luo, Y., Hurwitz, J. and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature* **375**, 159-61.

Ma, J. and Ptashne, M. (1988). Converting a eukaryotic transcriptional inhibitor into an activator. *Cell* **55**, 443-6.

Makela, T.P., Tassan, J.P., Nigg, E.A., Frutiger, S., Hughes, G.J. and Weinberg, R.A. (1994). A cyclin associated with the CDK-activating kinase MO15. *Nature* **371**, 254-7.

Masui, Y. and Markert, C.L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* **177**, 129-45.

Matsuoka, M., Kato, J.Y., Fisher, R.P., Morgan, D.O. and Sherr, C.J. (1994). Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. *Mol Cell Biol* **14**, 7265-75.

Matsuoka, S., Edwards, M.C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J.W. and Elledge, S.J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* **9**, 650-62.

Matsushime, H., Roussel, M.F., Ashmun, R.A. and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65**, 701-13.

Matsuura, I. and Wang, J.H. (1996). Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus. *J Biol Chem* **271**, 5443-5450.

May, P.C., Lampert-Etchells, M., Johnson, S.A., Poirier, J., Masters, J.N. and Finch, C.E. (1990). Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat. *Neuron* **5**, 831-9.

McGeer, P.L., Kawamata, T. and Walker, D.G. (1992). Distribution of clusterin in Alzheimer brain tissue. *Brain Res* **579**, 337-41.

Mendenhall, M.D. (1993). An inhibitor of p34CDC28 protein kinase activity from Saccharomyces cerevisiae. *Science* **259**, 216-9.

Meyerson, M., Enders, G.H., Wu, C.L., Su, L.K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.H. (1992). A family of human cdc2-related protein kinases. *Embo J* **11**, 2909-17.

Meyerson, M. and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol* **14**, 2077-86.

Michel, D., Gillet, G., Volovitch, M., Pessac, B., Calothy, G. and Brun, G. (1989). Expression of a novel gene encoding a 51.5 kD precursor protein is induced by different retroviral oncogenes in quail neuroretinal cells. *Oncogene Res* **4**, 127-36.

Millar, J.B., Lenaers, G. and Russell, P. (1992). Pyp3 PTPase acts as a mitotic inducer in fission yeast. *Embo J* **11**, 4933-41.

Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R. and Russell, P. (1991). p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. *Embo J* **10**, 4301-9.

Miller, J.H. (1972) Experiments in Molecular Genetics. 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Minden, A., Lin, A., Claret, F.X., Abo, A. and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**, 1147-57.

Moreno, S. and Nurse, P. (1994). Regulation of progression through the G1 phase of the cell cycle by the rum1+ gene. *Nature* **367**, 236-42.

Morgan, D.O. (1995). Principles of CDK regulation. [Review]. *Nature* **374**, 131-4.

Motokura, T., Keyomarsi, K., Kronenberg, H.M. and Arnold, A. (1992). Cloning and characterization of human cyclin D3, a cDNA closely related in sequence to the PRAD1/cyclin D1 proto-oncogene. *J Biol Chem* **267**, 20412-5.

Mueller, P.R., Coleman, T.R., Kumagai, A. and Dunphy, W.G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* **270**, 86-90.

Murray, A. (1995). Cyclin ubiquitination: the destructive end of mitosis. [Review]. *Cell* **81**, 149-52.

Murray, A.W. and Kirschner, M.W. (1989). Dominoes and clocks: the union of two views of the cell cycle. [Review]. *Science* **246**, 614-21.

Murray, A.W. and Kirschner, M.W. (1991). What controls the cell cycle? *Sci Am* **264**, 56-63.

Nagata, A., Igarashi, M., Jinno, S., Suto, K. and Okayama, H. (1991). An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. *New Biol* **3**, 959-68.

Nakanishi, M., Robetorye, R.S., Adami, G.R., Pereira-Smith, O.M. and Smith, J.R. (1995). Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. *Embo J* **14**, 555-63.

Nobes, C.D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K. and Carson, D.A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368**, 753-6.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M. and Smith, J.R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* **211**, 90-8.

Norbury, C., Blow, J. and Nurse, P. (1991). Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. *Embo J* **10**, 3321-9.

Norbury, C. and Nurse, P. (1992). Animal cell cycles and their control. [Review]. *Annu Rev Biochem* **61**, 441-70.

Nourse, J., Firpo, E., Flanagan, W.M., Coats, S., Polyak, K., Lee, M.H., Massague, J., Crabtree, G.R. and Roberts, J.M. (1994). Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* **372**, 570-3.

Nugroho, T.T. and Mendenhall, M.D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. *Mol Cell Biol* **14**, 3320-8.

O'Bryan, M.K., Cheema, S.S., Bartlett, P.F., Murphy, B.F. and Pearse, M.J. (1993). Clusterin levels increase during neuronal development. *J Neurobiol* **24**, 421-32.

Okamoto, A., Demetrick, D.J., Spillare, E.A., Hagiwara, K., Hussain, S.P., Bennett, W.P., Forrester, K., Gerwin, B., Serrano, M., Beach, D.H. *et al.* (1994). Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci U S A* **91**, 11045-9.

Olson, M.F., Ashworth, A. and Hall, A. (1995). An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* **269**, 1270-2.

Palmer, D.J. and Christie, D.L. (1990). The primary structure of glycoprotein III from bovine adrenal medullary chromaffin granules. Sequence similarity with human serum protein-40,40 and rat Sertoli cell glycoprotein. *J Biol Chem* **265**, 6617-23.

Parker, L.L., Atherton-Fessler, S., Lee, M.S., Ogg, S., Falk, J.L., Swenson, K.I. and Piwnica-Worms, H. (1991). Cyclin promotes the tyrosine phosphorylation of p34cdc2 in a wee1+ dependent manner. *Embo J* **10**, 1255-63.

Parker, L.L., Walter, S.A., Young, P.G. and Piwnica-Worms, H. (1993). Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase. *Nature* **363**, 736-8.

Parry, D., Bates, S., Mann, D.J. and Peters, G. (1995). Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. *Embo J* **14**, 503-11.

Paudel, H.K., Lew, J., Ali, Z. and Wang, J.H. (1993). Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. *J Biol Chem* **268**, 23512-8.

Peeper, D.S., Parker, L.L., Ewen, M.E., Toebes, M., Hall, F.L., Xu, M., Zantema, A., van der Eb, A.J. and Piwnica-Worms, H. (1993). A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. *Embo J* **12**, 1947-54.

Peter, M., Gartner, A., Horecka, J., Ammerer, G. and Herskowitz, I. (1993). FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. *Cell* **73**, 747-60.

Peter, M. and Herskowitz, I. (1994). Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**, 1228-31.

Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M. and Koff, A. (1994a). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* **8**, 9-22.

Polyak, K., Lee, M.H., Erdjument-Bromage, H., Koff, A., Roberts, J.M., Tempst, P. and Massague, J. (1994b). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59-66.

Poon, R.Y. and Hunter, T. (1995). Dephosphorylation of Cdk2 Thr160 by the cyclin-dependent kinase-interacting phosphatase KAP in the absence of cyclin. *Science* **270**, 90-3.

Poon, R.Y., Yamashita, K., Adamczewski, J.P., Hunt, T. and Shuttleworth, J. (1993). The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33cdk2 and p34cdc2. *Embo J* **12**, 3123-32.

Qi, Z., Huang, Q.Q., Lee, K.Y., Lew, J. and Wang, J.H. (1995). Reconstitution of neuronal Cdc2-like kinase from bacteria-expressed Cdk5 and an active fragment of the brain-specific activator. Kinase activation in the absence of Cdk5 phosphorylation. *J Biol Chem* **270**, 10847-54.

Reeves, S.A., Helman, L.J., Allison, A. and Israel, M.A. (1989). Molecular cloning and primary structure of human glial fibrillary acidic protein. *Proc Natl Acad Sci U S A* **86**, 5178-82.

Reynhout, J.K. and Smith, L.D. (1974). Studies on the appearance and nature of a maturation-inducing factor in the cytoplasm of amphibian oocytes exposed to progesterone. *Dev Biol* **38**, 394-400.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor induced membrane ruffling. *Cell* **70**, 401-10.

Rosenblatt, J., Gu, Y. and Morgan, D.O. (1992). Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. *Proc Natl Acad Sci U S A* **89**, 2824-8.

Roy, R., Adamczewski, J.P., Seroz, T., Vermeulen, W., Tassan, J.P., Schaeffer, L., Nigg, E.A., Hoeijmakers, J.H. and Egly, J.M. (1994). The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. *Cell* **79**, 1093-101.

Sadhu, K., Reed, S.I., Richardson, H. and Russell, P. (1990). Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. *Proc Natl Acad Sci U S A* **87**, 5139-43.

Schiestl, R.H. and Gietz, R.D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16**, 339-46.

Schneider, K.R., Smith, R.L. and O'Shea, E.K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. *Science* **266**, 122-6.

Schwob, E., Bohm, T., Mendenhall, M.D. and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. *Cell* **79**, 233-44.

Serizawa, H., Makela, T.P., Conaway, J.W., Conaway, R.C., Weinberg, R.A. and Young, R.A. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. *Nature* **374**, 280-2.

Serrano, M., Gomez-Lahoz, E., DePinho, R.A., Beach, D. and Bar-Sagi, D. (1995). Inhibition of ras-induced proliferation and cellular transformation by p16INK4. *Science* **267**, 249-52.

Serrano, M., Hannon, G.J. and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-7.

Sherr, C.J. (1993). Mammalian G1 cyclins. [Review]. *Cell* **73**, 1059-65.

Sherr, C.J. and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. [Review]. *Genes Dev* **9**, 1149-63.

Shiekhattar, R., Mermelstein, F., Fisher, R.P., Drapkin, R., Dynlacht, B., Wessling, H.C., Morgan, D.O. and Reinberg, D. (1995). Cdk-activating kinase complex is a component of human transcription factor TFIIH. *Nature* **374**, 283-7.

Shivji, M.K., Grey, S.J., Strausfeld, U.P., Wood, R.D. and Blow, J.J. (1994). Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. *Curr Biol* **4**, 1062-8.

Shuttleworth, J., Godfrey, R. and Colman, A. (1990). p40MO15, a cdc2-related protein kinase involved in negative regulation of meiotic maturation of Xenopus oocytes. *Embo J* **9**, 3233-40.

Smith, D.B. and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M. and Kirschner, M.W. (1990). Cyclin activation of p34cdc2. *Cell* **63**, 1013-24.

Solomon, M.J., Harper, J.W. and Shuttleworth, J. (1993). CAK, the p34cdc2 activating kinase, contains a protein identical or closely related to p40MO15. *Embo J* **12**, 3133-42.

Solomon, M.J., Lee, T. and Kirschner, M.W. (1992). Role of phosphorylation in p34cdc2 activation: identification of an activating kinase. *Mol Biol Cell* **3**, 13-27.

Steinert, P.M. and Roop, D.R. (1988). Molecular and cellular biology of intermediate filaments. *Annu Rev Biochem* **57**, 593-625.

Strausfeld, U., Labbe, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P. and Doree, M. (1991). Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein. *Nature* **351**, 242-5.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60-89.

Tam, S.W., Shay, J.W. and Pagano, M. (1994). Differential expression and cell cycle regulation of the cyclin-dependent kinase 4 inhibitor p16Ink4. *Cancer Res* **54**, 5816-20.

Tassan, J.P., Jaquenoud, M., Leopold, P., Schultz, S.J. and Nigg, E.A. (1995). Identification of human cyclin-dependent kinase 8, a putative protein kinase partner for cyclin C. *Proc Natl Acad Sci U S A* **92**, 8871-5.

Terada, Y., Tatsuka, M., Jinno, S. and Okayama, H. (1995). Requirement for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation. *Nature* **376**, 358-62.

Toyoshima, H. and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74.

Tsai, L.H., Delalle, I., Caviness, V.S., Jr., Chae, T. and Harlow, E. (1994). p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419-23.

Tsai, L.H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. (1993a). The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* **8**, 1593-602.

Tsai, L.H., Takahashi, T., Caviness, V.S., Jr. and Harlow, E. (1993b). Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119**, 1029-40.

Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993). Complex formation between RAS and RAF and other protein kinases. *Proc Natl Acad Sci U S A* **90**, 6213-7.

Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-14.

Waga, S., Hannon, G.J., Beach, D. and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**, 574-8.

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118**, 197-203.

Wu, L. and Russell, P. (1993). Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature* **363**, 738-41.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701-4.

Xiong, Y., Zhang, H. and Beach, D. (1992). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71**, 505-14.

Xu, Z.S., Liu, W.S. and Willard, M.B. (1992). Identification of six phosphorylation sites in the COOH-terminal tail region of the rat neurofilament protein M. *J Biol Chem* **267**, 4467-71.

Zervos, A.S., Gyuris, J. and Brent, R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites [published erratum appears in Cell 1994 Oct 21;79(2):following 388]. *Cell* **72**, 223-32.

Zhang, H., Hannon, G.J. and Beach, D. (1994). p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev* **8**, 1750-8.

Zhang, H., Xiong, Y. and Beach, D. (1993). Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. *Mol Biol Cell* **4**, 897-906.