Proteins regulating cyclin dependent kinases Cdk4 and Cdk5

Promotor: Dr. C. Veeger
Emeritus hoogleraar in de Biochemie
Landbouwuniversiteit Wageningen

Co-promotoren: Dr. B. Chaudhuri
Program Team Head, Oncology Research
Novartis Pharma te Basel

Dr. ir. J. Visser
Universitair hoofddocent
sectie Moleculaire Genetica van Industriële Micro-organismen
Landbouwuniversiteit Wageningen

Mark Johannes Magdalena Willibrordus Moorthamer

Proteins regulating cyclin dependent kinases Cdk4 and Cdk5

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C. M. Karssen,
in het openbaar te verdedigen
op maandag 27 september 1999
des namiddags te vier uur in de Aula.

The research described in this thesis was carried out at and financed by the Oncology Research Department, Novartis Pharma AG, Basel, Switzerland.

ISBN 90-5808-084-6

Stellingen

1. De hypothese dat neuroblasten vertraagd of gestopt worden in G_1 door verlengde expressie van cyclin D2 is niet correct.
   M.E. Ross & M. Risken (1995) J. Neurosci. 14, 6384-6391.

2. De gefosforyleerde band bij ~31 kDa in figuur 3b, waarvan wordt beweerd dat het bacterieel eiwit is, is hoogst waarschijnlijk gefosforyleerd Cdk5.
   J. Lew, Q.-Q. Huang, Z. Qi, R.J. Winkfein, R. Aebersold, T. Hunt & J.H. Wang (1994) Nature 371, 423-426.

3. Telomerase activiteit is karakteristiek voor kanker. Het kan echter kanker niet verklaren en het wordt ook niet exclusief bij kankercellen alleen aangetroffen.
   X.R. Jiang, G. Jimenez, E. Chang, M. Frolkis, B. Kusler, M. Sage, M. Beeche, A.G. Bodnar, G.M. Wahl, T.D. Tlsty & C.P. Chiu (1999) Nature Genet. 21, 111-114.
   C.P. Morales, S.E. Holt, M. Ouellette, K.J. Kaur, Y. Yan, K.S. Wilson, M.A. White, W.E. Wright & J.W. Shay (1999) Nature Genet. 21, 115-118.

4. Expressie van CDC6 door E2F en het uit de kern brengen van CDC6 via fosforylatie door Cdk2-cyclin A is een voorbeeld van precisie regulatie van DNA synthese, alhoewel E2F ook betrokken is bij de expressie van andere genen, die nodig zijn voor de inductie van DNA synthese in zoogdiercellen.
   B.O. Petersen, J. Lukas, C.S. Sorensen, J. Bartek & K. Helin (1999) EMBO J. 18, 396-410.
   K. Ohtani, A. Tsujimoto, M. Ikeda & M. Nakamura (1998) Oncogene 17,1777-1785.

5. Door de vooronderstellingen, die men moet maken over de te zoeken sequenties alsmede de beperkte gevoeligheid, zal de microarray technologie voorlopig de bestaande screeningsmethoden nog niet kunnen vervangen.

6. Geneesmiddelen zijn voor de farmaceutische industrie pas echt aantrekkelijk als de patient ze zijn hele leven lang moet gebruiken.

7. Het verblijven in den vreemde verschaft kennis over het eigene in den vreemde en het vreemde in het eigene.

8. Ik vrees diegenen die slechts één boek als richtlijn voor het leven erkennen.
   Muurschrift in bibliotheek universiteit van Genève

Stellingen bij het proefschrift
Proteins regulating cyclin dependent kinases Cdk4 and Cdk5

Mark J.M.W. Moorthamer
Wageningen, 27 september 1999

"If seven maids with seven mops
Swept it for half a year,
Do you suppose," the Walrus said,
"That they could get it clear?"
"I doubt it," said the Carpenter,
And shed a bitter tear.

(Lewis Carroll in *Through the Looking Glass*)

aan Mary, voor-alles

Voorwoord

(Preface)

Dit proefschrift dat nu voor U ligt draagt naast de titel ook mijn naam. Het beschrijft het promotieonderzoek dat ik heb verricht gedurende drie jaar, welke ik heb doorgebracht bij de Onkologie onderzoeksgroep van Novartis Pharma in Basel. Het spreekt voor zich dat al dit werk niet alleen het resultaat is van de inspanningen van één persoon, maar dat vele personen hebben bijgedragen aan het positief afronden van mijn promotieonderzoek. Een woord van dank is hier danook zeker op zijn plaats.

First of all I would like to thank my co-promotor Dr. Bob Chaudhuri for guiding me through my doctoral research. His enthusiasm for research and everlasting new ideas have inspired me a lot.

Een goede afronding van mijn proefschrift is mogelijk gemaakt door mijn promotor Prof. Dr. C. Veeger en mijn co-promotor Dr. ir. J. Visser. Het was mij een waar genoegen beiden in Basel te mogen begroeten om hen op de hoogte te kunnen brengen van de voortgang van mijn onderzoek.

I am thankful to Dr. William Greenhalf for helping me through the first stages of my doctoral research. You have not only been an enormous support in research but also a very good friend. Dr. Marjo Simonen, Dr. Karen Huebscher and Dr. Janet Lee have given me a lot of advice and support as well. In this respect I also want to say thank you to Dr. Rajeev Soni for helping me during my writing up and creating a good atmosphere in our lab. I would like to thank Sabine Zumstein-Mecker, Christine Stephan, Lionel Muller and Dominique Klein for their assistance and companionship in our research group. Furthermore I need to say that the work and presence of Mayur Panchal and Dominique Sarsfield who have done their practical period in Bob's group has contributed in a positive manner.

Nederlanders vind je overal, zo ook in Basel. Vele uurtjes zijn mijmerend in Mr. Pickwick's pub doorgebracht onder het genot van enkele liters bier. Ivo Baijens, John Evers, Ewoud Seip, Harmen Fredrikze, Wil & Petra Spooren, Ine Kietz, Alfred van der Pol en Arnout Calmeyn (de enige Belg in ons midden) bedankt voor vele gezellige hollandse avonden en weekenden!

Lieve Mary, jou wil ik speciaal bedanken voor alle steun en hulp, die je me hebt gegeven gedurende deze tijd. Gelukkig heb je zowel een stage als ook een project in Zwitserland kunnen doen, zodat we tenminste gedurende deze tijd in staat waren elkaar elk weekend te zien. Hopelijk kunnen we binnenkort voor altijd bij elkaar zijn.

Tenslotte wil ik mijn ouders bedanken voor het feit dat ze mij altijd gestimuleerd hebben in elke vrije keuze, die ik heb mogen maken. Het is fijn te weten, dat jullie mijn leven voor mij hebben mogelijk gemaakt. Bedankt, omdat jullie alles voor me wilden betekenen en altijd met me hebben meegeleefd.

Contents

Symbols and abbreviations

ADH1	alcohol dehydrogenase
β-gal	β-galactosidase
cAMP	cyclic AMP
CAK	Cdk-activating kinase
Cdc	cell division cycle gene
Cdk	cyclin-dependent kinase
Cdki	Cdk inhibitor protein
cDNA	complementary DNA
CKS	cyclin-dependent kinase regulatory subunit
CLN	yeast cyclin
CLB	yeast cyclin B
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
EDTA	ethylene-diamine tetraacetic acid
EGF	epidermal growth factor
EGTA	bis-(aminoethyl)-glycolether-N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
G_0	quiescent state cells enter the cell cycle
G_1	first gap phase of the cell cycle
G_2	second gap phase of the cell cycle
GSK	glycogen synthase kinase
GST	gluthathione-S-transferase
HA	human hemaglutinin antigen
HBS	Hepes buffered saline
Hepes	4-[2-hydroxyethyl]-1-piperazineethane sulphonic acid
IC50	50% inhibitory concentration
IL-2	interleukin-2
M	mitosis
MAP	mitogen-activated protein
MOI	multiplicity of infection

NF	neurofilament
NF-L, -M and -H	NF of low, medium and high molecular mass respectively
NP-40	Nonidet P-40
p35	35 kDa protein which activates Cdk5
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PHF	paired helical filaments
PMSF	phenylmethanesulfonylfluoride
p-NPP	4-nitrophenyl phosphate disodium salt
pol δ	DNA polymerase δ
pRb	retinoblastoma protein
R	restriction point
S	synthesis
TGF-β	transforming growth factor β
TPK	tau protein kinase
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
tRNA	transfer RNA
wt	wildtype
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. General introduction

1.1 The Cell Cycle

Completion of the cell cycle requires coordination of a variety of macromolecular syntheses, assemblies, and movements. The chromosomes must be replicated, condensed, segregated, and decondensed. The spindle poles must duplicate, separate, and migrate to opposite ends of the nucleus. In metazoans, the nuclear membrane is disassembled and reassembled, the spindle is assembled and disassembled, and the cell membranes invaginate to complete cytokinesis. Coordination of these complex processes is thought to be achieved by a series of changes (phase transitions) in the cell cycle (1,2).

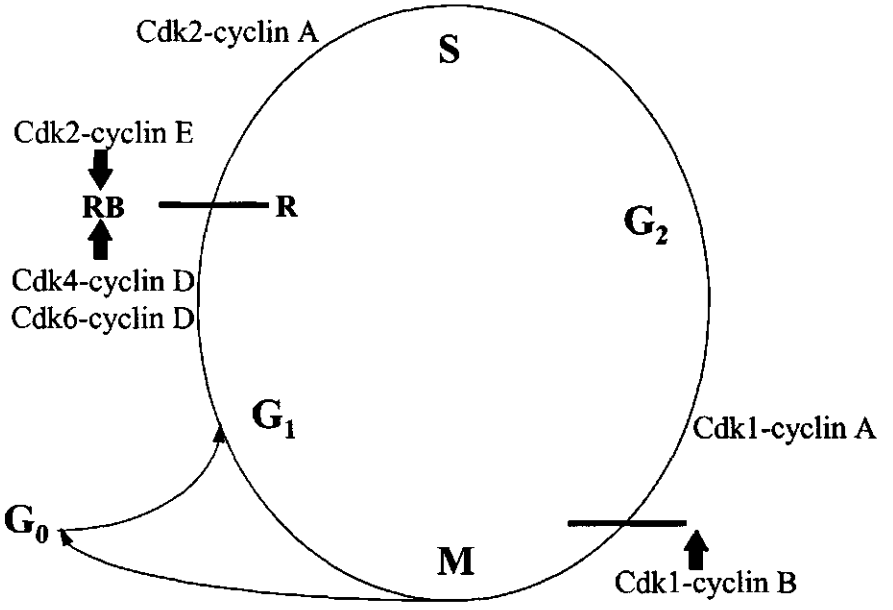


Figure 1. The Cell Cycle in higher eukaryotes
G_1: first gap, S: DNA replication phase, G_2: second gap, M: mitosis, G_0: quiescent state, R: restriction point, RB: retinoblastoma protein. Horizontal lines illustrate G_1/S and G_2/M phase transitions. Filled-in arrows indicate kinases active at phase transitions.

The cell cycle, divided into phases designated G_1 (first gap), S (synthesis), G_2 (second gap) and M (mitosis), is regulated at two major decision points: prior to the G_1/S transition (designated "START" in yeast and "restriction point" (R) in mammalian cells) and the G_2/M transition (3). G_0 is described as the quiescent state of the cells. In this phase cells are in a dormant state (Fig. 1). In multicellular eukaryotes, the cell cycle is driven by the complex interplay of regulatory cyclin proteins-cyclins A, B, C, D, E and H-with protein serine/threonine kinases, designated Cdc (cell division cycle gene) 2, and Cdk (cyclin-dependent kinase) 2,3,4,5,6,7 and 8 (Table 1). Cdc2 is also known as Cdk1. In unicellular eukaryotes such as budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeast, a single protein kinase (CDC28 or $cdc2^+$, respectively) fulfills the function of the family of proteins found in multicellular organisms. At distinct stages of the cell cycle different yeast cyclins activate the protein kinase: CLN1, 2 and 3 at START, CLB5 and 6 in S phase and CLB1 and 2 at mitosis (the roles of CLB3 and 4 are less well defined) (4-8).

Table 1. Cdk-s and their cyclin partners

Cdk	**cyclin**
Cdk1	cyclin A,B
Cdk2	cyclin A,E
Cdk3	unknown
Cdk4	cyclin D
Cdk5	cyclin D,E
Cdk6	cyclin D
Cdk7	cyclin H
Cdk8	cyclin C

The cell cycle stage is determined by the constellation of proteins activated or inactivated by phosphorylation as a result of the activity of the Cdk-s during that stage. The passage of cells from one stage of the cell cycle to another is tightly regulated by a wealth of controls that act on the transcription of cyclin genes, the degradation of cyclin proteins, and the modification of the kinase subunits by phosphorylation. A

number of positive and negative feedback loops also contribute to cell cycle progression (1).

1.2 Cdk activity regulation

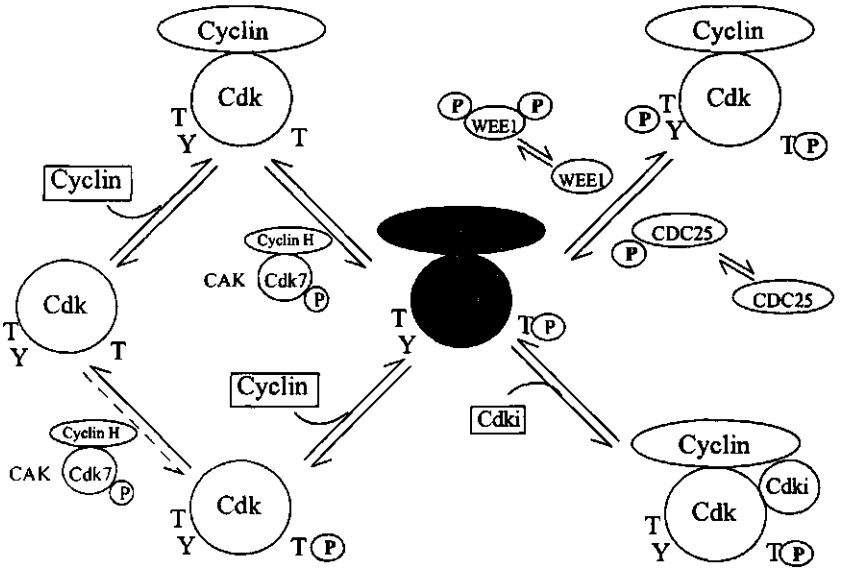


Figure 2. Principles of Cdk regulation (6)
At center right lies the active Thr160/161-phosphorylated Cdk-cyclin complex. Moving outward along any of the four pathways results in inactivation; moving inward leads to activation. This is a highly simplified scheme from which many pathways and alternative states have been omitted.

Presumably, the larger number of Cdk-s in higher eukaryotes reflects the increased regulatory capacity required to execute the complex instructions of development. These Cdk-s are closely related in size (~33 kDa) and sequence (> 40% identity). Specific cyclins, with their Cdk partners, have been shown to promote the advancement of different phases of the cell cycle (4,9). Thus, G_1 active cyclins A, C, D1, D2, D3, and E have been shown to mediate the progression through G_1/S (10-13), while G_2 cyclins A, B1, B2 and B3 usher the cell through G_2/M (14). Cellular Cdk levels tend to remain in constant excess throughout the normal cell cycle, and regulation of catalytic activity is primarily post-translational. The activity of the Cdk enzymes is controlled both by their association with cyclins and by phosphorylation of

a threonine residue (Thr161 in Cdk1 and Thr160 in Cdk2) of the Cdk polypeptide itself which obscures the substrate-binding site in its inactivated state. Cdk-s and their regulators are found in specific locations, but little is known about the mechanisms responsible or whether localization is regulated. One of the enduring mysteries of Cdk regulation is the role of the *S. pombe* protein, $p13^{suc1}$, and its homologues, the CKS proteins, in *S. cerevisiae* and vertebrates. These small proteins, which are essential for cell-cycle progress in yeast, have long been known to bind tightly to certain Cdk-s *in vivo*. They do not, however, have any distinct effect on Cdk function *in vitro*. The crystal structure of one family member, CksHs2, suggests that these proteins may be involved in Cdk oligomerization in the cell. Figure 2 shows the principles of Cdk regulation (4,6-8).

1.2.1 Cyclins

The cyclins possess a short life span. The persistence of the gene expression or stability of the protein may determine the length of the cell cycle phase in which the cyclin is expressed. Constant synthesis during the cell cycle results in a roughly linear increase in cyclin concentration, until at mitosis cyclin degradation abruptly increases, producing a rapid decline in cyclin levels. Mitotic cyclin degradation involves the ubiquitin-dependent proteolytic machinery, and requires a small sequence motif (the destruction box) near the amino terminus of mitotic cyclins. The trigger for destruction may be the increased Cdk1 activity at mitosis and the activation of components of the ubiquitin system by Cdk1. In *S. cerevisiae* activation of CDC28 by the G_1 cyclins (CLN-s) leads to activation of CLN1 and CLN2 transcription in a positive feedback loop. Activation of CDC28 by CLNs also inactivates the machinery responsible for the degradation of the CLB mitotic cyclins, allowing CLB levels to rise after G_1. CLB proteins then help to repress CLN transcription, while at the same time stimulating their own transcription in another positive feedback loop. The resulting increase in CLB levels leads to mitotic activation of CDC28, which then triggers CLB destruction. Cyclins are thought to contain regions that target the Cdk to specific substrates or subcellular locations. In addition to simply activating the associated Cdk, they can thus promote activity toward specific substrates. The enhanced activity of certain complexes is probably due to positive interactions between the cyclin and the substrate (6-9).

1.2.2 CAK

The activation of Cdk-s via phosphorylation of Thr160/161 is mediated by an enzyme complex termed Cdk-activating kinase (CAK), which itself is composed of a Cdk (termed variously MO15 or Cdk7), a cyclin (cyclin H) and a RING finger protein subunit MAT1 (15-20). This Cdk activation is reversed by a specific phosphatase (21). Since Cdk7 is also dependent upon an activating phosphorylation event (Thr170 in human Cdk7) by yet another kinase, this suggests the possibility of positive control by an extended kinase cascade, a notion that at present lacks direct experimental support. During the normal vertebrate cell cycle, phosphorylation of Thr160/161 tends to rise and fall in parallel with cyclin binding. Changes in phosphorylation are probably not due to changes in CAK activity, which does not change during the cell cycle, but appear to reflect the ability of cyclin binding to stimulate Cdk phosphorylation (4,6-8).

1.2.3 Wee1, Myt1 and Cdc25

Negative regulation of Cdk activity occurs via inhibitory phosphorylation of Tyr15 and Thr14 close to the ATP-binding region through Wee1 (22,23) and the dual-specificity kinase Myt1 (24) respectively. This inhibition is part of a negative-feedback pathway that prevents cells from entering mitosis with damaged or incompletely replicated DNA. Cdk1-cyclin B complexes are maintained in an inactive state, until Thr14-Tyr15 dephosphorylation at the end of G_2 activates Cdk1. This abrupt dephosphorylation is brought about by coordinated changes in the activities of kinases and phosphatases acting at these sites. Wee1 activity declines during mitosis, contributing to the fall in inhibitory phosphorylation at this stage. Decreased kinetic activity during mitosis is due to phosphorylation of Wee1. In *S. pombe*, the protein kinase Nim1 inhibits Wee1 by phosphorylating its C-terminal catalytic domain. Thr14 and Tyr15 are both dephosphorylated by the dual-specificity phosphatase termed Cdc25 (25-28). Cdc25 activity increases during mitosis, largely because of increased phosphorylation in its N-terminal half. During mitosis, the kinase responsible for this phosphorylation is activated and the corresponding phosphatase is inhibited. The Cdc25-stimulatory kinase is Cdk1 itself, forming the basis of an elegant positive-feedback system that induces the abrupt mitotic dephosphorylation of Cdk1. Positive feedback is also achieved by coordinated effects on other components: Cdk1 phosphorylates and inactivates Wee1 and may inhibit the phosphatase(s) that inactivates Cdc25 and activates Wee1 (4-8).

1.2.4 Cdk inhibitors

Different Cdk inhibitor proteins (Cdki) could form barriers at different control points in the cell cycle, for example G_1 and G_2 phases. Thus as cyclins are synthesized, they will bind a Cdk partner and a Cdki, such that the cyclin-Cdk complexes will remain inactive until the amount of cyclin-Cdk complex exceeds the amount of Cdki. At this point there will be insufficient Cdki to bind all the complexes, leaving active cyclin-Cdk-s to promote the next phase of the cell cycle (7,29).

Negative growth signals mobilize Cdki-s that associate either with a Cdk or with a cyclin-Cdk complex and block their function. The universality of negative cell cycle control via Cdk inhibitors is indicated by the identification of inhibitors in budding and fission yeast, as well as in mammalian cells (4,8,29,30).

One of the inhibitors known to be expressed in yeast is FAR1, found in *S. cerevisiae*, which inhibits CLN-CDC28 activity and blocks the cell cycle in G_1 in response to the pheromone α-factor. The pheromone-response pathway stimulates a mitogen-activated protein (MAP) kinase cascade that then activates a transcription factor, Ste12, to induce transcription of several genes such as the *FAR1* gene. After expression FAR1 is rapidly phosphorylated by the MAP kinase FUS3. The antimitogen α-factor thus appears to amplify FAR1 activity by two mechanisms, one transcriptional and the other posttranslational. SIC1 (p40) which is found in the same organism regulates entry into S phase by inhibiting CLB5,6-CDC28 kinase activity. Synthesis of FAR1 and SIC1 is under cell cycle control such that their levels peak in G1. This pattern results from transcriptional activation in a periodic manner and specific degradation after the G1/S transition. The temporal appearance ensures that these Cdki-s will inhibit only the kinases that are present during this time period. The Cdki-s are degraded by the ubiquitin-dependent proteolysis machinery. Studies indicate that SIC1 reduces both the V_{max} and the K_m of CDC28 for protein substrate. The inhibitor rum1+ is expressed in *S. pombe* and has the same function as SIC1 by inhibiting cdc13-cdc2 (31,32). Cdk-s are now known to participate in more than just cell cycle regulation. The Cdk PHO85-PHO80 expressed in *S.cerevisiae* regulates transcription of the acid phosphatase gene *PHO5*. Genetic analysis identified the *PHO81* gene, coding for a protein with homology to $p16^{INK4A}$, which functions upstream of PHO85-PHO80. PHO81 has recently been shown to inhibit PHO85-PHO80 activity in vitro by functioning as a Cdki (4,8,29,30).

In higher eukaryotes the CIP/KIP inhibitor family ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$) (33-38) are known to inhibit all Cdk-s by binding to the cyclin-Cdk complex whereas the INK4 inhibitor family ($p14^{INK4B}$, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$) (39,40) specifically inhibit Cdk4 and Cdk6 by competitively preventing binding of cyclin D to the Cdk. Cdki-s are phosphorylated by their Cdk target suggesting an interaction with the protein substrate binding site. The stoichiometry of Cdki-Cdk binding is unclear (4,6,8,29,30,41-43).

1.2.4.1 $p21^{CIP1/WAF1}$

Radiation and other DNA-damaging agents block pRb phosphorylation through use of the Cdki named $p21^{CIP1/WAF1}$. Steady-state levels of p53 increase rapidly upon DNA damage. p53 is a transcription factor that is involved in at least three functions: cell cycle arrest in G_1 in response to DNA damage; transcriptional activation of genes responding to DNA damage; and regulation of 'programmed cell death', apoptosis. p53 activates expression of $p21^{CIP1/WAF1}$, GADD45 and MDM2. Once DNA lesions are erased, the pRb-imposed block may be lifted and the cell permitted to advance into S and replicate its now-restored genome. Alternatively, in the face of irreparable lesions, the cell may choose to commit itself to an apoptotic death. Normal diploid fibroblasts differ from transformed cells in that many Cdk-cyclin complexes contain proliferating cell nuclear antigen (PCNA) in addition to $p21^{CIP1/WAF1}$. PCNA plays a positive role in cell proliferation in that it is required for processive DNA replication carried out by DNA polymerase δ (pol δ). $p21^{CIP1/WAF1}$ inhibits PCNA function by directly binding to the protein. Although not yet verified *in vivo*, these data suggest that $p21^{CIP1/WAF1}$ plays a dual role in the DNA-damage checkpoint by both arresting the cell cycle through inhibition of Cdk-s and blocking DNA replication. Levels of mRNA encoding $p21^{CIP1/WAF1}$ rise 10-20-fold when cells become senescent, and also rise as cells become quiescent (4,8,29,30). Normal human fibroblasts do not express telomerase, the enzyme that replicates the repeated sequences at the ends of chromosomes; thus, telomere length decreases as cells proliferate. It has been suggested that chromosome ends with shortened telomeres may activate a checkpoint pathway that inhibits cell proliferation and that senescence occurs as a result of the loss of telomeric sequences. One gene thought to be necessary for the arrest of proliferation in senescent cells is $p21^{CIP1/WAF1}$ (1). Association of $p21^{CIP1/WAF1}$ with Cdk-s is greatly enhanced by cyclin

binding. This property is shared by the structurally related inhibitor $p27^{KIP1}$, suggesting a common biochemical mechanism for inhibition. With respect to Cdk2 and Cdk4 complexes, $p27^{KIP1}$ shares the inhibitory potency of $p21^{CIP1/WAF1}$ but has slightly different kinase specificities. In normal diploid fibroblasts, the vast majority of active Cdk2 is associated with $p21^{CIP1/WAF1}$, but this active kinase can be fully inhibited by addition of exogenous $p21^{CIP1/WAF1}$. Reconstruction experiments using purified components indicate that multiple molecules of $p21^{CIP1/WAF1}$ can associate with Cdk/cyclin complexes and inactive complexes contain more than one molecule $p21^{CIP1/WAF1}$. Together, these data suggest a model whereby $p21^{CIP1/WAF1}$ functions as an inhibitory buffer whose levels determine the threshold kinase activity required for cell cycle progression (34).

1.2.4.2 $p27^{KIP1}$

At least three molecular mechanisms have been proposed to explain the ability of the extracellular signal protein, transforming growth factor (TGF)-β, to obstruct pRb phosphorylation. The first of these involves $p27^{KIP1}$, a Cdki that interacts with Cdk2, Cdk4 and Cdk6 and prevents their functional activation at the hands of CAK. The total amount of $p27^{KIP1}$, however, is unaffected by TGF-β treatment or during the cell cycle. Rather, $p27^{KIP1}$ appears to be sequestered in a heat-labile compartment, from which it is released upon TGF-β treatment. Contact inhibition and cAMP also act through their ability to mobilize $p27^{KIP1}$ (37). TGF-β also acts via p53 to dramatically reduce the translational level of Cdk4 (44-46) leading to a reduction in Cdk4-cyclin D complexes that harbor the majority of $p27^{KIP1}$ in cycling cells and potentially freeing $p27^{KIP1}$ to inhibit other Cdk-s (e.g. Cdk2; 44,45,47). Alterations in the balance of active Cdk-cyclin complexes through redistribution of inhibitors may be a common theme in cellular growth control. $p27^{KIP1}$ is inactivated by the mitogen interleukin-2 (IL-2) (4,8,29,30). Cyclin A mRNA induction by Myc in fibroblasts is inhibited by $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ whereas Myc-induced apoptosis is insensitive to these Cdk inhibitors (48).

1.2.4.3 $p15^{INK4B}$ and $p16^{INK4A}$

An alternative mechanism is suggested by the recent discovery that TGF-β can induce expression of $p15^{INK4B}$ (49), another Cdki. $p15^{INK4B}$ targets Cdk4/Cdk6, competing with

D cyclins for binding to Cdk4/Cdk6. In normal cells, Cdk4 is associated with a D-type cyclin, PCNA and $p21^{CIP1/WAF1}$. In some transformed cells, however, Cdk4 is found associated with $p16^{INK4A}$ and these complexes lack a cyclin and PCNA. Binding of $p16^{INK4A}$ to Cdk4 blocks and disrupts cyclin D association (4,8,30,50,51).

1.3 Structure of the Cdk-cyclin complex

Cyclin dependent kinases are a member of the highly conserved protein-serine/threonine kinase family (52). The typical Cdk catalytic subunit contains a 300 amino acid catalytic core that is completely inactive when monomeric and unphosphorylated. The crystal structure of the human Cdk2 apoenzyme shows that it is held in an inactive state by at least two major structural restraints: first, the substrate binding site is blocked by an extended loop termed the T-loop; and second, side chains in the ATP-binding site are oriented so that the ATP phosphates are poorly positioned for efficient phospho-transfer (53). Cyclin A binds to one side of Cdk2's catalytic cleft, inducing large conformational changes in its PSTAIRE helix and T-loop. These changes activate the kinase by realigning active site residues and relieving the steric blockade at the entrance of the catalytic cleft (54, 55). Homology among cyclins is often limited to a relatively conserved domain of about 100 amino acids, the cyclin box, which is responsible for Cdk binding and activation. In Cdk2, Thr160 lies in the T loop; its side chain is inaccessible to solvent. Upon phosphorylation the T-loop moves by as much as 7 Å, and this affects the putative substrate binding site as well as resulting in additional cyclin contacts (56). Phosphorylation thus affects the cyclin binding site and it enhances the binding of some Cdk-cyclin pairs; conversely, cyclin binding may enhance phosphorylation. In Cdk2, the side chains of the Thr14 and Tyr15 residues hang from the ceiling of the ATP-binding site and are certainly in a position to affect kinase activity when phosphorylated. The mechanism of inhibition is unknown, but phosphorylation of Tyr15 does not appear to inhibit ATP binding. Both residues are buried beneath the T loop and are inaccessible to solvent, suggesting that the T loop must be moved to allow phosphorylation. This may be accomplished by cyclin binding, as phosphorylation is thought to be cyclin-dependent (6).

1.4 Substrate specificity of cyclin-dependent kinases

Cyclin-dependent kinases are specifically phosphorylating serine/threonine residues preceding a proline residue. Such a substrate determinant has been found in other protein kinases and was termed proline-directed phosphorylation specificity. The Cdk phosphorylation site consensus sequence as deduced from substrate sequences is: **-X-S/T-P-X-K/R-X-** (57). In addition to the carboxyl-terminal proline, a basic amino acid, lysine or arginine, at the third position carboxyl-terminal to the phosphorylation target residue also appears to be an important substrate determinant. The proto-oncogene c-Src was among the earliest indentified Cdk substrates (58).

1.5 G_1/S transition

1.5.1 The Rb protein

pRb, the product of the retinoblastoma tumour suppressor gene, operates at the restriction point (R) of the cell cycle. Its main role is to act as a signal transducer connecting the cell cycle with the transcriptional machinery (59). The protein becomes hyperphosphorylated during the G_1 phase and maintains this configuration until the emergence from M. As cells enter anaphase, pRb dephosphorylation begins and continues, stepwise, until G_1 when pRb is once again hypophosphorylated. G_1 cyclins, serving as regulatory subunits of their partner Cdk-s, direct these enzymes to the pRb substrate, resulting in its phosphorylation. Cyclins of the D class (D1, D2 and D3) are most prominently implicated in the phosphorylation of pRb early in G_1 (60-63). These cyclins serve as activators of the Cdk4 and Cdk6 kinases. At the end of G_1 Cdk2 which complexes with cyclin E and cyclin A phosphorylates pRb at different sites driving the cell into the S phase. Without functional pRb, certain cell types are susceptible to apoptosis. This apoptosis depends upon the p53 protein. In this way pRb plays an important role in the decision whether a cell should proliferate, differentiate (64), undergo senescence or even apoptosis. Finally, in addition to a major role in the G_1/S transition, pRb may act at other stages in the cell cycle. For example, it has been shown that overexpression of pRb during S phase of the cell cycle may result in G_2 arrest (8,30).

1.5.2 The E2F transcription factor

When pRb is hypophosphorylated, it is bound to the E2F transcription factor. The term E2F subsumes a group of at least five distinct, closely related transcription factors that are all targeted to variations of the consensus nucleotide sequence TTTCGCGC. E2F-1 (65,66), E2F-2 (66,67) and E2F-3 (66) are under the direct control of pRb. E2F-4 and E2F-5 are under the control of p107 which is a pRb family member (68). Phosphorylation of pRb causes it to lose its grip on E2F, enabling the latter to proceed with the activation of a cohort of genes whose transcription it controls. The genes which are under the control of E2F include *c-myc*, *B-myb*, cyclin E, cyclin A, *Cdk1*, dihydrofolate reductase, thymidylate synthetase, ribonucleotide reductase, thymidine kinase and the *E2F-1* gene itself. Activation of these genes drives the cell into the S phase where DNA replication takes place. pRb binds to other proteins as well, such as Elf-1, MyoD, PU.1, ATF-2, and c-Abl proteins. Most intriguing of these is the nuclear tyrosine kinase encoded by the cellular *abl* proto-oncogene. Hypophosphorylated pRb is reported to bind directly to the active catalytic domain of the c-Abl kinase blocking its function. As expected, hyperphosphorylated pRb loses this binding ability. By binding multiple effectors such as E2Fs and Abl, pRb may be able to modulate simultaneously the activity of a number of downstream growth-controlling pathways. Physiological signals that favour cell proliferation should encourage pRb phosphorylation. Most of these signals originate with mitogens that impinge on cell surface receptors and in turn activate cytoplasmic signal transduction pathways that convey growth stimulatory signals to the nucleus (1,8). Despite an inability to bind to E2F, pRb mutants associated with a low risk of retinoblastoma, unlike high-risk mutants, retained the ability to activate transcription and promote differentiation. Thus, the pRb pocket participates in dual tumour suppressor functions, one linked to cell cycle progression and the other to differentiation control, and these functions can be genetically and mechanistically dissociated (69).

1.6 G_2/M transition

The G_2/M transition is prevented by DNA damage and by incompletely replicated DNA. This checkpoint prevents chromosome segregation if the chromosome is not intact. Genetic studies in *S. cerevisiae* and *S. pombe* have identified a number of genes

necessary for this control. In *S. cerevisiae*, the *RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC2*, and *MEC3* genes prevent mitosis in the presence of DNA damage or if replication is blocked in late S phase. The *MEC1* and *MEC2* genes also prevent mitosis if replication is blocked in the early S phase. One double-strand break in the DNA will activate this checkpoint and prevent the cell from undergoing mitosis. Some of the *S. cerevisiae* genes show sequence homology to the *S. pombe* genes, which indicates that this pathway evolved early and is likely to be present in human cells as well. Few gene products that control the G_2/M transition have been identified in mammalian cells. *S. cerevisiae* encodes at least six genes that prevent the cell from initiating a new cell cycle in the presence of microtubule poisons: *MAD1*, *MAD2*, *MAD3*, *BUB1*, *BUB2*, and *BUB3* (1).

1.7 Tumours

Perhaps the single most defining feature of cancer cells is uncontrolled growth. Tumours expand and invade with impunity, ignoring the mechanisms that maintain the integrity of normal tissue. They take over the body like a parasite, proliferating until life is sapped from the host. Cells from many advanced cancers traverse the cell cycle at breakneck speed. Furthermore, cells that would normally arrest their growth in response to specific environmental cues (such as contact with neighboring cells) disregard these stimuli when transformed into the cancerous state. To prevent loss of growth control, multiple pathways are probably involved. Some of these pathways may be quite general, functioning in many tissues, while others may be much more specific, operating in only few cell types. The increased evidence of cancer as a function of age has long been interpreted to suggest that multiple genetic changes are required for tumourigenesis, an interpretation borne out by recent systematic analysis of genetic changes during the evolution of colon cancer cells (1).

As cancer is a disease characterized by loss of cellular growth control, it is not surprising that the molecular machinery of the cell cycle is involved in tumourigenesis. In addition, surveillance of telomeric sequences as they are lost during senescence may be important in signalling somatic cells to stop proliferation. Senescing cells exhibit an increased number of chromosomal aberrations, many of which appear to involve telomere-telomere associations. Thus, the normal senescent program may generate

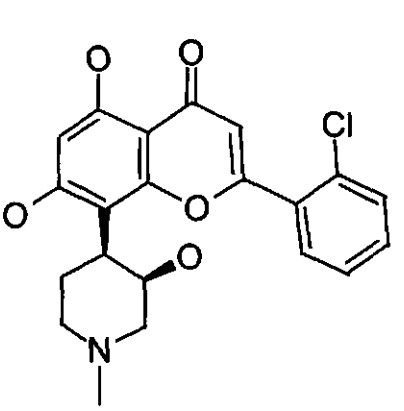
chromosome instability (1). Recent discoveries have brought several cell-cycle regulators into sharp focus as factors in human cancer. Among the most conspicuous types of molecules to emerge from ongoing studies in this field are the cyclin-dependent kinase inhibitors such as $p16^{INK4A}$. These molecules have several hallmarks of tumour suppressors and are perfectly positioned to regulate critical decisions in cell growth. The *$p16^{INK4A}$* gene appears to be a particularly significant target for mutation in sporadic tumours and in at least one form of hereditary cancer (51,70). As many as 70% of tumour cell lines and primary tumours from various tumour types and familial melanomas contain homozygous deletions or point mutations of the $p16^{INK4A}$ (*p16, MTS1* or *CDKN2*) locus. $p15^{INK4B}$ may be deleted or present in a mutant form in carcinomas. The majority of homozygous deletions that remove $p16^{INK4A}$ in tumour cell lines also remove $p15^{INK4B}$. It is unclear whether inactivation of $p15^{INK4B}$ in tumour cell lines is causal or merely a bystander effect of its proximity to $p16^{INK4A}$ (4,6,8,29,70). Many human tumour cell genomes have been found to have lost pRb function through chromosomal gene mutation (71). In other tumours the *cyclin D* gene (72-74) and/or the *Cdk4* gene (75,76) is amplified. Abnormal expression of cyclins A, B and E and Cdk1 have been reported (1,77). The fact that p53 is one of the most commonly mutated genes in human cancer points to the potential importance of $p21^{CIP1/WAF1}$ in negative growth control (1,4,6,8,29,78-80). Alterations in expression of the p53 binding protein MDM2 can result in adaptation of the G_1/S checkpoint (75,76). MDM2 is an endogenous inhibitor of p53 function (81,82). The *c-mos* proto-oncogene, a regulator of meiotic metaphase, produces polyploidy when it is expressed abnormally in mitotic cells, as it is during tumourigenesis (1).

The frequent deregulation of Cdk proteins and their regulators in cancer and the therapeutic potential of natural inhibitors has stimulated an active search for chemical Cdk inhibitors (83). Seven chemical inhibitors which are specifically inhibiting Cdk-s have been described so far: butyrolactone (84), flavopiridol, 9-hydroxy-ellipticine, olomoucine (85,86), roscovitine, staurosporine (87,88) and suramin (5,89,90). The IC50 values (50% inhibitory concentration) of these inhibitors with respect to the different Cdk-s are shown in Table 2. Figure 3 shows the structure of flavopiridol and staurosporine which both have been used in the experiments depicted in this thesis.

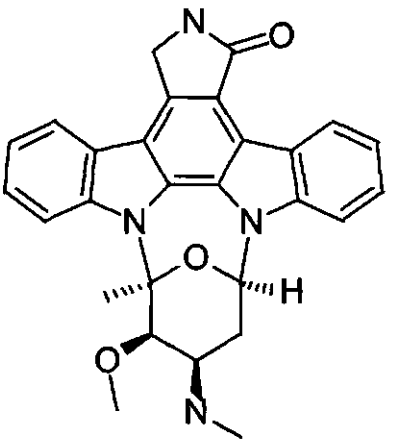
Table 2. Specificity of known chemical Cdk inhibitors towards different Cdk-s*

Inhibitor	Cdk1	Cdk2	Cdk4	Cdk5
butyrolactone	0.60	1.50	no effect	ND
flavopiridol	0.40	0.40	0.40	ND
9-hydroxy-ellipticine	1	ND	ND	ND
olomoucine	7	7	>1,000	3-8
roscovitine	0.65	0.70	>100	0.20
staurosporine	0.003-0.009	0.007	0.020	0.039
suramin	4	ND	ND	ND

*IC50 values (μM) of inhibitors tested against the specific Cdk-s; ND, not determined.



Flavopiridol



Staurosporine

Figure 3. Molecular structure of flavopiridol and staurosporine

1.8 Cdk5 and its role in differentiation

It has become clear that not all cyclins and Cdk-s function in cell cycle control. Examples of cyclins and Cdk-s that function in non-cell cycle regulating events are mounting; for instance, Cdk7-cyclin H and Cdk8-cyclin C in the TFIIH subunit of the RNA polymerase II holoenzyme have possible roles in phosporylating the C-terminal domain of RNA polymerase II (19,91,92). In the next paragraphs the role of Cdk5 will be discussed. Cdk5 has been found to play a role in differentiation of nerve and muscle cells. Malignant function of Cdk5 may lead to Alzheimer's or Lewy body diseases.

1.8.1 Neuronal development

The differentiation of a nerve cell is a process that begins early in embryonic development and continues through subsequent cell divisions, migrations, and maturations. It culminates in the acquisition of the form and function that define each specific adult cell type. Neurons are an exceptional class of cells that both attain mature morphology only after cessation of mitotic activity and persist in the fully differentiated state for the lifetime of the organism. The cell cycle arrest is one of the earliest events in the neuronal maturation process, although the expression of pattern formation genes as well as certain differentiation markers can precede it. The molecular events that accomplish this seemingly irreversible transition into the postmitotic state and formation of neuronal morphology are as yet largely unknown (9,30).

It has been observed that the duration of the cell cycle in the developing nervous system progressively lengthens during differentiation. This has been attributed to, predominantly, increases in the length of G_1 (9). In differentiating cells in general it has been shown that pRb protein levels increase whereas Cdk4 and cyclin D levels drop. $p18^{INK4C}$ and $p19^{INK4D}$ have been reported to be expressed during mouse brain development (93). Stopping cells in mid-to-late G_1 appears to be a prerequisite to their subsequent egress from the cell cycle into a more differentiated state (8). Several cell cycle regulators, including tumour suppressor protein pRb (30,64,94), transcription factor E2F-1 (95), c-Src (96), cyclins D1 (97,98), D2 (9) and E (99), cyclin dependent kinase Cdk5 and p35 (100-102), have been reported to be expressed in immature postmitotic and adult neurons whereas the expression of Cdk1, Cdk2, cyclins A and B is dramatically downregulated upon terminal differentiation of neurons *in vivo* (101-

103). p53 mRNA expression is also downregulated but to a lesser extend (103). In pRb deficient mutants expression patterns of certain neurotrophin receptors are low compared to the wildtype. Neurotrophins form a family of closely related proteins that play important roles in the development and maintenance of the nervous system. This provides support for the possibility that certain cell cycle proteins may well be involved at this critical juncture between proliferation and acquisition of mature morphology in the differentiation of specific neurons within the central nervous system (30,104).

1.8.2 Cdk5

Cdk5 was identified as a Cdk-related protein PSSALRE and shows about 60% amino acid sequence identity to both Cdk1 and Cdk2 (105). Tyr15 and Thr14 are conserved in Cdk5 and the amino acid residue of Cdk5 corresponding to Thr161 of Cdk1 is conservatively substituted by a serine residue (Ser159) (Fig. 4; 106). Both human and mouse *cdk5* genes have been localized on chromosome 5 at 7q36 and the centromeric region, respectively (107,108). While the location, 12q13, for human *cdk2* and *cdk4* genes is known to be associated with chromosome alteration in solid tumours, the location for human *cdk5* gene, 7q36, is not a major site of chromosome alteration in tumours (107). The *cdk5* gene, unlike *cdk1*, *cdk2* and *cdk3* genes, is incapable of complementing CDC28 mutants of *S. cerevisiae*. While Cdk1 and Cdk2 undergo high affinity association with the protein product of the *suc1* gene, Cdk5 shows no specific interaction with $p13^{suc1}$ (109). Instead, Cdk5 has been found to interact with an analogous protein of 15 kDa from starfish, $p15^{cdk\text{-}BP}$, which also binds to Cdk4 (110).

Cdk5 is the first example of a cyclin dependent kinase with neuronal function. Cdk5 is found to be expressed relatively equally in all human tissues with the exception of the central nervous system and peripheral nervous system, in which expression is several-fold greater in both nucleus and cytoplasm (100,102,109,111-114). During maturation of neurons the expression of Cdk5 increases and reaches highest levels in adult cerebral cortex and spinal cord (99,101,115). p35 which is expressed exclusively in the central nervous system is the only activator of Cdk5 known thusfar (113,116-118). This protein has first been isolated as a smaller proteolytic product p25 (113,118-120). Several truncated forms of p35, including p25 and two further truncated forms p23 and

p21, were expressed as GST-fusion proteins in *Escherichia coli* and found to activate bacterially expressed Cdk5 (121).

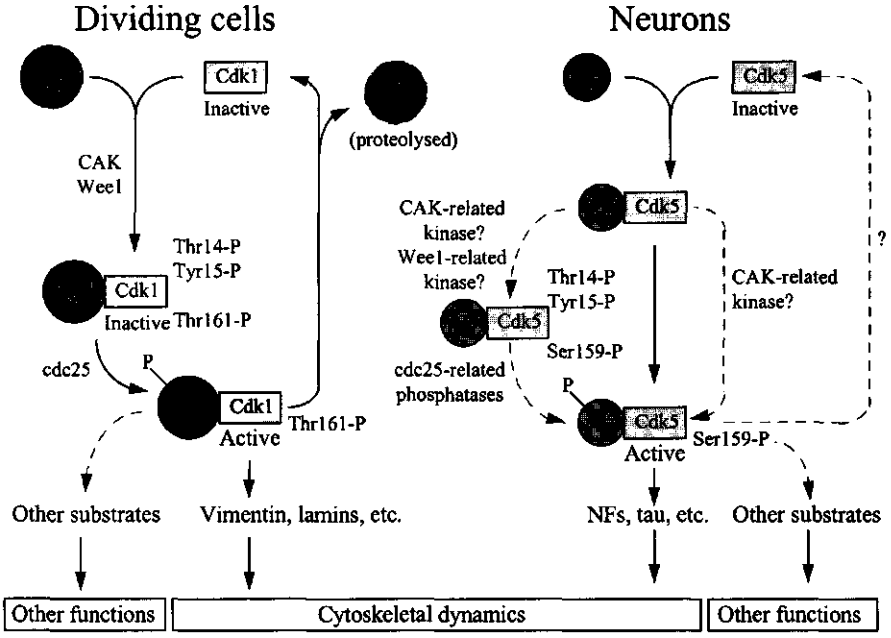


Figure 4. Regulation and function of Cdk1-cyclin B in the cell cycle of higher eukaryotes and Cdk5-p35 in neurons (106).
The active form of Cdk5 is a heterodimer, consisting of a catalytic subunit corresponding to Cdk5 and a neuron-specific regulatory protein, p35. *In vitro*, p35 alone can activate Cdk5, resulting in full kinase activity. However, it is unclear whether or not Cdk5-p35 regulation *in vivo* involves phosphorylation. As with Cdk1-cyclin B, one possibility may involve the phosphorylation of Cdk5 at Ser159 and the subsequent phosphorylation of Thr14 and Tyr15, by putative CAK- and Wee1-related kinases, respectively, followed by the subsequent dephosphorylation of Thr14 and Tyr15 by a cdc25-related phosphatase. Alternatively, activation may involve only Ser159 phosphorylation. Functionally, both Cdk1-cyclin B and Cdk5-p35 appear to phosphorylate cytoskeletal proteins and may ultimately function to control cytoskeletal dynamics. Broken arrows indicate putative pathways.

p35 has no significant homology to cyclins, with only eight out of a hundred identical residues in the central conserved cyclin box, and failing to match four out of five residues highly conserved among cyclins. Only the sequence of 17 residues (residues 222 to 238) LQAVLLTCLYLSYSYSYMG shows any similarity to the equivalent region of the cyclin box consensus sequence, or to other distantly related cyclin-like proteins (113). There is evidence however of the existence of a cyclin fold in this neuronal Cdk5 activator (122). p35 is a highly specific regulatory partner for Cdk5 and

does not activate any other Cdk. It can directly activate bacterially expressed Cdk5 in the absence of phosphorylation by other protein kinases, despite the fact that the surrounding sequences of Ser-159 in Cdk5 (the Thr161-equivalent residue) are similar to other Cdk-s. Moreover, no autophosphorylation of Cdk5 was observed under those conditions. This fact does not exclude the involvement of other proteins in the activation process *in vivo* (113,118,121,123). Recently a p35 isoform has been discovered which can also activate Cdk5. This protein which is called p39 is both necessary and sufficient for neurite outgrowth in hippocampal cells (124). p25, p35 and p39 may represent a subfamily of protein kinase activator proteins distinct from cyclins, which are regulators for a subfamily of Cdk5-related Cdk-s (125-128).

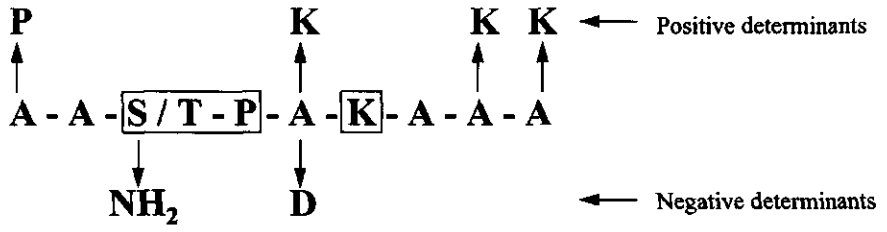


Figure 5. Structural determinants of Cdk5 substrate peptide (127).
Positive or negative determinants are the amino acid residues whose substitution for alanine results in an increase or a decrease in substrate activity of the peptide, respectively. Substitution of any of the boxed amino acid residues by alanine abolishes the substrate activity of the peptide.

The Cdk5/p35 complex is phosphorylating histone H1 (102,118,129), pRb (130) and p35 *in vitro*. The best *in vitro* substrate of the enzyme identified to date is histone H1. A peptide derived from the phosphorylation site sequence of histone H1 is as good a substrate as histone H1 itself (129) suggesting that the substrate activity determinants are restricted to the primary sequence around the phosphorylation sites. In comparison with a peptide called pro-Src, based on the phosphorylation site sequence of the proto-oncogene c-Src (57,58,96) which was used to purify Cdk5 from bovine brain (109), the histone H1 peptide is over 50 fold more efficient as a substrate for Cdk5/p35. Using a set of synthetic peptides systematically modified from the histone H1 peptide, the substrate determinants of the peptide, both positive and negative, have been elucidated as schematically represented in figure 5 (126,127).

Activated Cdk5 kinase phosphorylates a number of cytoskeletal proteins including neurofilament protein NF-M, NF-H (106, 131-137), the neuron-specific microtubule associated protein tau (138-140) and the actin binding protein caldesmon *in vitro* (127). Phosphorylation of cytoskeletal proteins may play an important role in the polymerization and assembly of cytoskeletal elements which, in turn, may effect the growing neurites suggesting that Cdk5 is involved in the growth and maintenance of neurites. The phosphorylation status of tau and NF-H/NF-M on at least some of the putative Cdk5 phosphorylated residues is dynamic: Tau is more heavily phosphorylated during development than in the adult (141-144) and NF-H/NF-M side-arms are largely nonphosphorylated in perikarya but are increasingly more heavily phosphorylated on serine and threonine residues in the carboxyl-terminal regions as NFs move down the axon (145-149).

In addition, Cdk5 phosphorylates neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies (150,151), i.e. diffuse Lewy body disease (dementia), Parkinson's disease (152,153) and amyothrophic lateral sclerosis (154,155), whereas Cdk5 phosphorylates tau protein at sites phosphorylated in Alzheimer's disease (138-140, 156-158). Comparison of tau protein kinase (TPK) II and Cdk5/p35 showed that these two kinases are identical or highly homologous (113,118,158). Cdk5 kinase activity correlates with the extend of differentiation of neuronal cells (99,113,118,159-161) and colocalizes with neurofilament tracts in the axons of neuronal cells in culture. Cdk5/p35 has been demonstrated to play a key role in neurite outgrowth (162) and neuronal migration during neuronal differentiation (163). This makes Cdk5 a strong candidate for catalyzing neurofilament and tau phosphorylation *in vivo* (113,117,164). Whilst Cdk5 activity is important during differentiation, hyperphosphorylation of neurofilaments and tau because of uncontrolled Cdk5 activity may be the cause of several neurodegenerative diseases.

It has been reported that cyclin D (35,165-167) and E (99) bind to Cdk5 but these complexes have never been shown to be enzymatically active. p67 (Munc-18), which is exclusively expressed in neurons and highly enriched in axons, binds to Cdk5 and has a positive effect on its kinase activity (87,161). Recently a novel role for Cdk5 has been demonstrated in regulating myogenesis in the early embryo (168,169). Unlike most other Cdk-cyclin complexes, Cdk5/p35 is not inhibited by the $p21^{CIP1/WAF1}$ (34) or

$p27^{KIP1}$ (35) Cdk inhibitors. There is some evidence, however, that a population of Cdk5/p35 exists as an inactive form within a macromolecular structure, suggesting Cdk5/p35 may bind to inhibitors in the brain (125). Presence of Cdk5 in reproductive organs provide new insight into the possible function of this Cdk during both differentiation and apoptosis (170,171). Activation of Cdk5 by p35 can be blocked by coexpression of dominant-negative mutants of Cdk5. This technique has widely been used in lots of experiments demonstrating the possible roles of Cdk5 (118,128,137,162,168,169,172).

1.8.3 Neurofilaments

Neurofilaments, the neuron-specific intermediate filaments represent the major cytoskeletal organelle in axons in terms of mass and volume (173-175). In mature mammalian brains, neurofilaments are composed of three subunits of different molecular weights which are referred to as low (68 kDa), medium (95 kDa) and high molecular weight (115 kDa) neurofilament proteins: NF-L, NF-M and NF-H respectively (176). Each of the subunits contains an alpha-helical rich conserved central core which is involved in the coiled-coil assembly of the filamentous structure (177). The amino terminal side of the core domain is a conserved globular domain which contains multiple second messenger regulated phosphorylation sites and is therefore suggested to play a role in regulating the filament assembly (178-185). The carboxyl terminal regions of the neurofilament subunits are the regions discriminating the three neurofilament subunits (149,175). NF-M and NF-H but not NF-L contain long carboxyl-terminal extensions which are rich in proline-directed serine/threonine residues (186-189). This is especially true for NF-H which has more than 50 such sites (189). These sites are heavily phosphorylated in neurons. Several studies have shown that NF-H and NF-M can be phosphorylated by Cdk5 or related kinases (100,114,175,190-192). Furthermore functional association of Cdk5 with neurofilaments has been shown (193).

The proline-directed phosphorylation appears to play important roles in neuron-skeleton structure and function. Dephosphorylated NF-H displays specific binding to microtubules *in vitro* whereas the phosphorylated form of the protein does not bind microtubules (136). The phosphorylation is under strict spatial and temporal control. The assembly of neurofilaments occurs in the cell body (194,195); once assembled, the filaments are transported along the axon. During the axonal transport, the subunits NF-

M and NF-H become heavily phosphorylated on the proline-directed sites (196,197). It has been suggested that the phosphorylation depends on the presence of myelin sheath (197). A mouse strain with defective myelin, trembling mouse, has been found to have greatly reduced neurofilament phosphorylation. Abnormality in NF-H and NF-M phosphorylation has also been implicated in Lewy body pathologies (150,151,154). Hypo- or hyperphosphorylation of neurofilaments are important symptomes in neurodegenerative diseases. Malfunction of the Cdk5 kinase may be intrumental in this.

1.8.4 Alzheimer's disease and tau protein

The mechanisms that result in neuronal loss in Alzheimer's disease are not clearly understood. The discovery of several different mutations in the gene for the amyloid precursor protein in some pedigrees with familial Alzheimer's disease has given support to the "amyloid cascade" hypothesis, in which the extracellular deposition of β-amyloid is an early pathogenic event. A consensus has not yet been reached, however, on how deposition of β-amyloid in the brain results in neurodegeneration, and hence dementia (198). On the other hand, neurons containing neurofibrillary tangles are almost certainly unable to function normally and many such neurons die, as is evident from the presence of "ghost" tangles, the residue of dead, tangle-bearing neurons, in the brains of patients with Alzheimer's disease. Neurofibrillary tangles are composed of paired helical filaments (PHFs) and, indeed, PHF pathology is widespread in Alzheimer's disease, giving rise also to neurophil threads and filling many dystrophic neurites that surround the β-amyloid cores of senile plaques. PHFs are composed of hyperphosphorylated tau (199-207), and appear to be the residue of a disintegrated neuronal cytoskeleton because microtubules and neurofilaments are absent from neuronal cytoplasm rich in PHFs (208-211). Most of the phosphorylated residues in PHF-tau are serine and threonine residues that are amino-terminal to a proline residue (200,212-216). In foetal brain, however, tau is also hyperphosphorylated, and the phosphorylation sites identified in foetal tau are also phosphorylated in PHF-tau. This suggests that PHF-tau may arise from a loss of regulatory control of tau phosphorylation in degenerating neurons that results in the reappearance of a foetal phosphorylation pattern (217,218).

Tau protein exists in adult neurons as a set of six isoforms derived from a single gene by alternative mRNA splicing (219,220). One property of tau is to stabilize assembled microtubules (221). This function is likely to be modulated by phosphorylation, as phosphorylated tau has a reduced ability to promote microtubule assembly *in vitro*. Indeed, PHF-tau does not bind to microtubules (222). Consequently, hyperphosphorylation of tau in Alzheimer's disease may lead to disruption of the neuronal cytoskeleton, and this could be a crucial event contributing to neurodegeneration.

A number of kinases, including MAP kinase (223-226), glycogen synthase kinase (GSK)-3α, GSK-3β (159,226-229) and Cdk5 (138-140,156-158), phosphorylate recombinant tau *in vitro* so that it resembles PHF-tau. A detailed study of tau protein phosphorylation in crude brain extracts has led to the suggestion that Cdk5 is the major kinase phosphorylating tau proteins *in vivo* (138,190). Recently is has been proposed that disregulation of various components of the cell cycle is a significant contributor to regionally specific neuronal death in Alzheimer's disease (230).

1.9 *Saccharomyces cerevisiae* as a useful tool in cell cycle research

The long association of yeast with human culture stems from the ability of certain yeast species to produce efficiently two important metabolites, ethanol for alcoholic beverages, and carbon dioxide for the leavening of bread, without simultaneously producing toxic by-products. Commercial exploitation of yeast has led to highly developed, technology-based industries involved in brewing, baking, wine making, distilling, industrial ethanol production and the manufacture of yeast-based foods.

In industrial production it is desirable, and often necessary, to adapt the performance of the organism to manufacturing needs. In yeast these adaptations can be brought about either by physiological conditioning of cultures or through genetic changes, traditionally done by strain selection. Today, DNA manipulation itself presents a short cut to the desired aim.

Recombinant DNA technology, i.e. the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell, has offered the possibility of developing the capabilities of yeast and dramatically extending the

industrial potential of these organisms. Commercial yeast species can be specifically modified to enhance their suitability for existing processes and to generate strains for new manufacturing applications. Furthermore, predefined modifications of this type are not subject to the undesirable side effects frequently encountered in hybrids and mutagenised cells.

Stable inheritance of introduced genes depends on their insertion into yeast chromosomes by recombination, or their linkage to a piece of DNA, a plasmid, which replicates autonomously and segregates to daughter cells. The pool of genetic material available for manipulation is almost unlimited since it is possible not only to remove, modify and reintroduce yeast genes but also to take genes from other organisms and make them function in yeast.

Morphologically, yeasts are unicellular fungi that reproduce by budding or fission. Taxonomically, the budding yeast *Saccharomyces cerevisiae*, generally known as baker's yeast, belongs to the class Hemiascomycetes (ascomycotina that lack ascocarps and ascogenous hyphae), and the family of Saccharomycetaceae of the order Endomycetales. The biochemistry and molecular biology of baker's yeast have been studied in detail over the years, although many aspects of its metabolism and life cycle remain unclear. However, as baker's yeast has been characterized better than any other fungus, the techniques of genetic engineering are especially well suited for the manipulation of this yeast (231).

1.9.1 The yeast-two-hybrid system

The two-hybrid system uses the efficacy of yeast genetic assays to identify protein-protein interactions. It permits the rapid cloning of genes encoding products that interact with a given protein of interest. While the current assay is carried out in yeast, genes from any organism may be used in the screen.

The conceptual basis for the two-hybrid system, as reported by Fields and Song (232), was established by studies on the regulation of eukaryotic transcription. Certain transcription factors, such as the yeast GAL4 and GCN4 proteins, were shown to comprise two distinct and separable domains: a site-specific DNA-binding domain, and an acidic region that is required for transcriptional activation. Functionally competent chimeric proteins were generated that consist of the DNA-binding region of one factor and the activation domain of another. In addition, heterodimeric complexes,

consisting of an activation domain and a DNA-binding domain encoded by separate genes, could activate transcription.

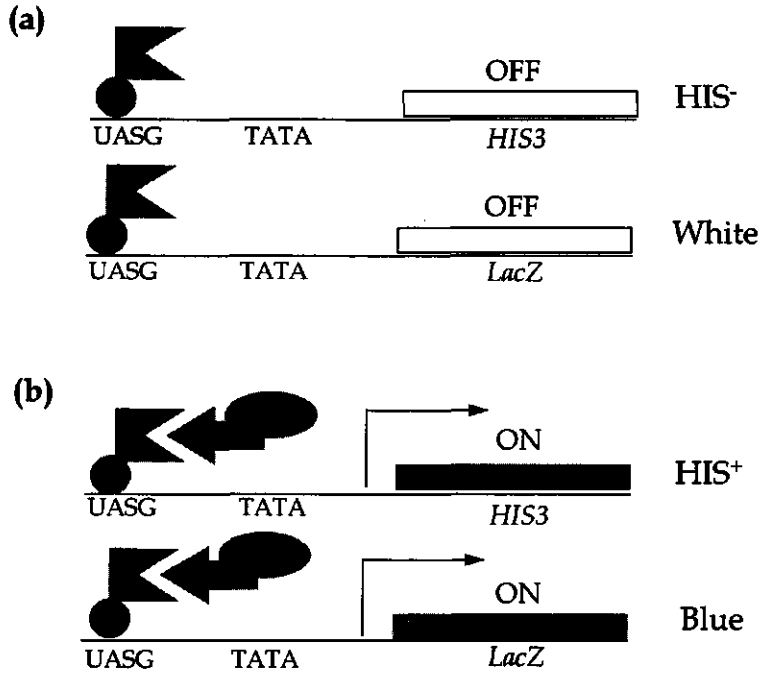


Figure 6. The basic two-hybrid system using a dual selection/screen (234)

(a) A protein (A) fused to the DNA-binding domain of LEXA is expressed in a yeast strain carrying two reporter genes, *HIS3* and *lacZ*, under the control of the LEXA promoter. Since the hybrid protein lacks transcriptional activation potential, the reporter genes are not transcribed and the strain remains His^- and does not hydrolyse the chromogenic indicator X-Gal. **(b)** A second plasmid that expresses a hybrid protein consisting of a protein (B) fused to the GAL4 transcriptional activation domain (GAD) is introduced into the strain. If protein B and protein A physically associate, the GAD will be localized to the promoters of the reporter genes, thereby activating their transcription and producing *HIS3* and *lacZ* transcripts.

In its current form, the yeast-based two-hybrid system utilizes hybrid genes to detect protein-protein interactions via the activation of reporter-gene expression. This gene expression occurs as a result of reconstitution of a functional transcription factor caused by the association of two hybrid proteins. Typically, a gene encoding a protein of interest is fused to the DNA-binding domain of a well-characterized transcription factor (e.g. GAL4, LEXA) while another gene is fused to a transcriptional activation domain (GAL4, VP16) (233). The activation-domain hybrid is introduced into a yeast

strain expressing the DNA-binding-domain hybrid and the resulting strain is assayed for association of the two proteins. A productive interaction between the proteins of interest localizes the activation domain to the DNA-binding domain. This results in transcription of an adjacent reporter gene, typically a nutritional marker, which provides a scorable phenotype or *lacZ* (Fig. 6).

There are several advantages of dual screen/selection assays. First, the ability to select for nutritional prototrophy greatly increases the number of library transformants that can be screened for *lacZ* activity. This is particularly important when large, high-quality libraries are available and low-abundance binding partners are sought. In addition, the requirement for expression of two reporter genes with different promoter contexts significantly reduces the number of false-positive signals since separate transciptional events must be maintained at distinct chromosomal loci (234).

Searches have been carried out with a large number of proteins of disparate subcellular location and function. Cytoplasmic, nuclear and membrane-associated proteins from a variety of species have been used successfully. A few examples include interactions between yeast RAP1 and RIF1 (235), $p21^{CIP1/WAF1}$ with Cdk2 (33) and $p16^{INK4A}$ with Cdk4 (236).

Library screens can select for proteins that are initially found to activate reporter-gene expression in the presence of a specific DNA-binding-domain hybrid protein, but that are later observed to promote transcription in concert with a number of different hybrid partners. While the biophysical basis of these 'false-positive' interactions is not clear, some proteins may contain regions with surfaces that have low affinities for many different proteins, for example, large hydrophobic surfaces. These proteins may form complexes with a number of other proteins that are stable enough to result in a recognizable phenotype.

A rapid genetic assay has been designed to eliminate false positives isolated in the system (33). Briefly, a collection of false-positive detector strains that express different DNA-binding-domain hybrids are used in mating assays with strains expressing putative interactors. A nonspecific hybrid interactor will generally activate reporter-gene expression in one or more of the false-positive detectors. This allows many activation-domain hybrids to be quickly screened for specific interaction with a given target protein (Fig. 7).

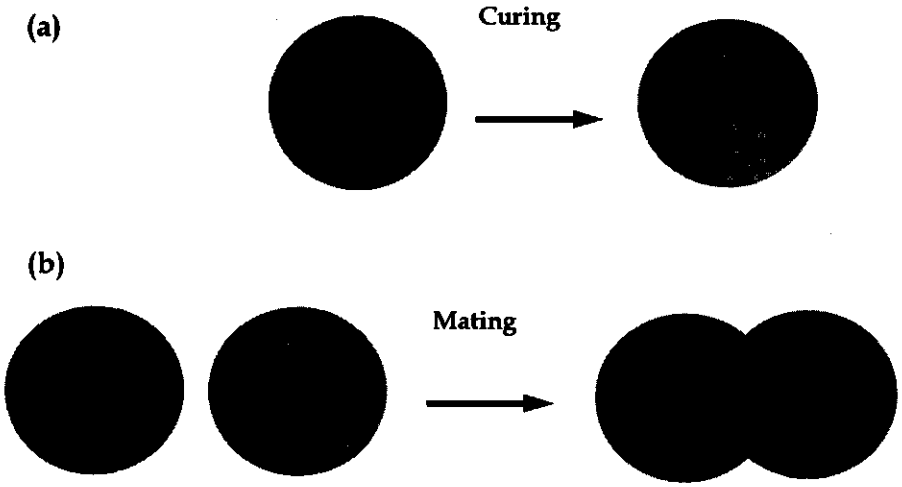


Figure 7. Curing and mating of the positives

(a) The positive (strain L40, Mat a) is cured from the pLEXA plasmid by selecting for the pGAD plasmid only using the appropriate auxotrophic markers. The cured positive should not express the reporter genes **(b)** This cured positive is mated with strains of the opposing sex (strain AMR70, Mat α) containing the pLEXA plasmid expressing putative interactors. The resulting diploids are assayed for nonspecific hybrid interactions in order to eliminate false-positives.

Another method to generate supporting evidence that a given interaction is real involves testing the ability of hybrid proteins to associate when the target protein is switched to the activation domain and the library-derived protein is fused to the DNA-binding domain. Observed interaction between reciprocal hybrids is a strong indication of true physical association. However, failure to detect association in this assay may not reflect a false-positive result since different fusion proteins probably have different properties (i.e. conformation and stability) that might prevent productive association. Importantly, the reciprocal transfer of sequences between the DNA-binding domain and the activation domain might not be experimentally trivial (234).

1.10 Outline of the thesis

In order to understand the different roles the cyclin dependent kinases could have in either proliferating or differentiating human cells we investigated the roles of both Cdk4 and Cdk5. The Cdk4 kinase phosphorylates the Rb protein and therefore plays a very important role in the G_1/S transition of the cell cycle. Whether a proliferating cell would undergo differentiation or not could thus be dependent on Cdk4 activity. Cdk5 activity on the other hand has been shown only in differentiating cells although Cdk5 is present in proliferating cells as well. With different activators Cdk5 could play different roles in cycling cells. Although D (35,165-167) and E (99) type cyclins are able to bind to Cdk5 no activity of the complex has been shown to date. It would be interesting to speculate on the requirement of Cdk4 for the onset of proliferation and on Cdk5 for the onset of differentiation. If this would be true the question arises why Cdk4 is expressed in differentiating cells and Cdk5 in proliferating cells (237). There is definitely much more to the fate of a cell than what is known till date.
Aberrant activity of both cyclin dependent kinases due to overexpression of the catalytic subunit or its activating partner and misfunction of specific Cdk inhibitors may lead to hyperplasia in the case of Cdk4 and apoptosis in the case of Cdk5 linking malignant function of both kinases to cancer and neurodegenerative diseases respectively.

Since $p16^{INK4A}$ is missing or mutated in a lot of tumours and the Cdk4/cyclin D complex upregulated (70), molecules mimicing $p16^{INK4A}$ or other molecules specifically inhibiting Cdk4 activity could be potential antineoplastic drugs. If these compounds specifically inhibit Cdk4 activity, the malignant cells could be stopped at the G_1/S checkpoint without interfering with other processes in the cell cycle. The arrested cells may be killed off or forced to undergo apoptosis by chemotherapeutic agents or irradiation enhancing the therapeutic effects of the currently used anti-tumour agents (1,5). In this context, the Cdk inhibitor flavopiridol is entering phase 3 studies as an anti-tumour agent. However flavopiridol is not a Cdk4 specific antagonist (Table 2). Other compounds are still under investigation.
Chapter 2 of this thesis shows a method developed in yeast to screen possible antineoplastic agents. Cdk4 is expressed in the yeast *S. cerevisiae* under the control of

the *GAL1-GAL10* promoter and inhibits cell growth when the yeast is grown on galactose. Known Cdk4 inhibitors like $p16^{INK4A}$ and flavopiridol restore cell growth.

Cdk5 is present in both cycling and non-cycling cells and has been found in many tissues (102). The highest concentration however is found in neuronal cells. p35 which is expressed solely in the brain can activate Cdk5. Cdk5 is a likely candidate to catalyze the *in vivo* phosphorylation of the neurofilament proteins and tau. Cdk5 phosphorylates neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies. In nerve cells of patients suffering from Alzheimer's disease tau protein is hyperphosphorylated at the Cdk5 phosphorylation sites (106,127). Cdk5 activity is suggested to be involved in terminal neuron and muscle differentiation.
In order to investigate about this interesting protein a search for new protein interactions in proliferating cells using the yeast-two-hybrid system was performed. A HeLa cervical carcinoma cDNA library was screened and DNA binding protein dbpA and 60S ribosomal protein L34 were found to interact with Cdk5. DbpA and L34 are not only binding to Cdk5 but also to Cdk4 and not to Cdk2 or Cdk1. Kinase assays show that both proteins inhibit the kinase activities of both Cdk5/p35 and Cdk4/cyclin D1 kinases and not that of Cdk2/cyclin A. These results are depicted in chapters 3 and 4.
In chapter 5 an endogenously present kinase-defective Cdk5 isoform is presented. This protein lacks subdomain VI, a domain highly conserved amongst the serine/threonine protein kinases. The isoform has lost its ability of autophosphorylation and is very weakly active. Furthermore it can compete with wildtype Cdk5 for binding to p35.

In this thesis a screen is presented to search for Cdk4 antagonists. Furthermore the Cdk5 interacting proteins presented and the Cdk5 isoform could be tested for their ability to regulate the Cdk5 kinase *in vivo*.

References

1. Hartwell, L.H. and Kastan M.B. (1994) Cell Cycle Control and Cancer. *Science* **266**, 1821-1828.
2. Murray, A. and Hunt, T. (1993) The Cell Cycle, Oxford University Press, Oxford, U.K.
3. Hartwell, L. and Weinert, T. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**, 629-634.
4. Elledge, S.J. and Harper J.W. (1994) Cdk inhibitors: on the threshold of checkpoints and development. *Curr. Opin. Cell Biol.***6**, 847-852.
5. Meijer, L., Borgne, A., Mulner, O., Chong, J.P.J., Blow, J.J., Inagaki, N., Inagaki, M., Delcros, J.-G. and Moulinoux, J.-P. (1997) Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243**, 527-536.
6. Morgan, D.O. (1995) Principles of CDK regulation. *Nature* **374**, 131-134.
7. Pines, J. (1994) Arresting developments in cell-cycle control. *TIBS* **19**, 143-145.
8. Weinberg, R.A. (1995) The Retinoblastoma Protein and Cell Cycle Control. *Cell* **81**, 323-330.
9. Ross, M.E. and Risken, M. (1994) MN20, a D2 Cyclin Found in Brain, Is Implicated in Neural Differentiation. *J. Neurosci.* **14**, 6384-6391.
10. Heichman, K.A. and Roberts, J.M. (1994) Rules to replicate by. *Cell* **79**, 557-562.
11. Ohtsubo, M. and Roberts, J.M. (1993) Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science* **259**, 1908-1912.
12. Sherr, C.J. (1993) Mammalian G1 cyclins. *Cell* **73**, 1059-1065.
13. Sherr, C.J. (1994) G1 phase progression: cycling on cue. *Cell* **79**, 551-555.
14. King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Mitosis in transition. *Cell* **79**, 563-571
15. Solomon, M. Lee, T. and Kirschner, M.W. (1992) The role of phosphorylation in $p34^{CDC2}$ activation: identification of an activating kinase. *Mol. Biol. Cell* **3**, 13-27.
16. 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 Thr 161 and its homologues. *EMBO J.* **12**, 3111-3121.

17. Poon, R.Y.C., Yamashita, K., Adamczewski, J.P., Hunt, T. and Shuttleworth, J. (1993) The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{cdk2}$ and $p34^{cdc2}$. *EMBO J.* **12**, 3123-3132.
18. Fischer, R.P. and Morgan D.O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78**, 713-724.
19. Poon, R.Y.C. and Hunter, T. (1995) Cell regulation. Innocent bystanders or chosen collaborators? *Curr. Biol.* **5**, 1243-1247.
20. Devault, A., Martinez, A.-M., Fesquet, D., Labbe, J.-C., Morin, N., Tassan, J.P., Nigg, E.A., Cavadore, J.-C. and Doree, M. (1995) MAT1 ('menage a trois') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and Xenopus CAK. *EMBO J.* 14, 5027-5036.
21. Poon, R.Y.C. and Hunter, T. (1995) Dephosphorylation of Cdk2 Thr160 by the cyclin-dependent kinase-interacting phosphatase KAP in the absence of cyclin. *Science* **270**, 90-93.
22. Parker, L.L. and Piwnica-Worms, H. (1992) Inactivation of the $p34^{CDC2}$ cyclin B complex by the human Weel tyrosine kinase. *Science* **257**, 1955-1957.
23. Watanabe, N., Broome, M. and Hunter, T. (1995) Regulation of the human Wee1Hu CDK tyrosine 15-kinase during the cell cycle. *EMBO J.* **14**, 1878-1891.
24. Meuller, 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.
25. Dunphy, W.G. and Kumagai, A. (1991) The cdc25 protein contains an intrinsic phosphatase activity. *Cell* **167**, 189-196.
26. 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 $p34^{cdc2}$. *Cell* **67**, 197-212.
27. Strausfeld, U., Labbe, J.C:, Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russel, P. and Doree, M. (1991) Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex in vitro by human CDC25 protein. *Nature* **351**, 242-244.

28. Sebastian, B., Kakizuka, A. and Hunter, T. (1993) Cdc25M2 activation of cyclin-dependent kinase by dephosphorylation of threonine-14 and tyrosine-15. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3521-3524.

29. Peter, M. and Herskowitz, I. (1994) Joining the Complex: Cyclin-Dependent Kinase Inhibitory Proteins and the Cell Cycle. *Cell* **79**, 181-184.

30. Lee, E.Y.-H.P., Hu, N., Yuan, S.-S.F., Cox, L.A., Bradley, A., Lee, W.-H. and Herrup, K. (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes & Dev.* **8**, 2008-2021.

31. Moreno, S. and Nurse, P. (1994) Regulation of progression through the G1 phase of the cell cycle by the rum+ gene. *Nature* **367**, 236-242.

32. Moreno, S., Labib, K., Correa, J. and Nurse, P. (1994)) Regulation of the cell cycle timing of Start in fission yeast by the rum+ gene. *J.Cell Sci.* (Suppl.) **18**, 63-68.

33. 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**, 805-816.

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

35. Lee, M.H., Nikolic, M., Baptista, C.A., Lai, E., Tsai, L.H. and Masagué, J. (1996) The brain-specific activator p35 allows Cdk5 to escape inhibition by $p27^{KIP1}$ in neurons. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3259-3263.

36. 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.

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

38. 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**, 701-704.

39. 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 $p16^{INK4/MTS1}$- and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes & Dev.***8**, 2939-2952.

40. 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**, 2672-2681.

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

42. Sherr, C.J. and Roberts, J.M. (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes & Dev.* **9**, 1149-1163.

43. Pines, J. (1995) Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem. J.* **308**, 697-711.

44. 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-β-induced G_1 cell-cycle arrest. *Genes & Dev.* **9**, 402-217.

45. Ewen, M.E. (1996) p53-dependent repression of cdk4 synthesis in transforming growth factor-beta-induced G1 cell cycle arrest. *J. Lab. Clin. Med.* **128**, 355-360.

46. Ewen, M.E. and Miller, S.J. (1996) p53 and translational control. *Biochim. Biophys. Acta* **1242**, 181-184.

47. Polyak, K., Kato, J.-Y., Solomon, M.J., Sherr, C.J., Massagué, J., Roberts, J.M. and Koff, A. (1994) $p27^{Kip1}$, a cyclin-cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes & Dev.* **8**, 9-22.

48. Rudolph, B., Saffrich, R., Zwicker, J., Henglein, B., Müller, R., Ansorge, W. and Eilers, M. (1996) Activation of cyclin-dependent kinases by Myc mediates induction of cyclin A, but not apoptosis. *EMBO J.* **15**, 3065-3076.

49. Hannon, G.J. and Beach, D. (1994) $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371**, 257-261.

50. Serrano, M., Gómez-Lahoz, E., DePinho, R.A., Beach, D. and Bar-Sagi, D. (1995) Inhibition of Ras-Induced Proliferation and Cellular Transformation by $p16^{INK4}$. *Science* **267**, 249-252.

51. Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D. and DePinho, R.A. (1996) Role of the *INK4a* Locus in Tumor Suppression and Cell Mortality. *Cell* **85**, 27-37.

52. Hanks, S.K. and Hunter, T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576-596.

53. 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.

54. Brown, N.R., Noble, M., Endicott, J.A., Garman, E.F., Wakatsuki, S., Mitchell, E., Rasmussen, B., Hunt, T. and Johnson, L.N. (1995) The crystal structure of cyclin A. *Structure* **3**, 1235-1247.

55. Jeffrey, P.D., Russo, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massagué, J. and Pavletich, N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* **376**, 313-320.

56. Russo, A.A., Jeffrey, P.D. and Pavletich, N.P. (1996) Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nat. Struct. Biol.* **3**, 696-700.

57. Tang, D., Lee, K.-Y., Qi, Z., Matsuura, I. and Wang, J.H. (1996) Neuronal Cdc2-like kinase: from cell cycle to neuronal function. *Biochem. Cell Biol.* **74**, 419-429.

58. Shenoy, S., Choi, J.-K., Bagrodia, S., Copeland, T.D., Maller, J.L. and Shalloway, D. (1989) Purified maturation promoting factor phosphorylates pp60c-src at the sites phosphorylated during fibroblast mitosis. *Cell*, **57**, 763-744.

59. Ewen, M.E. (1994) The cell cycle and the retinoblastoma protein family. *Cancer Metastasis Rev.* **13**, 45-66.

60. Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A. and Weinberg, R.A. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**, 499-511.

61. Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J.-Y. and Livingston D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**, 487-497.

62. Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I. and Weinberg, R.A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**, 993-1006.

63. Kato, J.-Y., Matsushime, H., Hiebert, S.W., Ewen, M.E. and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase cdk4. *Genes & Dev.* **7**, 331-342.

64. Kim, T.A., Lim, J. Ota, S., Raja, S., Rogers, R., Rivnay, B., Avraham, H. and Avraham, S. (1998) NRP/B, a Novel Nuclear Matrix Protein, Associates With p110^{RB} and Is Involved in Neuronal Differentiation. *J. Cell Biology* **141**, 553-566.

65. Helin, K., Wu, C.L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C. and Harlow, E. (1993) Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes & Dev.* **7**, 1850-1861.

66. Lees, J.A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N. and Helin K. (1993) The retinoblastoma protein binds to a family of E2F transcription factors. *Mol. Cell. Biol.* **13**, 7813-7825.

67. Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A. and Heimbrook, D.C. (1993) Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. *Mol. Cell. Biol.* **13**, 7802-7812.

68. Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M. Livingston, D., Dyson, N. and Harlow, E. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes & Dev.* **7**, 1111-1125.

69. Sellers, W.R., Novitch, B.G., Miyake, S., Heith, A., Otterson, G.A., Kaye, F.J., Lassar, A.B. and Kaelin, W.G.Jr. (1998) Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. *Genes & Dev.* **12**, 95-106.

70. Kamb, A. (1995) Cell-cycle regulators and cancer. *TIG* **11**, 136-140.

71. Hartwell, L. (1992) Defects in cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* **71**, 543-546.

72. Hinds, P.W., Dowdy, S.F., Eaton, E.N., Arnold, A. and Weinberg, R.A. (1994) Function of a human cyclin gene as an oncogene. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 709-713.

73. Leach, F.S., Elledge, S.J., Sherr, C.J., Willson, J.K.V., Markowitz, S., Kinzler, K.W. and Vogelstein, B. (1993) Amplification of cyclin genes in colorectal carcinomas. *Cancer Res.* **53**, 1986-1989.

74. Motokura, T. and Arnold, A. (1993) Cyclin D and oncogenesis. *Curr. Opin. Genet. Dev.* **3**, 5-10.

75. Khatib, Z.A., Matsushime, H., Valentine, M., Shapiro, D.N., Sherr, C.J. and Look, A.T. (1993) Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. *Cancer Res.* **53**, 5535-5541.

76. Reifenberger, G., Reifenberger, J., Ichimura, K., Meltzer, P.S. and Collins V.P. (1994) Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: Preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. *Cancer Res.* **54**, 4299-4303.

77. Keyomarsi, K. and Pardee, A.B. (1993) Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1112-1116.

78. Eliyahu, D., Michalovitz, D. and Oren, M. (1985) Overproduction of p53 antigen makes established cells highly tumorigenetic. *Nature* **316**, 158-160.

79. Gerwin, B.I., Spillare, E., Forrester, K., Lehman, T.A., Kispert, J., Welsh, J.A., Pfeifer, A.M.A., Lechner, J.F., Baker, S.J., Vogelstein, B. and Harris, C.C. (1992) Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β_1. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2759-2763.

80. Kern, S.E., Pietenpol, J.A., Thiagaligam, S., Seymour, A., Kinzler, K.W. and Vogelstein, B. (1992) Oncogenic forms of p53 inhibit p53 regulated gene expression. *Science* **256**, 827-830.

81. Momand, J., Zambetti, G.P., Olson, D.C., George, D. and Levine, A.J. (1992)) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell* **69**, 1237-1245.

82. Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes & Dev.* **7**, 1126-1132.

83. Yang, Z.-Y., Perkins, N.D., Ohno, T., Nabel, E.G. and Nabel, G.J. (1995) The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity *in vivo*. *Nature Med.* **1**, 1052-1056.

84. Nishio, K., Ishida, T., Arioka, H., Kurokawa, H., Fukuoka, K., Nomoto, T., Fukumoto, H., Yokote, H. and Saijo, N. (1996) Antitumor effects of butyrolactone I, a selective cdc2 kinase inhibitor, on human lung cancer cell lines. *Anticancer Res.* **16**, 3387-3395.

85. Schulze-Gahmen, U., Brandsen, J., Jones, H.D., Morgan, D.O., Meijer, L., Veseley, J. and Kim, S.H. (1995) Multiple modes of ligand recognition: crystal structures of cyclin-dependent protein kinase 2 in complex with ATP and two inhibitors, olomoucine and isopentenyladenine. *Proteins* **22**, 378-391.

86. Veselý, J., Havlicek, L. Strnad, M., Blow, J.J., Donella-Deana, A., Pinna, L., Letham, D.S., Kato, J.-Y., Detivaud, L., Leclerc, S. and Meijer, L. (1994) Inhibition of cyclin-dependent kinases by purine analogues. *Eur. J. Biochem.* **224**, 771-786.

87. Veeranna, Shetty, K.T., Amin, N., Grant, P., Albers, R.W. and Pant, H.C. (1996) Inhibition of Neuronal Cyclin-Dependent Kinase-5 by Staurosporine and Purine Analogs Is Independent of Activation by Munc-18. *Neurochem. Res.* **21**, 629-636.

88. Orr, M.S., Reinhold, W., Yu, L., Schreiber-Agus, N. and O'Connor, P.M. (1998) An Important Role for the Retinoblastoma Protein in Staurosporine-induced G_1 Arrest in Murine Embryonic Fibroblasts. *J. Biol. Chem.* **273**, 3803-3807.

89. De Azevedo, W. F., Leclerc, S., Meijer, L, Havlicek, L., Strnad, M. and Kim, S.-H. (1997) Inhibition of cyclin-dependent kinases by purine analogues Crystal structure of human cdk2 complexed with roscovitine. *Eur. J. Biochem.* **243**, 518-526.

90. Meijer, L. (1996) Chemical inhibitors of cyclin-dependent kinases. *Trends in Cell Biol.* **6**, 393-397.

91. Tassan, J.P., Jaquenoud, M., Fry, A.M., Frutiger, S., Hughes, G.J. and Nigg, E.A. (1995) In vitro assembly of a functional human CDK7-cyclin H complex requires MAT1, a novel 36 kDa RING finger protein. *EMBO J.* **14**, 5608-5617.

92. 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**, 8871-8875.

93. Zindy, F., Soares, H., Herzog, K.H., Morgan, J., Sherr, C.J. and Roussel, M.F. (1997) Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development. *Cell Growth Differ.* **8**, 1139-1150.

94. Okano, H.J., Pfaff, D.W. and Gibbs, R.B. (1993) RB and Cdc2 expression in brain: correlations with 3H-thymidine incorporation and neurogenesis. *J. Neurosci.* **13**, 2930-2938.

95. Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E. and Fattaey, A. (1992) A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. *Cell* **70**, 337-350.

96. Martinez, R., Mathey-Prevot, B., Bernards, A. and Baltimore, D. (1987) Neuronal pp60$^{c\text{-}src}$ contains a six-amino acid insertion relative to its non-neuronal counterpart. *Science*, **237**, 411-415.

97. Tamaru, T., Okada, M. and Nakagawa, H. (1994) Differential expression of D type cyclins during neuronal maturation. *Neurosci. Lett.* **168**, 229-232.

98. Tamaru, T., Trigun, S.K., Okada, M. and Nakagawa, H. (1993) Identification of cells expressing a D type G1 cyclin in matured brain: implication for its role in neuronal function. *Neurosci. Lett.* **153**, 169-172.

99. Miyajima, M., Nornes, H.O. and Neuman, T. (1995) Cyclin E is expressed in neurons and forms complexes with Cdk5. *NeuroReport* **6**, 1130-1132.

100. Hellmich, M.R., Pant, H.P., Wada, E. and Battey, J.F. (1992) Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10867-10871.

101. Ino, H., Ishizuka, T., Chiba. T. and Tatibana, M. (1994) Expression of CDK5 (PSSALRE kinase), a neuronal cdc2-related protein kinase, in the mature and developing mouse central and peripheral nervous systems. *Brain Res.* **661**, 196-206.

102. Tsai, L.-H., Takahashi, T., Caviness, V.S.Jr. and Harlow, E. (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119**, 1029-1040.

103. Hayes, T.E., Valtz, N.L. and McKay, R.D. (1991) Downregulation of CDC2 upon terminal differentiation of neurons. *New Biol.* **3**, 259-269.

104. Hopkins, S.J. and Rothwell, N.J. (1995) Cytokines and the nervous system I: expression and recognition. *TINS* **18**, 83-88.

105. 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**, 2909-2917.

106. Lew, J. and Wang, J.H. (1995) Neuronal cdc-2-like kinase. *TIBS* **20**, 33-37.

107. Demetrick, D.J., Zhang, H. and Beach, D.H. (1994) Chromosomal mapping of human CDK2, CDK4, and CDK5 cell cycle genes. *Cytogenet. Cell. Genet.* **66**, 72-74.

108. Ohshima, T., Nagle, J.W., Pant, H.C., Joshi, J.B., Kozak, C.A., Brady, R.O. and Kulkarni, A.B. (1995) Molecular cloning and chromosomal mapping of the mouse cyclin-dependent kinase 5 gene. *Genomics* **28**, 585-588.

109. Lew, J., Beaudette, K., Litwin, C.M. and Wang, J.H. (1992) Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J. Biol. Chem.* **267**, 13383-13390.

110. Azzi, L., Meijer, L., Ostvold, A.-C., Lew, J. and Wang, J.H. (1994) Purification of a 15-kDa cdk4- and cdk5-binding protein. *J. Biol. Chem.* **269**, 13279-12288.

111. Ino, H. and Chiba, T. (1996) Intracellular localization of cyclin-dependent kinase 5 (CDK5) in mouse neuron: CDK5 is located in both nucleus and cytoplasm. *Brain Res.* **732**, 179-185.

112. Lew, J., Winkfein, R.J., Paudel, H.K. and Wang, J.H. (1992) Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. *J. Biol. Chem.* **267**, 25922-25926.

113. Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R.J., Aebersold, R., Hunt, T., Wang, J.H. (1994)) A brain-specific activator of cyclin-dependent kinase 5. *Nature* **371**, 423-426.

114. Shetty, K.T., Link, W.T. and Pant, H.C. (1993) Cdc2-like kinase from rat spinal cord specifically phosphorylates KSPXK motifs in Neurofilament proteins: Isolation and characterization. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6844-6848.

115. Matsushita, M., Matsui, H., Itano, T., Tomizawa, K., Tokuda, M., Suwaki, H., Wang, J.H. and Hatase O. (1995) Developmental changes of cyclin dependent kinase 5 subcellular localization in the rat cerebellum. *Neuroreport* **6**, 1267-1270.

116. Dellale, I, Bhide, P.G., Caviness, V.S.Jr. and Tsai, L.H. (1997) Temporal and spatial patterns of expression of p35, a regolatory subunit of cyclin-dependent kinase 5, in the nervous system of the mouse. *J. Neurocytol.* **26**, 283-296.

117. Tomizawa, K., Matsui, H., Matsushita, M. Lew, J., Tokuda, M., Itano, T., Konishi, R., Wang, J.H. and Hatase, O. (1996) Localization and developmental changes in the neuron-specific cyclin-dependent kinase 5 activator (p35nck5a) in the rat brain. *Neuroscience* **74**, 519-529.

118. 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**, 419-423.

119. 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**, 203.208.

120. Uchida, T., Ishiguro, K., Ohnuma, J., Takamatsu, M., Yonekura, S. and Imahori, K. (1994) Precursor of cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental change in brain. *FEBS Lett.* **355**, 35-40.

121. 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 active fragment of the brain-specific activator. *J. Biol. Chem.* **270**, 10847-10854.

122. Tang, D., Chun, A.C.S., Zhang, M. and Wang, J.H. (1997) Cyclin-dependent kinase 5 (Cdk5) activation domain of neuronal Cdk5 activator. Evidence of the existence of cyclin fold in neuronal Cdk5a activator. *J. Biol. Chem.* **272**, 12318-12327.

123. Poon, R.Y.C., Lew, J. and Hunter, T. (1997) Identification of functional domains in the neuronal Cdk5 activator protein. *J. Biol. Chem.* **272**, 5703-5708.

124. Tang, D., Yeung, J. Lee, K.-Y, Matsushita, M. Masui, H. Tomizawa, K., Hatase, O. and Wang, J.H. (1995) An isoform of the neuronal cyclin-dependent kinase 5 (cdk5) activator. *J. Biol. Chem.* **270**, 26897-26903.

125. 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**, 1538-1542.

126. Lew, J., Qi, Z., Huang, Q.-Q., Paudel, H., Matsuura, I., Matsushita, M., Zhu, X. and Wang, J.H. (1995) Structure, function, and regulation of neuronal Cdc2-like protien kinase. *Neurobiol. Aging* **16**, 263-278; discussion 268-270.

127. Tang, D. and Wang, J.H. (1996) Structure, function, and regulation of neuronal Cdc2-like protien kinase. *Prog. Cell Cycle Res.* **2**, 205-216.

128. Xiong, W., Pestell, R. and Rosner, M.R. (1997) Role of cyclins in neuronal differentiation of immortalized hippocampal cells. *Mol. Cell. Biol.* **17**, 6585-6597.

129. 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**, 20825-20830.

130. Lee, K.-Y., Helbing, C.C., Choi, K.-S., Johnston, R.N. and Wang, J.H. (1997) Neuronal Cdc2-like Kinase (Nclk) Binds and Phosphorylates the Retinoblastoma Protein. *J. Biol. Chem.* **272**, 5622-5626.

131. Bajaj, N.P.S. and Miller, C.C.J. (1997) Phosphorylation of neurofilament heavy-chain side-arm fragments by cyclin-dependent kinase-5 and glycogen synthase kinase-3α in transfected cells. *J. Neurochem.* **69**, 737-743.

132. Guidato, S., Bajaj, N.P.S. and Miller, C.C.J. (1996) Cellular phosphorylation of neurofilament heavy-chain by cyclin-dependent kinase-5 masks the epitope for monoclonal antibody N52. *Neurosci. Lett.* **217**, 1-4.

133. Guidato, S., Tsai, L.-H., Woodgett, J. and Miller, C.C.J. (1996) Differential cellular phosphorylation of neurofilament heavy side-arms by glycogen synthase kinase-3 and cyclin-dependent kinase-5. *J. Neurochem.* **66**, 1698-1706.

134. Hisanaga, S., Ishiguro, K., Uchida, T., Okumura, E., Okano, T. and Kishimoto, T. (1993) Tau protein kinase II has a similar characteristic to cdc2 kinase for phosphorylating neurofilament proteins. *J. Biol. Chem.* **268**, 15056-15060.

135. Hisanaga, S., Uchiyama, M., Hosoi, T., Yamada, K., Honma, N., Ishiguro, K., Uchida, T., Dahl, D., Ohsumi, K. and Kishimoto, T. (1995) Porcine brain neurofilament-H tail domain kinase: its identification as cdk5/p26 complex and comparison with cdc2/cyclin B kinase. *Cell Motil. Cytoskel.* **31**, 283-297.

136. Miyasaka, H., Okabe, S., Ishiguro, K., Uchida, T. and Hirokawa, N. (1993) Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by τ protein kinase II. *J. Biol. Chem.* **268**, 22695-22702.

137. Sun, D., Leung, C.L. and Liem, R.K.H. (1996) Phosphorylation of the high molecular weight neurofilament protein (NF-H) by Cdk5 and p35. *J. Biol. Chem.* **271**, 14245-14251.

138. Baumann, K., Mandelkow, E.M., Biernat, J., Piwnica-Worms, H, and Mandelkow, E. (1993) Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. *FEBS Lett.* **336**, 417-424.

139. 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**, 23512-23518.

140. Sengupta, A., Wu, Q., Grundke-Iqbal, I, Iqbal, K. and Singh, T.J. (1997) Potention of GSK-3-catalyzed Alzheimer-like phosphorylation of human tau by cdk5. *Mol. Cell. Biochem.* **167**, 99-105.

141. Bramblett, G.T., Goedert, M., Jakes, R., Merrick, S.E., Trojanowski, J.Q. and Lee, V.M.-Y. (1993) Abnormal tau phosphorylation at ser^{396} in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron* **10**, 1089-1099.

142. Brion J.-P., Smith, C., Couck, A.-M., Gallo, J.-M. and Anderton, B.H. (1993) Developmental changes in τ phosphorylation: fetal τ is transiently phosphorylated in a manner similar to paired helical filament-τ characteristic of Alzheimer's disease. *J. Neurochem.* **61**, 2071-2080.

143. Goedert, M., Jakes, R., Crowther, R.A., Six, J., Lübke, U., Vandermeeren, M., Cras, P., Trojanowski, J.Q. and Lee V.M.-Y. (1993) The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5066-5070.

144. Mawal-Dewan, M., Henley, J., Van de Voorde, A., Trojanowski, J.Q. and Lee, V.M.-Y. (1994) The phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases. *J. Biol. Chem.* **269**, 30981-30987.

145. Carden, M.J., Trojanowski, J.Q., Schlaepfer, W.W. and Lee V.M.-Y. (1987) Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci.* **7**, 3489-3504.

146. Jones, S., and Williams, R. (1982) Phosphate content of mammalian neurofilaments. *J. Biol. Chem.* **257**, 9902-9905.

147. Julien, J.P. and Mushinski, W.E. (1983) The distribution of phosphorylation sites among identified proteolytic fragments of mammalian neurofilaments. *J. Biol. Chem.* **258**, 4019-4025.

148. Nixon, R.A., Paskevich P.A., Sihag, R.K. and Thayer C. (1994) Phosphorylation on carboxy terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament spacing, and axon caliber. *J. Cell Biol.* **126**, 1031-1046.

149. Nixon, R.A. and Sihag, R.K. (1991) Neurofilament phosphorylation: a new look at regulation and function. *Trends Neurosci.* **14**, 501-506.

150. Pollanen, M.S., Dickson, D.W. and Bergeron, C. (1993) Pathology and biology of the Lewy body. *Neuropathol. Exp. Neurol.* **52**, 183-191.

151. Xu, Z.S., Cork, L.C., Griffin, J.W. and Cleveland D.W. (1993) Involvement of neurofilaments in motor neuron disease. *J. Cell Sci.* **106** (Suppl. 17), 101-108.

152. Henchcliffe, C. and Burke R.E. (1997) Increased expression of cyclin-dependent kinase 5 in induced apoptotic neuron death in rat substantia nigra. *Neurosci. Lett.* **230**, 41-44.

153. Brion, J.-P. and Couck, A.-M. (1995) Cortical and Brainstem-Type Lewy Bodies Are Immunoreactive for the Cyclin-Dependent Kinase 5. *Am. J. Pathol.* **147**, 1465-1476.

154. Hirano, A. (1991) Cytopathology of amyotrophic lateral sclerosis. *Adv. Neurol.* **56**, 91-102.

155. Nakamura, S., Kawamoto, Y., Nakano, S., Ikemoto, A., Akiguchi, I. and Kimura, J. (1997) Cyclin-dependent kinase 5 in Lewy body-like inclusions in anterior horn cells of a patient with sporadic amyotrophic lateral sclerosis. *Neurology* **48**, 267-270.

156. Imahori, K. and Uchida, T. (1997) Physiology and pathology of tau kinases in relation to Alzheimer's disease. *J. Biochem.-Tokyo* **121**, 179-188.

157. Ishiguro, K., Omori, A., Sato, K., Tomizawa, K., Imahori, K. and Uchida, T. (1991) A serine/threonine proline kinase activity is included in the tau protein kinase fraction forming a paired helical filament epitope. *Neurosci. Lett.* **128**, 195-198.

158. Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K. and Uchida, T. (1993) A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDA subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. *FEBS Lett.* **335**, 171-175.

159. Lovestone, S., Reynolds, C.H., Latimer, D., Davis, D.R., Anderton, B.H., Gallo, J.-M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., Woodgett, J.R. and Miller, C.C.J. (1994) Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4**, 1077-1086.

160. Ohshima, T., Ward, J.M., Huh, C.-G., Longenecker, G., Veeranna, Pant, H.C., Brady, R.O., Martin, L.J. and Kulkarni, A.B. (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11173-11178.

161. Shetty, K.T., Kaech, S., Link, W.T., Jaffe, H., Flores, C.M., Wray, S., Pant, H.C. and Beushausen, S. (1995) Molecular Characterization of a Neuronal-Specific Protein that Stimulates the Activity of Cdk5. *J. Neurochem.* **64**, 1988-1995.

162. Nikolic, M., Dudek, H., Kwon, Y.T., Ramos, Y.F. and Tsai, L.H. (1996) The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes & Dev.* **10**, 816-825.

163. Chae, T., Kwon, Y.T. Bronson, R., Dikkes, P., Li, E. and Tsai, L.H. (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* **18**, 29-42.

164. Matsushita, M., Tomizawa, K., Lu, Y.F., Moriwaki, A., Tokuda, M., Itano, T., Wang, J.H., Hatase, O. and Matsui, H. (1996) Distinct cellular compartment of cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activator protein (p35nck5a) in the developing rat cerebellum. *Brain Res.* **734**, 319-322.

165. 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**, 505-514.

166. Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C. and Peters, G. (1994) CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene* **9**, 71-79.

167. Guidato, S., McLoughlin, D.M., Grierson, A.J. and Miller, C.C.J. (1998) Cyclin D2 interacts with cdk-5 and modulates cellular cdk-5/p35 activity. *J. Neurochem.* **70**, 335-340.

168. Lazaro, J.B., Kitzmann, M. Poul, M.A., Vandromme, M., Lamb, N.J. and Fernandez, A. (1997) Cyclin dependent kinase 5, cdk5, is a positive regulator of myogenesis in mouse C2 cells. *J. Cell Sci.* **110**, 1251-1260.

169. Philpott, A., Porro, E.B., Kirschner, M.W. and Tsai, L.H. (1997) The role of cyclin-dependent kinase 5 and a novel regulatory subunit in regulating muscle differentiation and patterning. *Genes & Dev.* **11**, 1409-1421.

170. Ahuja, H.S., Zhu, Y. and Zakeri, Z. (1997) Association of cyclin-dependent kinase 5 and its activator p35 with apoptotic cell death. *Dev.-Genet.* **21**, 258-267.

171. Zhang, Q., Ahuja, H.S., Zakeri, Z.F. and Wolgemuth, D.J. (1997) Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. *Dev. Biol.* **183**, 222-233.

172. Van den Heuvel, S. and Harlow, E. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**, 2050-2054.

173. Liem, R.K. (1993) Molecular biology of neuronal intermediate filaments. *Curr. Opin. Cell Biol.* **5**, 12-16.

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

175. Pant, H.C. and Veeranna (1995) Neurofilament phosphorylation. *Biochem. Cell Biol.* **73**, 575-592.

176. Fliegner, K.H. and Liem, R.K. (1991) Cellular and molecular biology of neuronal intermediate filaments. *Int. Rev. Cytol.* **131**, 109-167.

177. Parry, D.A. and Steinert, P.M. (1992) Intermediate filament structure. *Curr. Opin. Cell Biol.* **4**, 94-98.

178. Gill, S.R., Wong, P.C., Monteiro, M.J. and Cleveland, D.W. (1990) Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *J. Cell Biol.* **111**, 2005-2019.

179. Sihag, R.K. and Nixon, R.A. (1989) In vivo phosphorylation of distinct domains of the 70-kilodalton neurofilament subunit involves different protein kinases. *J. Biol. Chem.* **264**, 457-464.

180. Sihag, R.K. and Nixon, R.A. (1990) Phosphorylation of the amino-terminal head domain of the middle molecular mass 145-kDa subunit of neurofilaments. Evidence for regulation by second messenger-dependent protein kinases. *J. Biol. Chem.* **265**, 4166-4171.

181. Sihag, R.K. and Nixon, R.A. (1991) Identification of Ser-55 as a major protein kinase A phosphorylation site on the 70-kDa subunit of neurofilaments. Early turnover during axonal transport. *J. Biol. Chem.* **266**, 18861-18867.

182. Hisanaga, S., Gonda, Y., Inagaki, M., Ikai, A. and Hirokawa, N. (1990) Effects of phosphorylation of the neurofilament L protein on filamentous structures. *Cell Regul.* **1**, 237-248.

183. Nakamura, Y., Takeda, M. Angelides, K.J., Tanaka, T., Tada, K. and Nishimura, T. (1990) Effect of phosphorylation on 68 kDa neurofilament subunit protein

assembly by the cyclic AMP dependent protein kinase in vitro. *Biochem. Biophys. Res. Commun.* **169**, 744-750.

184. Dosemeci, A., Floyd, C.C. and Pant, H.C. (1990) Characterization of neurofilament-associated protein kinase activities from bovine spinal cord. *Cell. Mol. Neurobiol.* **10**, 369-382.
185. Dosemeci, A. and Pant, H.C. (1992) Association of cyclic-AMP-dependent protein kinase with neurofilaments. *Biochem. J.* **282**, 477-481.
186. Lee, V.M.-Y., Carden, M.J., Schlaepfer, W.W. and Trojanowski, J.Q. (1987) Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. *J. Neurosci.* **7**, 3474-3488.
187. Lee, V.M.-Y., Otvos, L.Jr., Carden, M.J., Hollosi, M., Dietzschold, B. and Lazzarini, R.A. (1988) Identification of the major multiphosphorylation site in mammalian neurofilaments. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1998-2002.
188. 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**, 4467-4471.
189. Elhanany, E., Jaffe, H., Link, W.T., Sheeley, D.M., Gainer, H. and Pant, H.C. (1994) Identification of endogenously phosphorylated KSP sites in the high-molecular-weight rat neurofilament protein. *J. Neurochem.* **63**, 2324-2335.
190. Hosoi, T., Uchiyama, M., Okumura, E., Saito, T., Ishiguro, K., Uchida, T., Okuyama, A., Kishimoto, T. and Hisanaga, S.-I. (1995) Evidence for cdk5 as a major activity phosphorylating tau protein in porcine brain extract. *J. Biochem.* **117**, 741-749.
191. Pant, A.C., Veeranna, Pant, H.C. and Amin, N. (1997) Phosphorylation of human high molecular weight neurofilament protein (hNF-H) by neuronal cyclin-dependent kinase 5 (cdk5). *Brain Research* **765**, 259-266.
192. Qi, Z., Tang, D., Matsuura, I., Lee, K.Y., Zhu, X., Huang, Q.Q. and Wang J.H. (1995) Regulatory properties of neuronal cdc2-like kinase. *Mol. Cell. Biochem.* **149-150**, 35-39.
193. Lee, K.-Y. and Johnston, R.N. (1997) Neurofilaments are part of the high molecular weight complex containing neuronal cdc2-like kinase (nclk). *Brain Research* **773**, 197-202.

194. Lasek, R.J., Garner, J.A. and Brady, S.T. (1984) Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* **99**, 212-221.

195. Hollenbeck, P.J. (1989) The transport and assembly of the axonal cytoskeleton. *J. Cell Biol.* **108**, 223-227.

196. Ksiezak-Reding, H. and Yen, S.H. (1987) Phosphatase and carbocyanine dye binding define different types of phosphate groups in mammalian neurofilaments. *J. Neurosci.* **7**, 3554-3560.

197. De Waegh, S.M., Lee, V.M. and Brady, S.T. (1992) Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. *Cell*, **68**, 451-463.

198. Kosik, K.S. (1992) Alzheimer's disease: a cell biological perspective. *Science* **256**, 780-783.

199. Anderton, B.H. (1993) Expression and processing of pathologic proteins in Alzheimer's disease. *Hippocampus* **3**, 227-237.

200. Lee, V.M.-Y., Balin, B.J., Otvos, L.Jr. and Trojanowski, J.Q. (1991) A68-a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* **251**, 675-678.

201. Grenberg, S.G. and Davies, P. (1990) A preparation of Alzheimer paired helical filaments that displays distinct τ proteins by polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5827-5831.

202. Wischik, C.M., Novak, M., Thogersen, H.C., Edwards, P.C., Runswick, M.J., Jakes, R., Walker, J.E., Milstein, C., Roth, M. and Klug, A. (1988) Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4506-4510.

203. Kosik, K.S. and Greenberg, S.M. (1992) Tau protein and Alzheimer disease. In *Alzheimer Disease* (Terry, R.D., Katzmann, R. and Bick, K.L., Eds.) Raven Press, New York, U.S.A., pp. 335-344.

204. Grundke-Iqbal, I., Iqbal, K., Tung, Y., Quinlan, M., Wisniewski, H. and Binder, L. (1986) Abnormal phosphorylation of the microtubule-associated tau in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4913-4917.

205. Ksiezak-Reding, H., Liu, W.K. and Yen, S.H. (1992) Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. *Brain. Res.* **597**, 209-219.

206. Kondo, J. Honda, T., Mori, H., Hamada, Y., Miura, R., Ogawara, M. and Ihara, Y. (1988) The carboxyl third of tau is tightly bound to paired helical filaments. *Neuron* **1**, 827-834.

207. Mandelkow, E.M. and Mandelkow, E. (1993) Tau as a marker for Alzheimer's disease. *Trends Biochem. Sci.* **18**, 480-483.

208. Goedert, M. (1993) Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Trends Neurosci.* **16**, 460-465.

209. Lee, V.M.-Y (1995) Disruption of the cytoskeleton in Alzheimer's disease. *Curr. Opin. Neurobiol.* **5**, 663-668.

210. Lee, V.M.-Y. and Trojanowski, J.Q. (1992) The disordered neuronal cytoskeleton in Alzheimer's disease. *Curr. Opin. Neurobiol.* **2**, 653-656.

211. Trojanowski, J.Q., Schmidt, M.L., Shin, R.-W., Bramblett, G.T., Rao, D. and Lee V.M.-Y. (1993) Altered tau and neurofilament proteins in neurodegenerative diseases: diagnostic implications for Alzheimer's and Lewy body dementias. *Brain Pathol.* **3**, 45-54.

212. 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**, 17047-17054.

213. Biernat, J., Mandelkow, E.M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Merken, M., Vandermeeren, A., Goedert, M. and Mandelkow, E. (1992) The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *EMBO J.* **11**, 1593-1598.

214. Kosik, K.S., Orecchio, L.D., Binder, L., Trojanowski, J.Q., Lee, V.M.-Y. and Lee, G. (1988) The carboxyl third of tau is tightly bound to paired helical filaments. *Neuron* **1**, 817-825.

215. Lichtenberg-Kraag, B., Mandelkow, E.-M., Biernat, J., Steiner, B., Schroter, C., Gustke, N., Meyer, H.E. and Mandelkow, E. (1992) Phosphorylation dependent interaction of neurofilament antibodies with tau protein: epitopes, phosphorylation sites, and relationship with Alzheimer tau. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5384-5388.

216. Vulliet, R., Halloran, S.M., Braun, R.K., Smith, A.J. and Lee, G. (1992) Proline-directed phosphorylation of human Tau protein. *J. Biol. Chem.* **267**, 22570-22574.

217. Kanamaru, K., Takio, K., Miura, R., Titani, K. and Ihara, Y. (1992) Fetal-type phosphorylation of tau in paired helical filaments. *Neurochemistry* **58**, 1667-1675.

218. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y. (1995) Proline-directed and Non-proline-directed Phosphorylation of PHF-tau. *J. Biol. Chem.* **270**, 823-829.

219. Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D. and Crowther, R.A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3**, 519-526.

220. Himmler, A., Drechsel, D., Kirschner, M.W. and Martin, D.W.Jr. (1989) Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Mol. Cell. Biol.* **9**, 1381-1388.

221. Mandelkow, E.M., Biernat, J., Drewes, G., Gustke, N., Trinczek, B. and Mandelkow, E. (1995) Tau domains, phosphorylation, and interactions with microtubules. *Neurobiol. Aging* **16**, 355-363.

222. Biernat, J., Gustke, N., Drewes, G., Mandelkow, E.M. and Mandelkow, E. (1993) Phosphorylation of Ser262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron* **11**, 153-163.

223. Drewes, G., Lichtenberg-Kraag, B., Döring, F., Mandelkow, E.-M., Biernat, J., Goris, J., Dorée M. and Mandelkow E. (1992) Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. *EMBO J.* **11**, 2131-2138.

224. Goedert, M., Cohen, E.S., Jakes, R. and Cohen, P (1992) p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1: implications for Alzheimer's disease. *FEBS Lett.* **312**, 95-99.

225. Ledesma, M.D., Correas, I., Avilla, J. and Díaz-Nido, J. (1992) Implication of brain cdc2 and MAP2 kinases in the phosphorylation of tau protein in Alzheimer's disease. *FEBS Lett.* **303**, 218-224.

226. Mandelkow, E.M., Biernat, J., Drewes, G., Steiner, B., Lichtenberg-Kraag, B., Wille, H., Gustke, N. and Mandelkow, E. (1993) Microtubule-associated protein

tau, paired helical filaments, and phosphorylation. *Ann. N.Y. Acad. Sci.* **695**, 209-216.

227. Hanger, D.P., Hughes, K. Woodgett, J.R., Brion, J.P. and Anderton, B.H. (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filaments epitopes and neuronal localization of the kinase. *Neurosci. Lett.* **147**, 58-62
228. Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T. and Imahori, K. (1993) Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325**, 167-172.
229. Mandelkow, E.M., Drewes, G., Biernat, J., Gustke, N., Van Lint, J., Vandenheede, J.R. and Mandelkow, E. (1992) Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett.* **314**, 315-321.
230. Busser, J., Geldmacher, D.S. and Herrup, K. (1998) Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. *J. Neurosci.* **18**, 2801-2807.
231. Tunnela, O. (1994) Trehalose synthesis in Baker's yeast. Academic dissertation, Research laboratories of ALKO Ltd, Helsinki and Department of Biochemistry, University of Helsinki, pp. 1-23.
232. Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245-246.
233. Sadowski, I., Bell, B., Broad, P. and Hollis, M. (1992) GAL4 fusion vectors for expression in yeast or mammalian cells. *Gene* **118**, 137-141.
234. Allen, J.B., Walberg, M.W., Edwards, M.C. and Elledge, S.J. (1995) Finding prospective partners in the library: the two-hybrid system and phage display find a match. *TIBS* **20**, 511-516.
235. Hardy, C.F., Sussel, L. and Shore, D. (1992) A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. *Genes & Dev.* **6**, 801-814.
236. 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**, 704-707.

237. Li, Z.-R., Hromchak, L.Z. and Bloch, A. (1997) Differential expression of proteins regulating cell cycle progression in growth vs. differentiation. *Biochim. Biophys. Acta* **1356**, 149-159.

2. The $p16^{INK4A}$ protein and flavopiridol restore yeast cell growth inhibited by Cdk4

Mark Moorthamer, Mayur Panchal, William Greenhalf and Bhabatosh Chaudhuri

(1998) *Biochem. Biophys. Res. Commun.* **250**, 791-797

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 250, 791–797 (1998)
ARTICLE NO. RC989348

The p16^{INK4A} Protein and Flavopiridol Restore Yeast Cell Growth Inhibited by Cdk4

Mark Moorthamer, Mayur Panchal, William Greenhalf, and Bhabatosh Chaudhuri[1]
Oncology Research, Novartis Pharma AG, Basel, Switzerland

Received August 7, 1998

Cyclin-dependent kinase 4 (Cdk4) activity is misregulated in most cancers. Loss of Cdk4 regulation can occur through overexpression of Cdk4 catalytic subunit or its regulatory partner cyclin D1, or if the Cdk4-specific inhibitory protein p16^{INK4A} is inactive. We have attempted to express the two human subunits, Cdk4 and cyclin D1, in the yeast *Saccharomyces cerevisiae*. Surprisingly, expression of Cdk4 alone, under control of the strong *GAL* promoter, inhibits cell growth. Coexpression of both subunits allows formation of an active Cdk4-cyclin D1 complex which accentuates growth arrest. In cells expressing Cdk4 only, growth is restored by overexpressing human Cdc37, a Cdk4-binding molecular chaperone. Interestingly, the effect of Cdk4 on yeast is also overcome by both p16- and p21-families of Cdk-inhibitory proteins. Moreover, flavopiridol, a compound which inhibits Cdk4 enzyme activity, restores cell division. The fact that p16^{INK4A} and flavopiridol negate Cdk4-mediated suppression of yeast cell growth implies that this simple system can be used as a screen for identifying Cdk4-specific antagonists which may mimic p16^{INK4A} in the cancer cell cycle. © 1998 Academic Press

Key Words: **Cdc37; Cdk4; Cdk4 Inhibitor; Flavopiridol; p16^{INK4A}; *Saccharomyces cerevisiae*.**

Quiescent cells at G0 enter the G1 phase of the cell cycle when stimulated by mitogens. The progress of the cell cycle is governed by the sequential activation-inactivation of different cyclin-dependent kinases (Cdk-s). Cdk4 is the first Cdk that is activated in G1 (1). Activation of Cdk4 is followed by induction of Cdk2 activity which permits entry into the DNA synthesis (S) phase of the cell cycle.

Cdk4 is activated by binding to one of three D type cyclins and its activity is specifically inhibited by the p16-family of proteins (1). The tumor suppressor retinoblastoma protein pRb is a natural substrate of Cdk4 (1-4). Underphosphorylated pRb represses the activity of different transcription factors, for example, the function of the E2F family of proteins. When Cdk4 phosphorylates pRb, hyperphosphorylated pRb dissociates from E2F. Free E2F can then transcribe a set of genes that allow entry of the cell from G1 to S phase of the cell cycle. Cyclins E and A, two of the genes induced by E2F, activate Cdk2 which can also phosphorylate pRb (1). The activity of Cdk2 is regulated by the p21-family of inhibitory proteins that can inhibit Cdk4 too. Thus, active Cdk4 initiates a complex cascade of events that culminates in cell proliferation through the initial phosphorylation of pRb.

Cdk4 activity is misregulated in most malignant cells (1). Besides Cdk4 and cyclin D1 overexpression in different tumors, it is estimated that the Cdk4-specific inhibitory activity of p16^{INK4A} (one of four proteins of the p16-family that do not inhibit Cdk2) is absent in the majority of cancers (5). Mice lacking a functional copy of the p16^{INK4A} protein are extremely susceptible to tumor formation confirming that p16^{INK4A} is a tumor suppressor (6). Expression of p16^{INK4A} has been shown to selectively block the growth of p16^{INK4A}-deficient tumor cells (7) and it is suspected that this may lead to senescence (8–10). Oncogenic Ras-mediated cell proliferation, as observed in different tumors, can also be blocked by the Cdk4 inhibitor p16^{INK4A} (11). Therefore, it is of great interest to identify a small molecular weight compound that may mimic p16^{INK4A} and selectively inhibit Cdk4 in a cancer cell.

We set out to investigate if coexpression of human

[1] Correspondence address: B. Chaudhuri, Oncology Research, Novartis Pharma AG, K-125.13.17, Basel, Switzerland. Fax: (41) (61) 696 38 35. E-mail: bob.chaudhuri@pharma.novartis.com.

Abbreviations: Cdk4, human cyclin dependent kinase; CEN, yeast centromere; *CYC1t*, transcription terminator from the yeast *CYC1* gene; EGY48_CDK4, yeast strain EGY48 transformed with CEN-URA3 plasmid encoding *GAL1p-cdk4-CYC1t* expression cassette; EGY48_CDK4_GSTD1, yeast strain EGY48_CDK4 transformed with CEN-LEU2 plasmid encoding *GAL1p-GST-cyclin_D1-CYC1t* expression cassette; Gal, galactose; *GAL1p*, promoter from the *GAL1-GAL10* gene; Glu, glucose; GST, glutathione-S-transferase; HT444_CDK4, yeast strain HT444 transformed with CEN-URA3 plasmid encoding *GAL1p-cdk4-CYC1t* expression cassette; PCR, polymerase chain reaction; pRb, human retinoblastoma protein.

0006-291X/98 $25.00

Cdk4 (12) and cyclin D1 (13) in the yeast *Saccharomyces cerevisiae* would yield an active enzyme and if this would help in identifying unique Cdk4 antagonists. In order to obtain activity, it is essential that the catalytic subunit be phosphorylated on a conserved Thr-residue (viz. Thr161 of Cdk1) (14-15). Phosphorylation is mediated by the Cdk-activating kinase (CAK) (15). There exists a CAK-like enzyme in yeast (CAK/Civ1) (15) which could permit phosphorylation of the catalytic subunit of Cdk4 and thus provide activity to Cdk4 bound to cyclin D1. Therefore, it is possible that yeast would produce an active kinase by coexpression of Cdk4 and cyclin D1. We thought it would be interesting to know the effects of this activity and to find if this would be inhibited in the same way as in mammalian cells by known Cdk inhibitors.

MATERIALS AND METHODS

Plasmid constructs. An *EcoRI-SalI* PCR fragment of human *CDK4* (12) was first subcloned in pBluescriptKS(+) (Stratagene). A *BamHI-SalI* fragment of *CDK4* from the resultant plasmid was then cloned in pRS416Gal (ATCC), a pCEN-URA3 plasmid containing the *GAL1* promoter and the *CYC1* terminator, for expression in yeast. The *CDK4* gene lies directly downstream of the *GAL1* promoter.

An *EcoRI-SalI* PCR amplified fragment of the human *cyclin D1* gene (13) was first subcloned in pBluescriptKS(+). For expression of cyclin D1 in yeast, a *BamHI-SalI* fragment from the pBluescriptKS(+) derived plasmid was cloned in pRS415Gal (a pCEN-LEU2 plasmid from ATCC). Furthermore, a *BglII-EcoRI* PCR fragment of GST (glutathione S-transferase fragment from pGEX4T-1, Pharmacia) and the *EcoRI-SalI* fragment of cyclin D1 was subcloned in pBluescriptKS(+). The *BglII-SalI* fragment, which contains the fusion gene GST-cyclin_D1, was then cloned in pRS415Gal (pCEN-LEU2) for further expression in yeast.

A *BamHI-NsiI* PCR fragment of human *CDC37* (h_*CDC37*) (16) was first subcloned in pGEM11z(+) (Promega). Linkers which encode EQKLISEEDLING (the *c-myc* epitope) were tagged to the 5'-end of the gene. This created a *BamHI* site at the 5'-end of the *c-myc* tag. A *BamHI-HindIII* fragment of *c-myc*-h_*CDC37* was cloned in both pRS415Gal (pCEN-LEU2) and pRS414Gal (a pCEN-TRP1 plasmid; ATCC).

The Cdk inhibitors were cloned in the following manner for further expression in yeast. An *EcoRI-XhoI* PCR fragment of $p21^{CIP1}$ (17) which contains a 3'-end *SalI-XhoI* fragment encoding YPYDVPDYA (the HA-epitope tag), was subcloned in pBluescriptKS(+) to yield the plasmid BluKSp/p21-flu. *EcoRI-SalI* PCR fragments of $p16^{INK4A}$ (18), $p18^{INK4C}$ (19), $p19^{INK4D}$ (20), and $p27^{KIP1}$ (21) were subcloned in the vector BluKSp/p21-flu, digested with *EcoRI-SalI* to yield the plasmids BluKSp/p16-flu, BluKSp/p18-flu, BluKSp/p19-flu and BluKSp/p27-flu, respectively. *BamHI-XhoI* fragments containing the genes for the inhibitors were then cloned either in pRS415Gal (pCEN-LEU2) or pRS414Gal (pCEN-TRP1).

Sequences of all PCR fragments were confirmed by automated DNA sequencing (LiCor).

Yeast strains and media. The *S. cerevisiae* strains HT444 (*MATa leu2-3 leu2-112 his4-519 ura3 lys2 Gal*$^+$) (22) and EGY48 (*Mata ura3, trp1, his3, leu2 Gal*$^+$) (23) were used for yeast transformation. HT444 was used for stepwise transformation of pCEN-URA3 and pCEN-LEU2 plasmids whereas EGY48 was similarly used for transformation of pCEN-URA3, pCEN-LEU2 and pCEN-TRP1 plasmids. Plasmids were introduced into HT444 by electroporation while the lithium acetate protocol (23) allowed transformation of EGY48. The strain resulting from transformation of HT444 with pCEN-URA3/CDK4 is referred to as HT444_CDK4 whereas the one obtained from transformation of EGY48 has been named EGY48_CDK4. HT444_CDK4 has been transformed with pCEN-LEU2 plasmids encoding either (a) h_*CDC37* or (b) the Cdk inhibitors ($p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{CIP1}$, $p27^{KIP1}$). On the other hand, EGY48_CDK4 was first transformed with pCEN-LEU2 plasmids encoding (a) h_*CDC37* or (b) the Cdk inhibitor $p16^{INK4A}$ or (c) *cyclin D1*, or *GST-cyclin D1*. The EGY48 derivative which contains both *CDK4* and *GST-cyclin D1* was named EGY48_CDK4_GSTD1. pCEN-TRP1 plasmids which carry (a) the Cdk inhibitors ($p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{CIP1}$, $p27^{KIP1}$) or (b) h_*CDC37* were then transformed in EGY48_CDK4_GSTD1.

The growth media used were mainly as described earlier (22). YEP being a rich medium and SD a defined minimal medium. For simplicity, all defined media have been described simply as SD and the added supplements have not been specified (supplements being all those required for growth of the specific strain). The carbon sources were 2% (w/v) glucose or galactose.

Spot tests. $\sim 10^5$ cells were suspended in 100 μl of water and 5-μl aliquots were spotted on appropriate minimal SD medium containing appropriate carbon sources.

Affinity purification of Cdk4/GST-cyclin D1, Cdk4, and kinase assay. The Cdk4/GST-cyclin D1 complex and Cdk4 alone were immunoprecipitated using published procedures (24 and GST-handbook, Pharmacia). In essence, 150 A_{600} units of cells were harvested (either from EGY48_CDK4_GSTD1 or from EGY48_CDK4) after a 5 h induction with galactose in SD medium. The cells were lysed in PBS, containing a protease inhibitor cocktail (Boehringer) and 0.2 mM sodium orthovanadate (Sigma), by vortexing with glass beads (0.45-0.5 mm diameter, Sigma). The supernatants were clarified through high-speed centrifugation. GSH-Sepharose (100 μl; Pharmacia) or the Cdk4 antibody (Santa Cruz Biotechnology) followed by protein A-Sepharose (100 μl; Pharmacia) were used for immunoprecipitation.

Preparation of samples for SDS-PAGE and Western blot analysis. Cells in late exponential phase were harvested, washed twice with 0.9% NaCl and were resuspended in an equal volume of medium containing 2% galactose. Cultures were incubated at 30°C for 5 h with shaking. 150 A_{600} units of cells were harvested and resuspended in 200 μl of 2x sample buffer (4% SDS w/v, 0.1 M Tris, 4 mM EDTA, 20% glycerol v/v, 33% bromophenol blue w/v) containing a cocktail of protease and phosphatase inhibitors (see above).

1 μl cell lysates, pretreated with 30 mM dithiothreitol, were loaded on 12.5 % SDS-polyacrylamide gels along with 10 μl of prestained marker (SDS-PAGE broad range standard; Bio-Rad) using a Mini Protean chamber system (Bio-Rad). The gels were blotted onto Immobilon P (Millipore) using a semi-dry electroblotter (Sartorius). After blotting, proteins were visualized using the ECL kit (Amersham). A HA-specific polyclonal antibody raised in rabbits (Boehringer) was used to recognize the Cdk-inhibitory proteins expressed in yeast. The mouse monoclonal antibody (myc1-9E10.2) raised against the *c-myc* epitope was obtained from ATCC (#CRL-1729; kindly provided by M. Geiser; Novartis) and was used to detect h_Cdc37. The Cdk4 and cyclin D1 antibodies were available from Santa Cruz Biotechnology.

Microscopic analysis of yeast cell morphology. A pre-culture of yeast cells was prepared using SD-glucose (SD-Glu) and grown overnight to stationary phase at 30°C. An appropriate amount of the stationary culture was then diluted to achieve an A_{600} of ~0.5 in SD-Glu after overnight growth, i.e early exponential phase growth. Cells were then harvested and washed three times with sterile water before induction with galactose. The compound flavopiridol (1 μM or 10 μM) was added at this stage. The galactose-induced cells were analysed under a microscope using bright-field and 500-fold magnification, at different time points. At each time point, a sample of cells were treated with methylene blue as described by Pierce (25).

Briefly, cells were resuspended in an appropriate volume of 0.9 M KCl and were treated with the dye solution (final concentration of 0.5 g/l).

A minimum of 5 (usually 10) separate frames were counted. The Standard Error of Mean (SEM) was calculated for each cell type and the errors were combined so as to give a maximum and minimum value for each percentage.

RESULTS AND DISCUSSION

The *S. cerevisiae* yeast strains HT444 and EGY48 were transformed with pRS416Gal/*CDK4*. The plasmid pRS416Gal is a single-copy centromere plasmid (pCEN-URA3, encoding the yeast *URA3* gene as selection marker). The human *CDK4* gene (12) is under the control of the galactose-inducible *GAL1* promoter. Transformants were spotted on minimal medium plates which contained either glucose or galactose. Unexpectedly, we find that upon induction with galactose, yeast expressing wild type *CDK4* stop growing (Fig. 1A; the results with the strain EGY48 are not shown). In contrast, a catalytically inactive Cdk4 mutant has no effect on growth, nor has expression of Cdk4 with a weaker promoter (e.g. *ADH1* as in the yeast-two-hybrid system) (results not shown). One transformant from each of the two strains, bearing *CDK4*, is referred to as HT444_CDK4 and EGY48_CDK4.

In order to find if Cdk4-inhbitory proteins would restore cell growth, pCEN-LEU2 plasmids encoding the genes $p16^{INK4A}$ (18), $p18^{INK4C}$ (19), $p19^{INK4D}$ (20), $p21^{CIP1}$ (17), $p27^{KIP1}$ (21), were transformed in HT444_CDK4 and EGY48_CDK4. Transformants grow in galactose-containing medium, implying that the inhibitory proteins restore growth to *CDK4*-expressing cells (Fig. 1B; the results with EGY48_CDK4, which are not shown, are identical). The presence of HA-tagged Cdk-inhibitory proteins and Cdk4 in these transformants is confirmed by Western blotting (Fig. 1C, top and bottom panels). It can be concluded that $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{CIP1}$, $p27^{KIP1}$ proteins can abolish the growth-inhibitory effect of Cdk4 in yeast.

The observed inhibition of growth induced by Cdk4 can be due to the inactivation of a protein that is essential for growth. The human Cdc37 protein (h_Cdc37) has been recently identified as a Cdk4-binding protein and is postulated to act as a Cdk4-specific molecular chaperone (16, 26-27). On the other hand, yeast Cdc37 (a protein similar in its primary sequence to h_Cdc37) is known to be vital for the assembly of the yeast protein kinase Cdc28, the prototype of all known Cdk-s (28). Moreover, a mutant yeast Cdc37 (therefore probably inactivated) causes a G1 block in the cell cycle (28 and references therein). It is possible that expression of Cdk4 in yeast nullifies the function of the endogenous Cdc37. If this were to be true, then overexpression of h_Cdc37 (16) would negate the effect of Cdk4 in yeast. Hence, HT444_CDK4 and

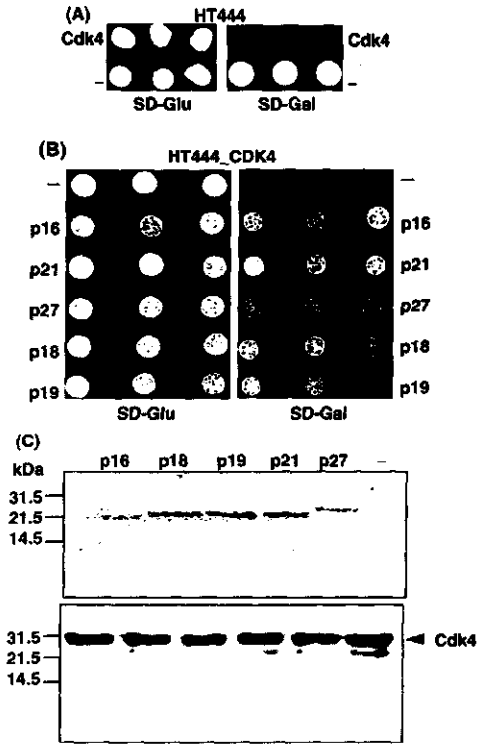


FIG. 1. Cdk4 blocks yeast cell growth whereas the Cdk-inhibitory proteins restore growth in the presence of Cdk4. (A) The yeast strain HT444 was transformed with the pCEN-URA3 plasmid (pRS416Gal) encoding *CDK4*. An equal number of cells from three individual transformants were spotted on minimal SD medium plates, in the presence of glucose (Glu) or galactose (Gal), as described under Materials and Methods. One such transformant is referred to as HT444_CDK4. (B) HT444_CDK4 was transformed with pCEN-LEU2 plasmids (pRS415Gal) encoding genes for the Cdk-inhibitors. An equal number of cells from three individual transformants were spotted as in (A). (C) Western blot analysis of cells coexpressing Cdk4 and one of the Cdk-inhibitory proteins. 150 A_{600} cells were harvested and lysed in 200 µl of lysis buffer (Materials and Methods). After blotting, 1µl of each lysate was probed either with the HA-antibodies (upper panel) or with the Cdk4 antibodies (lower panel) using the ECL kit (Amersham). '—' denotes the transformants of centromere plasmids which contain no expression cassette.

EGY48_CDK4 were transformed with a plasmid encoding human *cdc37*. Expression of h_Cdc37 does indeed restore cell growth (Fig. 2A and B). The growth restoring ability of h_Cdc37 in HT444_CDK4 are similar (results not shown).

In contrast to h_Cdc37, coexpression of cyclin D1 with Cdk4 continues to inhibit growth (Fig. 3A) whereas coexpression of cyclin D1 with catalytically inactive Cdk4 has no effect on yeast. Precipitation of the GST-cyclin_D1/Cdk4 complex with GSH-

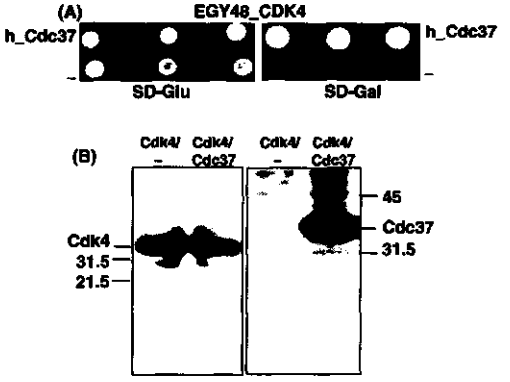


FIG. 2. Human Cdc37 restores yeast growth blocked by Cdk4. (A) The yeast strain EGY48 was transformed with the pCEN-URA3 plasmid (pRS416Gal) encoding *CDK4*. One such transformant is referred to as EGY48_CDK4 which was then transformed with a pCEN-LEU2 plasmid (pRS415Gal) encoding h_Cdc37. An equal number of cells from three individual transformants were spotted on minimal SD medium plates, in the presence of glucose (Glu) or galactose (Gal) as in Fig. 1. (B) Western blot analysis of cells coexpressing Cdk4 and h_Cdc37, as described above. After blotting, 1 μl of each lysate was probed with either the Cdk4 antibodies (left panel) or the *c-myc* antibodies (right panel) using the ECL kit (Amersham). '—' denotes the transformants of centromere plasmids which contain no expression cassette.

Sepharose (Fig. 3B) from cell lysates shows that an active enzyme is produced in yeast (Fig. 3C, lanes 5-6). Although $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{CIP1}$, $p27^{KIP1}$ overcome Cdk4/cyclin_D1 mediated growth inhibition, h_Cdc37 is unable to do the same (Fig. 3D). Probably, h_Cdc37 can neither displace cyclin D1 from Cdk4 like $p16^{INK4A}$ nor can it act as an ATP-competitive inhibitor like $p21^{CIP1}$ or $p27^{KIP1}$. Thus h_Cdc37 cannot inactivate active Cdk4 like the p16 and p21-families of Cdk-inhibitory proteins (27).

In yeast, temperature sensitive alleles of *cdc28* (that do not grow at the restrictive temperature of 37°C, but grow at the permissive temperature of 25°C) are complemented at 37°C by human Cdk2 but not by human Cdk4 (29-30). It is also known that the mammalian cyclins E (which activates Cdk2) and D1 (which activates Cdk4) are similar to specific yeast cyclins (13, 31). This may indicate that, at least in yeast, Cdk2 and Cdk4 may have fundamentally different properties (viz. only Cdk2 can be considered as a true Cdc28 analog).

Our results show that overexpression of Cdk4 or Cdk4-cyclin D1 in yeast blocks cell growth. Coexpression of $p16^{INK4A}$ nullifies this effect, as seen in plate assays (Fig. 1B, 3D) and in liquid cultures (Fig. 4A, left panel; similar results are obtained when $p16^{INK4A}$ is coexpressed with Cdk4 and cyclin D1). We have therefore attempted to corroborate these unusual findings through microscopic analysis of cells growing in liquid cultures. This was achieved by counting the number of budded cells against unbudded ones, at different time points of growth, in strains which express Cdk4 only.

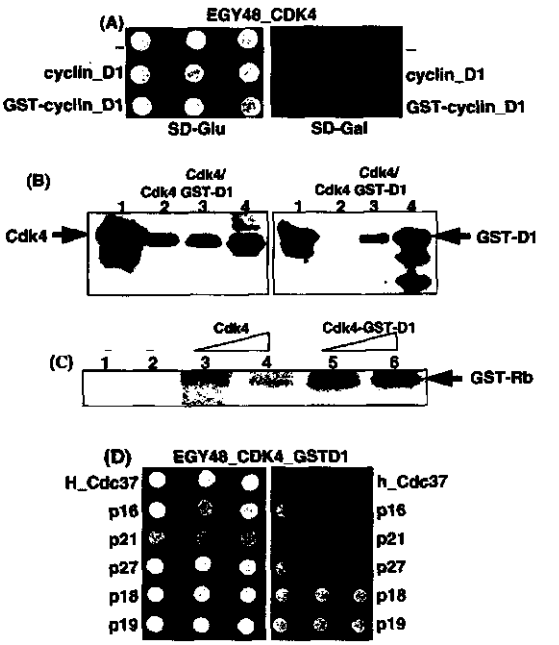


FIG. 3. Coexpression of cyclin D1 with Cdk4 forms an active kinase and blocks yeast cell growth that is relieved by the Cdk-inhibitory proteins but not h_Cdc37. (A) The yeast strain EGY48_CDK4 was transformed with a pCEN-LEU2 plasmid (pRS415Gal) encoding cyclin D1 and GST-cyclin D1. An equal number of cells from three individual transformants were spotted on minimal SD medium plates, in the presence of glucose (Glu) or galactose (Gal) as in Fig. 1. (B) Western blot analysis of cells expressing Cdk4 alone or coexpressing Cdk4 and GST-cyclin D1. After blotting, filters were probed either with Cdk4 antibodies (left panel) or with cyclin D1 antibodies (right panel), as in Fig. 1. Lane 1, 500 ng *E. coli* expressed His-tagged Cdk4 (left panel) or 500 ng *E. coli* expressed GST-tagged cyclin D1 (right panel); lane 2, 10 μl of protein A-Sepharose beads after immunoprecipitating cell lysates from EGY48_CDK4 with Cdk4 antibodies (see Materials and Methods); lane 3, 10 μl of GSH-Sepharose beads used for precipitating GST-cyclin D1/Cdk4 complex from EGY48_CDK4::pCENLEU2/GST-cyclin D1 (see Materials and Methods); lane 4, 1 μl of GSH-Sepharose beads used for precipitating GST-cyclin D1/Cdk4 from $\sim 2 \times 10^7$ Sf9 insect cells. (C) Kinase assay using 10 μl of beads with GST-Rb (Rb = C-terminal 773-924 amino-acid residues of human pRb) as substrate (24). (D) The yeast strain EGY48_CDK4 transformed with a pCEN-LEU2 plasmid encoding GST-cyclin D1 is referred to as EGY48_CDK4_GSTD1. This strain is transformed with pCEN-TRP1 plasmids encoding the Cdk-inhibitors or h_Cdc37. An equal number of cells from three individual transformants were spotted on minimal SD medium plates, in the presence of glucose (Glu) or galactose (Gal) as in Fig. 1. '—' denotes the transformants of centromere plasmids which contain no expression cassette.

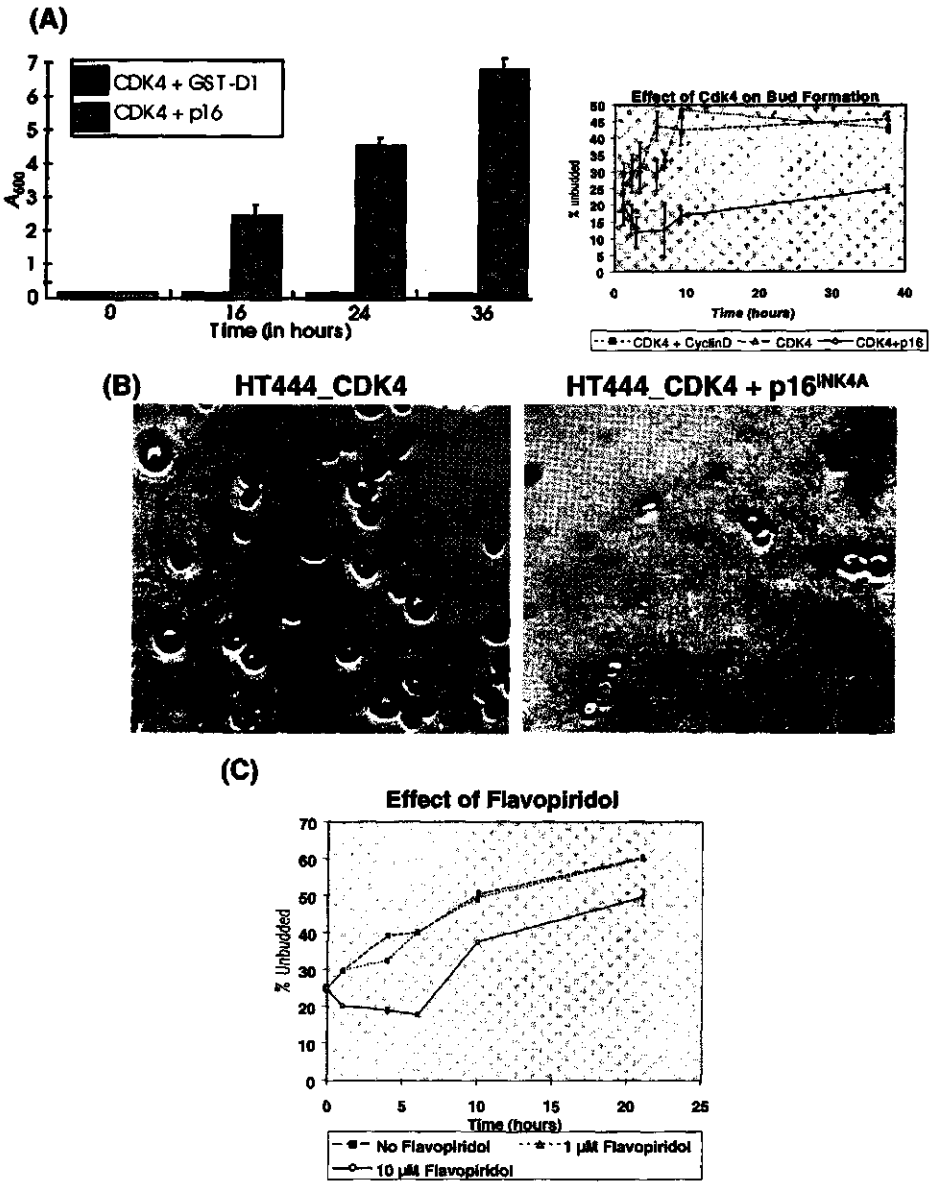


FIG. 4. Expression of Cdk4 and coexpression of Cdk4/GST-cyclin D1 cause cell cycle arrest in yeast, whereas $p16^{INK4A}$ and flavopiridol restore cell division. (A) Left panel, restoration of cell division as monitored by A_{600} measurements at different time points. Right panel, analysis of bud formation through microscopic analysis (see Materials and Methods). (B) Left panel, an example of the unbudded phenotype of Cdk4-expressing cells; right panel, during restoration of cell division by $p16^{INK4A}$, bipolar buds form. Cells with a bipolar bud pattern are indicated by arrows. Such a budding pattern is not normally seen with haploid yeast nor is it seen in any of the other cultures described here. (C) Effect of flavopiridol on cells whose growth is arrested by Cdk4.

The majority of living cells are unbudded, as clearly visualized in Fig. 4B (left panel; the figure depicts HT444_CDK4 after 10 h of induction with galactose). The phenomenon of cell cycle arrest observed in a strain expressing Cdk4 alone is compared with strains which either coexpress Cdk4 and GST-cyclin D1 or Cdk4 and $p16^{INK4A}$ (Fig. 4A, right). Although no significant change in A_{600} is seen with cells lacking $p16^{INK4A}$ (Fig. 4A, left), cells with or without cyclin D1 were able to complete mitosis. This is shown by the accumulation of unbudded cells in the 2 upper curves (Fig. 4A, right). Cells with $p16^{INK4A}$, as would be expected from growing cells, maintain a high level of budding. Even though coexpression of cyclin D1 makes no significant difference to the final percentage of cells which undergo mitosis there is a significant kinetic difference. While in the absence of cyclin D1 there is an immediate decrease in budding, budding levels with cyclin D1 remain constant for the first 8 hours. This delay could be due to an immediate effect of the Cdk4-cyclin D1 complex inhibiting mitosis whereas the less severe effect of Cdk4 is probably manifested only after mitosis. Cdk4 on its own prevents further bud formation but allows cells in G2/M to complete their cell cycle unhindered. It seems that simultaneous induction of cyclin D1 only accentuates the growth-inhibitory effect of Cdk4 at early time points of induction and imposes a delay in the cells' entry into mitosis. In stark contrast, when galactose induces $p16^{INK4A}$ levels in Cdk4-containing yeast, the percentage of unbudded cells remains unaltered during growth which is typical of an exponentially growing culture. This also corresponds with the observed increase in cell density (Fig. 4A, left panel). Identical observations on budding have been made when $p16^{INK4A}$ is coexpressed in a yeast cell along with Cdk4 and cyclin D1.

Interestingly, during $p16^{INK4A}$-mediated restoration of growth, a significant percentage (>13% of cells) of dividing cells form bipolar buds (Fig. 4B, right panel). Yeast cells display either an axial (for haploid Mata or Matα cells) or bipolar (for diploid Mata/α cells) pattern of bud-site selection. Choice of bud site is controlled by a series of genes, notably *BUD1* to *BUD5* and *AXL1* and *AXL2*. Mutations in *BUD3, BUD4, AXL1* and *AXL2* all cause the budding pattern to be altered from axial to bipolar (32–34). *BUD3, BUD4* and *AXL2* have a cellular localisation at the bud site and are possibly all involved in recruitment of other proteins to that site. *AXL1* encodes a protein similar in sequence to the human and Drosophila insulin-degrading enzymes (33). It could be speculated that $p16^{INK4A}$ not only inactivates Cdk4 in yeast but may also inhibit the *AXL1* gene product thereby causing bipolar bud formation. Further studies should reveal if $p16^{INK4A}$ is indeed an inhibitor of the human insulin-degrading enzyme.

Flavopiridol is an inhibitor of Cdk4 and Cdk2 kinases and in mammalian cells can inhibit cell cycle progression in either G1 or G2 (35-36). We have enquired if this compound would have any effect on yeast cell cycle arrest induced by Cdk4 and Cdk4-cyclin D1 at 1 and 10 μM concentrations (Fig. 4C; although results for a strain expressing Cdk4 are only shown, similar observations have been made with strains expressing Cdk4-cyclin D1). Using pRb as substrate, we find that flavopiridiol (the racemic form) may be an equipotent inhibitor of Cdk4/GST-cyclin D1 and Cdk2/GST-cyclin A (the active kinases are produced from insect cells; unpublished observations). An increase in budding is clearly observed in the presence of 10 μM concentrations of flavopiridol (Fig. 4C). This suggests that, for a limited time period (5 h), the cells are able to continue dividing normally. This is in contrast to (a) the sharp decrease in budding which is typical for Cdk4-expressing yeast, and (b) to the initial delay followed by more rapid accumulation of unbudded cells seen in Cdk4-cyclin D1 expressing cells (Fig. 4A, right). Even after 20 h of flavopiridol treatment, the level of budding remains higher than in the absence, or with low levels, of the compound. Since the effects of the compound are observed only at 10 μM concentrations, it may be that flavopiridol is efficiently metabolized by yeast and therefore its growth stimulatory effect is not clearly perceived at lower concentrations. It is also possible that flavopiridol does not induce more pronounced cell division because it is a Cdk2 inhibitor too, and therefore can inhibit yeast Cdc28 whose activity is critical for growth. It is very likely that a compound which inhibits Cdk4 but not Cdk2 (and therefore also not Cdc28) will be able to restore growth fully, possibly like $p16^{INK4A}$.

We have shown in this communication that Cdk4-cyclin D1 complex in yeast forms an active enzyme, and its activity blocks cell growth indicating that active Cdk4 is toxic to yeast. The block is relieved by coexpression of $p21^{CIP1}$, $p27^{KIP1}$ (proteins belonging to the p21-family of Cdk-inhibitors), and $p16^{INK4A}$, $p18^{INK4c}$ and $p19^{INK4d}$ (proteins belonging to the $p16^{INK4A}$-family of Cdk-inhibitors) (1). We also observe that expression of Cdk4 without its activating partner stops cell growth. However, overexpression of human Cdc37 (16) relieves these cells from growth arrest. Cdk4-mediated inhibition of growth can be also abrogated by expression of the same kinase inhibitors ($p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, and $p21^{CIP1}$, $p27^{KIP1}$) which nullify the effect of the Cdk4-cyclin D1 complex. Perhaps even more significantly, flavopiridol (35-36), a compound which inhibits Cdk4, can restore growth to Cdk4-cyclin D1 inhibited cells.

It can be considered anomalous that Cdk4 should inhibit cell growth in yeast yet cause proliferation in mammalian cells. However, it must be remembered that the main target for Cdk4 in mamalian cells is pRb which is clearly absent in yeast cells. Furthermore,

unlike mammalian cells, yeast cells pass through continuous cycles of mitosis unless acted upon by a specific "anti-mitogenic signal" like the α-factor (37). Therefore, Cdk4 activity in normally dividing yeast is probably inappropriate and may thus have catastrophic consequences. We suspect that expression of Cdk4 alone gives rise to an active enzyme (Fig. 3C, lanes 3-4) which may imply that there is a cyclin D-like molecule in yeast (may be Cln3) that activates Cdk4 (16). Furthermore coexpression of Cdk4 with cyclin D1 clearly yields an active kinase (Fig. 3C, lanes 5-6). We expect that compounds which destroy this activity in yeast would permit growth again. These chemical entities would behave either like $p16^{INK4A}$ or would be novel ATP-competitive inhibitors. Such compounds would not inhibit Cdk2 because, if it did so, it would inactivate yeast Cdc28 and would cause cell cycle arrest. This leads us to believe that true mimics of $p16^{INK4A}$, unable to inhibit Cdk2, would be identified using this simple cellular system. This new class of compounds would compensate for the functionally inactive $p16^{INK4A}$ present in a variety of known tumors.

It seems that this speculation can be aptly corroborated (Lionel Muller, Rajeev Soni and B.C.; unpublished observations). An inhibitor has been identified using this system that also inhibits the recombinant enzyme produced in baculovirus infected insect cells (24). The compound does not inhibit Cdk2. The inhibitor also blocks growth of mammalian pRb-positive cancer cells in early G1. The identified compound is currently undergoing further preclinical tests. These results definitely confirm that this simple yeast-based system devised for identifying Cdk4-specific inhibitors could be an invaluable addition to the very difficult protocols (i.e. baculovirus-produced recombinant enzyme assay or an ELISA, based on the interaction of Cdk4 and cyclin D1) currently used for identifying Cdk4 antagonists in a high throughput format.

ACKNOWLEDGMENTS

We are indebted to S. Zumstein, L. Muller, and C. Stephan for sharing their reagents with us.

REFERENCES

1. Sherr, C. J. (1996) *Science* **274,** 1672–1677.
2. Herwig, S., and Strauss, M. (1997) *Eur. J. Biochem.* **246,** 581–601.
3. Bartek, J., Bartkova, J., and Lukas, J. (1996) *Curr. Opin. Cell Biol.* **8,** 805–814.
4. Weinberg, R. A. (1995) *Cell* **81,** 323–330.
5. Palmero, I., and Peters, G. (1996) *Cancer Surv.* **27,** 351–367.
6. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996) *Cell* **85,** 27–37.
7. Suzuki-Takahashi, I., Higashi, H., Yoshida, E., Nishimura, S., and Kitagawa, M. (1997) *Biochem. Biophys. Res. Commun.* **234,** 386–392.
8. Uhrbom, L., Nister, M., and Westermark, B. (1997) *Oncogene* **15,** 505–514.
9. Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13742–13747.
10. Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996) *Mol. Cell. Biol.* **16,** 859–867.
11. Serrano, M., Gomez, Lahoz, E., DePinho, R. A., Beach, D., and Bar-Sagi, D. (1995) *Science* **267,** 249–252.
12. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J.-Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992) *Cell* **71,** 323–334.
13. Xiong, Y., Connolly, T., Futcher, B., and Beach, D. (1991) *Cell* **65,** 691–699.
14. Morgan, D. O. (1996) *Curr. Opin. Cell Biol.* **8,** 767–772.
15. Lew, D. J., and Kornbluth, S. (1996) *Curr. Opin. Cell Biol.* **8,** 795–804.
16. Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996) *Genes Dev.* **10,** 1491–1502.
17. Harper, J. W., Adami, G. R., Wei, N., and Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75,** 805–816.
18. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366,** 704–707.
19. Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994) *Genes Dev.* **8,** 2939–2952.
20. Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. (1995) *Mol. Cell. Biol.* **15,** 2672–2681.
21. Toyoshima, H., and Hunter, T. (1994) *Cell* **78,** 67–74.
22. Greenhalf, W., Stephan, C., and Chaudhuri, B. (1996) *FEBS Lett.* **380,** 169–175.
23. Zervos, A. S., Gyuris, J., and Brent, R. (1993) *Cell* **72,** 223–232.
24. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. (1994) *Mol. Cell. Biol.* **14,** 2066–2076.
25. Pierce, J. (1970) *J. Inst. Brew.* **76,** 442–443.
26. Hunter, T., and Poon, R. Y. C. (1997) *Trends Cell Biol.* **7,** 157–161.
27. Lamphere, L., Fiore, F., Xu, X., Brizuela, L., Keezer, S., Sardet, C., Draetta, G. F., and Gyuris, J. (1997) *Oncogene* **14,** 1999–2004.
28. Gerber, M. R., Farrell, A., Deshaies, R. J., Herskowitz, I., and Morgan, D. O. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4651–4655.
29. Ninomiya-Tsuji, J., Nomoto, S., Yasuda, H., Reed, S. I., and Matsumoto, K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9006–9010.
30. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. (1992) *EMBO J.* **11,** 2909–2917.
31. Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M., and Roberts, J. M. (1991) *Cell* **66,** 1217–1228.
32. Chant, J., and Herskowitz, I. (1991) *Cell* **65,** 1203–1212.
33. Fujita, A. C., Oka, Y., Arikawa, T., Katagi, A., Tonouchi, S., Kuhara, S., and Misumi, Y. (1994) *Nature* **372,** 567–570.
34. Roemer, T., Madden, K., Chang, J., and Snyder, M. (1996) *Genes Dev.* **10,** 777–793.
35. Carlson, B. A., Dubay, M. M., Sausville, E. A., Brizuela, L., and Worland, P. J. (1996) *Cancer Res.* **56,** 2973–2978.
36. Bible, K. C., and Kaufmann, S. H. (1996) *Cancer Res.* **56,** 4856–4861.
37. Peter, M., and Herskowitz, I. (1994) *Cell* **79,** 181–184.

3. DNA binding protein dbpA binds Cdk5 and inhibits its activity

Mark Moorthamer, Sabine Zumstein-Mecker and Bhabatosh Chaudhuri

(1999) *FEBS Lett.* **446**, 243-350

FEBS 21716 FEBS Letters 446 (1999) 343–350

DNA binding protein dbpA binds Cdk5 and inhibits its activity

Mark Moorthamer, Sabine Zumstein-Mecker, Bhabatosh Chaudhuri*

Oncology Research, Novartis Pharma AG, K-125.13.17, Basel, Switzerland

Received 19 December 1998

Abstract Progress in the cell cycle is governed by the activity of cyclin dependent kinases (Cdks). Unlike other Cdks, the Cdk5 catalytic subunit is found mostly in differentiated neurons. Interestingly, the only known protein that activates Cdk5 (i.e. p35) is expressed solely in the brain. It has been suggested that, besides its requirement in neuronal differentiation, Cdk5 activity is induced during myogenesis. However, it is not clear how this activity is regulated in the pathway that leads proliferative cells to differentiation. In order to find if there exists any Cdk5-interacting protein, the yeast two-hybrid system was used to screen a HeLa cDNA library. We have determined that a C-terminal 172 amino acid domain of the DNA binding protein, dbpA, binds to Cdk5. Biochemical analyses reveal that this fragment (dbpA(CΔ)) strongly inhibits p35-activated Cdk5 kinase. The protein also interacts with Cdk4 and inhibits the Cdk4/cyclin D1 enzyme. Surprisingly, dbpA(CΔ) does not bind Cdk2 in the two-hybrid assay nor does it inhibit Cdk2 activated by cyclin A. It could be that dbpA's ability to inhibit Cdk5 and Cdk4 reflects an apparent cross-talk between distinct signal transduction pathways controlled by dbpA on the one hand and Cdk5 or Cdk4 on the other.

Key words: Cyclin dependant kinase 5; DNA binding protein A; Kinase inhibitor; Yeast two-hybrid system

1. Introduction

Cyclin dependent kinases (Cdks) are a class of serine/threonine protein kinases which regulate important transitions in the cell cycle. Cdk5 was initially identified on the basis of its sequence similarity to the family of cyclin dependent kinases [1,2]. However, Cdk5 is thought be a cyclin dependent kinase with neuron-specific function only, since its catalytic subunit is predominantly expressed in post-mitotic cells of the nervous system, cells which have permanently exited the cell cycle [2,3]. Moreover, the protein p35 which activates the Cdk5 enzyme is exclusively expressed in the central nervous system (CNS) [3,4].

Nevertheless, the catalytic subunit of Cdk5 is widely expressed at basal levels, in both cycling and non-cycling cells, in most human tissues [2,3]. Interestingly, cyclins D and E, activator molecules which regulate Cdk activity in proliferating cells, bind Cdk5 ([5,6]; our own observations). It could be argued that Cdk5 bound to cyclin D or cyclin E phosphorylates substrates which are not yet known.

The 35 kDa p35 protein, the only known activator of Cdk5, which was originally isolated as a smaller 25 kDa proteolytic product, bears no significant homology to the family of cyclins [3,4]. As a Cdk activator, p35 is unique since it has no influence on the activity of other Cdks [2,3].

Histone H1 and the retinoblastoma protein (pRb) are substrates that are frequently used to confirm in vitro activity of the Cdks. Heterodimeric Cdk5/p35 and Cdk5/p25 complexes can phosphorylate both histone H1 and pRb [3,7]. Activated Cdk5 also phosphorylates its activating partner p35 [8]. Besides, Cdk5 is known to phosphorylate in vitro a number of neuron-specific cytoskeletal proteins that includes the neurofilament proteins NF-M, NF-H, the microtubule associated protein tau and the actin binding protein caldesmon [2,3]. Phosphorylation of cytoskeletal proteins is thought to play an important role in the polymerization and assembly of cytoskeletal elements which, in turn, may affect growth of neurites. Indeed, Cdk5 activity has been demonstrated to play a key role in neurite outgrowth [9] and neuronal migration during differentiation of neurons [10]. Recently, Cdk5 has been shown to participate in the regulation of myogenesis in the early embryo [11,12]. These observations may provide new insight into the possible function of Cdk5 during differentiation.

Cdk5 is known to phosphorylate neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies, i.e. diffuse Lewy body disease (dementia), Parkinson's disease [13,14] and amyotrophic lateral sclerosis [2,15], and tau protein at sites phosphorylated in Alzheimer's disease [2,3,16,17]. Moreover, it has been reported that the kinase activity of Cdk5 correlates with the extent of differentiation of neuronal cells [2,3,18] and colocalizes with neurofilament tracts in the axons of neuronal cells in culture.

It is therefore possible that Cdk5 activity is tightly regulated in pathways that lead from cell proliferation towards differentiation. Hence, we have inquired whether if there are any proteins that could be involved in the regulation of Cdk5 (i.e. may activate or inhibit the kinase) in cycling cells. With this in mind, a cDNA library, constructed from total RNA obtained from HeLa cells, was screened using the yeast two-hybrid system. Amongst other proteins (unpublished observations), we find that a C-terminal fragment of the DNA binding protein dbpA (in this communication referred to as dbpA(CΔ)) interacts with the bait protein Cdk5, in the two-hybrid assay. Conversely, when dbpA(CΔ) was used as bait, the interaction between dbpA(CΔ) and Cdk5 was equally efficient. The validity of these interactions was corroborated using the *Escherichia coli* expressed GST-dbpA(CΔ) fusion protein. The purified protein precipitates not only in vitro transcribed/translated Cdk5 but also the protein which is expressed in

*Corresponding author. Fax: (41) (61) 696 38 35.
E-mail: bob.chaudhuri@pharma.novartis.com

Abbreviations: β-gal, β-galactosidase; Cdk, human cyclin dependent kinase; CSD, cold shock domain; DBD, DNA binding domain; GSH-Sepharose, glutathione Sepharose; GST, glutathione *S*-transferase; p35, 35 kDa protein which activates Cdk5; PCR, polymerase chain reaction; pRb, human retinoblastoma protein; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAD, transcriptional activation domain

PII: S0014-5793(99)00248-3

COS-1 cells. Similar experiments reveal that dbpA(CΔ) can bind Cdk4 as well, but not Cdk2 or Cdk1. Surprisingly, dbpA(CΔ) appears to be an efficient inhibitor of Cdk5/p35 and Cdk4/cyclin D1 kinase activities but fails to inhibit Cdk2/cyclin A.

2. Materials and methods

2.1. Plasmid constructs for yeast expression

*Eco*RI-*Bgl*II PCR fragments of human *Cdk1*, *Cdk2* and *Cdk4* and *Eco*RI-*Sal*I PCR fragments of bovine *Cdk5* and human *p35* were cloned in the pLEX-a vector [19] downstream of the *LEX-a* DNA binding domain (DBD). Expression in yeast from pLEX-a plasmids is controlled by the constitutive alcohol dehydrogenase promoter (*ADH1*), and the plasmid encodes the *TRP1* and *ADE2* genes for stable propagation in yeast. Furthermore, the *Eco*RI-*Sal*I fragments of *Cdk5* and *p35* were also cloned in the vector pGAD424 (Clontech) in such a way that they lie directly downstream of the *GAL4* transcriptional activation domain (TAD). Expression from the pGAD424 plasmid, which encodes *LEU2* as a marker gene, is also driven by the *ADH1* promoter.

A *Bam*HI-*Sal*I PCR fragment of *dbpA*(CΔ) (the isolated HeLa cDNA) was cloned in pLEX-a for expression in yeast. Furthermore, a *Bam*HI-*Xho*I fragment of the *dbpA*(CΔ) was cloned in pACT2 (Clontech) whereas a *Bam*HI-*Not*I fragment was cloned in pVP16 [19]. The pACT2 plasmid encodes $GAL4_{TAD}$ whereas the pVP16 plasmid encodes $VP16_{TAD}$. The TADs in both plasmids are under the control of the constitutive *ADH1* promoter. The plasmids encode *LEU2* for selection in yeast. The *dbpA*(CΔ) fragment has been cloned directly downstream of $GAL4_{TAD}$ and $VP16_{TAD}$.

Plasmids pLEX-a/lamin and pLEX-a/MyoD were gifts from S.M. Hollenberg [19].

2.2. Plasmid constructs for E. coli expression

A *Bam*HI-*Sal*I fragment of the *dbpA*(CΔ) cDNA was cloned in (a) pGEX4T-1 (Amersham-Pharmacia Biotech) for expression of a GST-dbpA(CΔ) fusion protein and (b) pQE32 (Qiagen) for expression of a His-tagged dbpA(CΔ) protein.

2.3. Plasmid constructs for COS-1 cell expression and in vitro transcription/translation

A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pcDNA3.1− (Invitrogen). The *Eco*RI-*Xho*I fragments of *Cdk1*, *Cdk2* and *Cdk4* were cloned into pcDNA3.1+ (Invitrogen). The plasmid pcMV-β-gal was obtained from H.-J. Keller (Novartis).

2.4. Plasmid constructs for insect cell expression

A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pFastBac1 (Gibco BRL) for construction of a recombinant baculovirus. The pFastBac1 vector has a baculovirus-specific promoter from *Autographa californica* nuclear polyhedrosis virus (AcNPV) for heterologous expression of proteins in insect cells. Similarly, *Bam*HI-*Eco*RI fragments of human *Cdk2* and *Cdk4* were cloned in pFastBac1 for construction of recombinant baculoviruses.

*Eco*RI-*Sal*I fragments of human *p35*, human *cyclin A*, murine *cyclin D2* and murine *cyclin D3* were cloned in pFastBac1 downstream of a *Bgl*II-*Eco*RI fragment of GST (PCR fragment from pGEX4T-1; Amersham-Pharmacia Biotech) for construction of recombinant baculoviruses. Similarly, an *Eco*RI-*Xho*I fragment of human *cyclin D1* and a *Bam*HI-*Sal*I fragment of *dbpA*(CΔ) were cloned as *GST* fusions in pFastBac1.

2.5. Yeast strains and media

The *Saccharomyces cerevisiae* strains L40 (Mata, *his3*Δ200, *trp1*-901, *leu2*-3,112, *ade2*, *lys2*-801am, *URA3*::$(lexAop)_8$-lacZ, *LYS*::$(lexAop)_4$-*his3*) and AMR70 (Matα, *his3*Δ200, *trp1*-901, *leu2*-3,112, *ade2*, *lys2*-801am, *URA3*::$(lexAop)_8$-*lacZ*) [19] were used for yeast transformation and for mating assays. In order to perform the yeast two-hybrid screen, L40 was transformed sequentially with (a) pLEX-a/cdk5 and (b) a human HeLa S3 Matchmaker cDNA library cloned unidirectionally into the *Eco*RI and *Xho*I sites of pGADGH (Clontech, HL4000AA). The pGADGH vector contains the $GAL4_{TAD}$ under the control of the *ADH1* promoter and also encodes *LEU2* as a marker gene. In order to mate a L40 strain that was cured of bait plasmid but still contains the prey plasmid (from the cDNA library), transformants of AMR70 (bearing plasmids pLEX-a/cdk5 or pLEX-a/lamin) were used. Both L40 and AMR70 contain the *lacZ* reporter gene linked to the *lexA* operon. Besides, L40 also contains the *HIS3* reporter gene downstream of the *lexA* operon. Untransformed yeast strains were grown in YPAD whereas transformed yeast strains were grown in minimal medium that allow maintenance of plasmids in a particular strain.

The *S. cerevisiae* strain L40 was also used for transformation of pLEX-a plasmids that encode *Cdk1*, *Cdk2*, *Cdk4*, *Cdk5*, *p35*, *dbpA*(CΔ), *lamin* or *MyoD*. A pACT2 or pVP16 plasmid that encodes *dbpA*(CΔ) or pGAD424 plasmids that encode *Cdk5* and *p35* were used to perform yeast two-hybrid assays on selected transformants.

2.6. Yeast two-hybrid screen

A pLEX-a/cdk5 plasmid-bearing strain of L40 was transformed with the HeLa cDNA library (Clontech) and transformants were selected exactly as described earlier [19]. His^+ colonies were lysed in liquid nitrogen and assayed for β-galactosidase activity on filters. Only those colonies, which do not express β-galactosidase after loss of the pLEX-a/cdk5 bait plasmid, were selected for further analysis. The plasmids pLEX-a/cdk5 or pLEX-a/lamin were reintroduced into L40 (a) by direct transformation of L40 strains that already contain the prey plasmid or (b) by mating prey plasmid-containing L40 strains with AMR70 transformants of pLEX-a/cdk5 or pLEX-a/lamin. Later, the prey plasmids were isolated by transforming total yeast DNA into HB101 and by selecting for leucine prototrophy on minimal medium plates (manufacturer's protocol; Clontech). Plasmids, that contain defined inserts, were then retransformed into L40 strains that already harbor pLEX-a/cdk5 or pLex-a/lamin. Plasmids, which express proteins that do not bind lamin but reproducibly interact with Cdk5, were sequenced using an automated DNA sequencer (LiCor). Inserts were identified by comparing translated DNA sequences with the SWISS PROT database.

2.7. Pull-down assay with [^{35}S]methionine-labeled Cdk1, Cdk2, Cdk4 and Cdk5

The pcDNA3.1 plasmids (1 μg) carrying the genes *Cdk1*, *Cdk2*, *Cdk4* or *Cdk5* were linearized uniquely at the 3′-end of the gene inserts. The linearized plasmids were used as templates for in vitro transcription/translation. The Cdk proteins were radioactively labeled with [^{35}S]methionine (20 μCi of an in vivo cell labeling grade; Amersham-Pharmacia Biotech). The transcription and translation was performed using the TNT-T7 in vitro transcription/translation kit (Promega).

Bacterially expressed glutathione *S*-transferase (GST) and GST-dbpA(CΔ) fusion protein were bound to glutathione Sepharose 4B (Amersham-Pharmacia Biotech) by incubating protein and beads at RT for 1 h. After four washing steps with 10 volumes of ice-cold PBS, the beads were resuspended in 200 μl 1% w/v BSA-PBS solution and kept on ice. 5 μl of the [^{35}S]methionine-labeled Cdk proteins (from 100 μl of an in vitro transcribed/translated product) diluted in 200 μl 1% w/v BSA-PBS solution, which contained 4 μl of a 100× solution of universal protease inhibitors (tablet dissolved in redistilled H_2O; Boehringer Mannheim), was kept on ice for 15 min. GSH-Sepharose 4B beads, bound to GST or GST-dbpA(CΔ), were incubated with labeled Cdks at 4°C for 2 h with gentle mixing. The beads were washed three times with 1 ml of bead-binding buffer (50 mM potassium phosphate pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100) containing universal protease inhibitors (Boehringer Mannheim). The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer (4% w/v SDS, 0.1 M Tris, 4 mM EDTA, 20% v/v glycerol, 33% w/v bromophenol blue) containing 50 mM DTT, and were analyzed by 12.5% SDS-PAGE. The gels were fixed (10% v/v glacial acetic acid, 30% v/v methanol solution) for 10 min, and the signal was enhanced by soaking the gel in EN^{3}HANCE (NEN) for 1 h at RT. The dried gel was exposed to Kodak X-OMAT AR film.

2.8. Transfection of pcDNA 3.1+/cdk2, pcDNA 3.1+/cdk4 and pcDNA 3.1-/cdk5 in COS-1 cells and precipitation of COS-1 expressed Cdks by GST-dbpA(CΔ)

COS-1 cells in six-well plates, grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) that contained 10% v/v fetal bovine serum (FBS, Gibco BRL), were transfected with 2 μg of pcDNA 3.1

plasmids that carry *Cdk2*, *Cdk4* or *Cdk5*, using calcium phosphate. In order to control transformation efficiency, pcMV-β-gal was used.

Harvested cells ($\sim 5\times10^5$) were washed once with PBS and then lysed in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% v/v Nonidet P-40) that contained 1 mM DTT and universal protease inhibitors (Boehringer Mannheim). Total protein in cell lysates was measured by the Bradford assay (Bio-Rad) and was stored at −70°C before use.

400 μl of a COS-1 cell lysate was first preincubated for 2 h at 4°C with 30 μl of Sepharose 4B (to remove unspecific binding of proteins to beads). This was followed by incubation with 30 μl of GSH-Sepharose 4B that was already bound to bacterially expressed GST-dbpA(CΔ). Pull-down of the Cdks in COS-1 cell lysates was performed as described above for the [^{35}S]methionine-labeled Cdks. The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer and were analyzed by Western blotting. The blots were probed with polyclonal antibodies raised against Cdk2 and N-terminal fragments of Cdk4 and Cdk5 and were detected by ECL (Amersham-Pharmacia Biotech).

2.9. Northern blotting

25 ng of pcDNA3.1+ encoding the *dbpA(CΔ)* gene was used to make a labeled probe for Northern blotting (1.9×10^9 dpm/μg) using the rediprime DNA labeling system (Amersham-Pharmacia Biotech, RPN1633) with Redivue [^{32}P]dCTP (specific activity = 3000 Ci/mmol; Amersham-Pharmacia Biotech). The probe was used to hybridize a human multiple tissue Northern blot according to the manufacturer's protocol (Clontech). The blot was quantified using a phosphorimager (Molecular Devices).

2.10. Expression of baculoviruses

Recombinant baculoviruses encoding different genes were constructed using protocols provided by the manufacturer (Gibco BRL). The viruses were harvested and amplified until a desired titer was reached (viz. 1×10^8 pfu/ml).

2×10^7 Sf9 cells were used to co-infect baculoviruses carrying *Cdk5*, *Cdk4* or *Cdk2* with *GST-p35*, *GST-cyclin A*, *GST-cyclin D1*, *GST-cyclin D2* or *GST-cyclin D3* in 25 ml of SF900 II SFM medium (Gibco BRL). Triple infections were performed with the above mentioned viral combinations and baculoviruses carrying *GST-dbpA(CΔ)*. After 72 h, cells were harvested and lysed by sonication in NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 05% v/v NP-40, 5 mM NaF, 30 mM p-NPP, 1 mM PMSF, 1 μg/ml antipain) that contained universal protease inhibitors (Boehringer Mannheim). HBT buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% v/v Tween 20, 1 mM DTT, 5 mM NaF, 30 mM p-NPP, 25 mM β-glycerolphosphate, 1 mM PMSF, 1 μg/ml antipain) was used to lyse cells that were co-infected with *GST-cyclin D1*, *D2* or *D3* viruses. Heterodimeric enzyme complexes were isolated by binding to 100 μl of GSH-Sepharose 4B, overnight at 4°C. The beads were washed seven times with ice-cold NETN buffer (or HBT buffer) and three times with ice-cold kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$). The beads were resuspended in 100 μl kinase buffer and kept on ice before a kinase assay was performed.

2.11. Kinase assays

Phosphorylation of 0.1 μg histone H1 (type III-S: from calf thymus, Sigma H-5505) or 0.25 μg GST-Rb152 (i.e. C-terminal 152 amino acid residues of pRb covalently linked to GST; Santa Cruz Biotechnology) was performed in kinase buffer for 40 min at 30°C using 2 μl of beads, 10 μM ATP and 3 μCi [γ-^{33}P]ATP (specific activity = 3000 Ci/mmol; Amersham-Pharmacia Biotech) in a total volume of 10 μl. The reactions were boiled in Laemmli sample buffer and were analyzed by 12.5% SDS-PAGE. The gel was fixed and the phosphorylation was quantified using a phosphorimager (Molecular Devices).

3. Results

3.1. A C-terminal fragment of dbpA binds to Cdk5 in a yeast two-hybrid screen

Cdk5 has been used as a bait in the yeast two-hybrid system to screen for proteins that may interact with Cdk5. Since Cdk5 is expressed in both cycling and non-cycling cells, we thought it would be interesting to know if there are any Cdk5 binding proteins in cycling cells. Hence, a HeLa cDNA library in the yeast vector pGADGH was transformed into the yeast strain L40 [19] that already contained the bait plasmid pLEX-a/cdk5 (which encodes $Lexa_{DBD}$-Cdk5). Genes encoding interacting proteins were identified by selecting for His$^+$ prototrophs and subsequently through induction of β-galactosidase (β-gal) activity. Colonies that were His$^+$ and blue were considered positive and were used for further analysis (results not shown). It was later found that one such positive yeast colony harbored a cDNA library plasmid (i.e. the prey plasmid) encoding $GAL4_{TAD}$ fused to a C-terminal fragment of the human gene for the DNA binding protein, dbpA (in this communication referred to as dbpA(CΔ); Fig. 2A).

In order to eliminate the possibility that an identified positive colony was an artifact of the two-hybrid system (frequently referred to as 'false positives') [20], the yeast strain was cured of the bait plasmid pLEX-a/cdk5 by growing the strain in a non-selective medium (50 generations of growth in minimal medium that contained tryptophan and adenine but lacked leucine; the prey plasmid contains *LEU2*). The resulting L40 strain (MATa) that contains the prey plasmid, pGADGH/dbpA(CΔ), was mated with strains of AMR70 (MATα) that had been transformed either with pLEX-a/cdk5 or with pLEX-a/lamin. β-Gal assays performed on mated diploid strains showed that dbpA(CΔ) specifically interacts with Cdk5 but not with lamin (Fig. 1A).

At this stage, the pGADGH/dbpA(CΔ) plasmid was isolated from yeast (via transformation of total yeast DNA in *E. coli*; see Section 2). The insert was sequenced and it revealed that the cloned gene encoded 172 amino acids of the C-terminal fragment of human dbpA (Fig. 2A). The *dbpA(CΔ)* cDNA was amplified by PCR from the pGADGH plasmid and was subcloned into pACT2, another yeast vector often used for two-hybrid assays. The plasmid pACT2/dbpA(CΔ) (which encodes $GAL4_{TAD}$-dbpA(CΔ) fusion protein) was transformed into the strain L40 that already contained pLEX-a/cdk5, pLEX-a/lamin, pLEX-a/MyoD or pLEX-a/p35 (all plasmids code for $Lexa_{DBD}$ fusion proteins). The results reiterate that, at least in the two-hybrid system, Cdk5 not only binds p35 (Cdk5's known activator) but also interacts with dbpA(CΔ) (Fig. 1B, left and right panels). It was also found that interaction of dbpA(CΔ) with Cdk5 does not depend on the specific TAD to which dbpA(CΔ) is linked. This was supported by the observation that a $VP16_{TAD}$-dbpA(CΔ) fusion protein binds as well as $GAL4_{TAD}$-dbpA(CΔ) to $Lexa_{DBD}$-Cdk5, in the two-hybrid assay (data not shown). Furthermore, the *dbpA(CΔ)* was cloned in pLEX-a and *cdk5* in pGAD424. It was seen that interchanging the two domains, TAD and DBD, did not affect the interaction between dbpA(CΔ) and Cdk5 (i.e. $Lexa_{DBD}$-dbpA(CΔ) fusion protein bound strongly to $GAL4_{TAD}$-Cdk5; Fig. 1C). All these observations prove beyond doubt that neither a TAD nor a DBD has any influence on the Cdk5-dbpA(CΔ) interaction.

We also wanted to find out if Cdk5 was the only Cdk to which dbpA(CΔ) has affinity. In order to test this, yeast strain L40 was first transformed with pLEX-a/cdk5, pLEX-a/cdk4, pLEX-a/cdk2 or pLEX-a/cdk1. The plasmid pACT2/dbpA(CΔ) (which encodes $GAL4_{TAD}$-dbpA(CΔ)) was then transformed into L40 strains that already had the ability to express Lexa-Cdk5, Lexa-Cdk4, Lexa-Cdk2 or Lexa-Cdk1 fusion proteins. A β-gal assay (Fig. 1D) depicts that, in the yeast-two-hybrid

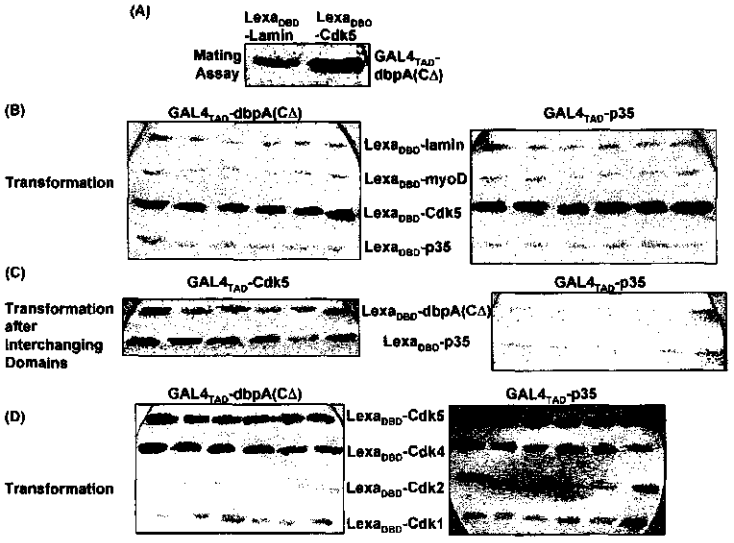


Fig. 1. In a yeast two-hybrid assay the C-terminal fragment of DNA binding protein dbpA binds to Cdk5 and Cdk4. Interactions were monitored by analyzing six individual colonies by colorimetric β-galactosidase assays. A: After curing the bait plasmid, a L40 strain that retains the prey plasmid (which encodes GAL4$_{TAD}$-dbpA(CΔ) fusion protein) was mated with strain AMR70 that had already been transformed with pLEX-a/lamin or pLEX-a/Cdk5 (these plasmids express Lex-a-lamin and Lex-a-Cdk5 fusion proteins, respectively). The results show that dbpA(CΔ) interacts with Cdk5 but not with the unspecific protein, lamin. B: Left panel, plasmid pACT2/dbpA(CΔ) (which expresses GAL4$_{TAD}$-dbpA(CΔ)) was transformed in strain L40 that already contains pLEX-a/lamin, pLEX-a/MyoD, pLEX-a/Cdk5 or pLEX-a/p35. The results show that dbpA(CΔ) interacts specifically with Cdk5. Right panel, plasmid pGAD424/p35 (expressing GAL4$_{TAD}$-p35) was transformed in the same L40 yeast strains (see left panel), as a control. C: Plasmids pGAD424/Cdk5 (left panel) and pGAD424/p35 (right panel, as negative control) were transformed into L40 strains that already harbor pLEX-a/dbpA(CΔ) or pLEX-a/p35. Results show that Cdk5 and dbpA(CΔ) can still interact with each other even after interchanging the two domains (GAL4$_{TAD}$ and Lex-a$_{DBD}$) which has to be linked to two putative interacting proteins, in the two-hybrid assay. D: Plasmids pACT2/dbpA(CΔ) (left panel) and pACT2/p35 (right panel, as control), which express GAL4$_{TAD}$-dbpA(CΔ) and GAL4$_{TAD}$-p35 respectively, were transformed in L40 strains that already harbor pLEX-a/Cdk5, pLEX-a/Cdk4, pLEX-a/Cdk2 or pLEX-a/Cdk1. Results show that dbpA(CΔ) interacts specifically with Cdk5 and Cdk4.

system, dbpA(CΔ) can interact with Cdk5 and Cdk4 but not with Cdk2 and Cdk1.

3.2. The dbpA(CΔ) protein precipitates ^{35}S-labeled Cdk5 and Cdk4

In order to confirm some of the yeast two-hybrid data, a set of in vitro pull-down experiments were employed. We found that bacterially expressed GST-dbpA(CΔ) fusion protein (Fig. 2B) was able to pull down [^{35}S]methionine-labeled Cdk5 and Cdk4 but not Cdk2 and Cdk1 (all proteins were transcribed/translated in vitro). We were sure that the GST moiety in GST-dbpA(CΔ) did not play a role in the precipitation of Cdk5 or Cdk4 since the GST protein alone is not able to pull down Cdk5 (Fig. 2C).

3.3. The dbpA(CΔ) protein precipitates Cdk5 and Cdk4 expressed in COS-1 cells

As an alternative to the in vitro transcription/translation procedure to obtain desired proteins, Cdk5, Cdk4 and Cdk2 were expressed in COS-1 cells. Lysates from transfectants were incubated with the *E. coli* expressed GST-dbpA(CΔ) fusion protein. The bound proteins were detected by Western blotting, using antibodies specific to Cdk5, Cdk4 or Cdk2. Similar to our earlier observations with labeled proteins, we observed that GST-dbpA(CΔ) was able to bind COS-1-expressed Cdk5 and Cdk4 whereas it was unable to pull-down Cdk2 (Fig. 2D).

3.4. Tissue specific expression of dbpA

The *dbpA*(CΔ) cDNA was labeled with ^{32}P and a human multiple tissue Northern blot was probed. The *dbpA* mRNA was relatively abundant in skeletal muscle tissue and the heart (Fig. 3), corroborating earlier findings of Kudo et al. [21]. Since Cdk5 has been demonstrated to play a role in muscle differentiation [11,12] we were interested to find out what effect dbpA(CΔ) would have on the Cdk5 kinase.

3.5. Inhibition of the Cdk5 kinase by bacterially expressed GST-dbpA(CΔ) and His-dbpA(CΔ)

The Cdk5/GST-p35 heterodimeric complex was expressed in insect cells using the baculovirus expression system. The enzyme was purified by binding to GSH-Sepharose 4B. The bead-bound enzyme was tested for activity (see Section 2) using histone H1 as a substrate. Fig. 4A (lane 1) portrays the phosphorylation of histone H1 by GST-p35 activated Cdk5 kinase. Kinase assays performed on GST-dbpA(CΔ) demonstrate that dbpA(CΔ) is not phosphorylated by Cdk5 and therefore cannot be a Cdk5 substrate (Fig. 4A, lane 3). We then asked if dbpA(CΔ) could be an inhibitor of Cdk5. Fig. 4B shows a dose-dependent inhibition of Cdk5 by GST-dbpA(CΔ). It should be noted that, whereas 10 μg of GST did not have any effect on the kinase (Fig. 4B, lane 8), 0.4 μg of GST-dbpA(CΔ) distinctly inhibited the complex (> 50%; Fig. 4B, lane 5). Staurosporine (CGP39360, Novartis), which

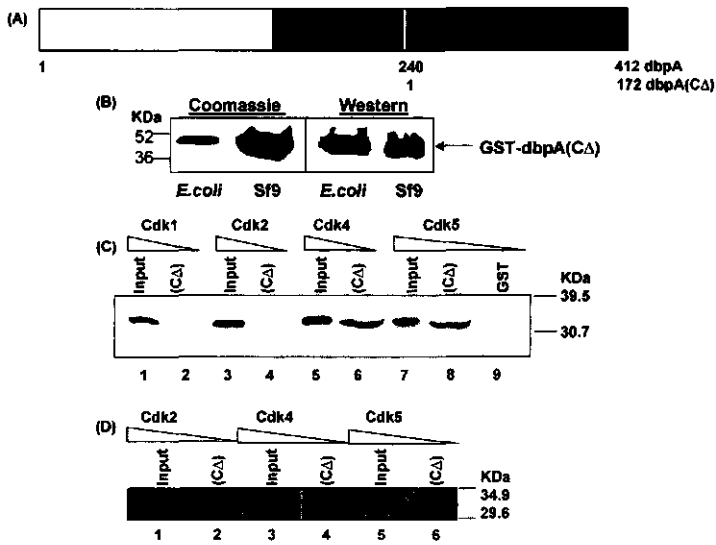


Fig. 2. A: Model of DNA binding protein dbpA. The gray area is the C-terminal fragment of dbpA (172 aa) which we have found to interact with Cdk5 and Cdk4. This area contains α-helices, basic and acidic stretches which are important in protein-protein interactions. The black area is the cold shock domain, this region is homologous in all Y-box binding proteins and is presumed to be the DNA binding domain [24]. B: Coomassie stain of 0.1 μg *E. coli* and 1 μg insect cell (Sf9) expressed GST-dbpA(CΔ) and Western blot of 0.1 μg *E. coli* and 0.1 μg insect cell (Sf9) expressed GST-dbpA(CΔ) using a monoclonal antibody against GST (Clontech). C: GST-dbpA(CΔ) (referred to as (CΔ)) binds to ^{35}S-labeled Cdk4 and Cdk5 and not to ^{35}S-labeled Cdk1 and Cdk2. Lane 1, 10% of the ^{35}S-labeled Cdk1 input used for the pull-down assay with (CΔ) (as in lane 2); lane 3, 10% of the ^{35}S-labeled Cdk2 input used for the pull-down assay with (CΔ) (as in lane 4); lane 5, 10% of the ^{35}S-labeled Cdk4 input used for the pull-down assay with (CΔ) (as in lane 6); lane 7, 10% of the ^{35}S-labeled Cdk5 input used for the pull-down assay with (CΔ) (as in lane 8) and the pull-down assay with GST alone (as in lane 9). D: GST-dbpA (CΔ) binds to Cdk4 and Cdk5 and not to Cdk2 overexpressed in COS-1 cells. Each lane shows a Western blot of proteins fractionated on a 10% SDS-polyacrylamide gel. Polyclonal antibodies raised against Cdk2 and the N-termini of Cdk4 and Cdk5 were used to detect the respective proteins. Lanes 1, 3 and 5, 15 μl of lysates from COS-1 cells expressing Cdk2, Cdk4 or Cdk5. Lanes 2, 4 and 6, 400 μl of same cell lysates, used in pull-down assays with GST-dbpA(CΔ) (referred to as (CΔ)).

strongly inhibits most Cdks including Cdk5, was used as a control inhibitor [22].

It can be argued that the relatively large GST moiety causes a steric hindrance in the fusion protein so that it interferes with dbpA(CΔ)'s ability to act as a substrate for Cdk5 or that the presence of GST causes an artifactual inhibition of Cdk5. Therefore, dbpA(CΔ) was expressed in *E. coli* as a His-tagged protein (six consecutive histidines attached to the protein at its N-terminus, as in pQE32; see Section 2). His-dbpA(CΔ) was used to probe if it is a substrate or an inhibitor of the Cdk5/p35 kinase. Like GST-dbpA(CΔ), His-dbpA(CΔ) is not phosphorylated by Cdk5/GST-p35 (Fig. 4D, right panel, lane 4). Again similar to GST-dbpA(CΔ), His-dbpA(CΔ) inhibits the Cdk5/GST-p35 complex in a dose-dependent manner. His-dbpA(CΔ) completely abolishes the Cdk5-mediated phosphorylation of histone H1 (Fig. 4C, left panel). The His-tagged protein can also completely inhibit the phosphorylation of GST-Rb152 by the Cdk5/GST-p35 complex (Fig. 4C, right panel, lane 4 and 5). Kinase assays performed with Cdk5/GST-p35 enzyme eluted from the GSH-Sepharose 4B beads gave identical results (results not shown).

3.6. Activation of Cdk5 with D-type cyclins

Since D-type cyclins are reported to bind Cdk5 [5], we wanted to find out if cyclin D formed a complex with Cdk5 when both proteins are expressed in insect cells, and if it did, whether the complex formed an active kinase by phosphorylating either histone H1 or GST-Rb152. One could speculate that an active cyclin D/Cdk5 enzyme, with no known cellular substrate, may phosphorylate the Cdk5 binding protein, dbpA(CΔ). Hence, Sf9 insect cells were co-infected with baculoviruses encoding Cdk5 and GST-cyclin D1, GST-cyclin D2 or GST-cyclin D3. The cyclin D-bound Cdk5 proteins obtained from insect cells were precipitated with GSH-Sepharose 4B and the bead-bound complexes (as confirmed by Western blotting using antibodies specific for Cdk5 and D-type cyclins; results not shown) were used directly for kinase assays. We observed that neither GST-cyclin D1 nor GST-cyclin D3 can activate Cdk5 (results not shown). Surprisingly, like cyclin D2-activated Cdk2 (Fig. 4D, left panel, lane 2) and Cdk4 (Fig. 4D, left panel, lane 4), kinases that are known to phos-

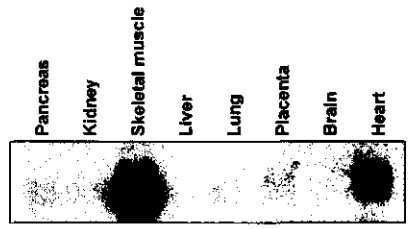


Fig. 3. Tissue-specific expression of dbpA. Human multiple tissue Northern blot (Clontech) hybridized with dbpA(CΔ).

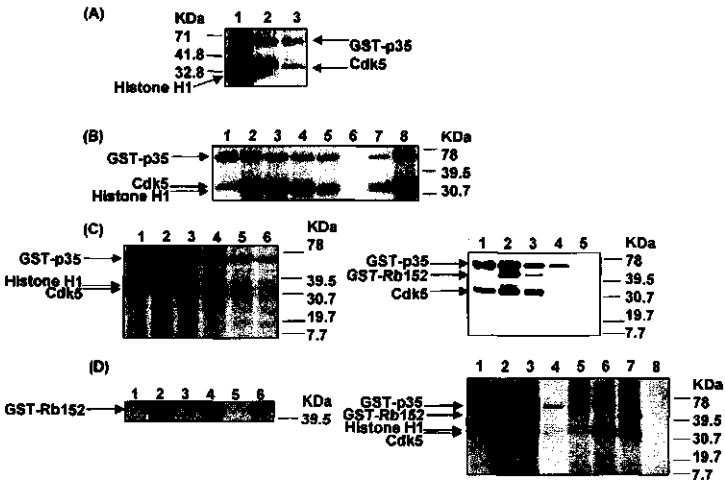


Fig. 4. Kinase assays with histone H1 and GST-Rb152, as substrates, show that both GST-dbpA(CΔ) and His-dbpA(CΔ) proteins are not substrates for Cdk5 that is activated by p35 or cyclin D2. However, GST-dbpA(CΔ) and His-dbpA(CΔ) inhibit Cdk5-mediated phosphorylation of histone H1 and GST-Rb152 in a dose-responsive manner. A: GST-dbpA(CΔ) is not phosphorylated by p35 activated Cdk5 kinase. Lane 1, Cdk5/GST-p35+10 μg histone H1 as substrate; lane 2, Cdk5/GST-p35+10 μg GST as substrate; lane 3, Cdk5/GST-p35+0.4 μg GST-dbpA(CΔ) as substrate. B: Dose response to GST-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of histone H1 (1 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.4 μg GST-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+0.8 μg GST-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+1.6 μg GST-dbpA(CΔ); lane 6, Cdk5/GST-p35+substrate+300 nM staurosporine; lane 7, Cdk5/GST-p35+substrate+40 nM staurosporine; lane 8, Cdk5/GST-p35+substrate+10 μg GST. C: Left panel, dose response to His-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of histone H1 (0.1 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+1 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+3 μg His-dbpA(CΔ); lane 6, Cdk5/GST-p35+substrate+6 μg His-dbpA(CΔ). Right panel, dose response to His-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of GST-Rb152 (0.25 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+1 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+6 μg His-dbpA(CΔ). D: His-dbpA(CΔ) is not a substrate for Cdk5/GST-p35 and Cdk5/GST-cyclin D2 kinases. Left panel, GST-cyclin D2-activated kinases were prepared and were tested for their ability to phosphorylate 0.25 μg GST-Rb152. Lane 1, Cdk2/GST-cyclin D2+no substrate; lane 2, Cdk2/GST-cyclin D2+substrate; lane 3, Cdk4/GST-cyclin D2+no substrate; lane 4, Cdk4/GST-cyclin D2+substrate; lane 5, Cdk5/GST-cyclin D2+no substrate; lane 6, Cdk5/GST-cyclin D2+substrate. Right panel, both Cdk5/GST-p35 and Cdk5/GST-cyclin D2 were tested for their ability to phosphorylate histone H1, GST-Rb152 and His-dbpA(CΔ). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35 incubated with 1 μg histone H1; lane 3, Cdk5/GST-p35 kinase incubated with 0.25 μg GST-Rb152; lane 4, Cdk5/GST-p35 incubated with 5 μg His-dbpA(CΔ); lane 5, Cdk5/GST-cyclin D2+no substrate; lane 6, Cdk5/GST-cyclin D2 incubated with 1 μg histone H1; lane 7, Cdk5/GST-cyclin D2 incubated with 0.25 μg GST-Rb152; lane 8, Cdk5/GST-cyclin D2 incubated with 5 μg His-dbpA(CΔ).

phorylate pRb, cyclin D2-activated Cdk5 also has the ability to phosphorylate GST-Rb152 (Fig. 4D, left panel, lane 6). However, His-dbpA(CΔ) is not phosphorylated by cyclin D2 and p35 activated Cdk5 (Fig. 4D, right panel, lanes 4 and 8).

3.7. Inhibition of the Cdk4 kinase by bacterially expressed His-dbpA(CΔ)

Since dbpA(CΔ) interacts not only with Cdk5 but also with Cdk4 in both two-hybrid and in vitro binding assays, the effect of His-dbpA(CΔ) on active Cdk4/cyclin D1 complex was investigated. We see that the His-tagged protein completely inhibits Cdk4/GST-cyclin D1 mediated phosphorylation of GST-Rb152 (Fig. 5A, lane 4). Furthermore, dbpA(CΔ) is definitely not a substrate for the Cdk4/GST-cyclin D1 enzyme (Fig. 5A, lane 5).

3.8. Specificity of inhibition of the Cdk5 kinase compared to the Cdk2 kinase

Although His-dbpA(CΔ) inhibits the Cdk5/GST-p35 mediated phosphorylation of histone H1 (there is at least a 20-fold decrease in activity; compare lanes 3 and 4 in Fig. 5B), it seems that phosphorylation of the same substrate by Cdk2/GST-cyclin A is not affected at all (Fig. 5B, compare lanes 1 and 2). This seems to corroborate our earlier two-hybrid data which indicated that dbpA(CΔ) does not interact with Cdk2 but binds Cdk4 and Cdk5.

3.9. Triple infections with GST-DbpA(CΔ) baculoviruses do not yield active Cdk5 or Cdk4 kinases

Sf9 insect cells were infected with three baculoviruses encoding (a) Cdk5, GST-p35 and GST-dbpA(CΔ), (b) Cdk4, GST-cyclin D1 and GST-dbpA(CΔ), or (c) Cdk2, GST-cyclin A and GST-dbpA(CΔ). Proteins were precipitated using GSH-Sepharose. Western blot analysis showed that all three proteins were expressed in each precipitate. Kinase assays performed on the GSH-Sepharose purified enzymes (according to Section 2) yielded inactive kinases for Cdk5 and Cdk4 whereas the Cdk2 kinase was still active (results not shown).

3.10. Inhibition of the Cdk5 and Cdk4 kinase by insect cell expressed GST-DbpA(CΔ)

GST-dbpA(CΔ) was purified from Sf9 insect cells using the baculovirus expression system (Fig. 2B) and used in Cdk5/GST-p35, Cdk4/GST-cyclin D1 and Cdk2/GST-cyclin A kin-

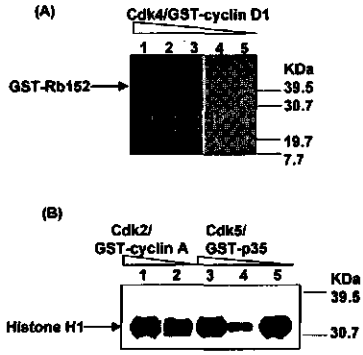


Fig. 5. His-dbpA(CΔ) is not a substrate for Cdk4/GST-cyclin D1 but instead inhibits the kinase. Comparison of the inhibition of Cdk5/GST-p35 and Cdk2/GST-cyclin A kinases by His-dbpA(CΔ). A: His-dbpA(CΔ) inhibits Cdk4/GST-cyclin D1-mediated phosphorylation of GST-Rb152 (0.5 μg) and is not phosphorylated by the kinase. Lane 1, Cdk4/GST-cyclin D1+no substrate; lane 2, Cdk4/GST-cyclin D1+substrate (the lower band is a breakdown product of GST-Rb152); lane 3, Cdk4/GST-cyclin D1+substrate+1 μg His-dbpA(CΔ); lane 4, Cdk4/GST-cyclin D1+substrate+6 μg His-dbpA(CΔ); lane 5, Cdk4/GST-cyclin D1+6 μg His-dbpA(CΔ). B: Comparison of inhibition of the Cdk5/GST-p35 and Cdk2/GST-cyclin A-mediated phosphorylation of histone H1 (1 μg) by His-dbpA(CΔ). Lane 1, Cdk2/GST-cyclin A+substrate; lane 2, Cdk2/GST-cyclin A+substrate+0.5 μg His-dbpA(CΔ); lane 3, Cdk5/GST-p35+substrate; lane 4, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+6 μg His-FKBP12 (an unspecific protein). The results show that His-dbpA(CΔ) inhibits Cdk5/GST-p35 but not the Cdk2/GST-cyclin A kinase.

ase assays. The insect cell expressed GST-dbpA(CΔ) protein gave the same results as the bacterially expressed GST-dbpA(CΔ) in its inhibition of the Cdk5/GST-p35 and the Cdk4/GST-cyclin D1 kinase and not the Cdk2/cyclin A kinase, whereas it was not a substrate for any of the kinases tested (results not shown). Eukaryotic expression of GST-dbpA(CΔ) did therefore not give any drastic difference in the folding or post-translational modification of the polypeptide.

4. Discussion

DNA binding protein DbpA belongs to the Y-box protein family. These proteins contain three domains: N-terminal domain, cold shock domain (CSD) and C-terminal domain. The cold shock domain, comprising of 70 amino acids, is highly conserved from prokaryotes to eukaryotes [23] and can bind so called Y-boxes on DNA. The DNA binding depends on the presence of inverted CCAAT motifs and flanking bases in these Y-boxes. The N-terminal region of dbpA is rich in proline and alanine residues which could potentially function as a transcriptional regulation domain [24]. Several eukaryotic genes contain a Y-box in their regulatory region. Ishii and colleagues were the first to isolate dbpA via interaction with the enhancer of the human epidermal growth factor receptor gene and the promoter of the human *c-erbB-2* gene [25]. The dbpA protein has also been found to bind to the promoter sequence of the leukosialin gene [21]. Furthermore, the dbpA protein represses the expression of the *I-Aβ* gene of the major histocompatibility complex [24] and the expression of the stress-inducible gene *grp78* [26]. The C-terminal domain of dbpA is hydrophilic due to an alteration of groups of basic and acidic amino acids, termed the charged zipper domain, that presumably contributes to interactions with other proteins [24]. This is the region we have isolated using the yeast two-hybrid system (Fig. 2A). Surprisingly, it inhibits both Cdk5 and Cdk4 activity. Further experiments should reveal whether full length dbpA has the same effect on Cdk5 and Cdk4 as its C-terminal fragment.

We have not found the consensus sequence (X-S/T-P-X-K/R-X-) [27], which is typical of a site phosphorylated by a Cdk, in dbpA(CΔ) (EMBL-PROSITE database). Our data confirm that dbpA(CΔ) is neither a substrate for Cdk5 nor for Cdk4 (Fig. 4D, right panel, lane 4 and Fig. 5A, lane 5 respectively). However, it should be noted that the protein has three potential protein kinase C phosphorylation sites. We do not know if these sites are utilized at all.

Although Cdk5 is expressed in both proliferative and differentiated cells, its expression is relatively abundant in nerve and muscle cells. Since dbpA mRNA is present in HeLa cells, the dbpA protein must also be present in proliferating cells. DbpA may therefore play a role in inhibiting Cdk5 during the cell cycle.

The p35 protein, the only known activator of Cdk5, is solely expressed in differentiated nerve cells. Cdk5 kinase activity (with p35 as its activator) could therefore be responsible for the onset of differentiation of nerve cells and possibly of muscle cells [9–12]. However, Cdk5 kinase activity should be blocked during terminal differentiation. Since both Cdk5 and p35 are present in terminally differentiated cells, other proteins should be involved in preventing this complex to be active in these cells. We would like to propose that dbpA is probably a specific inhibitor of Cdk5. This assumption is supported by results from Northern blot analyses which show that dbpA is highly expressed in skeletal muscle and heart as compared to other tissues ([21]; and our results, Fig. 3). Cdk5 is reported to phosphorylate neurofilament proteins and tau protein in vitro. Neurofilament proteins are also hyperphosphorylated in patients suffering from Lewy body pathologies, whereas the tau protein is hyperphosphorylated in patients suffering from Alzheimer's disease. Since the dbpA transcript was not detected in the brain ([21]; and our results, Fig. 3) other proteins may be responsible for inhibition of Cdk5 in the brain. It is also possible that dbpA is expressed in a particular section of the brain whose mRNA was not present in the commercial blot used for Northern analysis.

It is known that the Cdk4 kinase is responsible for phosphorylation of pRb early in the G1 phase of the cell cycle and has a very important function in cycling cells [28]. One can speculate that binding of dbpA to the Cdk4 protein allows inhibition of its kinase activity which thereby causes cell cycle arrest. Probably, this is what occurs in differentiated cells where it is essential that Cdk4 activity is inhibited. In cycling cells however, dbpA should be bound to Cdk5 to prevent the premature onset of differentiation. When Cdk5 is activated during differentiation, dbpA may switch its binding allegiance to Cdk4, resulting in the latter's inhibition and causing a cell cycle block. Later, terminally differentiated cells may again have dbpA bound to Cdk5 to maintain the kinase in its inactive form.

It has been reported that dbpA activates the transcription of thymidine kinase [29]. This may also suggest that dbpA is expressed at the beginning of S phase of the cell cycle and

may thus block Cdk4 activity at a point in the cell cycle where Cdk4 activity is no longer needed. This assumption may be equally valid if Cdk5 were to be responsible for the onset of differentiation. It is noteworthy that interactions between dbpA and Cdk5 or Cdk4 provide a link between unique signal transduction pathways and allow for alteration in gene expression. However, the true role of dbpA in cycling and post-mitotic cells needs to be evaluated through further cellular analyses.

References

[1] Meyerson, M., Enders, G.H., Wu, C.L., Su, L.K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.H. (1992) EMBO J. 11, 2909–2917.
[2] Lew, J. and Wang, J.H. (1995) Trends Biochem. Sci. 20, 33–37.
[3] Tang, D. and Wang, J.H. (1996) Prog. Cell Cycle Res. 2, 205–216.
[4] Dellale, I., Bhide, P.G., Caviness Jr., V.S. and Tsai, L.H. (1997) J. Neurocytol. 26, 283–296.
[5] Guidato, S., McLoughlin, D.M., Grierson, A.J. and Miller, C.C.J. (1998) J. Neurochem. 70, 335–340.
[6] Miyajima, M., Nornes, H.O. and Neuman, T. (1995) NeuroReport 6, 1130–1132.
[7] Lee, K.-Y., Helbing, C.C., Choi, K.-S., Johnston, R.N. and Wang, J.H. (1997) J. Biol. Chem. 272, 5622–5626.
[8] Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R.J., Aebersold, R., Hunt, T. and Wang, J.H. (1994) Nature 371, 423–426.
[9] Nikolic, M., Dudek, H., Kwon, Y.T., Ramos, Y.F. and Tsai, L.H. (1996) Genes Dev. 10, 816–825.
[10] Chae, T., Kwon, Y.T., Bronson, R., Dikkes, P., Li, E. and Tsai, L.H. (1997) Neuron 18, 29–42.
[11] Lazaro, J.B., Kitzmann, M., Poul, M.A., Vandromme, M., Lamb, N.J. and Fernandez, A. (1997) J. Cell Sci. 110, 1251–1260.
[12] Philpott, A., Porro, E.B., Kirschner, M.W. and Tsai, L.H. (1997) Genes Dev. 11, 1409–1421.
[13] Henchcliffe, C. and Burke, R.E. (1997) Neurosci. Lett. 230, 41–44.
[14] Brion, J.-P. and Couck, A.-M. (1995) Am. J. Pathol. 147, 1465–1476.
[15] Nakamura, S., Kawamoto, Y., Nakano, S., Ikemoto, A., Akiguchi, I. and Kimura, J. (1997) Neurology 48, 267–270.
[16] Imahori, K. and Uchida, T. (1997) J. Biochem. (Tokyo) 121, 179–188.
[17] Sengupta, A., Wu, Q., Grundke-Iqbal, I., Iqbal, K. and Singh, T.J. (1997) Mol. Cell. Biochem. 167, 99–105.
[18] Ohshima, T., Ward, J.M., Huh, C.-G., Longenecker, G., Veeranna, Pant, H.C., Brady, R.O., Martin, L.J. and Kulkami, A.B. (1996) Proc. Natl. Acad. Sci. USA 93, 11173–11178.
[19] Hollenberg, S.M., Sternglanz, R., Cheng, P.F. and Weintraub, H. (1995) Mol. Cell. Biol. 15, 3813–3822.
[20] Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) Cell 75, 805–816.
[21] Kudo, S., Mattei, M.-G. and Fukuda, M. (1995) Eur. J. Biochem. 231, 72–82.
[22] Veeranna, Shetty, K.T., Amin, N., Grant, P., Albers, R.W. and Pant, H.C. (1996) Neurochem. Res. 21, 629–636.
[23] Graumann, P.L. and Marahiel, M.A. (1998) Trends Biochem. Sci. 23, 286–290.
[24] Lloberas, J., Soler, C. and Celada, A. (1997) Immunobiology 198, 249–263.
[25] Sakura, H., Maekawa, T., Imamoto, F., Yasuda, K. and Ishii, S. (1988) Gene 71, 499–507.
[26] Li, W.W., Hsiung, Y., Wong, V., Galvin, K., Zhou, Y., Shi, Y. and Lee, A.S. (1997) Mol. Cell. Biol. 17, 61–68.
[27] Tang, D., Lee, K.-Y., Qi, Z., Matsuura, I. and Wang, J.H. (1996) Biochem. Cell Biol. 74, 419–429.
[28] Weinberg, R.A. (1995) Cell 81, 323–330.
[29] Kim, E.C., Lau, J.S., Rawlings, S. and Lee, A.S. (1997) Cell Growth Differ. 8, 1329–1338.

4. Identification of Ribosomal Protein L34 as a novel Cdk5 inhibitor

Mark Moorthamer and Bhabatosh Chaudhuri

(1999) *Biochem. Biophys. Res. Commun.* **255**, 631-638

Biochemical and Biophysical Research Communications 255, 631–638 (1999)
Article ID bbrc.1999.0145, available online at http://www.idealibrary.com on IDEAL®

Identification of Ribosomal Protein L34 as a Novel Cdk5 Inhibitor

Mark Moorthamer and Bhabatosh Chaudhuri[1]
Oncology Research, Novartis Pharma AG, Basel, Switzerland

Received November 27, 1998

The cell cycle is regulated by sequential activation, inactivation of cyclin dependent kinases (Cdk-s). Like all other Cdk-s, the catalytic subunit of Cdk5 is present in cycling cells. However, its highest concentration is found in differentiated neurons, and the only known protein that activates Cdk5 (i.e., p35) is expressed solely in the brain. Active Cdk5 is thought to be involved in the *in vivo* phosphorylation of the neurofilament proteins and tau which are hyperphosphorylated in neurodegenerative diseases. Recent reports suggest that Cdk5 may also contribute to cellular differentiation. Therefore, it would not be unusual to surmise that there exist specific proteins that regulate Cdk5 activity in cycling cells. In order to find if this was true, a cDNA library prepared from HeLa cells was screened using the yeast-two-hybrid system. The 60S ribosomal protein, L34, was identified as a Cdk5-interacting protein. Biochemical analyses reveal that L34 cannot activate Cdk5 but potently inhibits the p35-activated kinase. L34 also interacts with Cdk4 and, in parallel, inhibits the Cdk4/cyclin D1 activity. Interestingly, L34 does not interact with Cdk2 in the two-hybrid assay nor does it inhibit the Cdk2/cyclin A enzyme. The fact that a ribosomal protein inhibits Cdk5 and Cdk4 may suggest that these two kinases have a cellular role in translational regulation. © 1999 Academic Press

Key Words: **Cdk5; kinase inhibitor; L34; p35; yeast-two-hybrid system.**

Cyclin dependent kinase 5 (Cdk5) was initially identified on the basis of its sequence similarity to the family of cyclin dependent kinases (Cdk-s), a class of serine/threonine kinases which regulate important transitions in the cell cycle (1, 2). However, Cdk5 is an unusual member of the Cdk-family. Its catalytic subunit is predominantly expressed in post-mitotic cells of the nervous system, cells which have permanently exited the cell cycle (2, 3). Moreover, the protein p35 which activates the Cdk5 enzyme is exclusively expressed in the central nervous system (CNS) (3, 4). These observations have led to the belief that Cdk5 may be a cyclin dependent kinase with only neuron-specific function.

Nevertheless, the catalytic subunit of Cdk5 is widely expressed at basal levels in most human tissues, that is, in both cycling and non-cycling cells (2, 3). Interestingly, cyclins D and E, activator molecules which regulate Cdk activity in proliferating cells, bind Cdk5 (5, 6; our own observations). It could be argued that Cdk5 bound to cyclin D or cyclin E phosphorylates substrates which are not yet known.

The p35 protein, the only known activator of Cdk5, bears no significant homology to the family of cyclins (3, 4). As a Cdk-activator, p35 is unique since it has no influence on the activity of other Cdk-s (2, 3). The 35 kDa p35 protein was originally isolated as a smaller 25 kDa proteolytic product.

Not only can heterodimeric Cdk5/p35 and Cdk5/p25 complexes phosphorylate histone H1 but they also phosphorylate the retinoblastoma protein (pRb). Histone H1 and pRb are substrates that are frequently used to confirm *in vitro* activity of the Cdk-s (3, 7). Activated Cdk5 also phosphorylates its activating partner p35 (8). Besides, Cdk5 is known to phosphorylate *in vitro* a number of neuron-specific cytoskeletal proteins that includes the neurofilament proteins NF-M, NF-H, the microtubule associated protein tau and the actin binding protein caldesmon (2, 3). Phosphorylation of cytoskeletal proteins is thought to play an important role in the polymerization and assembly of

[1] Correspondence address: B. Chaudhuri, Oncology Research, Novartis Pharma AG, K-125.13.17, Basel, Switzerland. Fax: (41) (61) 696 38 35. E-mail: bob.chaudhuri@pharma.novartis.com.

Abbreviations: β-gal, beta-galactosidase; Cdk, human cyclin dependent kinase; DBD, DNA-binding domain; GSH-Sepharose, gluthathione Sepharose; GST, gluthathione-S-transferase; p35, 35 kDa protein which activates Cdk5; ORF, open reading frame; PCR, polymerase chain reaction; pRb, human retinoblastoma protein; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAD, transcriptional activation domain.

0006-291X/99 $30.00

cytoskeletal elements which, in turn, may affect growth of neurites. Indeed, Cdk5 activity has been demonstrated to play a key role in neurite outgrowth (9) and neuronal migration during differentiation of neurons (10). Recently, Cdk5 has been shown to participate in the regulation of myogenesis in the early embryo (11, 12). These observations may provide new insight into the possible function of Cdk5 during differentiation.

Cdk5 is known to phosphorylate neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies, i.e., diffuse Lewy body disease (dementia), Parkinson's disease (13, 14) and amyothrophic lateral sclerosis (2, 15), and tau protein at sites phosphorylated in Alzheimer's disease (2, 3, 16, 17). Moreover, it has been reported that the kinase activity of Cdk5 correlates with the extent of differentiation of neuronal cells (2, 3, 18) and colocalizes with neurofilament tracts in the axons of neuronal cells in culture.

It is therefore possible that Cdk5 activity is tightly regulated in pathways that lead from cell proliferation towards differentiation. Hence, we have inquired if there are any proteins that could be involved in the regulation of Cdk5 (i.e., may activate or inhibit the kinase) in cycling cells. With this in mind, a cDNA library, constructed from total RNA obtained from HeLa cells, was screened using the yeast-two-hybrid system. Amongst other proteins (unpublished observations), we find that the 60S ribosomal protein L34 interacts with the bait protein Cdk5, in the two-hybrid assay. Conversely, when L34 was used as bait, the interaction between L34 and Cdk5 was equally efficient. The validity of these interactions were corroborated by using the *Escherichia coli* expressed GST-L34 fusion protein. The purified protein precipitates not only *in vitro* transcribed/translated Cdk5 but also the protein which is expressed in COS-1 cells. Similar experiments reveal that L34 can bind Cdk4 as well, but not Cdk2 or Cdk1. Surprisingly, L34 appears to be an efficient inhibitor of Cdk5/p35 and Cdk4/cyclin D1 kinase activities but fails to inhibit Cdk2/cyclin A.

MATERIALS AND METHODS

Plasmid constructs for yeast expression. *Eco*RI-*Bgl*II fragments of human *Cdk1*, *Cdk2* and *Cdk4* and *Eco*RI-*Sal*I PCR fragments of bovine *Cdk5* and human *p35* were cloned in the pLEX-a vector (19) downstream of the *LEX-a* DNA-binding domain (DBD). Expression in yeast from pLEX-a plasmids is controlled by the constitutive alcohol dehydrogenase promoter (*ADH1*), and the plasmid encodes the *TRP1* and *ADE2* genes for stable propagation in yeast. Furthermore, the *Eco*RI-*Sal*I fragments of *Cdk5* and *p35* were also cloned in the vector pGAD424 (Clontech) in such a way that they lie directly downstream of the *GAL4* transcriptional activation domain (TAD). Expression from the pGAD424 plasmids, that encodes *LEU2* as a marker gene, is also driven by the *ADH1* promoter.

A *Bam*HI-*Sal*I fragment of the *L34* ORF was cloned in pLEX-a for expression in yeast. Furthermore, a *Bam*HI-*Xho*I fragment of L34 was cloned in pACT2 (Clontech) whereas a *Bam*HI-*Kpn*I fragment was cloned in pVP16 (19). The pACT2 plasmid codes for $GAL4_{TAD}$ whereas the pVP16 plasmid codes for $VP16_{TAD}$. The TAD-s in both plasmids are under the control of the constitutive *ADH1* promoter. The plasmids encode *LEU2* for selection in yeast. The *L34* gene has been cloned directly downstream of $GAL4_{TAD}$ and $VP16_{TAD}$.

Plasmids pLEX-a/lamin and pLEX-a/MyoD were gifts from S. M. Hollenberg (19).

Plasmid constructs for E. coli expression. A *Bam*HI-*Sal*I fragment of the *L34* open reading frame (ORF) was cloned in (a) pGEX4T-1 (Amersham Pharmacia Biotech) for expression of GST-L34 fusion protein and (b) pQE32 (Qiagen) for expression of a His-tagged L34 protein.

Plasmid constructs for COS-1 cell expression and in vitro transcription/translation. A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pcDNA3.1- (Invitrogen). Similarly, *EcoRI-XhoI* fragments of *Cdk1*, *Cdk2* and *Cdk4* were cloned into pcDNA3.1+ (Invitrogen). The plasmid pcMV-βgal was obtained from H.-J. Keller (Novartis).

Plasmid constructs for insect cell expression. A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pFastBac1 (Gibco-BRL) for construction of a recombinant baculovirus. The pFastBac1 vector has a baculovirus-specific promoter from *Autographa californica* nuclear polyhedrosis virus (AcNPV) for heterologous expression of proteins in insect cells. Similarly, *Bam*HI-*Eco*RI fragments of human *Cdk2* and *Cdk4* were cloned in pFastBac1 for construction of recombinant baculoviruses.

*Eco*RI-*Sal*I fragments of human *p35*, human *cyclin A*, murine *cyclin D2* and murine *cyclin D3* were cloned in pFastBac1 downstream of a *Bgl*II-*Eco*RI fragment of GST (PCR fragment from pGEX4T-1; Amersham Pharmacia Biotech) for construction of recombinant baculoviruses. Similarly, an *Eco*RI-*Xho*I fragment of human *cyclin D1* was cloned as a *GST* fusion in pFastBac1.

Yeast strains and media. The *S. cerevisiae* strains L40 (Mat **a**, *his*3Δ200, *trp*1-901, *leu*2-3,112, *ade*2, *lys*2-801am, $URA3::(lexAop)_8$-lacZ, $LYS::(lexAop)_4$-*his*3 and AMR70 (Mat α, *his*3Δ200, *trp*1-901, *leu*2-3,112, *ade*2, *lys*2-801am, $URA3::(lexAop)_8$-*lacZ* (19) were used for yeast transformation and for mating assays. In order to perform the yeast-two-hybrid screen, L40 was transformed sequentially with (a) pLEX-a/cdk5 and (b) a human HeLa S3 Matchmaker cDNA library cloned unidirectionally into the *Eco*RI and *Xho*I sites of pGADGH (Clontech, HL4000AA). The pGADGH vector contains the $GAL4_{TAD}$ under the control of the *ADH1* promoter and also encodes *LEU2* as a marker gene. In order to mate a L40 strain that was cured of bait plasmid but still contains the prey plasmid (from the cDNA library), transformants of AMR70 (bearing plasmids pLEX-a/cdk5 or pLEX-a/lamin) were used. Both L40 and AMR70 contain the *lacZ* reporter gene linked to the *lexA* operon. Besides, L40 also contains the *HIS3* reporter gene downstream of the *lexA* operon. Untransformed yeast strains were grown in YPAD whereas transformed yeast strains were grown in minimal medium that allow maintenance of plasmids in a particular strain.

The *S. cerevisiae* strain L40 was also used for transformation of pLEX-a plasmids that encode *Cdk1*, *Cdk2*, *Cdk4*, *Cdk5*, *p35*, *L34*, *lamin* or *MyoD*. A pACT2 or pVP16 plasmid that encodes *L34* or pGAD424 plasmids that encode *Cdk5* and *p35* were used to perform yeast-two-hybrid assays on selected transformants.

Yeast-two-hybrid screen. A pLEX-a/cdk5 plasmid-bearing strain of L40 was transformed with the HeLa cDNA library (Clontech) and transformants were selected exactly as described earlier (19). His^+ colonies were lysed in liquid nitrogen and assayed for β-galactosidase activity on filters. Only those colonies, which do not express β-galactosidase after loss of the pLEX-a/cdk5 bait plasmid, were selected for further analysis. The plasmids pLEX-a/cdk5 or pLEX-a/lamin were reintroduced into L40 (a) by direct transformation of L40 strains that already contain the prey plasmid or (b) by

mating prey plasmid-containing L40 strains with AMR70 transformants of pLEX-a/cdk5 or pLEX-a/lamin. Later, the prey plasmids were isolated by transforming total yeast DNA into HB101 and by selecting for leucine prototrophy on minimal medium plates (manufacturer's protocol; Clontech). Plasmids, that contain defined inserts, were then retransformed into L40 strains that already harbor pLEX-a/cdk5 or pLex/lamin. Plasmids, which express proteins that do not bind lamin but reproducibly interact with Cdk5, were sequenced using an automated DNA sequencing machine (LiCor). Inserts were identified by comparing translated DNA sequences with the SWISS PROT database.

^{35}S-methionine labeling of Cdk1, Cdk2, Cdk4 and Cdk5. 1 μg of the pcDNA3.1 plasmids carrying the genes *Cdk1, Cdk2, Cdk4* or *Cdk5* were linearized uniquely at the 3'-end of the gene inserts. The linearized plasmids were used as templates for *in vitro* transcription/translation. The Cdk proteins were radioactively labeled with ^{35}S-methionine (20 μCi of an *in vivo* cell labeling grade; Amersham Pharmacia Biotech). The transcription and translation was performed using the TNT-T7 *in vitro* transcription/translation kit (Promega).

Pull-down assay with ^{35}S labeled Cdk proteins. Bacterially expressed glutathione-S-transferase (GST) and GST-L34 fusion protein were bound to gluthathione Sepharose 4B (Amersham Pharmacia Biotech) by incubating protein and beads at RT for 1 h. After 4 washing steps with 10 volumes of ice-cold PBS, the beads were resuspended in 200 μl 1% w/v BSA-PBS solution and kept on ice. 5 μl of the ^{35}S-methionine labeled Cdk proteins (from 100 μl of an *in vitro* transcribed/translated product) diluted in 200 μl 1% w/v BSA-PBS solution, that contains 4 μl of a 100x solution of universal protease inhibitors (tablet dissolved in redist. H_2O; Boehringer Mannheim), was kept on ice for 15 min. GSH-Sepharose 4B beads, bound to GST or GST-L34, were incubated with labeled Cdk-s at 4°C for 2 h with gentle mixing. The beads were washed three times with 1 ml of bead-binding buffer (50 mM potassium phosphate pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100) that contain universal protease inhibitors (Boehringer Mannheim). The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer (4% w/v SDS, 0.1 M Tris, 4 mM EDTA, 20% v/v glycerol, 33% w/v bromophenol blue) that contains 50 mM DTT, and were analyzed by 12.5% SDS-PAGE. The gels were fixed (10% v/v glacial acetic acid, 30% v/v methanol solution) for 10 min, and the signal was enhanced by soaking the gel in EN^{3}HANCE (NEN) for an hour at RT. The dried gel was exposed to Kodak X-OMAT AR film.

Transfection of pcDNA 3.1+/cdk2, pcDNA 3.1+/cdk4 and pcDNA 3.1-/cdk5 in COS-1 cells. COS-1 cells in six-well plates, grown in Dulbecco's modified Eagle Medium (DMEM, Gibco BRL) that contained 10% v/v fetal bovine serum (FBS, Gibco BRL), were transfected with 2 μg of pcDNA 3.1 plasmids that carry *Cdk2, Cdk4* or *Cdk5,* using calcium phosphate. In order to control transformation efficiency, pcMV-βgal was used.

Precipitation of COS-1 expressed Cdk-s by GST-L34. Harvested cells (~5 × 10^5) were washed once with PBS and then lysed in 1 ml of hypotonic lysis buffer (10 mM Tris/HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% v/v Nonidet P-40) that contained 1 mM DTT and universal protease inhibitors (Boehringer Mannheim). Total protein in cell lysates was measured by the Bradford assay (Bio-Rad) and was stored at −70°C before use.

400 μl of a COS-1 cell lysate was first preincubated for 2 h at 4°C with 30 μl of Sepharose 4B (to remove unspecific binding of proteins to beads). This was followed by incubation with 30 μl of GSH-Sepharose 4B that was already bound to bacterially expressed GST-L34. Pull-down of the Cdk-s in COS-1 cell lysates was performed as described above for the ^{35}S-methionine labeled Cdk-s. The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer and were analyzed by Western blotting. The blots were probed with polyclonal antibodies raised against Cdk2 and N-terminal fragments of Cdk4 and Cdk5 and were detected by ECL (Amersham Pharmacia Biotech).

Expression of baculoviruses. Recombinant baculoviruses encoding different genes were constructed using protocols provided by the manufacturer (Gibco-BRL). The viruses were harvested and amplified until a desired titer was reached (viz. 1 × 10^8 pfu/ml).

2 × 10^7 *Sf9* cells were used to coinfect bacoluviruses carrying *Cdk5, Cdk4* or *Cdk2* with *GST-p35, GST-cyclin A, GST-cyclin D1, GST-cyclin D2* or *GST-cyclin D3* in 25 ml of SF900 II SFM medium (GibcoBRL). After 72 h, cells were harvested and lysed by sonication in NETN buffer (20 mMTris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 05% v/v NP-40, 5 mM NaF, 30 mM p-NPP, 1 mM PMSF, 1μg/ml Antipain) that contained universal protease inhibitors (Boehringer Mannheim). HBT buffer (50 mM Hepes pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% v/v Tween 20, 1 mM DTT, 5 mM NaF, 30 mM p-NPP, 25 mM β-glycerolphosphate, 1 mM PMSF, 1 μg/ml Antipain) was used to lyse cells that were co-infected with *GST-cyclin D1, D2* or *D3* viruses. Heterodimeric enzyme complexes were isolated by binding to 100 μl of GSH-Sepharose 4B, overnight at 4°C. The beads were washed 7 times with ice-cold NETN buffer (or HBT buffer) and 3 times with ice-cold kinase buffer (20 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$). The beads were resuspended in 100 μl kinase buffer and kept on ice before a kinase assay was performed.

Kinase assays. Phosphorylation of 0.1 μg histone H1 (Type III-S: from calf thymus, Sigma H-5505) or 0.25 μg GST-Rb152 (i.e., C-terminal 152 amino acid residues of pRb covalently linked to GST; Santa Cruz Biotechnology) was performed in kinase buffer for 40 min at 30°C using 2 μl of beads, 10 μM ATP and 3 μCi [γ-^{33}P] ATP (specific activity = 3000 Ci/mmol; Amersham Pharmacia Biotech) in a total volume of 10 μl. The reactions were boiled in Laemmli sample buffer and were analyzed by 12.5% SDS-PAGE. The gel was fixed and the phosphorylation was quantified using a phosphorimager (Molecular Devices).

RESULTS AND DISCUSSION

Cdk5 has been used as a bait in the yeast-two-hybrid system to screen for proteins that may interact with Cdk5. Since Cdk5 is expressed in both cycling and non-cycling cells, we thought it would be interesting to know if there are any Cdk5-binding proteins in cycling cells. Hence, a HeLa cDNA library in the yeast vector pGADGH was transformed into the yeast strain L40 (19) that already contained the bait plasmid pLEX-a/cdk5 (which codes for $Lexa_{DBD}$-Cdk5). Genes coding for interacting proteins were identified by selecting for His^+ prototrophs and subsequently through induction of β-galactosidase (β-gal) activity. Colonies that were His^+ and blue were considered positives and were used for further analysis (results not shown). It was later found that one such positive yeast colony harbored a cDNA library plasmid (i.e. the prey plasmid) encoding $GAL4_{TAD}$ fused to the human gene for the 60S ribosomal protein, L34.

In order to negate the possibility that an identified positive colony was an artifact of the two-hybrid system (frequently referred to as 'false positives') (20), the yeast strain was cured of the bait plasmid pLEX-a/cdk5 by growing the strain in a non-selective medium (50 generations of growth in minimal medium that contained tryptophan and adenine but

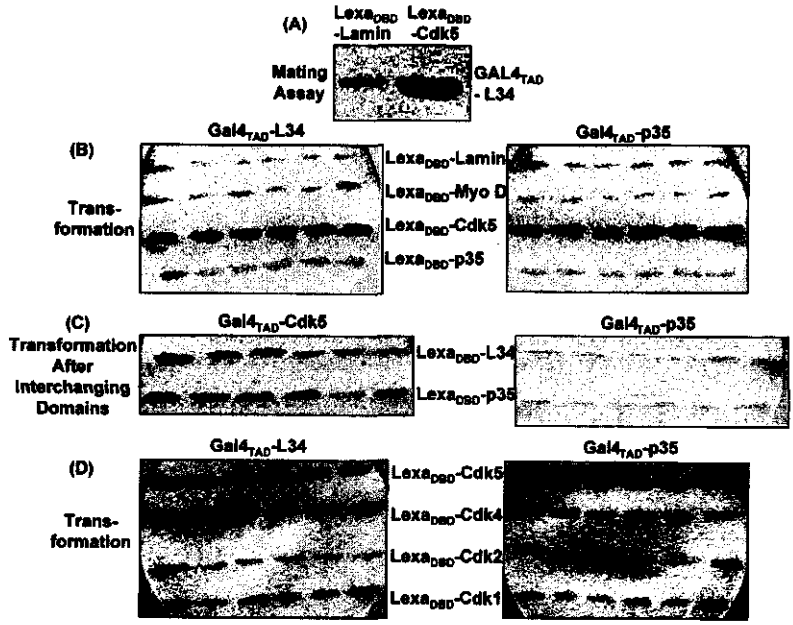


FIG. 1. In a yeast-two-hybrid assay the 60S ribosomal protein L34 binds to Cdk5 and Cdk4. Interactions were monitored by analyzing six individual colonies by colorometric β-galactosidase assay. (A) After curing the bait plasmid, an L40 strain that retains the prey plasmid (that codes for $GAL4_{TAD}$-L34 fusion protein) was mated with strain AMR70 that had already been transformed with pLEX-a/lamin or pLEX-a/Cdk5 (these plasmids express Lex-a-lamin and Lex-a-Cdk5 fusion proteins, respectively). The results show that L34 interacts with Cdk5 but not with the unspecific protein, lamin. (B) Left panel, plasmid pACT2/L34 (that expresses $GAL4_{TAD}$-L34) was transformed in strain L40 that already contains pLEX-a/lamin, pLEX-a/MyoD, pLEX-a/Cdk5 or pLEX-a/p35. The results show that L34 interacts specifically with Cdk5. Right panel, plasmid pGAD424/p35 (expressing $GAL4_{TAD}$-p35) was transformed in the same L40 yeast strains (see left panel), as a control. (C) Plasmids pGAD424/Cdk5 (left panel) and pGAD424/p35 (right panel, as negative control) were transformed into L40 strains that already harbor pLEX-a/L34 or pLEX-a/p35. Results show that Cdk5 and L34 can still interact with each other even after interchanging the two domains ($GAL4_{TAD}$ and Lex-a_{DBD}) which has to be linked to two putative interacting proteins, in the two-hybrid assay. (D) Plasmids pACT2/L34 (left panel) and pACT2/p35 (right panel, as control), that express $GAL4_{TAD}$-L34 and $GAL4_{TAD}$-p35 respectively, were transformed in L40 strains that already harbor pLEX-a/Cdk5, pLEX-a/Cdk4, pLEX-a/Cdk2 or pLEX-a/Cdk1. Results show that L34 interacts specifically with Cdk5 and Cdk4.

lacked leucine; the prey plasmid contains *LEU2*). The resulting L40 strain (Mat **a**) that contains the prey plasmid, pGADGH/L34, was mated with strains of AMR70 (Mat α) that had been transformed either with pLEX-a/cdk5 or with pLEX-a/lamin. Beta-galactosidase assays performed on mated diploid strains showed that L34 specifically interacts with Cdk5 but not with lamin (Fig. 1A).

At this stage, the pGADGH/L34 plasmid was isolated from yeast (via transformation of total yeast DNA in *E. coli;* see Materials and Methods). The insert was sequenced and it revealed that the cloned gene was full-length human *L34*. The ORF of the *L34* gene was amplified by PCR from the vector pGADGH/L34 and was subcloned into pACT2, another yeast vector often used for two-hybrid assays. The plasmid pACT2/L34 (which codes for $GAL4_{TAD}$-L34 fusion protein) was transformed into the strain L40 that already contained pLEX-a/cdk5, pLEX-a/lamin, pLEX-a/MyoD or pLEX-a/p35 (all plasmids code for $Lexa_{DBD}$ fusion proteins). The results reiterate that, at least in the two-hybrid system, Cdk5 not only binds p35 (Cdk5's known activator) but also interacts with L34 (Fig. 1B, left and right hand panels). It was also found that interaction of L34 with Cdk5 does not depend on the specific TAD to which L34 is linked. This was supported by the observation that a $VP16_{TAD}$-L34 fusion protein binds as well as $GAL4_{TAD}$-L34 to $Lexa_{DBD}$-Cdk5, in the two-hybrid assay (data not shown). Furthermore, the gene *L34* was cloned in pLEX-a and *cdk5* in pGAD424. It was seen that interchanging the two domains, TAD and DBD, did not affect the interaction between L34 and Cdk5 (i.e., $Lexa_{DBD}$-L34 fusion protein bound strongly to $GAL4_{TAD}$-Cdk5; Fig. 1C). All these observations proved beyond doubt that neither a TAD nor a DBD has any influence on the Cdk5-L34 interaction.

We also wanted to find out if Cdk5 was the only Cdk to which L34 has an affinity. In order to test this, yeast strain L40 was first transformed with pLEX-a/cdk5, pLEX-a/cdk4, pLEX-a/cdk2 or pLEX-a/cdk1. The plasmid pACT2/L34 (that codes for $GAL4_{TAD}$-L34) was then transformed into L40 strains that already have the ability to express Lexa-Cdk5, Lexa-Cdk4, Lexa-Cdk2 or Lexa-Cdk1 fusion proteins. A β-galactosidase assay (Fig. 1D) depicts that, in the yeast-two-hybrid system, L34 can interact with Cdk5 and Cdk4 but not with Cdk2 and Cdk1.

In order to confirm some of the yeast-two-hybrid data, a set of *in vitro* pull-down experiments were employed. We found that bacterially expressed GST-L34 fusion protein was able to pull-down ^{35}S-methionine-labeled Cdk5 and Cdk4 but not Cdk2 and Cdk1 (all proteins were transcribed/translated *in vitro*). We were sure that the GST moiety in GST-L34 did not play a role in the precipitation of Cdk5 or Cdk4 since GST protein alone is not able to pull-down Cdk5 (Fig. 2A).

As an alternative to the *in vitro* transcription/translation procedure to obtain desired proteins, Cdk5, Cdk4 and Cdk2 were expressed in COS-1 cells. Lysates from transfectants were incubated with the *E. coli* expressed GST-L34 fusion protein. The bound proteins were detected by Western blotting, using antibodies specific to Cdk5, Cdk4 or Cdk2. Similar to our earlier observations with labeled proteins, we observed that GST-L34 was able to bind COS-1-expresssed Cdk5 and Cdk4 whereas it was unable to pull-down Cdk2 (Fig. 2B).

The Cdk5/GST-p35 heterodimeric complex was expressed in insect cells using the baculovirus expression system. The enzyme was purified by binding to GSH-Sepharose 4B. The bead-bound enzyme was tested for activity (see Materials and Methods) using histone H1 as substrate. Fig. 3A (lane 1) portrays the phosphorylation of histone H1 by GST-p35 activated Cdk5 kinase. Kinase assays performed on GST-L34 demonstrate that L34 is not phosphorylated by Cdk5 and therefore cannot be a Cdk5 substrate (Fig. 3A, lanes 2 & 3). We then asked if L34 could be an inhibitor of Cdk5. Fig. 3B shows a dose dependent inhibition of Cdk5 by GST-L34. However, it should be noted that 10 μg of GST did not have any affect on the kinase (Fig. 3B, lane 8) whereas 0.4 μg of GST-L34 distinctly inhibited the complex (>50%; Fig. 3B, lane 5). Staurosporine (CGP 39360, Novartis), which strongly inhibits most Cdk-s including Cdk5, was used as a control inhibitor (21).

It can be argued that the relatively large GST moiety causes a steric hindrance in the fusion protein so that it interferes with L34's ability to act as a substrate for Cdk5 or that the presence of GST causes an artifactual inhibition of Cdk5. Therefore, L34 was expressed in *E.*

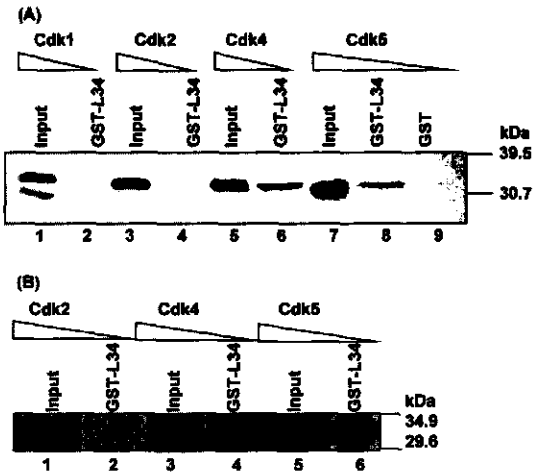


FIG. 2. L34 binds to Cdk5 and Cdk4 but not to Cdk2 and Cdk1 in *in vitro* binding assays. L34 was expressed as a GST fusion in *E. coli*. (A) GST-L34 binds to ^{35}S-labeled Cdk4 and Cdk5 and not to ^{35}S-labeled Cdk1 and Cdk2. Lane 1, 10% of the ^{35}S-labeled Cdk1 input used for the pull-down assay with GST-L34 (as in lane 2); lane 3, 10% of the ^{35}S-labeled Cdk2 input used for the pull-down assay with GST-L34 (as in lane 4); lane 5, 10% of the ^{35}S-labeled Cdk4 input used for the pull-down assay with GST-L34 (as in lane 6); lane 7, 10% of the ^{35}S-labeled Cdk5 input used for the pull-down assay with GST-L34 (as in lane 8); lane 9, 100% of the ^{35}S-labeled Cdk5 input used for the pull-down assay with GST alone. (B) GST-L34 binds to Cdk4 and Cdk5 and not to Cdk2 overexpressed in COS-1 cells. Each lane shows a Western blot of proteins fractionated on a 10% SDS-polyacrylamide gel. Polyclonal antibodies raised against Cdk2 and the N-terminii of Cdk4 and Cdk5 were used to detect the respective proteins. Lanes 1, 3 and 5, 15 μl of lysates from COS-1 cells that express Cdk2, Cdk4 or Cdk5. Lanes 2, 4 and 6, 400 μl of same cell lysates, used in pull-down assays with GST-L34.

coli as a His-tagged protein (six consecutive histidines attached to the protein at its N-terminus, as in pQE32; see Materials and Methods). His-L34 has been used to probe if it is a substrate or an inhibitor of the Cdk5/p35 kinase. Like GST-L34, His-L34 is not phosphorylated by Cdk5/GST-p35 (Fig. 3D right panel, lane 4). Again similar to GST-L34, His-L34 inhibits the Cdk5/GST-p35 complex in a dose dependent manner. His-L34 completely abolishes the Cdk5-mediated phosphorylation of histone H1 (Fig. 3C, left panel). The His-tagged protein can also completely inhibit the phosphorylation of GST-Rb152 by the Cdk5/GST-p35 complex (Fig. 3C right panel, lane 5).

Since D-type cyclins are reported to bind Cdk5 (5), we wanted to find if cyclin D would form a complex with Cdk5 when both proteins are expressed in insect cells, and if it did, whether the complex would form an active kinase by phosphorylating either histone H1 or GST-Rb152. One could speculate that an active cyclin D/Cdk5 enzyme, with no known cellular substrate,

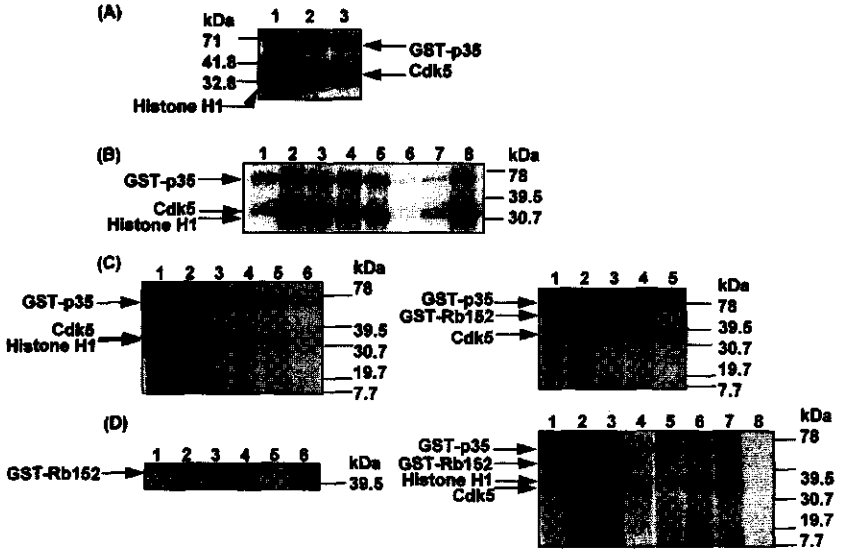


FIG. 3. Kinase assays with histone H1 and GST-Rb152, as substrates, show that both GST-L34 and His-L34 proteins are not substrates for Cdk5 that is activated by p35 and cyclin D2. However, GST-L34 and His-L34 inhibit Cdk5-mediated phosphorylation of histone H1 and GST-Rb152 in a dose-responsive manner. (A) GST-L34 is not phosphorylated by p35 activated Cdk5 kinase. Lane 1, Cdk5/GST-p35 + 10 μg histone H1 as substrate; lane 2, Cdk5/GST-p35 + 10 μg GST as substrate; lane 3, Cdk5/GST-p35 + 0.4 μg GST-L34 as substrate. (B) Dose response to GST-L34 in the inhibition of Cdk5-mediated phosphorylation of histone H1 (1 μg). Lane 1, Cdk5/GST-p35 + no substrate; lane 2, Cdk5/GST-p35 + substrate; lane 3, Cdk5/GST-p35 + substrate + 0.4 μg GST-L34; lane 4, Cdk5/GST-p35 + substrate + 0.8 μg GST-L34; lane 5, Cdk5/GST-p35 + substrate + 1.6 μg GST-L34; lane 6, Cdk5/GST-p35 + substrate + 300 nM staurosporine; lane 7, Cdk5/GST-p35 + substrate + 40 nM staurosporine; lane 8, Cdk5/GST-p35 + substrate + 10 μg GST. (C) Left panel, dose response to His-L34 in the inhibition of Cdk5-mediated phosphorylation of histone H1 (0.1 μg). Lane 1, Cdk5/GST-p35 + no substrate; lane 2, Cdk5/GST-p35 + substrate; lane 3, Cdk5/GST-p35 + substrate + 0.5 μg His-L34; lane 4, Cdk5/GST-p35 + substrate + 1 μg His-L34; lane 5, Cdk5/GST-p35 + substrate + 3 μg His-L34; lane 6, Cdk5/GST-p35 + substrate + 6 μg His-L34. Right panel, dose response to His-L34 in the inhibition of Cdk5-mediated phosphorylation of GST-Rb152 (0.25 μg). Lane 1, Cdk5/GST-p35 + no substrate; lane 2, Cdk5/GST-p35 + substrate; lane 3, Cdk5/GST-p35 + substrate + 0.5 μg His-L34; lane 4, Cdk5/GST-p35 + substrate + 1 μg His-L34; lane 5, Cdk5/GST-p35 + substrate + 6 μg His-L34. (D) His-L34 is not a substrate for Cdk5/GST-p35 and Cdk5/GST-cyclin D2 kinases. Left panel, GST-cyclin D2 activated kinases were prepared and were tested for their ability to phosphorylate 0.25 μg GST-Rb152. Lane 1, Cdk2/GST-cyclin D2 + no substrate; lane 2, Cdk2/GST-cyclin D2 + substrate; lane 3, Cdk4/GST-cyclin D2 + no substrate; lane 4, Cdk4/GST-cyclin D2 + substrate; lane 5, Cdk5/GST-cyclin D2 + no substrate; lane 6, Cdk5/GST-cyclin D2 + substrate. Right panel, both Cdk5/GST-p35 and Cdk5/GST-cyclin D2 were tested for their ability to phosphorylate histone H1, GST-Rb152 and His-L34. Lane 1, Cdk5/GST-p35 + no substrate; lane 2, Cdk5/GST-p35 incubated with 1 μg histone H1; Lane 3, Cdk5/GST-p35 kinase incubated with 0.25 μg GST-Rb152; lane 4, Cdk5/GST-p35 incubated with 5 μg His-L34; lane 5, Cdk5/GST-cyclin D2 + no substrate; lane 6, Cdk5/GST-cyclin D2 incubated with 1 μg histone H1; lane 7, Cdk5/GST-cyclin D2 incubated with 0.25 μg GST-Rb152; lane 8, Cdk5/GST-cyclin D2 incubated with 5 μg His-L34.

may phosphorylate the Cdk5-binding protein, L34. Hence, Sf9 insect cells were co-infected with baculoviruses coding for Cdk5 and GST-cyclin D1, GST-cyclin D2 or GST-cyclin D3. The cyclin D-bound Cdk5 proteins obtained from insect cells were precipitated with GSH-Sepharose 4B and the bead-bound complexes (as confirmed by Western blotting using antibodies specific for Cdk5 and D-type cyclins; results not shown) were used directly for kinase assays. We observed that neither GST-cyclin D1 nor GST-cyclin D3 can activate Cdk5 (results not shown). Surprisingly, like cyclin D2-activated Cdk2 (Fig. 3D left panel, lane 2) and Cdk4 (Fig. 3D left panel, lane 4), kinases that are known to phosphorylate pRb, cyclin D2-activated Cdk5 also has the ability to phosphorylate the GST-Rb152 (Fig. 3D left panel, lane 6). However, His-L34 is not phosphorylated by cyclin D2 and p35 activated Cdk5 (Fig. 3D right panel, lanes 4 & 8).

Since L34 interacts not only with Cdk5 but also with Cdk4 in both two-hybrid and *in vitro* binding assays, the affect of His-L34 on active Cdk4/cyclin D1 complex was investigated. We see that the His-tagged protein completely inhibits Cdk4/GST-cyclin D1-mediated phosphorylation of GST-Rb152 (Fig. 4A, lane 4). Furthermore, L34 is definitely not a substrate for the Cdk4/GST-cyclin D1 enzyme (Fig. 4A, lane 5).

Although His-L34 inhibits the Cdk5/GST-p35-mediated phosphorylation of histone H1 (there is at

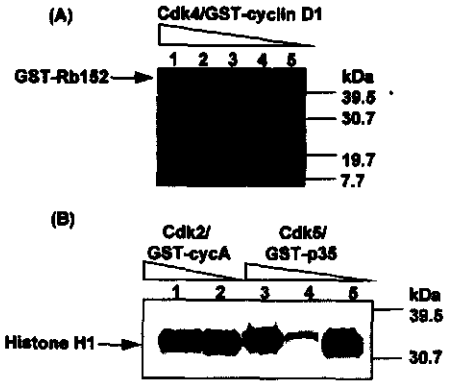


FIG. 4. His-L34 is not a substrate for Cdk4/GST-cyclin D1 but instead inhibits the kinase. Comparison of the inhibition of Cdk5/GST-p35 and Cdk2/GST-cyclin A kinases by His-L34. (A) His-L34 inhibits Cdk4/GST-cyclin D1-mediated phosphorylation of GST-Rb152 (0.5 μg) and is not phosphorylated by the kinase. Lane 1, Cdk4/GST-cyclin D1 + no substrate; lane 2, Cdk4/GST-cyclin D1 + substrate (the lower band is a breakdown product of GST-Rb152); lane 3, Cdk4/GST-cyclin D1 + substrate + 1 μg His-L34; lane 4, Cdk4/GST-cyclin D1 + substrate + 6 μg His-L34; lane 5, Cdk4/GST-cyclin D1 + substrate + 6 μg His-L34. (B) Comparison of inhibition of the Cdk5/GST-p35 and Cdk2/GST-cyclin A-mediated phosphorylation of histone H1 (1 μg) by His-L34. Lane 1, Cdk2/GST-cyclin A + substrate; lane 2, Cdk2/GST-cyclin A + substrate + 0.5 μg His-L34; lane 3, Cdk5/GST-p35 + substrate; lane 4, Cdk5/GST-p35 + substrate + 0.5 μg His-L34; lane 5, Cdk5/GST-p35 + substrate + 6 μg His-FKBP12 (an unspecific protein). The results show that His-L34 inhibits Cdk5/GST-p35 but not the Cdk2/GST-cyclin A kinase.

least a 20-fold decrease in activity; compare lanes 3 & 4 in Fig. 4B), it seems that phosphorylation of the same substrate by Cdk2/GST-cyclin A is not affected at all (Fig. 4B, compare lanes 1 & 2). This seems to corroborate our earlier two-hybrid data which indicated that L34 does not interact with Cdk2 but binds Cdk4 and Cdk5.

Interestingly, the *L34* gene lies within the *BRCA1* locus (22). *BRCA1*, a familial breast and ovarian cancer susceptibility gene encodes a nuclear phosphoprotein that functions as a tumor suppressor in human breast cancer cells. BRCA1 splice variants BRCA1a and BRCA1b associate with E2F, cyclins and cyclin dependent kinases (23). We have not found the consensus sequence (X-S/T-P-X-K/R-X-) (24), that is typical of a site phosphorylated by a Cdk, in L34 (PROSITE database). However, the protein has a cAMP- and a cGMP-dependent protein kinase phosphorylation site and five protein kinase C phosphorylation sites.

Although Cdk5 is expressed in both proliferative and differentiated cells, its expression is relatively abundant in nerve and muscle cells. Since L34 mRNA is present in HeLa cells, the L34 protein must be also present in proliferating cells. L34 may therefore play a role in inhibiting Cdk5 during the cell cycle.

The p35 protein, the only known activator of Cdk5, is solely expressed in differentiated nerve cells. Cdk5 kinase activity (with p35 as its activator) could therefore be responsible for the onset of differentiation of nerve cells and possibly of muscle cells (9–12). However, Cdk5 kinase activity should be blocked during terminal differentiation. Since both Cdk5 and p35 are present in terminally differentiated cells, other proteins should be involved in preventing this complex to be active in these cells. We would like to propose that L34 is probably one of the specific inhibitors of Cdk5. Since Cdk5 can phosphorylate neurofilament proteins and tau protein *in vitro* and neurofilament proteins are hyperphosphorylated in patients suffering from Lewy body pathologies, whereas the tau protein is hyperphosphorylated in patients suffering from Alzheimer's disease, it could be that L34 also plays a role in the aberrant phosphorylation of neurofilaments and tau.

On the other hand, the Cdk4 kinase is responsible for the phosphorylation of pRb early in the G1-phase of the cell cycle and has a very important function in cycling cells (25). One can speculate that binding of L34 to the Cdk4 protein allows inhibition of its kinase activity which thereby causes cell cycle arrest. Probably, this is what occurs in differentiated cells where it is essential that Cdk4 activity is inhibited. In cycling cells however, L34 should be bound to Cdk5 to prevent the premature onset of differentiation. When Cdk5 is activated during differentiation, L34 may switch its binding allegiance to Cdk4, resulting in the latter's inhibition and causing a cell cycle block. Later, terminally differentiated cells may again have L34 bound to Cdk5 to maintain the kinase in its inactive form.

Interaction of Cdk5 with the 60S ribosomal protein L34 may imply that Cdk5 activity is regulated at the translational level. It has been reported that L34 regulates polyamine biosynthesis by inhibiting ornithine decarboxylase (26). Polyamines are overexpressed in many cancers. The fact that L34 regulates polyamine biosynthesis by inhibiting ornithine decarboxylase may also suggest that L34 is expressed at the beginning of the S phase of the cell cycle and may thus block Cdk4 activity at a point in the cell cycle where Cdk4 activity is no longer needed. This assumption may be equally valid if Cdk5 were to be responsible for the onset of differentiation. However, the true role of L34 in cycling and post-mitotic cells needs to be evaluated through further cellular analyses.

REFERENCES

1. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. (1992) *EMBO J.* **11**, 2909–2917.

2. Lew, J., and Wang, J. H. (1995) *Trends Biochem. Sci.* **20,** 33–37.
3. Tang, D., and Wang, J. H. (1996) *Prog. Cell Cycle Res.* **2,** 205–216.
4. Dellale, I, Bhide, P. G., Caviness, V. S., Jr., and Tsai, L. H. (1997) *J. Neurocytol.* **26,** 283–296.
5. Guidato, S., McLoughlin, D. M., Grierson, A. J., and Miller, C. C. J. (1998) *J. Neurochem.* **70,** 335–340.
6. Miyajima, M., Nornes, H. O., and Neuman, T. (1995) *NeuroReport* **6,** 1130–1132.
7. Lee, K.-Y., Helbing, C. C., Choi, K.-S., Johnston, R. N., and Wang, J. H. (1997) *J. Biol. Chem.* **272,** 5622–5626.
8. Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) *Nature* **371,** 423–426.
9. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F., and Tsai, L. H. (1996) *Genes Dev.* **10,** 816–825.
10. Chae, T., Kwon, Y. T. Bronson, R., Dikkes, P., Li, E., and Tsai, L. H. (1997) *Neuron* **18,** 29–42.
11. Lazaro, J. B., Kitzmann, M., Poul, M. A., Vandromme, M., Lamb, N. J., and Fernandez, A. (1997) *J. Cell Sci.* **110,** 1251–1260.
12. Philpott, A., Porro, E. B., Kirschner, M. W., and Tsai, L. H. (1997) *Genes Dev.* **11,** 1409–1421.
13. Henchcliffe, C., and Burke R. E. (1997) *Neurosci. Lett.* **230,** 41–44.
14. Brion, J.-P., and Couck, A.-M. (1995) *Am. J. Pathol.* **147,** 1465–1476.
15. Nakamura, S., Kawamoto, Y., Nakano, S., Ikemoto, A., Akiguchi, I., and Kimura, J. (1997) *Neurology* **48,** 267–270.
16. Imahori, K., and Uchida, T. (1997) *J. Biochem. Tokyo* **121,** 179–188.
17. Sengupta, A., Wu, Q., Grundke-Iqbal, I, Iqbal, K., and Singh, T. J. (1997) *Mol. Cell. Biochem.* **167,** 99–105.
18. Ohshima, T., Ward, J. M., Huh, C.-G., Longenecker, G., Veeranna, Pant, H. C., Brady, R. O., Martin, L. J., and Kulkami, A. B. (1996) *Proc. Nat. Acad. Sci.USA* **93,** 11173–11178.
19. Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. (1995) *Mol. Cell. Biol.* **15,** 3813–3822.
20. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75,** 805–816.
21. Veeranna, Shetty, K. T., Amin, N., Grant, P., Albers, R. W., and Pant, H. C. (1996) *Neurochem. Res.* **21,** 629–636.
22. Rommens, J. M., Durocher, F., McArthur, J., Tonin, P., LeBlanc, J.-F., Allen, T., Samson, C., Ferri, L., Narod, S., Morgan, K., and Simard, J. (1995) *Genomics* **28,** 530–542.
23. Wang, H., Shao, N., Ding, Q. M., Cui, J.-q., Reddy, E. S. P., and Rao, V. N. (1997) *Oncogene* **15,** 143–157.
24. Tang, D., Lee, K.-Y., Qi, Z., Matsuura, I., and Wang, J. H. (1996) *Biochem. Cell Biol.* **74,** 419–429.
25. Weinberg, R. A. (1995) *Cell* **81,** 323–330.
26. Panagiotidis, C. A., Huang, S. C., and Canellakis, E. S. (1995) *Int. J. Biochem. Cell Biol.* **27,** 157–168.

5. Identification of a Human cDNA Encoding a Kinase-Defective Cdk5 Isoform

Mark Moorthamer, Sabine Zumstein-Mecker, Christine Stephan, Peer Mittl and Bhabatosh Chaudhuri

(1998) *Biochem. Biophys. Res. Commun.* **253**, 305-310

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 253, 305–310 (1998)
ARTICLE NO. RC989737

Identification of a Human cDNA Encoding a Kinase-Defective Cdk5 Isoform

Mark Moorthamer, Sabine Zumstein-Mecker, Christine Stephan, Peer Mittl, and Bhabatosh Chaudhuri[1]
Oncology Research, Novartis Pharma AG, Basel, Switzerland

Received October 22, 1998

The cyclin-dependent kinase 5 (Cdk5) catalytic subunit is expressed in both cycling and noncycling cells and is present in many tissues. Neuronal and muscle cells contain the highest amount of this protein. The p35 protein, which is expressed solely in the brain, activates Cdk5. Cdk5 activity is involved in terminal differentiation of neurons and muscle cells. We attempted to clone *cdk5* by PCR from a human fetal brain cDNA library. Surprisingly, we amplified two forms of the *cdk5* gene, the wild type and a *cdk5* variant that lacks the complete kinase domain VI. The variant is also found in SH-SY-5Y neuroblastoma cells but not in T-cells, HeLa cells, the thymus, and placental tissue. The protein encoded by the *cdk5* variant, the Cdk5 isoform (Cdk5i), purifies with p35 when coexpressed in insect cells. The activity associated with the heterodimer Cdk5i/p35 is found to be appreciably weaker than the wild-type Cdk5/p35 kinase. Moreover, Cdk5i/p35 cannot autophosphorylate its two subunits as with Cdk5/p35. Interestingly, kinase-defective Cdk5i can abolish the activity of wild-type Cdk5 when both are coexpressed with p35 in insect cells, suggesting that Cdk5i may have a function in regulating Cdk5 activity in human cells too. © 1998 Academic Press

Key Words: **p35; Cdk5; Cdk5 isoform; defective-kinase.**

Cyclin dependent kinase 5 (Cdk5) was initially identified on the basis of its sequence similarity to the cyclin dependent kinases (Cdk-s), enzymes which play a key role in cell cycle progression (1, 2). However, Cdk5 seems to have a function only in post-mitotic cells and is the first example of a cyclin dependent kinase with neuronal function (2, 3). Cdk5 is expressed at basal levels in most human tissues (i.e., in both cycling and non-cycling cells), with the exception of the central nervous system (CNS) and peripheral nervous system (PNS) where expression is several-fold greater (2, 3). It has been reported that cyclins D and E bind to Cdk5 but these complexes have not been shown to be enzymatically active (3, 4). The protein p35, which is expressed exclusively in the CNS, is the only known activator of Cdk5 (3, 5), and has no influence on the activity of other Cdk-s. Moreover, p35 has no significant homology to cyclins, with only eight out of a hundred residues identical in the central conserved cyclin box (2, 3). The Cdk5 activator p35 was originally isolated as p25, a smaller proteolytic product (2, 3).

The Cdk5/p35 complex can phosphorylate both histone H1 and the retinoblastoma protein (pRb), two substrates which are commonly used to test Cdk activity *in vitro* (3, 6). Active Cdk5 also phosphorylates p35, its activating partner (7). Furthermore, Cdk5 is known to phosphorylate *in vitro* a number of cytoskeletal proteins including the neurofilament proteins NF-M, NF-H, the neuron-specific microtubule-associated protein tau and the actin-binding protein caldesmon (2, 3). Phosphorylation of cytoskeletal proteins are thought to play an important role in the polymerization and assembly of cytoskeletal elements which, in turn, may effect growth of neurites. Indeed, Cdk5 activity has been demonstrated to play a key role in neurite outgrowth (8) and neuronal migration during neuronal differentiation (9). Recently, Cdk5 has been shown to participate in the regulation of myogenesis in the early embryo (10, 11). These observations may provide new insight into the possible function of Cdk5 during differentiation.

Cdk5 phosphorylates neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies, i.e., diffuse Lewy body disease (dementia), Parkinson's disease (12, 13) and amyothrophic lateral

[1] To whom correspondence should be addressed at Oncology Research, Novartis Pharma AG, WKL-125.13.17, Basel, Switzerland. Fax: (41) (61) 696 38 35. E-mail: bob.chaudhuri@pharma.novartis.com.

Abbreviations used: bp, base pair; Cdk5, human cyclin dependent kinase 5; Cdk5i, Cdk5 isoform; GSH–Sepharose, gluthathione–Sepharose; GST, glutathione-*S*-transferase; p35, 35-kDa protein which activates Cdk5; PHF, paired helical filament; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase PCR; pRb, human retinoblastoma protein; var, variant; wt, wild type.

0006-291X/98 $25.00

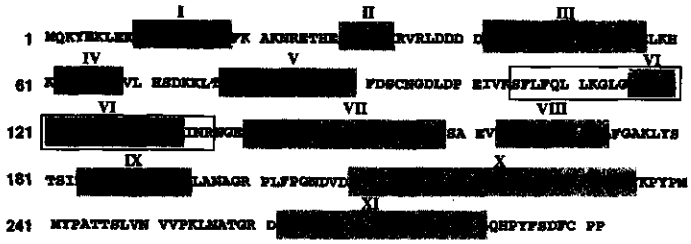


FIG. 1. The primary sequence of wt-Cdk5 which consists of 293 amino acid residues. The gray shaded areas indicate the 11 conserved subdomains of protein-serine/threonine kinases (19). The sequence (amino acids 105–136) in wt-Cdk5 which is deleted in Cdk5i (consisting of 261 residues) is boxed. Cdk5i completely lacks subdomain VI.

RESULTS AND DISCUSSION

To clone the gene which codes for Cdk5, PCR was performed using a fetal brain cDNA library as template. Unexpectedly, fragments of two distinct sizes were amplified using two oligonucleotide primers that should normally hybridize uniquely to *cdk5* (results not shown). Both fragments were subcloned and sequenced. DNA sequencing revealed that one of the products was a 879-base pair (bp) fragment which was identical to the wt-*cdk5* gene. The second fragment was smaller (783 bp in length) and coded for a *cdk5* variant (var-*cdk5*). One contiguous region (bases 313 to 409) in wt-*cdk5* (1) was completely missing in var-*cdk5*.

Figure 1 shows a comparison of the polypeptide sequences translated from the genetic sequence of wt-*cdk5* and var-*cdk5*. The shaded areas indicate the eleven conserved subdomains of protein-serine/threonine kinases. The subdomains are defined as regions never interrupted by large amino acid insertions and that contain characteristic patterns of conserved residues (18). Cdk5i completely lacks subdomain VI. Subdomain VI is not only thought to act as a support structure, but the hydrophobic β-strands with an intervening loop are considered to be the likely candidate for forming the catalytic base. This catalytic loop contains the consensus motif HRDLKxxN which is highly conserved within the protein kinase family. It has been predicted that, through an in-line phosphotransfer mechanism, the aspartic acid in this motif could accept the proton from the attacking hydroxyl group of the substrate.

In Fig. 2, the 3-dimensional Cdk5 structure, modeled on the basis of the published Cdk2 structure (20) and known similarities between the primary sequences of

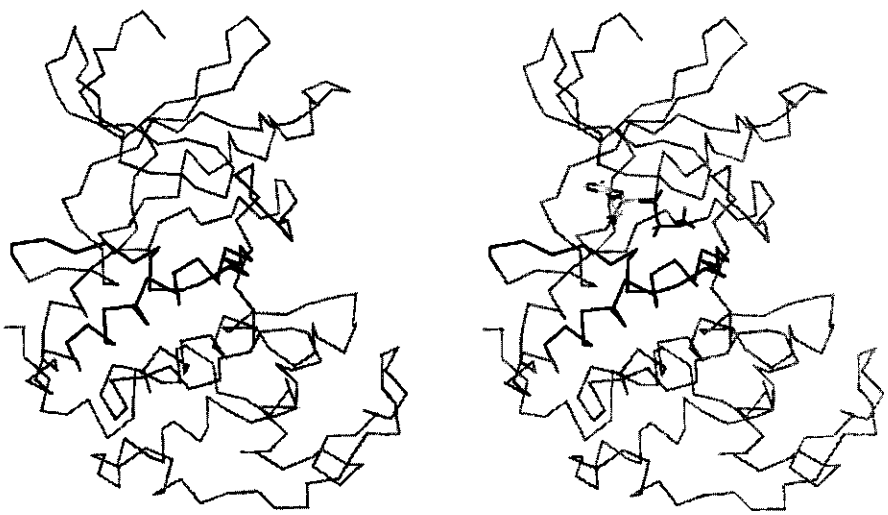


FIG. 2. Structure of Cdk5 obtained through homology modeling (on the basis of the known structure of Cdk2; Ref. 20). The darker region is the peptidic region which is missing in Cdk5i. The right panel shows the same model, with ATP lying in the binding pocket of Cdk5.

sclerosis (2, 14), and tau protein at sites phosphorylated in Alzheimer's disease (2, 3, 15,16). Since the kinase activity of Cdk5 correlates with the extent of differentiation of neuronal cells (2, 3, 17) and colocalizes with neurofilament tracts in the axons of neuronal cells in culture, Cdk5 is a strong candidate for catalyzing neurofilament and tau phosphorylation *in vivo* (2, 3).

In this communication, we report the identification of a naturally-occuring variant of the *cdk5* gene (var-*cdk5*). This was fortuitously identified during cloning of the *cdk5* gene (1) from a human fetal brain cDNA library. The protein encoded by this novel gene is referred to as the Cdk5 isoform (Cdk5i). The var-*cdk5* gene was not found in cDNA libraries constructed from human T-cells, HeLa cells, human thymus, human placenta and human cerebellum but was present in a cell line (SH-SY-5Y) derived from a human neuroblastoma. Although the gene lacks one of the conserved domains present universally in all kinases (18), the Cdk5i protein still binds p35 when coexpressed in insect cells. The heterodimeric complex formed between Cdk5i and p35 (Cdk5i/p35) has the ability to phosphorylate histone H1 and the retinoblastoma protein (pRb), although quite weakly compared to Cdk5/p35. However, the enzyme composed of Cdk5i and p35 can no longer phosphorylate Cdk5i or p35, its two subunits. Intriguingly, increasing amounts of Cdk5i can nullify the activity of wild type Cdk5 protein when both wild type and mutant proteins are coexpressed with p35 in insect cells. The kinase-defective Cdk5i is reminiscent of the artificially constructed dominant-negative mutants of Cdk5 which are known to block the activity of wild-type protein (3, 8, 10, 11, 19). One could speculate that Cdk5i may have a physiological role in the biochemical events that lead to neuronal differentiation.

MATERIALS AND METHODS

PCR. To clone a *Bam*HI–*Hind*III fragment of *cdk5* (1), PCR was performed on the following cDNA libraries (all obtained from Clontech): human fetal brain, human T-cells, HeLa cells, human thymus, human placenta and human cerebellum. The primers used were 5′-TAGGATCCGATGCAGAAATACGAGAGAAACTGGAAAAG-ATT-3′ (5′-end primer) and 5′-ATAAGCTTCTAGGGCGGACAGA-AGTCGGAGAAGTAGGG-3′ (3′-end primer).

Total RNA derived from SH-SY-5Y human neuroblastoma cells (using a kit from Qiagen) was first reverse transcribed using a cDNA synthesis kit (Boehringer) before PCR was performed using the above mentioned primers.

Plasmid constructs. *Bam*HI–*Hind*III fragments of human wild type (wt)-*cdk5* and var-*cdk5*, obtained from the fetal brain cDNA library (Clontech) and from SH-SY-5Y cells, were first subcloned in pBluescriptKS(+) (Stratagene). The cloned genes were sequenced using the Applied Bio-Systems 370A automated DNA sequencer (Microsynth, Switzerland).

The gene isolated from SH-SY-5Y cells was used for construction of recombinant baculovirus encoding var-*cdk5*, whereas the wt-*cdk5* containing baculovirus was constructed from the gene obtained from the fetal brain cDNA library. At first, *Bam*HI–*Sal*I fragments of wt- and var-*cdk5* were isolated from the corresponding pBluescriptKS(+) plasmids. They were then cloned in pFastBac1 (Gibco-BRL) to facilitate generation of the recombinant baculoviruses. The vector pFastBac1 carries a baculovirus-specific polyhedrin promoter from *Autographa californica* nuclear polyhedrosis virus (AcNPV) for heterologous expression of proteins in insect cells.

A *Bgl*II–*Eco*RI PCR fragment of GST (PCR template: pGEX4T-1, Pharmacia) was first subcloned in pBluescriptKS(+). The same fragment from the resultant plasmid was then cloned in pFastBac1. The *Eco*RI–*Sal*I fragments of human *p35*, human *cyclin A*, and an *Eco*RI–*Xho*I fragment of human *cyclin D1* were then cloned in the pFastBac1 vector downstream of the *Bgl*II–*Eco*RI fragment of *GST*.

Transposition and transfection. pFastBac1 constructs were transformed in DH10Bac competent cells (containing "bacmid" and helper; Gibco-BRL) in order to transpose the expression cassettes into the baculovirus genome. Recombinant bacmid DNA was isolated (according to the instructions from Gibco-BRL) and Sf9 cells were transfected with bacmid DNA using Cellfectin (Gibco-BRL). Recombinant baculoviruses were harvested and amplified until a desired titer was reached (viz. 1×10^8 pfu/ml).

Coinfections. Baculoviruses carrying the wt-*cdk5* and *GST–p35* genes, or the var-*cdk5* and *GST–p35* genes were used to coinfect 2×10^7 Sf9 insect cells (multiplicity of infection, MOI, of 5) in 25 ml of SF900 II SFM medium (Gibco-BRL). After 72 h, the cells were harvested and lysed by sonication in NETN buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 05% v/v NP-40, 5 mM NaF, 30 mM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml antipain; 50 ml of buffer contained 1 tablet of protease inhibitor cocktail Complete (Boehringer)]. The lysate was preincubated for 2 h at 4°C with 100 μl of Sepharose 4B (Amersham-Pharmacia Biotech) to reduce non-specific binding of proteins to GSH-Sepharose beads, which were used in the next step. Finally the lysate was incubated with 100 μl of glutathione(GSH)–Sepharose 4B (Amersham-Pharmacia Biotech), overnight at 4°C. The beads were washed seven times with ice-cold NETN buffer and 3 times with ice-cold kinase buffer (20 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$). The beads were resuspended in 100 μl kinase buffer and kept on ice before performing a kinase assay.

Kinase assays. The substrates used in the assays were either 100 ng of histone H1 (Type III-S from calf thymus, Sigma H-5505) or 250 ng of GST–Rb152 (i.e., C-terminal 152 amino acids of pRb covalently linked to GST; Santa Cruz Biotechnology, Cat. No. sc-4112, Lot. No. G287). The phosphorylation reaction was performed in kinase buffer for 40 min at 30°C using 2 μl of the GSH-Sepharose bead-bound enzyme, 10 μM ATP and 3 μCi [γ-^{33}P]ATP (specific activity = 3000 Ci/mmol, Amersham-Pharmacia Biotech) in a total volume of 10 μl. The reactions were boiled in Laemmli sample buffer containing 50 mM DTT before loading on to a 12.5% SDS–polyacrylamide gel along with the prestained molecular weight markers (Kaleidoscope; Bio-Rad). The gel was fixed (10% v/v glacial acetic acid, 30% v/v methanol) and dried on Whatman paper. The phosphorylation was quantified using a PhosphoImager (Molecular Devices).

Western blot analysis. Ten microliters of GSH–Sepharose beads, either bound to Cdk5wt/GST–p35 or Cdk5i/GST–p35 complex, was boiled in Laemmli sample buffer containing 50 mM DTT. The proteins in the supernatants, along with the prestained molecular weight markers (Kaleidoscope; Bio-Rad), were fractionated by 12.5% SDS–PAGE. Proteins were transferred to PVDF membranes (Immobilon; Millipore) using a semidry electroblotter (Pegasus, Germany). GST–p35 was detected using a monoclonal antibody against GST (Clonetech) whereas both Cdk5 and its isoform were detected with a monoclonal antibody against Cdk5 (Ab-1; Calbiochem) using the ECL kit, according to the manufacturer's instructions (Amersham-Pharmacia Biotech).

Cdk2 and Cdk5 (homology-modeling), is depicted. Like Cdk2, the Cdk5 kinase domain folds into a two-lobed structure. The smaller, NH_2-terminal lobe, which includes subdomains I-IV, is primarily involved in anchoring and orienting the nucleotide, ATP (Fig. 2, right panel). This lobe has a predominantly antiparallel β-sheet structure that is unique among nucleotide binding proteins. The larger COOH-terminal lobe, which includes subdomains VI-XI, is largely responsible for binding the peptide substrate and initiating phosphotransfer. It is predominantly α-helical in content. Subdomain V residues span the two lobes. The deep cleft between the two lobes is recognized as the site of catalysis. The darker region in the COOH-terminal lobe is the region (amino acid residues 105–136) missing in Cdk5i.

After having discovered the gene coding for Cdk5i in the fetal brain cDNA library, we asked if var-*cdk5* was present in all cells (i.e., also in proliferating cells and tissues). Hence, PCR was performed on cDNA libraries derived from HeLa cells, human T-cells, thymus and placenta using the unique *cdk5* primers. We isolated wt-*cdk5* from all the libraries. However, it was surprising that repeated attempts failed to amplify var-*cdk5* from any of these libraries (data not shown). The var-*cdk5* gene was also not obtained from a cDNA library constructed from the adult human cerebellum which should consist of post-mitotic, differentiated cells. It could be that var-*cdk5* exists only in neuronal cells which have not yet undergone differentiation (i.e., cells similar to those present in the fetal brain).

With this in mind, reverse transcriptase (RT)-PCR was performed on total RNA derived from dividing human neuroblastoma SH-SY-5Y cells. Like fetal brain cells, SH-SY-5Y cells in culture can readily undergo differentiation under specific conditions (21). It was indeed interesting to find that RT-PCR yielded two products similar to the ones obtained from the PCR amplification of the fetal brain cDNA library. Sequencing revealed that the two products were identical to wt-*cdk5* and var-*cdk5,* the two genes identified in the fetal brain library.

To find out if the mutant gene could form an active kinase, we coexpressed wt-Cdk5 with GST–p35 and, in parallel, Cdk5i (the protein encoded by var-*cdk5*) with GST–p35. Protein expression was obtained by identical means, that is, by coinfecting Sf9 insect cells with the respective baculoviruses (each with an MOI of 5; see Materials and Methods). Attempts were then made to precipitate the heterodimeric complexes from the cell lysates using equal amounts of GSH-Sepharose and also under exactly identical conditions. The precipitates (10 μl of GSH-Sepharose beads; see Materials and Methods) were analyzed by Western-blotting (Fig. 3). It seems that both complexes are pulled down by GSH–Sepharose beads, which implies that not only wt-Cdk5 protein binds to GST–p35 but that also Cdk5i

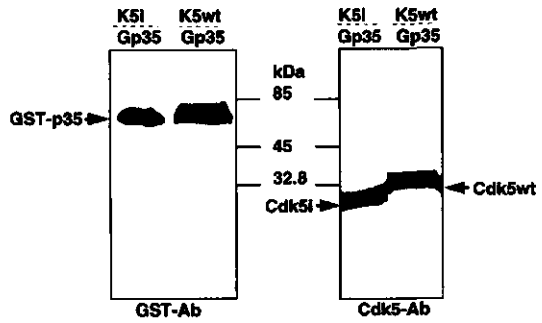


FIG. 3. Western blot analyses of Cdk5i/GST–p35 (K5i/Gp35) and the Cdk5/GST–p35 (K5wt/Gp35) complexes bound to GSH-Sepharose beads (10 μl each; see Materials and Methods). GST–p35 was detected with a monoclonal antibody against GST (left panel), whereas both Cdk5 and its isoform were detected with a monoclonal antibody against Cdk5 (right panel) using the ECL kit (Amersham-Pharmacia Biotech).

forms a complex with the activator protein (Fig. 3). We observe that levels of the two subunits (catalytic and regulatory) are roughly the same in Cdk5i/GST–p35 and Cdk5/GST–p35 (Fig. 3, compare left and right panels).

Activities of the two complexes, Cdk5i/GST–p35 and Cdk5/GST–p35, were tested in kinase assays using histone H1 and GST–Rb152 as substrates. Figure 4A depicts that wt-Cdk5 kinase is able to phosphorylate itself and its activating partner p35, even in the absence of any substrate (lane 2). In contrast, the Cdk5i kinase has totally lost the ability to autophosphorylate either of its two subunits (lane 5). However, the Cdk5i/GST–p35 complex can still phosphorylate histone H1 and GST–Rb152 (lanes 4 and 6). It should be mentioned that, since the kinase activity of the isoform is much weaker than that obtained from the wild type (Fig. 5; compare lanes 1 and 6; and unpublished results), the concentration of Cdk5i kinase used in these experiments was at least ten times more than that of the corresponding wt-Cdk5 kinase. This allowed us to make a clear distinction between the two purified kinases (compare lanes 1 and 3 with 4 and 6). Figure 4B depicts that GST–p35 alone does not precipitate a residual kinase activity which could have phosphorylated histone H1 or GST–Rb152. To further confirm the validity of our Cdk5 assay, we tried to activate Cdk5i and Cdk5 using GST–cyclin A and GST–cyclin D1 as regulatory partners. As known in the literature, neither cyclin A nor cyclin D1 yielded active kinases in characteristic phosphorylation assays using histone H1 and GST–Rb152 as substrates (data not shown).

We then questioned if Cdk5i could compete with wt-Cdk5 for binding to p35. Therefore, Sf9 insect cells were simultaneously infected with the three baculoviruses encoding var-*cdk5,* wt-*cdk5* and *GST–p35.* Dif-

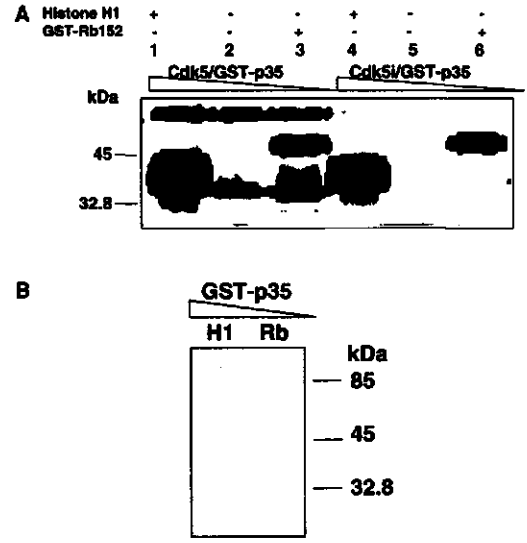


FIG. 4. (A) The Cdk5i/GST–p35 kinase has lost the ability to autophosphorylate its two subunits, Cdk5i and p35. Lane 1, Cdk5/GST–p35 kinase incubated with 100 ng of histone H1; lane 2, Cdk5/GST–p35 kinase without any substrate; lane 3, Cdk5/GST–p35 kinase incubated with 250 ng of GST–Rb152; lane 4, Cdk5i/GST–p35 kinase incubated with 100 ng of histone H1; lane 5, Cdk5i/GST–p35 kinase without any substrate; lane 6, Cdk5i/GST–p35 kinase incubated with 250 ng of GST–Rb. (B) GSH-Sepharose was used to precipitate proteins from insect cells which had been infected only with *GST–p35* encoding baculovirus. The precipitated proteins do not phosphorylate histone H1 (H1) or GST–Rb152 (Rb).

ferent MOI-s of var-*cdk5* virus were used in these triple infections whereas the MOI of the other two viruses was kept constant. The complexes were pulled down with GSH-Sepharose and their activities were tested in kinase assays using histone H1 and GST–Rb152 as substrates (Fig. 5; upper and lower panels). When the m.o.i. of var-*cdk5* in the triple infection assays was twice that of wt-*cdk5*, phosphorylation on the catalytic subunit was not detectable anymore (lane 3, lower panel; in the upper panel, phosphorylated Cdk5 protein co-migrates with the multiple histone H1 phosphorylation bands and, therefore, the disappearance of the Cdk5 band is not clearly perceived). When the MOI of var-*cdk5* virus in the triple infection assays was 4 times the MOI of the wt-*cdk5*, phosphorylation of the activating partner p35 decreased considerably (lane 4). When MOI of var-*cdk5* virus was 5 times greater than the wt-*cdk5* virus, the kinase activity of the precipitated complex was as if there was no wt-Cdk5 protein bound to GSH-Sepharose beads (i.e., activity was the same as the Cdk5i kinase; compare lanes 5 and 6).

The above results seem to suggest that Cdk5i is not really a "kinase-dead" protein (like the dominant negative mutants of the Cdk-s; Ref. 22) but can assemble with p35 as a defective kinase. This is indicated by the fact that Cdk5i has some activity when coexpressed with p35 in insect cells. We believe that absence of the kinase domain VI in Cdk5i does not prevent it to bind ATP. This has been proven by using staurosporine, a nonspecific ATP competitor for Cdk2, Cdk4 and Cdk5 (23). We observe that staurosporine can inhibit Cdk5i, just like Cdk5 (data not shown). Gradual increase of ATP concentrations in the kinase assay prevent this inhibition to occur. Therefore, kinase domain VI seems to have no role in ATP binding. However, the deleted domain in Cdk5i must have a function in substrate recognition. Even though the affinity of Cdk5i for p35 may be the same as wt-Cdk5, Cdk5i has lost the ability to autophosphorylate its two subunits. We also observe that, in insect cells, activity of Cdk5/p35 is abrogated by coinfecting increasing amounts of the var-*cdk5* baculovirus. This can only mean that the catalytic subunit Cdk5i, defective in its kinase activity, can displace wt-Cdk5 from a Cdk5/p35 heterodimeric complex. Although these conclusions are solely based on our experiments with only two substrates (the retinoblastoma protein and histone H1), we believe that the defective kinase Cdk5i/p35 would behave identically if tested for its ability to phosphorylate neurofilament proteins and tau *in vitro* (24, 25).

It has been widely suggested that misregulation of Cdk5 can cause a variety of neurodegenerative diseases, from Alzheimer's and Parkinson's diseases to amyotrophic lateral sclerosis (ALS). It could be that

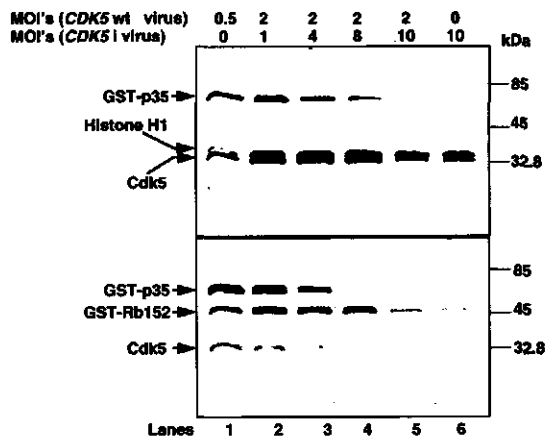


FIG. 5. Cdk5i competes with wt-Cdk5 for binding to GST–p35. Sf9 insect cells were infected with the 3 baculoviruses encoding wt-*cdk5*, var-*cdk5* and *GST–p35*. The amount of var-*cdk5* virus in the triple infections was raised stepwise, whereas the wt-*cdk5* and *GST–p35* viruses were kept constant (MOI as indicated). The complexes were purified via binding of Cdk5/GST–p35 and Cdk5i/GST–p35 to 100 μl GSH-Sepharose beads. 2 μl of beads were used for each kinase assay. Upper panel, phosphorylation of 100 ng of histone H1. Lower panel, phosphorylation of 250 ng GST–Rb152.

Cdk5i is differentially expressed in patients suffering from such diseases. In Alzheimer's disease, paired helical filaments (PHF) are composed of hyperphosphorylated tau (24). In the fetal brain, tau is also hyperphosphorylated and the phosphorylation sites identified in fetal tau are the same as for PHF-tau (26). This suggests that PHF-tau may arise from a loss of regulatory control of tau phosphorylation in degenerating neurons that results in the reappearance of a fetal phosphorylation pattern (26). Since the var-*cdk5* occurs specifically in the fetal brain and not in mature human cerebellum, it is possible that kinase-defective Cdk5i plays a specific role in the developing brain (27, 28). There is a similar precedent in the recent literature. It has been proposed that another naturally occuring defective kinase, the tyrosine kinase receptor ErbB-3, plays a specific role only in proliferating cells whereas the active kinase ErbB-4 correlates with only the differentiated phenotype (29).

ACKNOWLEDGMENT

We are grateful to Rainer Ortmann (Novartis Pharma) for providing the SH-SY-5Y neuroblastoma cells.

REFERENCES

1. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. (1992) *EMBO J.* **11,** 2909–2917.
2. Lew, J., and Wang, J. H. (1995) *Trends Biochem. Sci.* **20,** 33–37.
3. Tang, D., and Wang, J. H. (1996) *Prog. Cell Cycle Res.* **2,** 205–216.
4. Miyajima, M., Nornes, H. O., and Neuman, T. (1995) *Neuro-Report* **6,** 1130–1132.
5. Dellale, I, Bhide, P. G., Caviness, V. S., Jr., and Tsai, L. H. (1997) *J. Neurocytol.* **26,** 283–296.
6. Lee, K.-Y., Helbing, C. C., Choi, K.-S., Johnston, R. N., and Wang, J. H. (1997) *J. Biol. Chem.* **272,** 5622–5626.
7. Lew, J., Huang, Q.-Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) *Nature* **371,** 423–426.
8. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. and Tsai, L. H. (1996) *Genes Dev.* **10,** 816–825.
9. Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E., and Tsai, L. H. (1997) *Neuron* **18,** 29–42.
10. Lazaro, J. B., Kitzmann, M., Poul, M. A., Vandromme, M., Lamb, N. J., and Fernandez, A. (1997) *J. Cell Sci.* **110,** 1251–1260.
11. Philpott, A., Porro, E. B., Kirschner, M. W., and Tsai, L. H. (1997) *Genes Dev.* **11,** 1409–1421.
12. Henchcliffe, C., and Burke R. E. (1997) *Neurosci. Lett.* **230,** 41–44.
13. Brion, J.-P., and Couck, A.-M. (1995) *Am. J. Pathol.* **147,** 1465–1476.
14. Nakamura, S., Kawamoto, Y., Nakano, S., Ikemoto, A., Akiguchi, I., and Kimura, J. (1997) *Neurology* **48,** 267–270.
15. Imahori, K., and Uchida, T. (1997) *J. Biochem. Tokyo* **121,** 179–188.
16. Sengupta, A., Wu, Q., Grundke-Iqbal, I., Iqbal, K., and Singh, T. J. (1997) *Mol. Cell. Biochem.* **167,** 99–105.
17. Ohshima, T., Ward, J. M., Huh, C.-G., Longenecker, G., Veeranna, Pant, H. C., Brady, R. O., Martin, L. J., and Kulkami, A. B. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11173–11178.
18. Hanks, S. K., and Hunter, T. (1995) *FASEB J.* **9,** 576–596.
19. Tsai, L.-H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) *Nature* **371,** 419–423.
20. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) *Nat. Struct. Biol.* **3,** 696–700.
21. Påhlman, S., Hoehner, J. C., Nånberg, E., Hedborg, F., Fagerström, S., Gestblom, C., Johansson, I., Larsson, U., Lavenius, E., Ortoft, E., and Söderholm, H. (1995) *Eur. J. Cancer* **31A,** 453–458.
22. van den Heuvel, S., and Harlow, E. (1993) *Science* **262,** 2050–2054.
23. Lawrie, A. M., Noble, M. E., Tunnah, P., Brown, N. R., Johnson, L. N., and Endicott, J. A. (1997) *Nat. Struct. Biol.* **4,** 796–801.
24. Baumann, K., Mandelkow, E. M., Biernat, J., Piwnica-Worms, H., and Mandelkow, E. (1993) *FEBS Lett.* **336,** 417–424.
25. Sun, D., Leung, C. L., and Liem, R. K. H. (1996) *J. Biol. Chem.* **271,** 14245–14251.
26. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995) *J. Biol. Chem.* **270,** 823–829.
27. Matsushita, M., Tomizawa, K., Lu, Y. F., Moriwaki, A., Tokuda, M., Itano, T., Wang, J. H., Hatase, O., and Matsui, H. (1996) *Brain Res.* **734,** 319–322.
28. Tomizawa, K., Matsui, H., Matsushita, M., Lew, J., Tokuda, M., Itano, T., Konishi, R., Wang, J. H., and Hatase, O. (1996) *Neuroscience* **74,** 519–529.
29. Pinkas-Kramarski, R., Eilam, R., Alroy, I., Levkowitz, G., Lonai, P., and Yarden, Y. (1997) *Oncogene* **15,** 2803–2815.

6. Summary and conclusions

Cell division (proliferation) is necessary for growth, maintenance and multiplication of living organisms, consisting of one or more cells. Different cells can fulfill specific functions within one multicellular organism. These cells which do not divide anymore and have taken up a certain specialized function are called differentiated cells. Cells which neither differentiate nor proliferate are in the quiescent state G_0. Proliferating cells reside in different phases before they divide. After cell division a cell can enter the different phases again until another division takes place. This is what we call the cell cycle.

Each phase transition is controlled by specific enzymes. These enzymes which are called cyclin dependent kinases, Cdk-s, have to bind a cyclin in order to be active. The Cdk catalytic subunits are present during each phase of the cell cycle. This is in contrast with the cyclins, the activating partners of the Cdk-s, which have a shorter half life then the Cdk-s catalytic subunits and are expressed only at certain time points during the cell cycle. The temporal activation of individual Cdk-s is dictated in part by the timing of expression of their cognate cyclins together with both activating and inhibitory phosphorylation. Different cyclins can bind different Cdk-s giving rise to a whole set of Cdk-cyclin complexes each with a specific function. Cdk-s belong to the protein kinase family and their function is to phosphorylate protein substrates which play defined roles during the cell cycle. Cdk-s in turn are also inhibited by specific inhibitors.

During the first phase of the cell cycle which is called G_1 (first gap), certain conditions must be met before DNA can be replicated in the S (synthesis) phase. A few Cdk-s check the G_1/S phase transition which is called the 'restriction point' in mammals. At this point of no return the retinoblastoma protein, (pRb), plays a key role. Hypophosphorylated pRb binds to certain transcription factors amongst which E2F is the most known. When pRb is hyperphosphorylated by Cdk-s, the E2F transcription factor is released and activates the transcription of a range of genes driving the cell into the S phase. pRb phosphorylating Cdk-s acting at the G_1/S transition are Cdk6-cyclin D, Cdk4-cyclin D, Cdk2-cyclin E and Cdk2-cyclin A.

After the S phase the cell resides in G_2 (second gap). If the DNA is replicated correctly the cell can divide. The division phase is called M (mitosis). The G_2/M phase transition is controlled by Cdk1-cyclin B.

The exact passage through the eukaryotic cell cycle is therefore regulated by the progressive activation and inactivation of a family Cdk-s. Cell cycle diseases like for instance cancer have a genetic basis. Cancer cells evolve from normal cells when some essential processes in a dividing cell malfunction. This causes inappropriate replication, segregation and repair of the genome during progression of the cell cycle. Increased Cdk4 activity due to overexpression of Cdk4 and/or cyclin D, or because $p16^{INK4A}$, the Cdk4 specific inhibitor is missing from the cell, will cause the cell to cycle without a functional restriction point. The duration of the cell cycle shortens giving rise to mistakes in DNA replication. These malignant cells will grow out to form tumours sapping all life from the host.

Potential antineoplastic drugs against certain forms of cancer could be synthetic chemicals which would inhibit for instance Cdk4 activity. This chemical should of course not be lethal to the patient. Screening of compounds on their Cdk inhibitory effect is one example of how pharmaceutical industries are performing cancer research. In chapter 2 of this thesis a method is presented to facilitate Cdk4 compound screening. Human Cdk4 is expressed in the yeast *Saccharomyces cerevisiae* under the control of the *GAL1-GAL10* promoter and inhibits cell growth when the yeast is grown on galactose. Coexpression of $p16^{INK4A}$ restores yeast cell growth. Moreover flavopiridol, the Cdk inhibitor which has already entered phase 3 clinical trials, is also restoring growth when it is added to the growth medium of the yeast. Simple OD readings of this yeast transformation when grown on galactose could give useful information upon whether a compound added to the growth medium could be a Cdk4 inhibitor or not. This Cdk4 inhibition will be of certain specificity since the yeast Cdc28 has high homology with human Cdk1 and Cdk2.

Components, which normally would reside in the cell cycle, have recently been found to function atypically in non-proliferating neuronal cells. The cyclin dependent kinase Cdk5 for instance is identified via its homology with other Cdk-s and has not been shown to play a role in the cell cycle. Cdk5 has a ubiquitous tissue distribution in mammals with brains containing the highest amount of the transcript. On the other hand, the expression of p35, the only known activator of Cdk5, which has no homology to cyclins, is strictly confined to brains. Cyclin D and cyclin E have been

found to bind to Cdk5 but no activity of these complexes has been shown. The Cdk5/p35 complex can phosphorylate histone H1 and pRb, two proteins which are commonly used to test Cdk activity *in vitro*. Activated Cdk5 kinase phosphorylates a number of cytoskeletal proteins including neurofilaments and the neuron-specific microtubule associated protein tau *in vitro*, which are assumed to be the natural substrates. Phosphorylation of cytoskeletal proteins may play an important role in the polymerization and assembly of cytoskeletal elements which, in turn, may effect the growing neurites suggesting that Cdk5 is involved in the growth and maintenance of neurites. Recently Cdk5 has been shown to play a role in differentiation of muscles as well.

Nerve cells are terminally differentiated before the onset of birth. This is in contrast with other tissues where differentiated cells still are able to divide after tissue damage. Since nerves do not have any regeneration capacity, damaged or malignant cells will automatically undergo apoptosis. In the neurodegenerative diseases dementia (diffuse Lewy body disease), Parkinson's and amyotrophic lateral sclerosis neurofilament proteins are hyperphosphorylated whereas in Alzheimer's disease the tau protein is hyperphosphorylated. These malignant nerve cells undergo apoptosis leading to a certain death of the patient suffering from these diseases. Neurodegeneration possibly occurs as a result of inappropriate activation/deactivation of tissue-specific components of the cell cycle. It is very likely that Cdk5 is overactive in these malignant nerve cells.

Since Cdk5 has been found in both proliferative and differentiated cells it is interesting to search for protein interactions with Cdk5 and the effect of these proteins on the Cdk5 kinase. During these investigations attempts were made to search for protein interactions with Cdk5 in proliferative cells and the possible roles of these proteins towards the Cdk5 kinase.

In chapter 3 a C-terminal fragment of DNA binding protein, dbpA is described which can bind to Cdk5. This protein-protein interaction is shown via the yeast-two-hybrid system. Several *in vitro* experiments have confirmed that the C-terminal fragment of dbpA indeed specifically binds to Cdk5. Kinase assays have shown that this protein fragment inhibits the phosphorylation of both histone H1 and pRb by the Cdk5 kinase. DbpA is expressed in skeletal muscle and the heart. Since Cdk5 has been found to play a role in differentiation of muscles it is possible that dbpA plays a role in this phenomenon as well.

Chapter 4 depicts another Cdk5 binding protein, a 60S ribosomal protein, L34. This protein interaction is discovered via the yeast-two-hybrid system as well. L34 like dbpA blocks the phosphorylation of histone H1 and pRb by Cdk5.

Cloning of the *cdk5* gene has been done via PCR on cDNA libraries. PCR on cDNA from a fetal brain library revealed two PCR products. One of the PCR products had the size of the *cdk5* open reading frame whereas the other product was much smaller. Sequencing of both products showed the larger fragment coded indeed for the *cdk5* gene whereas the other product was identical with the *cdk5* open reading frame with bases 313 to 409 missing in the middle of the gene. In fetal brain this Cdk5 isoform could be expressed which lacks amino acids 105-136, which are thought to be responsible for catalyzing the phosphorylation reaction. Although this Cdk5 isoform (Cdk5i) can bind to p35, little kinase activity has been shown. Since this *cdk5* variant has been found in total RNA of SH-SY-5Y neuroblastoma cells as well, but not in cDNA libraries of T-cells, HeLa cells, thymus, placenta and cerebellum, this protein could play a specific role in the differentiation of human nerve cells too. Binding of Cdk5i in these respective cells to p35 could prevent the wildtype Cdk5 from being activated. In this way Cdk5i could modulate the activity of the Cdk5 wildtype kinase. Chapter 5 depicts the results of this research.

In this thesis a screen is presented to search for Cdk4 antagonists. Furthermore the Cdk5 interacting proteins presented and the Cdk5 isoform could be tested for their ability to regulate the Cdk5 kinase *in vivo*. The role of these Cdk5 regulating proteins in cycling and post-mitotic cells needs to be evaluated through further cellular analyses.

With these findings which have been revealed during my doctoral research I hope to have contributed in a positive manner to the elucidation of both cancer and neurodegenerative diseases.

Samenvatting

(Summary in Dutch)

Het delen van cellen (proliferatie) is nodig voor groei, instandhouding en vermeerdering van levende organismen, die uit een of meer cellen bestaan. Daarnaast kunnen cellen binnen een meercellig organisme specifieke functies vervullen, zonder zich voortdurend te delen. Deze cellen met specifieke functie, die zich niet meer delen, noemt men gedifferentiënteerde cellen. Een cel, die zich niet deelt, maar ook niet is gedifferentiëerd bevindt zich in een rustfase genaamd G_0 (quiescent state). Een zich delende cel doorloopt verschillende fasen voordat zij zich heeft gedeeld. Na celdeling kan een cel de verschillende fasen opnieuw volledig doorlopen, totdat een volgende celdeling kan plaatsvinden. Een delende, niet gedifferentiëerde, cel cycleert dus. Men spreekt over de celcyclus (cell cycle).

Elke fase overgang van de cel (phase transition) wordt gecontroleerd door een aantal enzymen. Deze enzymen worden cyclin dependent kinases, Cdk's, genoemd, omdat ze voor hun activiteit aan een cyclin moeten binden. Meestal zijn verschillende Cdk's tijdens elke fase aanwezig. De cyclins, de activerende partners voor de Cdk's echter niet. Zij worden tot expressie gebracht als hun functie tijdens de celcyclus gewenst is en worden ook afgebroken als hun aanwezigheid niet meer noodzakelijk is. Sommige cyclins kunnen verschillende Cdk's binden, zodat een heel scala aan Cdk-cyclin complexen gevormd kan worden, elk met hun eigen specifieke functie. Deze functie bestaat uit het fosforyleren van bepaalde eiwitten (Cdk's behoren tot de eiwit kinasen), zodat dat eiwit (het substraat) andere eigenschappen verkrijgt en daardoor een andere rol in de celcyclus zal gaan spelen. De geactiveerde Cdk's kunnen geremd worden door bepaalde andere eiwitten (Cdk inhibitors).

Voordat het erfelijk materiaal (DNA) tijdens de celcyclus wordt verdubbeld, moet aan een bepaald aantal voorwaarden worden voldaan. De cel bevindt zich nu in fase G_1 (first gap). Een aantal Cdk's controleren de overgang naar de DNA replicatie fase S (synthesis). Deze G_1/S overgang wordt bij zoogdieren 'restriction point' genoemd. Deze deur naar de DNA replicatie fase wordt geblokkeerd door een eiwit, genaamd pRb (retinoblastoma protein). Dit eiwit bindt onder andere aan E2F, een eiwit wat een aantal genen kan activeren (transcriptie factor) indien E2F niet aan pRb

gebonden is. Deze genen dienen geactiveerd te worden ten behoeve van de DNA replicatie. De Cdk-cyclin kinasen die de G_1/S fase overgang controleren kunnen pRb fosforyleren, waardoor het niet meer aan E2F bindt. E2F kan vervolgens zijn functie vervullen en de DNA replicatie op gang brengen. De Cdk-cyclin complexen die pRb kunnen fosforyleren zijn: Cdk6-cyclin D, Cdk4-cyclin D en Cdk2-cyclin E.

Na de S fase bevindt de cel zich in G_2 (second gap). Hier wordt gecontroleerd of het DNA juist gedupliceerd is, voordat de cel zich in tweeën deelt. Het uiteindelijke delen vindt plaats in de M fase (mitosis). De G_2/M fase overgang wordt gecontroleerd door Cdk1-cyclin B.

Het juiste verloop van de celcyclus wordt dus gereguleerd door Cdk's. Het is erg belangrijk dat deze enzymen op het juiste tijdstip de juiste activiteit vertonen. Helaas komt het in de natuur wel eens voor dat deze enzymen een te hoge activiteit vertonen, bijvoorbeeld doordat teveel Cdk4 of cyclin D tot expressie wordt gebracht of doordat het eiwit p16, wat het Cdk4-cyclin D kinase zou kunnen remmen, ontbreekt. Sommige cellen gaan zich hierdoor vaker delen dan gewenst is. De celcyclus wordt sneller doorlopen en sommige fouten in DNA replicatie worden niet meer hersteld. De zo ontstane cellen woekeren in het lichaam van het organisme zonder dat het lichaam er enige controle over heeft. De cellen parasiteren in de gastheer en zorgen er uiteindelijk voor dat het organisme aan deze genetisch bepaalde afwijking overlijdt. Deze ziekte is bij ons bekend onder de naam kanker.

Een medicijn tegen bepaalde soorten kanker zou dus een bepaalde stof kunnen zijn, dat in staat is om bijvoorbeeld Cdk4 activiteit te remmen. Vanzelfsprekend mag deze verbinding niet te giftig (toxisch) zijn voor de patiënt. Bij de farmaceutische industrie verricht men ondere andere op deze wijze onderzoek naar kanker. Men zoekt Cdk remmende verbindingen (compounds). In hoofdstuk 2 van dit proefschrift wordt een methode beschreven om het zoeken naar specifieke Cdk4 remmende compounds (compound screening) te vergemakkelijken. Humaan Cdk4 wordt tot expressie gebracht in bakkersgist. Daar deze Cdk voor de gist een vreemd eiwit is wat allerlei reacties in de gist zou kunnen veroorzaken, vermeerdert de gist zich niet meer. Als tezamen met Cdk4 ook p16 in de gist tot expressie wordt gebracht kan de gist zich weer normaal delen. Ook flavopiridol, een chemische verbinding die als kankermedicijn in overweging wordt genomen, heeft hetzelfde effect op de gist, die door Cdk4 wordt gekweld. De gist vermeerdert zich weer als flavopiridol aan het groeimedium van de gist wordt toegevoegd. De zieke gist met humaan Cdk4 als

ballast zou derhalve gebruikt kunnen worden bij het testen van verbindingen op hun Cdk4 kinase remmende activiteit. Zodra de gist zich weer gaat vermeerderen is dit een indicatie dat de stof een Cdk4 remmer zou kunnen zijn.

Er bestaat een Cdk, Cdk5 genaamd, waarvan geen rol binnen de celcyclus is aangetoond. Cdk5 komt in vele weefsels voor, maar wordt in grotere hoeveelheden tot expressie gebracht in zenuwcellen. Cyclin D en cyclin E kunnen met Cdk5 binden, maar er is nog geen activiteit van deze enzymcomplexen aangetoond. Het eiwit p35, wat geen homologie met cyclins heeft kan Cdk5 echter activeren. Er zijn verschillende substraten aangetoond. Zo kan de Cdk5 kinase histone H1 en pRb fosforyleren. Deze twee eiwitten worden vaak gebruikt tijdens onderzoek naar Cdk5 activiteit. De natuurlijke substraten van Cdk5 moeten echter gezocht worden onder de eiwitten die het skelet van de zenuwcel vormen. Cdk5 fosforyleert bijvoorbeeld neurofilamenten en tau. Van Cdk5 wordt gedacht dat het geen functie vervult tijdens de celcyclus, maar dat het betrokken is bij de differentiatie van zenuwcellen. Recentelijk is aangetoond dat Cdk5 ook een rol speelt bij de differentiatie van spiercellen.

Zenuwcellen differentiëren zich reeds voor de geboorte, totdat ze een bepaalde fase bereikt hebben (terminale differentiatie), waarbij ze zich nooit meer kunnen delen. Dit is in tegenstelling tot vele andere celtypen, die na beschadiging van een bepaald weefsel, hun gedifferentieerde toestand verlaten om zich weer te gaan delen. De zenuwcellen vervullen dus een bepaalde functie in het lichaam en behouden deze functie een leven lang. Beschadigde of zieke cellen kunnen niet vervangen worden, zodat het individu deze functie verliest. Er zijn een aantal zenuwziekten bekend waarbij zenuwcellen afsterven. Dit afsterven is een actief proces wat door de cel zelf wordt gestuurd. Zieke cellen plegen als het ware zelfmoord (apoptosis) om zo het individu tot dienst te zijn. Dementie (difuse Lewy body disease), Parkinson, amyothrophic lateral sclerosis en Alzheimer zijn voorbeelden van ziekten waarbij zenuwcellen afsterven. Bij zenuwonderzoek vraagt men zich af waarom dit het geval is. Bij restanten van zenuwcellen, die men gevonden heeft bij patienten, die aan dementie, Parkinson of amyothrophic lateral sclerosis lijden, heeft men ontdekt dat de neurofilamenten te veel gefosforyleerd zijn (gehyperfosforyleerd). Bij Alzheimer patienten heeft men juist ontdekt dat tau gehyperfosforyleerd is. Net als bij kanker waar door een genetische afwijking Cdk4 te actief zou kunnen zijn, zou bij deze zenuwziekten door een genetische afwijking Cdk5 overactief kunnen zijn. Ook bij

deze ziekten zou dus weer de mogelijkheid kunnen bestaan dat een natuurlijk eiwit, wat aan het eind van de differentiatie van zenuwcellen Cdk5 zou moeten remmen (Cdk5 inhibitor), ontbreekt.

Omdat Cdk5 in zo vele verschillende cellen van verschillende weefsels voorkomt in zowel prolifererende als gedifferentiëerde cellen is het interessant te onderzoeken welke eiwitten met Cdk5 kunnen binden in zowel prolifererende als gedifferentiëerde cellen en wat hun invloed zou kunnen zijn op de activiteit van dit enzym. Tijdens dit promotie onderzoek is getracht eiwitinteracties met Cdk5 aan te tonen in prolifererende cellen.

In hoofdstuk 3 wordt een DNA bindend eiwit, dbpA beschreven wat met humaan Cdk5 kan binden. Deze eiwitinteractie is aangetoond met behulp van een bepaald systeem in gist het zogenaamde yeast-two-hybrid systeem. Meerdere bindingsexperimenten hebben aangetoond dat dit eiwit, dbpA, inderdaad met Cdk5 associëert. DbpA schijnt met name in skeletspieren en de hartspier voor te komen. Daar Cdk5 ook een rol speelt bij de differentiatie van spiercellen is het zeer wel mogelijk dat dbpA ook een functie vervult tijdens de differentiatie van spiercellen. Kinase experimenten hebben aangetoond dat dbpA de activiteit van Cdk5 kan remmen. Door bepaalde hoeveelheden dbpA aan de Cdk5 kinase toe te voegen was dit enzym niet meer in staat histone H1 of pRb te fosforyleren.

Hoofdstuk 4 gaat over een 60S ribosomaal eiwit, L34. Dit eiwit kan ook een binding aangaan met Cdk5 en is op dezelfde wijze als dbpA aangetoond namelijk met behulp van het yeast-two-hybrid systeem. L34 kan net als dbpA fosforylering van histone H1 en pRb door Cdk5 remmen.

Om het gen coderend voor humaan Cdk5 in gist te kunnen inbrengen (transformatie) dient het vermeerderd te worden. Deze methode heet PCR. Na een PCR reactie verricht aan cDNA van fetale hersenen was niet alleen het Cdk5 gen vermeerderd, maar er ontstond ook een kleiner PCR produkt. Na sequencing van het DNA bleek dat dit PCR produkt identiek was aan het Cdk5 gen. Echter er ontbrak een deel van de DNA sequentie in het midden van het PCR produkt. In fetale hersenen zou dus een eiwit kunnen worden geproduceerd wat identiek is aan Cdk5 maar wat juist dat deel van het enzym ontbreekt, waarvan gedacht wordt dat het een belangrijke rol vervult bij de katalyse van de fosforyleringsreactie. Deze Cdk5 isoform (Cdk5i) kan met p35 binden, maar heeft vrijwel nauwelijks kinase activiteit. Omdat dit gentranscript ook in neuroblastoma cellen, maar niet in T-cellen, HeLa cellen, thymus,

placenta en cerebellum wordt gevonden, zou dit eiwit ook een specifieke rol kunnen spelen bij de differentiatie van humane zenuwcellen. Doordat dit kinase-defectieve eiwit met p35 kan binden, wordt verhinderd dat p35 met het normale Cdk5 (Cdk5 wildtype) bindt. Op deze wijze zou Cdk5i de activiteit van Cdk5 wildtype kunnen reguleren. In hoofdstuk 5 staan de resultaten van dit onderzoek vermeld.

Met deze resultaten, die naar voren zijn gekomen tijdens mijn promotie onderzoek, hoop ik een positieve bijdrage te hebben geleverd op het gebied van zowel kanker- als zenuwonderzoek.

Curriculum vitae

Mark Johannes Magdalena Willibrordus Moorthamer werd geboren op 15 juni 1970 te Hulst. In 1988 werd het V.W.O. diploma behaald aan het R.K. 'Jansenius-Lyceum', eveneens te Hulst. In hetzelfde jaar begon hij met de studie bedrijfskunde aan de 'Erasmus' universiteit te Rotterdam. Na de propaedeuse werd de militaire dienstplicht vervuld. In 1990 ving hij aan met de studie bioprocestechnologie aan de Landbouwuniversiteit te Wageningen. Het doctoraalprogramma omvatte afstudeervakken bij de vakgroepen Microbiologie (Dr. C. Dijkema, Dr. A.J.M. Stams, Prof. Dr. W.M de Vos), Biochemie (Prof. Dr. W.R. Hagen, Prof. Dr. C. Veeger) en de sectie Moleculaire Genetica van Industriële Micro-organismen (Dr. ir. J. Visser). Een stage werd uitgevoerd in Basel (Zwitserland) bij de 'Core Drug Discovery Technology' afdeling van Ciba-Geigy (Dr. B. Meyhack).

Van november 1995 tot januari 1999 verrichtte hij zijn promotie-onderzoek bij de 'Oncology Research' afdeling van Novartis Pharma in Basel (Zwitserland) wat heeft geleid tot dit proefschrift.

In januari 1999 trad Mark in dienst bij Promega Benelux B.V. als Field Applications Specialist voor de regio zuid- en oost-Nederland.

List of Publications

Full papers

Mark Moorthamer, Mayur Panchal, William Greenhalf and Bhabatosh Chaudhuri (1998) The $p16^{INK4A}$ protein and flavopiridol restore yeast cell growth inhibited by Cdk4. *Biochem. Biophys. Res. Commun.* **250**, 791-797.

Mark Moorthamer, Sabine Zumstein-Mecker and Bhabatosh Chaudhuri (1999) DNA binding protein dbpA complexes with Cdk5 and inhibits its activity. *FEBS Lett.* **446**, 243-350.

Mark Moorthamer and Bhabatosh Chaudhuri (1999) Identification of Ribosomal Protein L34 as a novel Cdk5 inhibitor. *Biochem. Biophys. Res. Commun.* **255**, 631-638.

Mark Moorthamer, Sabine Zumstein-Mecker, Christine Stephan, Peer Mittl and Bhabatosh Chaudhuri (1998) Identification of a Human cDNA Encoding a Kinase-Defective Cdk5 Isoform. *Biochem. Biophys. Res. Commun.* **253**, 305-310.

Abstracts

C. Dijkema, M.J.M.W. Moorthamer and A.J.M. Stams, ^{13}C, ^{23}Na and ^{31}P NMR study on the anaerobic propionate-forming bacterium *Veillonella alcalescens*. (1994) XVI th International Conference on Magnetic Resonance in Biological Systems, Veldhoven, The Netherlands, p. 68

Mark Moorthamer, Mayur Panchal, William Greenhalf and Bhabatosh Chaudhuri, The $p16^{INK4A}$ protein and flavopiridol restore yeast cell growth inhibited by Cdk4. (1998) Eighty-Ninth Annual Meeting of the American Association for Cancer Research, New Orleans, LA U.S.A., p. 253