Identification and characterization of a common protein association region in the neuronal Cdk5 activator

BY

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Identification and Characterization of a common protein association region in the neuronal Cdk5 activator

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AUG, 2000
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Xiujie Wang

Jun, 2000
Authorization

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Preface

This dissertation is written in a format consisting of four chapters. Chapter 1 describes the background of my work, the reasons for doing the research. Chapter 2 describes the methods and materials employed. Chapter 3 is devoted to the results of my thesis research, and Chapter 4 is the discussion.

Some of the studies described in Chapter 3 and 4 were submitted to Journal of Biological Chemistry for publication, with the title of “Identification of a common protein association region in the neuronal Cdk5 activator”. I’m very grateful to Prof. Jerry H. Wang’s thorough revision in these two parts.
Abstract

Cyclin-dependent protein kinase 5, Cdk5, achieves its full activity only upon the association of its neuronal specific activator, Nck5a or Nck5ai. To date, a variety of cellular proteins have been shown to undergo high affinity association with Nck5a. Three novel Nck5a-associated proteins, C42, C48 and C53 were isolated recently by using yeast two-hybrid system screening. In this MPhil thesis research, a 26-amino acid region of Nck5a was found as a common region which is capable of interacting with these three novel Nck5a-associated proteins and the binding of these Nck5a-associated proteins to Nck5a has no effect on Cdk5 kinase activity. In addition, the 26-amino acid binding region of Nck5a can also interact with Cdk5 with high binding affinity, thus forming ternary complex with Cdk5 and a Nck5a-associated protein. To understand the molecular basis of such interaction, different reaction conditions and site-directed mutagenesis were used during the investigation. Unlike the binding between Cdk5 and Nck5a which is via hydrophobic-hydrophobic interaction, the binging between Nck5a and these Nck5a associated proteins was proved to be charge-charge interaction. Glu157 of Nck5a was proved to be essential for such interactions; mutation of Glu157 to Gln totally abolished the binding of Nck5a and these Nck5a associated proteins.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>CAK</td>
<td>Cdk activating kinase</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>dopamine and cyclic AMP-regulated phospho-protein</td>
</tr>
<tr>
<td>dbpA</td>
<td>DNA binding protein A</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
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<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic Acid</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>MPF</td>
<td>maturation-promoting factor</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanr sulfonic acid</td>
</tr>
<tr>
<td>Nck5a</td>
<td>neuronal Cdk5 activator</td>
</tr>
<tr>
<td>Nck5ai</td>
<td>neuronal Cdk5 activator isoform</td>
</tr>
<tr>
<td>Ncklk</td>
<td>neuronal Cdc2-like kinase</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>RB</td>
<td>retinoblastoma protein</td>
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<tr>
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INTRODUCTION

1.1 The Cell Cycle

Cell division cycle is the fundamental means by which all living things are propagated. The cell cycle begins when two new cells are formed by the division of a single parental cell and ends when one of these cells then divides to form new cells. This is controlled by an intricate network of signal events which is regulated by mechanisms such as protein-protein interaction and protein modification events.

The cell cycle is traditionally divided into several phases, of which most dramatic is mitosis (M phase), the process of nuclear division, leading up to the moment of cell division itself. M phase comprises of several distinct stages, in which the chromosomes separate and two nuclei form in place of one. M phase ends in cytokinesis by the splitting of the cell as a whole into two (Albert et al., 1994).

In most cells the whole M phase takes only about an hour, which is only a small fraction of the total cycle time. The much longer period that elapses between one M phase and the next is known as interphase. Replication of the nuclear DNA usually occupies a portion of interphase, called the S phase of the cell cycle (S=synthesis). The interval between the completion of mitosis and the beginning of DNA synthesis is called the G\textsubscript{1} phase (G=gap), and the interval between the end of DNA synthesis and the beginning of mitosis is called the G\textsubscript{2} phase. G\textsubscript{1} and G\textsubscript{2} provide
additional time for growth: if interphase lasted only long enough for DNA replication, the cell would not have time to double its mass before it divided. During $G_1$ the cell monitors its environment and its own size and, when the time is ripe, takes a decisive step that commits it to DNA replication and completion of a division cycle. The $G_2$ phase provides a safety gap, allowing the cell to ensure that DNA replication is complete before it plunges into mitosis.

The duration of the cell cycle varies greatly from one cell type to another, from 8 minutes of the fly embryos to more than one year of the mammalian liver cells. The cycle time of typical dividing mammalian cell is about 24 hours. Most of this variation occurs in the $G_1$ phase. Cells in $G_1$, if they have not yet committed themselves to DNA replication, can pause in their progress around the cycle and enter a specialized resting state, often called $G_0$ (G zero), where they can remain for days, weeks, or even years before resuming proliferation (Baserga, 1985; Johnston, 1990).

1.2 Cyclin-dependent kinases

The cell-cycle control system is based on two key families of proteins. The first is the family of cyclin-dependent protein kinases (Cdk for short), which induce downstream processes by phosphorylating selected proteins on serines and threonines. The second is a family of specialized proteins, called cyclins, that bind to Cdk molecules and control their ability to phosphorylate appropriate target proteins. The cyclic assembly, activation, and disassembly of cyclin-Cdk complexes are the pivotal events driving the cell cycle (Pine, 1994; Morgan, 1995).
1.2.1 Cyclin dependent kinase family

It is believed that cell cycle progression is governed by a biochemical cycle in which a family of serine/threonine kinases is activated and inactivated in a precisely timed program. The members in cyclin-dependent kinase family share a high degree of sequence homology. Cdk5 have been numbered based on the chronological order of their discovery. Cdc2 is the founding member of this family. To date there are nine recognized members in this family: Cdc2 (Cdk1), Cdk2 to Cdk9. Eight of them (namely Cdk1-8) were isolated from human cells (Lew and Wang, 1995; Morgan, 1995; Tassan et al., 1995; Fisher, 1994). Recently, Cdk9 has been cloned from a Drosophila cDNA library (Wei et al., 1998).

The percentage of sequence identity of these homologues to Cdc2 (i.e. Cdk1) in the kinase domain ranges from 36-65% (Hutchison and Glover, 1995). Besides, a consensus amino acid sequence PSTAIRE close to the amino terminal of the kinase domain of Cdc2 is highly conserved among these Cdns. In spite of their sequence homology, different Cdns participate in different stages of cell cycle progression by associating with specific cyclins (Meyerson et al., 1992; Tassan et al. 1995).

The cdc2 gene product in fission yeast, whose functional counterpart in budding yeast is cdc28, is a protein serine/threonine kinase. In animal cell, while the regulation by Cdc2 of entry into and exit from mitosis is conserved, the distinct Cdc2 homologues, Cdk2, Cdk4 and Cdk6, are primarily involved in control of G1 progression and subsequent DNA synthesis (Fang and Newport, 1991; Koff et al., 1992; Rosenblatt et al., 1992; Tsai et al., 1993).
The monomeric Cdk5 do not display any kinase activity. Association with the primary regulatory subunit, which is cyclin, is essential for their catalytic activities (Solomon et al., 1993). Cdk-cyclin are subjected to an integrated regulatory mechanism of phosphorylation and dephosphorylation, as well as association with additional regulatory subunits.

1.2.2 Cyclins

Cyclins were initially discovered in clam embryos as proteins periodically synthesized during early embryonic developments (Evans et al., 1983). Cyclins are so called because they undergo a cycle of synthesis and degradation in each cell division cycle.

Studies using *Xenopus* oocytes provided evidence for biochemical mechanisms that control cell division (Murray and Kirchner, 1989). Microinjection of cytoplasms from mature oocytes into immature oocytes, which are naturally arrested in meiotic prophase, promotes their full maturation without requiring further protein synthesis (Masui and Markert, 1971; Reynhout and Smith, 1974). The material in the egg cytoplasm that promotes cell maturation in this way is called maturation-promoting factor (MPF) because it induces the maturation of an immature oocyte into a mature egg.

Cyclins were identified as one of the important components of MPF. In surf clams, cyclin levels were seen to peak at each M phase, with two types, A and B,
being distinguished by their gel mobilities and the slightly earlier appearance and
disappearance of the A type. Moreover, the \textit{cdc13} gene product of fission yeast was
identified as a homologue of the 45 kDa subunit of \textit{Xenopus} MPF, which is now
known as cyclin B (Booher and Beach, 1988; Gautier \textit{et al.}, 1990).

Cyclins compose a rapidly growing family, based on sequence homology and
functional complementation. In mammalian cells, there are now cyclins A-I identified
(Hutchison and Glover, 1995; Nakamura \textit{et al.}, 1997). Recently, there are two more
new members of cyclins found in human cells. Cyclin K was isolated from human
HepG2 hepatoblastoma cDNA library. Cyclin K is associated with the large subunit of
RNAPII as well as a potent RNAPII-CTD kinase (Edwards \textit{et al.}, 1998). Another new
member is cyclin T, originally identified in \textit{Drosophila} as the regulatory subunit of
DmP-TEFb. The human cyclin T (87 kDa), the counterpart of \textit{Drosophila} cyclin T
(124 kDa), activates human Cdk9 (previously called PITALRE) in the TAK complex
(Peng \textit{et al.}, 1998; Wei \textit{et al.}, 1998).

Cyclin function is primarily controlled by its protein level, which is regulated
by protein synthesis and turnover. The cyclin levels peak at particular stages of the
cell cycle corresponding to their functions. Indeed, they are categorized by the stages
in which they function, such as mitotic cyclins, which control the progression from G2
to M phase, and G1 cyclins, which control the progression from G1 to S transition
(Sherr, 1995). Cdc2/cyclin B is the well characterized complex that controls crossing
of the G2/M boundary. The quantity of cyclin B gradually accumulates during
interphase until its maximal level is reached in the late G2 phase. At that time, the
Cdc2/cyclin B complex is ready for activation. After induction of M phase and
cytokinesis, cyclin B is rapidly proteolyzed via a ubiquitination pathway; cyclin degradation is essential for cell exit from mitosis (Glotzer et al., 1991; Murray, 1993).

1.3 Cdk Regulation

Cell cycle is such an accurate process, therefore it must be regulated precisely at every step. The regulation of Cdk's is essential for the activity status of various Cdns during cell division which is critical in controlling the progression of different cell cycle phases.

1.3.1 Cdk regulation by cyclin binding

The sequence similarity in the cyclin family is restricted to an internal region of approximately 100 amino acid residues, which is termed the cyclin box (Hunt, 1991). It is thought that the cyclin box is the region responsible for Cdk binding and activation (Kobayashi et al., 1992; Lee and Harlow, 1993). Deletion or mutation in this region abolishes association with Cdns and their Cdk-activating activity. In addition to supporting Cdk activity, cyclin contributes to the substrate selection of the Cdk-cyclin complex. The interaction between cyclin and substrate facilitates phosphorylation of particular substrates (Dynlacht et al., 1994; Hoffmann et al., 1993; Peeper et al., 1993). The high degree homology among primary sequences in Cdk members is not observed among the members of cyclin family. The activation of Cdns require the association with their specific cyclin partners. For example, Cdc2 (Cdk1) binds specially to A- or B- type cyclins, Cdk2 specially binds to A- or E-type cyclins, Cdk4/6 bind to D-type cyclin, and Cdk8/9 bind C-type cyclin (Tassan, et al., 1995;
Rickert, et al., 1996; Wei, et al., 1998) to become active. Furthermore, the binding of Cdk to cyclin is in a one to one ratio (see reviews in Nurse, 1994; Pine, 1994; Morgan, 1995).

The crystal structures of monomeric Cdk2 and the Cdk2 complexed with an fragment of cyclin A provides an explanation of the mechanism of activation of most Cdk-cyclin complexes (Jeffrey et al., 1995; Russo et al., 1996). Cdk2 has a bilobal structure resembling that of the catalytic subunit of protein kinase A (PKA). However, there are variations between the structure of free Cdk2 and PKA. First, the core of the protein substrate binding cleft between the two lobes in Cdk2 is blocked by a large loop, which includes residues 152-170 and is called the T-loop. Secondly, the residues in the catalytic core required to coordinate ATP orientation are positioned quite differently in Cdk2 from those equivalent residues in PKA. With the misalignment of the catalytic residues, it is impossible for Cdk2 to catalyze the phosphate-transfer reaction. The binding of cyclin A induces dramatic changes of Cdk2 structure including the ATP-binding site, which restores the active conformation of Cdk2. Since sequences in the catalytic domain of Cdk members are well conserved and display extensive similarity to other protein serine/threonine kinases. The structure features of the Cdk2/cyclin A complex are most likely shared by other Cdk-cyclin complexes.

1.3.2 Cdk regulation by phosphorylation / dephosphorylation

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

Cyclin accumulates in cells in interphase and binds Cdc2. The cyclin binding induces immediate phosphorylation of Thr\textsuperscript{14}, Tyr\textsuperscript{15} and Thr\textsuperscript{161}, among which Thr\textsuperscript{14} and Tyr\textsuperscript{15} phosphorylation prevents premature activation of Cdc2/cyclin. When cells approach the G\textsubscript{2}/M transition, both Thr\textsuperscript{14} and Tyr\textsuperscript{15} are rapidly dephosphorylated to produce an active Cdc2/cyclin complex. After completion of cell division, cyclin is degradated and then phospho-Thr\textsuperscript{161} is dephosphorylated, and the cells exit from mitosis (Gu et al., 1992).

a. Activation by phosphorylation

In addition to cyclin association, Cdc2 activation shows absolute dependence on phosphorylation of Thr\textsuperscript{161} (Desai et al., 1992; Solomon et al., 1990). The binding of cyclin A to Cdk2 establishes a basal activity of Cdk2; full activity requires phosphorylation of Thr\textsuperscript{160}, which is the residue corresponding to Thr\textsuperscript{161} of Cdc2 (Connell-Crowley et al., 1993). In the Cdk2 structure, Thr\textsuperscript{160} is at the apex of the T-loop (De Bondt et al., 1993). Phosphorylation of Thr\textsuperscript{160} introduces ionic interactions between Phospho-Thr\textsuperscript{160} and a cationic pocket formed by the basic residues Arg\textsuperscript{50}, Arg\textsuperscript{126} and Arg\textsuperscript{150} (Jeffrey et al., 1995). These interactions are suggested to stabilized the active conformation of the T-loop introduced by association with cyclin A. Thus,
the requirement of both cyclin association and Thr\textsuperscript{160}-phosphorylation for the kinase activity is due to the unique features of the Cdk structure (Fig. 1-1).

Thr\textsuperscript{161} in Cdc2 and Thr\textsuperscript{160} in Cdk2 are phosphorylated by Cdk activating kinase (CAK) (Nortury, 1991). Initially identified from Xenopus egg extracts by virtue of its ability to phosphorylate and activate Cdc2 (Solomon et al., 1992), CAK is a multimeric enzyme complex composed of a distantly related Cdk-cyclin pair, Cdk7 (MO15) and cyclin H. In addition, CAK also contains a 36 kDa protein which is a new member of the RING finger family and named MAT1 (Fisher et al., 1995; Tassan et al., 1995; Devault et al., 1995).

b. Inhibition by phosphorylation

In Cdk2, Thr\textsuperscript{14} and Tyr\textsuperscript{15} are both in the middle of the glycine-rich loop that serves as a phosphate anchor in ATP binding. The hydroxyl group of Thr\textsuperscript{14} is very close to the γ-phosphate anchor in ATP and phosphorylation here may directly disrupt the conformation of the ATP phosphates. Phosphorylation of Tyr\textsuperscript{15} has been suggested to disrupt the adjacent ATP recognition site rather than prevent ATP binding (Marcote et al., 1993). The phosphorylation of these two sites are cyclin dependent and the T-loop must be removed to allow phosphorylation and it is accomplished by cyclin binding (Parker et al., 1991; De Bondt et al., 1993; Jeffrey et al., 1995).

Two kinases are for the phosphorylation at Thr\textsuperscript{14} and Tyr\textsuperscript{15}, Weel kinase and Myt1 kinase. Weel is a dose-dependent inhibitor for mitosis through its direct
Fig. 1-1 The activity of M phase kinase is regulated by phosphorylation, dephosphorylation and proteolysis.
phosphorylation of Cdc2. Wee1 can only phosphorylate Cdc2 at Tyr15 (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). As a member of the Wee1 family, Myt1 kinase was cloned from Xenopus by polymerase chain reaction strategy. Unlike Wee1, Myt1 is a dual-specificity kinase which can phosphorylate Cdc2 efficiently at both Thr\textsuperscript{14} and Tyr\textsuperscript{15} (Muelle et al., 1995).

c. Regulation by dephosphorylation

Cdc25 gene was first cloned by a complementation method in fission yeast (Russell and Nurse, 1986). Like Wee1, cdc25 acts in a dose-dependent manner. Studies have shown that Cdc25 is a dual-specificity phosphatase that directly dephosphorylates Thr\textsuperscript{14} and Tyr\textsuperscript{15} and activates Cdc2 kinase (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991). Dephosphorylation of Cdc2 in vivo and in vitro is a sequential process in which Thr\textsuperscript{14} dephosphorylation precedes Thr\textsuperscript{15} dephosphorylation (Borgne et al., 1996).

1.3.3 Cdk inhibitors

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

There are three categories of Cdk inhibitors demonstrated in mammalian cells, p21, p27\textsuperscript{kip1} and its homologue p57\textsuperscript{kip2}, and the INK4 family inhibitors. These inhibitors function in G1/S progression instead of G2/M transition, and provide important control mechanism for initiation of DNA synthesis (Fig. 1-2).

\textbf{a. p21}

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

The regulation of p21 occurs primarily at the transcriptional level (Firpo \textit{et al.}, 1994; Li \textit{et al.}, 1994; Nurse \textit{et al.}, 1994), and p21 is transcriptionally activated by the tumor suppressor gene product p53 (Li \textit{et al.}, 1994). Over-expression of p21 prevents growing cells from entering S phase and DNA synthesis in diploid fibroblasts and mouse NIH 3T3 cells, indicating an inhibitory role of p21 in cell cycle progression. This inhibition can be reversed by co-expression of SV40 T-antigen, which bind
Fig. 1-2 Putative roles for the cyclin-dependent kinase inhibitors p21^{G_{1p}}, p27^{K_{ip}}, and p16^{Ink4A} in the mammalian cell cycle. The restriction point $G_{1}$ phase is shown as \textit{R-point}. TGF, transforming growth factor.
the tumour suppressing gene products pRb and p53 and blocks their negative effects on cell cycle progression (Harper et al., 1993).

In normal cells, Cdk5 exist predominantly in quaternary complexes containing Cdk, cyclin, proliferating cell nuclear antigen (PCNA) and p21 (Xiong et al., 1993; Zhang et al., 1993). It has been shown by immunoprecipitation that p21 exists in multiple Cdk/cyclin complexes from human diploid fibroblasts involving Cdc2, Cdk2, Cdk4, Cdk5, cyclins A, B, D and E. p21 displays potent inhibitory activity towards Cdk2/cyclin A and E, and Cdk4/cyclin D, which are essential for G1 and S progression (Harper et al., 1993). However, cyclin B-containing complexes are affected much less efficiently by p21. p21 seems to be specified to control the progression through G1 and S by inhibition of the G1/S-related Cdk/cyclin complexes.

Cdk inhibition by p21 does not require any additional cellular proteins. The inhibitory mechanism does not involve disruption of Cdk/cyclin complexes, dephosphorylation of Cdk2 at phospho-Thr\(^{160}\) or the equivalent residues in other Cdks, or phosphorylation of Cdk2 at the negative regulatory sites (Harper et al., 1993; Xiong et al., 1993). It has been reported that most Cdk/cyclin complexes from proliferating cells contain p21 and still display kinase activities (Zhang et al., 1994). Addition of excess p21 to the active Cdk complexes containing endogenous p21 effectively inactivates the kinase, indicating that binding of multiple p21 to the Cdk/cyclin complexes is required to turn off the kinase activities (Harper et al., 1995; Zhang et al., 1994). Moreover, it has been shown that the p21 binding to Cdk/cyclin prevents phosphorylation of Cdk by CAK, providing a second mode of Cdk inhibition by p21.
In addition to binding to Cdk/cyclin, p21 binds directly to PCNA in the complex of Cdk-cyclin-p21-PCNA. PCNA is a cofactor of DNA polymerase δ (pol δ), which enhances processivity of pol δ. PCNA is also involved in nucleotide excision repair of DNA. Association of p21 with PCNA blocked SV40 DNA replication with pol δ in vitro, but not the excision repair function of PCNA (Flores-Rozas et al., 1994; Li et al., 1994; Shivji et al., 1994; Waga et al., 1994).

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

In studies of cell contact inhibition and cell cycle arrest in G1 by treatment with transforming growth factor β (TGFβ), Polyak et al., (1994) isolated and cloned a Cdk inhibitor from the inactive Cdk2/cyclin E complex, which is a 27 kDa thermostable protein, and designated it Kip1 for Cdk inhibitory protein 1. Independently, Toyoshima and Hunter (1994) identified $p27^{Kip1}$ in a yeast two-hybrid screen for cdk4/cyclin D-binding proteins.

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

p57Kip2 contains an N-terminal Cdk inhibitory domain homologous to those in Kip1 and p21, and a C-terminal region homologous to Kip1 (Lee et al., 1995; Matsuoka et al., 1995). Kip2 binds the cyclin complexes of Cdk2, Cdk3, Cdk4 and Cdk6, and blocks their activities. The chromosomal location of Kip2 was mapped to 11p15.5 on the human chromosome, which is a region with frequent abnormality in a number of human cancers, promoting the proposal of Kip2 as a tumour suppressor gene (Matsuoka et al., 1995).

c. INK4 family inhibitors

INK4 is a family of Cdk4- and Cdk6-specific inhibitors. The first described member in this family, p16INK4A, was originally uncovered as an inhibitor of Cdk4 (Serrano et al., 1993). Besides p16INK4A, there are three other polypeptides in INK4 family: p15INK4B, p18INK4C and p19INK4D (Guan et al., 1996; Hirai et al., 1995; Serrano et al., 1993). They are structurally related to one another, each containing four ankyrin-like repeats that are important for interaction with Cdks (Hirai et al., 1995; Pavletich, 1999).

They form complexes with Cdk4 or Cdk6 by displacing cyclin D, but have no effect on other Cdks. It is thought that association of the INK4 inhibitors with Cdk4 or Cdk6 in vivo interferes with cyclin binding and the subsequent activation of Cdk,
resulting in G₁ arrest. The association of the INK4 inhibitors with Cdk4 or Cdk6 will arrest cells in G₁ phase (Guan et al., 1994; Lukas et al., 1995; Serrano et al., 1995).

The expression of p16\textsuperscript{INK4A} is significantly elevated in tumor cells lacking functional pRb, such as SV40 T-antigen transformed cells, suggesting that pRb suppresses the expression of p16\textsuperscript{INK4A} (Okamoto et al., 1995; Parry et al., 1995; Tam et al., 1994). The expression of p15\textsuperscript{INK4B}, but not p16\textsuperscript{INK4A}, is greatly induced (30-fold) in human keratinocytes (HaCaT) cells treated with TGFβ, but this treatment does not affect Cdk4 expression, suggesting that p15\textsuperscript{INK4B} is an effector of TGFβ (Hannon and Beach, 1994a).

1.4 Neuronal Cdc2-like kinase

While Cdk's are predominantly involved in the control of cell proliferation, Northern blot analysis revealed the existence of Cdc2 homologues in specifically differentiated cells and tissues (Meyerson et al., 1992), suggesting that the physiological role of the Cdc2 family members may not be restricted in the cell cycle control. A Cdk2-like kinase, called neuronal Cdc2-like kinase (Nclk), has been identified in mammalian brain. The purified Nclk is a heterodimer of cyclin-dependent kinase 5 (Cdk5) and a 25 kDa regulatory subunit that is essential for kinase activity (Lew et al., 1995). Evidence has been accumulating to suggest that Cdk5 has important neuronal functions. The Cdk5 protein is enriched in neurons of adult brain (Hellmich et al., 1992) whereas Cdc2 and Cdk2 are essentially absent. Although Cdk5 protein appears to be ubiquitously distributed in mammalian tissues and cells (Tsai et
al., 1993), up to now, Cdk5 kinase activity has been demonstrated only in mammalian brains (Ishiguro et al., 1988; Ishiguro et al., 1992; Lew et al., 1992).

1.4.1 General Characterization of Nclk

a. Identification of Cdk5

Active Cdk5 was identified and cloned independently by four different laboratories. Using a synthetic peptide containing the consensus phosphorylation sequence motif of Cdc2 kinase (-S/T-P-X-K/R-) as a substrate, Lew et al. first purified Nclk from bovine brain extract (Lew et al., 1992). Three other groups also identified and cloned this protein independently (Meyerson et al., 1992; Hellmich et al., 1992; Xiong et al., 1992). Nclk appears to represent the predominant proline-directed kinase activity in bovine brain extracts. It catalyzes in vitro phosphorylation of NF-M, NF-H and tau at the sites identical to those phosphorylated by Cdc2 (Lew et al., 1992).

Alignment of amino acid sequences of Cdk5 with Cdc2 and Cdk2 has shown high degrees of sequence identity, with 58% to Cdc2 and 59% to Cdk2. Cdk5 homologue in human, rat and mouse were also cloned by complementary homologous library screening (Meyerson et al., 1992; Hellmich et al., 1992; Tsai et al., 1993). The highly conserved domains in Cdc2 and Cdk2 are also conserved in Cdk5. In addition, Cdk5 was identified as a Cdk that associates with cyclin D1 in human fibroblast cells, but no kinase activity could be demonstrated with the protein complex of the Cdk5 and cyclin D1 (Xiong et al., 1992). Similarly, although Cdk5 has been shown to
associate with cyclin E, kinase activity of such complexes has not been demonstrated (Miyajima et al., 1995).

b. Multiple Cdk5 activators

First purified from bovine brain, the active Cdk5 is a heterodimer of a Cdk5 catalytic subunit (33 kDa) and a 25 kDa regulatory subunit (p25\textsuperscript{Nck5a}) (Ishiguro et al., 1994; Lew et al., 1994). Further studies have shown that p25\textsuperscript{Nck5a} is a proteolytic derivative of a 35 kDa brain- and neuron-specific protein (p35\textsuperscript{Nck5a}) with a 98-amino acid truncation at the N-terminus (Lew et al., 1994; Tsai et al., 1994). Both p25\textsuperscript{Nck5a} and p35\textsuperscript{Nck5a} display high specific enzyme activity towards a synthetic peptide substrate modeled after the p34\textsuperscript{cyc2} phosphorylation site in histone H1 (Lew et al., 1992). p35\textsuperscript{Nck5a} is the predominant form of Nck5a in crude bovine extract but it is associated with Cdk5 in a macromolecular protein complex that has no observable kinase activity (Lee et al., 1996).

Despite of its cyclin-like activity, p35\textsuperscript{Nck5a} has no sequence homology to cyclins. Only a short stretch of 17 amino acids (residues 222-238) of p35\textsuperscript{Nck5a} displays marginal similarity to the equivalent region of cyclin box consensus sequence. The overall identity of p35\textsuperscript{Nck5a} to the cyclin box is only 8% (Lew et al., 1994; Tsai et al., 1994). The active domain of p35\textsuperscript{Nck5a} has been determined to residues 150 to 291 by using the truncation mutants, where Leu151 and Leu152 are the two residues that are essential for Cdk5 binding and activation (Tang et al., 1997). Recent experiment results obtained by site-directed mutagenesis studies and X-ray 3-dimentional
structure modeling have shown that the activation domain of Nck5a adopts a conformation of cyclin-fold structure (Brown et al., 1995; Chou et al., 1999).

A 39 kDa protein showing 57% sequence identity to p35\textsuperscript{nck5a} was cloned and named neuronal Cdk5 activator isoform (Nck5ai) by screening of a human hippocampus library with bovine p35 cDNA as a probe (Tang et al., 1995). p39\textsuperscript{nck5ai} is capable of activating monomeric Cdk5 to a similar extent as p35\textsuperscript{nck5a} does. Studies have shown that p39\textsuperscript{nck5ai} plays a role in neurite outgrowth in cells (Xiong et al., 1997; Paglini et al., 1998).

In addition to Nck5a or Nck5ai, another Cdk5 activator, p67 was co-purified with Cdk5 from rat spinal cord lysate by column chromatography (Shetty et al., 1995). p67 was originally identified to be Munc-18, the mammalian homologue of the C.elegans unc-18 gene (Hosono et al., 1992; Hata et al., 1993). Recombinant p67 can stimulate Cdk5 kinase activity \textit{in vitro} in a dose-dependent manner and it can co-precipitate Cdk5 from rat brain lysate (Shetty et al., 1995).

1.4.2 Tissue distribution and function of Nck5

Cdk5 is ubiquitously expressed in mammalian tissues and cultured cells, with the brain containing highest amount of the transcript (Tsai et al., 1993; Lew et al., 1994; Tsai et al., 1994). In contrast, the expression of Nck5a is detected exclusively in neurons and developing muscle tissues (Lew et al., 1994; Tsai et al., 1994). The restricted p35\textsuperscript{nck5a} expression explains why Cdk5-associated histone H1 kinase activity was only demonstrated in mammalian brain extracts during the original study
(Lew and Wang, 1995). Recent research results have shown that Cdk5 kinase activity can be detected in lens epithelial cells and differentiated lens fibers throughout the development of lens. The expression of Cdk5 and p35\textsuperscript{nck5a} mRNAs were also detected (Gao et al., 1997).

In developing mice forebrains, Cdk5 expression and its kinase activity correlate with terminal differentiation of neurons of the embryonic brain (Tsai et al., 1993). During the maturation of the rat cerebellum, the subcellular location of Cdk5 changes dramatically from the cell body to the axon with the originally predominant expression in neuronal cell bodies (Matsushita et al., 1995). The presence of Cdk5 was also found in nuclei and axons of mouse brain neurons (Tatibana et al., 1996). Like Cdk5, p35\textsuperscript{nck5a} also localizes to the soma and along the entire length of neurites in developing neurons (Nikolic et al., 1996). There is a change in p35\textsuperscript{nck5a} expression throughout the brain development. During early states of brain development, p35\textsuperscript{nck5a} localizes in axonal pathway such as the corpus callosum, whereas in the adult brain, p35\textsuperscript{nck5a} was mainly found in cell body and dendrites, with only very low amounts in axons (Tomizawa et al., 1996). By using Immunocytochemistry and subcellular fractionation, p35\textsuperscript{nck5a} was proved to be a membrane associated protein (Nikolic et al., 1998).

Cdk5 mainly functions in the regulation of neuro-cytoskeleton dynamics. Proteins involved in all three classes of cytoskeleton systems have been found to be phosphorylated \textit{in vitro} by Nclk, including microtubules, intermediate filaments and actin filaments (Lew et al., 1992b). Cdk5(-/-) mice exhibits perinatal mortality associated with abnormal corticogenesis and cerebellar defoliation. The brains of
Cdk5(-/-) mice lack cortical laminar structure and cerebellar foliation (Ohshima et al., 1996). Mice lacking p35nck5a displays defects in cortical lamination and fasciculation of axon fibers. The layering of cortical neurons is inverted in p35/- cortex (Kown et al., 1999; Kown and Tsai, 1998; Chae et al., 1997).

Deregulation of Nclk was implicated in neurodegenerative diseases like Alzheimer and Parkinson disease. Tau protein has been shown to be a good substrate, both in vitro an in vivo. Conversion of p35nck5a to p25nck5a forms p25/Cdk5 complex which hyperphosphorylates tau, which is probably involved in the pathogenesis of AD (Pant and Veeranna, 1995; Patrick et al., 1999; Ahlijianian et al., 2000). Increased expression of Cdk5 was found in apoptotic cells and may be related to the loss of dopaminergic neurons in Parkinson’s disease (Henchcliffe and Burke, 1997).

Recent research results have shown that p35/Cdk5 kinase is involved in signal transduction pathways mediated by a small GTPase Rac, as p35/Cdk5 interacts with Rac when it is in the active GTP-bound configuration (Nikolic et al., 1998). Cdk5 can also phosphorylate DARPP-32 (dopamine and cyclic AMP-regulated phospho-protein), converts it to an inhibitor of protein kinase A and alters the responses of striatal neurons to dopamine (Bibb et al., 1999).

Though the tissue-specific expression of p39nck5ai is similar to that of p35nck5a which is specific to post-mitotic neurons of the nervous system (Cai et al., 1997; Zheng et al., 1998), p39nck5ai temporal and spatial expression patterns are different from and complementary to that of p35nck5a. In particular, p39nck5ai is expressed later
in the CNS during embryonic development. The p39/Cdk5 complex may play a role in regulating actin cytoskeletal dynamics in cells (Humbert et al., 2000).

1.4.3 Regulation of cyclin dependent kinase 5

Like other Cdns whose activation depend on their association with their specific cyclin partners (Morgan, 1995), Cdk5 has to associate with one of its activators (p35\textsuperscript{nck5a}, p25\textsuperscript{nck5a} or p39\textsuperscript{nck5a}) to achieve its activity (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995). But Cdk5 is unique among Cdk family in that Cdk5 activation does not require the phosphorylation by CAK (Qi et al., 1995). Although the inhibitory phosphorylation sites of p34\textsuperscript{cdc2}, Thr-14 and Tyr-15, are also conserved in Cdk5, Nclk is only very slightly inhibited by, or refractory to Wee1 kinase (Tang et al., 1996; Poon et al., 1997). Cdk inhibitors, p21 and p27 also have no effect on Cdk5 kinase activity (Harper et al., 1995; Lee et al., 1996b).

A C-terminal 172 amino acid domain of the DNA binding protein, dbpA, binds to Cdk5 as well as Cdk4 and also inhibits their kinase activity (Moorthamer et al., 1999). The 60S ribosomal protein, L34, was identified as a Cdk5-interacting protein and can also inhibit Cdk5 kinase activity (Moorthamer and Chaudhuri, 1999). Overexpression of cyclin D2 has been shown to inhibit cellular Cdk5-p35\textsuperscript{nck5a} activity, suggesting that cyclin D2 is somehow a negative regulator of Cdk5-p35\textsuperscript{nck5a} (Guidato et al., 1998). A 29-amino acid peptide containing residues Gin\textsuperscript{145}-Asp\textsuperscript{173} of Nck5a was found to act as a non-competitive inhibitor of Cdk5:Nck5a complex which can bind to Cdk5 and inhibit its kinase activity without disrupting the association between Cdk5 and Nck5a (Chin et al., 1999).
A new Cdk5 interacting protein called CABLES has been reported recently. CABLES appears to promote association between Cdk5 and Src family tyrosine kinases, such as abl. In contrast to other mammalian Cdk5, Cdk5 is activated when phosphorylated by Src tyrosine kinase at Tyr15 (Hinds et al., 1999). On the other hand, the activator of Cdk5, p35^{nck5a}, is degraded by the ubiquitin-mediated proteolysis pathway (Patrick et al., 1998).

### 1.4.4 Cdk5-p35^{nck5a} associated proteins

Three forms of Cdk5 were found in bovine brain by gel-filtration chromatography: the monomeric Cdk5, a heterodimer of Cdk5 and p25^{nck5a}, and a 670 kDa macromolecular protein complex containing Cdk5 and p35^{nck5a} complex. The three forms of Cdk5 have different kinase activities with Cdk5 monomer showing no endogenous kinase activity but can be activated by the addition of bacterial expressed Nck5a. While the heterodimer of Cdk5 and p25^{nck5a} displays high kinase activity, the macromolecular protein complex containing Cdk5 and p35^{nck5a} has, surprisingly, no kinase activity, nor can it be activated by the addition of the activator (Lee et al., 1996).

The fact that Cdk5-p25^{nck5a} exists as a heterodimer whereas Cdk5-p35^{nck5a} is part of a macromolecular protein complex suggests that Nck5a may show high affinity binding to specific cellular proteins. Over the last few years, several laboratories have reported the identification of specific Nck5a-binding proteins, including neurofilament proteins, retinoblastoma protein (RB), a brain specific
microtubule-associated protein (MAP), and the versatile adhesion signaling molecule (Qi et al., 1998; Lee et al., 1997; Kwon et al., 2000). We have used the yeast two-hybrid system to screen for Nck5a-binding proteins, resulting in the identification in a human brain cDNA library of 7 Nck5a-binding proteins, including three novel proteins (Ching et al., 2000). Full-length clones of these novel Nck5a-binding proteins, called C42, C48 and C53, have been subsequently cloned from a rat brain cDNA library (Fig 1-3). The molecular weight if the protein products of the full-length C42, C48 and C53 are 66, 24 and 57 respectively. The mRNA of all three p35 binding proteins were detected in a wide range of tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. Homology search shows that the C42 protein is conserved through out the evolution, while the C48 protein is related to restin (Ching et al., 2000).

In this Mphil thesis research, I found that the three novel Nck5a-binding proteins, C48, C42 and C53, bind to Nck5a in a competitive manner suggesting that they share a common binding site or have overlapping binding sites. Using C48 as the model binding protein, a region of Nck5a spanning 26 amino acid residues has been found to be the minimal size required for C48 binding. The region of Nck5a is localized proximal to the N-terminal boundary of the kinase domain. It was shown previously to form an amphipathic α-helix that is involved in the interaction between Nck5a and Cdk5. While this helix of Nck5a interacts with Cdk5 using its hydrophobic side (Tang et al., 1997 and Chou et al., 1999), it depends on the charged side chains of the hydrophilic side for C48 binding.

25
Glial fibrillary acidic protein (GFAP)

N(α₂)-chimaerin
Clusterin
C42
C48
C53
Cdk5
p35

<table>
<thead>
<tr>
<th>Clone</th>
<th>cDNA insert size (kb)</th>
<th>Protein molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C42</td>
<td>0.7</td>
<td>66</td>
</tr>
<tr>
<td>C48</td>
<td>1.2</td>
<td>24</td>
</tr>
<tr>
<td>C53</td>
<td>1.6</td>
<td>57</td>
</tr>
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</table>

Fig. 1-3 p35nck5a-binding proteins from the yeast two-hybrid system screen.
Chapter 2

Experimental Procedures

2.1 Materials

FPLC Superose-12 column was obtained from Pharmacia Biotech Inc. [γ-32P]ATP (4500Ci/mmol) was purchased from ICN. Restriction enzymes and pfu polymerase were obtained from Stratagene. All chemicals were purchased from Sigma or Riedel-de. Oligonucleotide primers were synthesized at Life Technologies. Antibodies were obtained from Santa Crus Technology.

2.2 Protein Concentration Determination

Protein concentration was determined by directly measuring absorbance of protein sample at 280 nm, or by using Bradford method: 20 μl protein sample was mixed with 1 ml of 20% BioRad protein assay dye and the absorbance was measured at 595 nm using a spectrophotometer (Pharmais, Biohro 4060), with BSA as the standard.

2.3 Recombinant Protein Expression and Purification

2.3.1 Bacterial Strains, Plasmids and Enzymes
Fig. 2-1. Map of the pET32h Vector.
Plasmid pGEX4T-2 (Pharmacia Biotech) was used for the construction of expression plasmid for GST-fusion proteins and pET32H [modified from pET32a (Novagen) by Ms. Ming Li, see Fig. 2-1] for His-tagged proteins. *E.coli* strain XL-1 Blue was employed as the recipient in plasmid constructions. Plasmids DNA was then extracted and transformed into the *E.coli* host BL21 (DE3) (Novagen) for protein expression.

### 2.3.2 Constructions of Human \( p35^{nck5a} \) Deletion Mutants

The GST-fusion N-terminal and C-terminal deletion mutants of human \( p35^{nck5a} \) were constructed following the procedures described previously (Tang *et al.*, 1997). A set of different deletion mutants of human \( p35^{nck5a} \) was generated by polymerase chain reaction (PCR). PCR primers were designed according to different deletion with *BamHI* site and *EcoRI* site flanking the N-terminal and the C-terminal ends of primers, respectively. PCR was carried out in 50 μl of reaction mixture containing 100 ng of double-stranded DNA template, 200 μM deoxynucleoside triphosphate, 2.5 units of *Pyrococcus furiosus* DNA polymerase (*pfu* polymerase) using a DNA thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer) for 30 cycles. The PCR amplified fragments were gel purified with a Geneclean II kit (Bio101, Inc. from BCH Medical Co.). After digestion with *BamHI* and *EcoRI*, the fragments were inserted into *BamHI/EcoRI* linearized pGEX2T vector (Fig. 2-2).

\((\text{His})_6\)-tagged \( p35^{145-170} \) in pET32H was constructed by putting the *BamHI*- and *EcoRI*-digested \( p35^{145-170} \) fragment from pGEX4T-2 into *BamHI-* and *EcoRI-* digested pET32H.
Fig. 2-2. Different Deletion Mutants of Human p35<sub>ack5a</sub>.
2.3.3 Constructions of C48, C42 and C53*

Constructions of GST fused protein C48, C42 and C53 in pGEX4T were described previously (Ching et al., 2000). In brief, the full-length cDNA sequences of C48 and C42 were amplified by PCR using the library clones obtained from phage as templates and pfu polymerase as the amplification enzyme. The PCR products was cloned into pBluescript II KS (+) vector (Stratagen) for DNA sequencing. The full-length cDNA fragments were then subcloned into the pGEX4T-2 expression vector for protein expression. The full-length C53 expression vector was constructed by direct ligation of the library cDNA insert into pGEX4T-2.

C48α1 and C48α2 fragments in pGEX4T-2 were amplified by pfu polymerase using the sense primer 5'-CGGGATCCCACCACATGCTGTGCCTG-3' and antisense primer 5'-CGGAATTCAGCGGTGTTGGGAGACAG-3' for C48α1, and sense primer 5'-CGGGATCCAGGGGTGCTTTGCGACAGCG-3' and antisense primer 5'-GACAGGGGTGCTTTGCGACAGCG-3' for C48α2. For the C48 dual helix, the sense primer was 5'-CGGGATCCACCACATGCTGTGCCTG-3' and the antisense primer was 5'-CGGAATTCAGCGGTGTTGGGAGACAGCG-3'. Twenty five cycles were carried out in both of the amplification at an annealing temperature of 53°C. Purified PCR fragments with expected size were digested with BamHI and EcoRI restriction enzymes, and subcloned into pGEX4T-2 expression vector (Clontech Laboratories) for protein expression (Fig. 2-3).

* This work was kindly provided by Dr. Yick-Pang Ching and Mr. Wing-Ho Lam in our lab.
Fig. 2-3. C48 Sequence and Different C48 Deletion Constructs.

* This figure was kindly provided by Mr. Wing-Ho Lam.
C48 deletion clones were constructed by using the Exo Mung Bean Deletion Kit (Stratagene). Plasmid of full length C48 cloned in pGEX4T-2 vector was digested with *SmaI* and *XhoI* restriction enzymes. The digested plasmid containing full length C48 insert was progressively digested with exonuclease III from the C-terminal end. The opened plasmid was re-ligated and transformed into *E.Coli* strain BL-21 DE3 for protein expression (Fig. 2-2).

The (His)$_6$-tagged forms of these constructions in pET32h were constructed by putting the *BamHI*- and *EcoRI*- digested fragments in pGEX4T-2 into *BamHI*- and *EcoRI*-digested pET32h.

### 2.3.4 Protein Expression and Purification

For expression of the GST-fusion proteins, *E.coli* strain BL21(DE3) transformed with the DNA constructs were cultured to $A_{600} = 1.2$ and then stimulated with 0.2 mM of IPTG at room temperature overnight. Cells were then lysed with a French press (1100 p.s.i.) in MTPBS (150 mM NaCl, 16 mM Na$_2$HPO$_4$, 4 mM NaH$_2$PO$_4$, pH 7.5) containing 2 mM DTT, 2 µg/ml antipain, 2 µg/ml leupeptin, and 1 mM phenylmethysulfonyl fluoride. After centrifugation at $10,000 \times g$ for 30 min at 4 °C, the GST-fusion proteins were purified on glutathione-agarose.

For histidine-tagged proteins, cells were cultured to $A_{600} = 1.0$ before the addition of IPTG (0.4 mM) to induce protein expression. The cell culture was subsequently incubated for 10 hours at room temperature. The pelletted cells were
washed with a 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA. Cells were then lysed by a French press in 50 mM Tris-HCl buffer (pH 7.5), and the lysate was subjected to centrifugation at 10,000 \( \times \) g for 30 minutes. The resulting supernatant was adjusted to pH 7.9 and then incubated with 3 ml of Ni\(^{2+}\)-nitrilotriacetic acid-agarose beads (QIAGEN) for 1 hour with stirring. The resin was packed onto a column and then washed with 50 ml of binding buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole] and 30 ml of washing buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 30 mM imidazole]. Histidine-tagged proteins were then eluted with about 18 ml of elution buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 200 mM imidazole].

GST-free Cdk5 was purified by FPLC Superose-12 gel filtration chromatography column. GST-Cdk5 (5 mg) was incubated with 15 units of thrombin at 4\(^{\circ}\)C for three hours in a volume of 200 \( \mu \)l, and the sample was then loaded onto Superose-12 gel filtration chromatography column equilibrated in MTPBS. The column was washed with 40 ml of MTPBS at 0.5 ml/min, and 0.3-ml fractions were collected. The presence of GST-free Cdk5 fractions were determined by Western blot analysis with Cdk5-specific antibody (C-8) as described below.

### 2.4 In vitro Binding Assay

GST-fusion proteins (30 \( \mu \)g) were incubated with 30 \( \mu \)l of GSH-agarose in 500 \( \mu \)l of binding buffer (150 mM NaCl, 16 mM Na\(_2\)HPO\(_4\), 4 mM NaH\(_2\)PO\(_4\), pH 7.3, 1 \( \mu \)g/ml antipain, 1 \( \mu \)g/ml leupeptin, 5 mg/ml BSA). After incubation at 4\(^{\circ}\)C with end-to-end rotation for 1 h, the beads were washed three times with 1 ml of binding buffer.
(His)_6-tagged proteins (15 μg) together with 500 μl of 25 mM Tris-Cl (pH7.5) and 5 mg/ml BSA were then added to each sample. After incubation at 4°C with end-to-end rotation for 30 min, the beads were washed five times with 1 ml of 25 mM Tris-Cl (pH7.5). The samples were then dissolved in 20 μl of SDS-loading buffer, and the bound histidine-tagged proteins were then analyzed by Western blotting with p35^Nck5a-specific antibody (C-19) or Anti-His antibody (Fig. 2-3).

In the binding competition assay, increasing amounts of competitor were added together with 15 μg of (His)_6-tagged proteins in the secondary binding step, while other conditions remained the same. C48 protein was used as the example to elucidate the competition mechanism (Fig. 2-4).

2.5 SDS-PAGE, Immunoblots and Antibodies

SDS-PAGE was performed by the method of Lamml in 10 or 12% vertical slab gels. For Western blot analyses, samples were analyzed by SDS-PAGE followed by transfer to PVDF membrane (Bio-Rad Labotatories). The blots were probed with primary antibodies (1/500 dilution of a rabbit polyclonal p35^Nck5a-specific antibody (C-19), 1/1000 dilution of a rabbit polyclonal Cdk5-specific antibody (C-8) or 1/3000 dilution of a mouse monoclonal histidine-specific antibody), followed by incubation with a 1/3000 dilution of secondary antibodies (either goat anti-rabbit IgG or goat anti-mouse IgG conjugated with horseradish peroxidase). Signals were detected by ECL reagents as specified by the manufacturer (Amersham Pharmacia Biotech).

2.6 In vitro Kinase Assay
**Binding Procedure**

Glutathione agarose beads (wash many times before use) +

GST fusion p35 deletion mutants ↓

Binding for one and a half hours ↓

Wash with 1×MTPBS for 3 times ↓

Add His-tagged C48, binding for 45 minutes ↓

Wash with 25 mM Tris·Cl (pH7.5) for 5 times ↓

Run SDS PAGE and do western blotting ↓

Detected by Mono-His antibody

Positive control:
- glutathione agarose beads + GST·p25 + His-tag C48

Negative control:
- glutathione agarose beads + His-tag C48
- glutathione agarose beads + GST + His-tag C48

Reaction volume: 500 µl

Antibody: Mono-His

Fig. 2-4. The Procedure of Binding Assay.
Two kinds of kinase assay were used in this study: (1) Co-incubation: GST-p25\textsuperscript{nek5a} and GST-Cdk5 were co-incubated with GST-C48 full-length protein or GST-C48\textalpha1 fragment in the reconstitution step; (2) Pre-incubation: GST-p25\textsuperscript{nek5a} was pre-incubated with GST-C48 full-length protein or GST-C48\textalpha1 fragment for \( \frac{1}{2} \) h, after that GST-Cdk5 was added and incubated for another \( \frac{1}{2} \) h in the reconstitution step. The kinase assay was then performed as described previously (Lew et al., 1995).

2.7 Site-directed Mutagenesis

The site-directed substitution of Glu-157 with glutamic acid was performed by using a commercial kit (QuickChange\textsuperscript{TM} Site-Directed Mutagenesis Kit, Stratagene). A pair of complementary PCR primers was designed with the mutation in the middle of the primers. The sense primer was 5’-CGCTGCCTGGTCAATTTCTCTGCGCC-3’ and the antisense primer was 5’-GGCGGCAGAGAAATTGCCGCCAGGCAGCG-3’. Parental p25\textsuperscript{145-170} in pGEX4T-2 was amplified by polymerase chain reaction (PCR) using \textit{pfu} DNA polymerase with these primers for 18 cycles at an annealing temperature of 68°C. After digestion of the parental DNA with \textit{DpnI}, the amplified DNA incorporated with the nucleotide substitution was transformed into \textit{Escherichia coli} (XL 1-Blue strain). The mutation was confirmed by DNA sequencing.

2.8 DNA Sequencing
The nucleotide sequences of all the clones were determined by the ABI Prism 377 DNA Sequencer using ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The DNA was sequenced either in the pGEX4T-2 vector with sense primer (5’-CAGCAAGTATATAGCATGGC-3’) and antisense primer (5’-GGAGCTGATGTGTCAGAGG-3’) or in the pET32h vector with T3 (5’-GCAATTAACCCTCACTAAAG-3’) and T7 primer (5’-TAATACGACTCACTATAGGG-3’).

2.9 Secondary Structure Prediction

The secondary structure prediction of the α-helix regions of C48 protein was performed by using the program Consensus Secondary Structure Prediction, including methods SOPMA (Geourjon and Deleage, 1995), HNN (Guermeur, 1997), DPM (Deleage and Roux, 1987), DSC (King and Stenberg, 1996), GOR IV (Garnier et al., 1996), PHD (Rose et al., 1994), PREDATOR (Argos et al., 1996) and SIMPA96 (Levin et al., 1996).
Chapter 3

Results

3.1 Nck5a Binding Site of C48 Is In A Helical Region—

One of the novel Nck5a binding proteins, C48, was chosen as the model protein to study the molecular basis of interactions between Nck5a and its binding proteins. C48 has the lowest molecular weight among the Nck5a-binding proteins discovered in our yeast two-hybrid screen (Ching et al., 2000). The protein, upon expression in E. coli as a GST fusion protein or a (His)$_6$-tagged protein, can be recovered from the bacterial cytosol in reasonable amount, and readily purified by affinity chromatography procedures. By using the secondary structure prediction program, the Consensus Secondary Structure Prediction, three $\alpha$-helices were found in C48 protein (Fig. 3-1). The last $\alpha$-helix is not contained in the C48 protein fragment encoded by the cDNA from the original yeast two-hybrid clone, suggesting that this region of the protein is not required for Nck5a binding. To further define the region of C48 containing the Nck5a binding domain, constructs containing each of the first two $\alpha$-helix fragments, called C48$\alpha$1 and C48$\alpha$2, as well as one containing both helices (C48$\alpha$1-2) were produced and the GST fusion forms of these deletion mutant proteins were expressed in E. coli and affinity purified through a glutathione column. The purified protein samples were then tested for the ability to bind Nck5a. Samples of the GST fused forms of the three truncation mutants, the GST fused full length C48 and GST protein were separately incubated with (His)$_6$-tagged p35$^{nck5a}$ for 30 minutes and then affinity precipitated using GSH-beads. The affinity precipitates were
Fig. 3-1. Helical Regions of C48. The predicted helixes of C48 are highlighted with yellow boxes. The constructs of C48α1, C48α2 and C48α1-2 were also indicated.
analyzed by Western blotting using anti-Nck5a antibody (C-19). As shown in Fig. 3-2A, (His)$_6$-tagged p35$^{nck5a}$ was found in the samples containing GST-C48$\alpha$1, GST-C48$\alpha$1-2 as well as the GST-fused full length C48, whereas no (His)$_6$-tagged p35$^{nck5a}$ was detected in the sample containing GST-C48$\alpha$2, nor in GSH beads or GST protein sample that were used as negative controls. The result suggests that Nck5a binding site is contained in the first $\alpha$-helical region of C48. To further confirm that the binding of C48 to p35$^{nck5a}$ was in the first $\alpha$-helix of C48, dose dependent affinity binding was preformed. The amount of (His)$_6$-tagged p35$^{nck5a}$ found in the affinity precipitates was shown to increase with the increased amount of GST-C48$\alpha$1 in the binding assay, suggesting that the binding between C48$\alpha$1 and p35$^{nck5a}$ is specific (Fig. 3-2B).

3.2 The C48-binding Site of Nck5a Is Localized in A 26 Amino Acid Helical Region—

To map the C48 binding site of Nck5a, a series of N-terminal and C-terminal deletion mutants of Nck5a were constructed (Fig. 3-3), expressed as GST fusion proteins in E.coli and tested for the ability to bind C48. The various GST-fused deletion mutants of Nck5a, along with GST-p25$^{nck5a}$ (positive control) and GST (negative control) were pre-incubated separately with the (His)$_6$-tagged C48 and affinity precipitated by GSH-beads, the precipitates were then analysed by a monoclonal His-tag antibody to determine which of the deletion mutants of Nck5a could still bind C48. In this experiment, equal amounts (by mass) of the different GST-fusion proteins were used. Since the different deletion mutants have different molecular weights and the samples did not have uniform purity, the relative intensity
Fig. 3-2. Binding of C48 and its deletion mutants with p35nck5a. A. Defining of C48 α1 as the p35nck5a binding region. The presence of (His)_6-tagged p35nck5a was detected by p35nck5a-specific antibody (C-19) both in the supernatant(S) and on the glutathione-agarose precipitated protein complexes(P). In this experiment, the loading amount of supernatant is 5% of the total volume. B. Dose dependent binding between C48α1 and p35nck5a. Increasing amount of GST-fusion C48α1 (10 μg, 100 μg, 200 μg, 400 μg and 600 μg) was pre-bound to GSH-agarose beads. 15 μg (His)_6-tagged p35nck5a was subsequently added and the C48α1-bound form of (His)_6-tagged p35nck5a was analyzed by p35nck5a-specific antibody(C-19). GSH-agarose beads and GST protein were used as the negative controls.
Fig. 3-3. The Binding of Human p35\textsuperscript{nck5a} Deletion Mutants to C48. Schematic diagram showing the p35\textsuperscript{nck5a} truncation mutants used in the experiment. The C48 binding activities of the p35\textsuperscript{nck5a} mutants are also summarized in the figure.
of bands on the Western blot does not directly reflect the relative C48-binding affinities of the testing samples. For example, GST-p25\textsuperscript{Nck5a} has the highest molecular weight among testing samples and was less pure than most, it showed a staining signal weaker than most of the protein samples (Fig. 3-4). However, we still can calculate the relative C48-binding affinities of these Nck5a deletion mutants by comparing the intensity of bands on Western blot and the purity of proteins on SDS-PAGE (Fig. 3-3).

Fig. 3-4 shows that extensive deletion from either the N-terminus or the C-terminus did not abolish Nck5a of its C48 binding activity. The observation that in certain pairs of N- and C-terminal deletion mutants, such as deletion mutants Nck5a(Met1-Asp173) and Nck5a(Ser214-Glu292), both could bind C48 yet contain no overlapping sequences suggests that there are two C48-binding sites on Nck5a, designated here as a-site and b-site for the site close to the N-terminus and C-terminus of Nck5a, respectively. The smallest C48-binding fragment that contains the a-site is the deletion mutant Nck5a(Gln145-Asp170). The second site, b-site, appears to be localized in a region spanning residues Ser214 to Glu240, since both deletion mutants Nck5a(Leu187-Glu240) and Nck5a(Ser214-Glu292) showed high binding affinity for Nck5a.

Although two C48-binding sites were identified on Nck5a by using deletion mutants of the protein, it was not clear whether both sites were functional in the intact protein. To test this, we resorted to the use of truncation mutants of C48 that contain Nck5a binding site. It was reasoned that by using a binding fragment of the protein with regions not essential for binding eliminated, the possibility of non-specific
Fig. 3-4. Mapping of the C48-binding region in p35\textsuperscript{ack5a}. A. "Pull-down" assay of the binding between p35\textsuperscript{ack5a} mutants and C48. About 30 μg of GST-fusion p25\textsuperscript{ack5a} deletion mutants and 15 μg of (His)$_6$-tagged C48 protein were used to form complex. The existence of C48 in the GSH-agarose precipitated protein complexes was detected by monoclonal Anti-His antibody. GSH-agarose beads and GST protein were used as the negative controls. GST-p25\textsuperscript{ack5a} was used as positive control. B. The binding of p25\textsuperscript{145-170} and p25\textsuperscript{214-292} with His\textsuperscript{-C48α1}. About 30 μg of GST-fusion proteins and 15 μg of (His)$_6$-tagged C48α1 were used in the binding assay. The lane labeled with "Input" represents the amount of (His)$_6$-tagged C48 protein used in the experiment.
binding might be reduced. As C48α1 was the smallest Nck5a binding fragment of C48 available (Fig. 3-2), it was tested for binding to the two deletion mutants Nck5a(Gln145-Ser170) and Nck5a(Ser214-Glu292), which contain the N- and C-terminal C48 binding site respectively. Fig. 3-4 shows that only the deletion mutant containing the N-terminal binding site could bind C48α1, thus suggesting that the C-terminal binding site may not operate in the intact Nck5a.

3.3 Simultaneous Binding of Peptide Nck5a(Gln145-Ser170) to Cdk5 and C48—

Earlier studies have suggested that the region corresponding to peptide Nck5a(Gln145-Ser170) contains important sites for interaction between Nck5a and Cdk5 (Tang et al., 1997; Chin et al., 1999). The question of whether the binding of C48 will modulate the kinase activity of Cdk5/p25\textsuperscript{Nck5a} therefore arises. To address this question, two different conditions were used to test the effect of C48 on Cdk5 kinase activity. Under one of the conditions, Nck5a was pre-incubated with C48 before the addition of Cdk5 for kinase activation. The experiment was designed to test the effect of C48 on the kinase activation activity of Nck5a. The other condition involved pre-activation of Cdk5 by Nck5a and then C48 was added to test the effect on Cdk5 kinase activity. Under both conditions and over a wide range of C48 concentrations (up to 2 mg/ml), no change in Cdk5 activity was observed (Fig. 3-5). The lack of effect of C48 on either the kinase activation activity of Nck5a or the Nck5a-activated Cdk5 kinase activity suggests that Cdk5 and C48 do not compete with each other for Nck5a binding. This observation is also in agreement with earlier observation that C48 is capable of binding both free Nck5a and Nck5a in association with Cdk5 (Ching et al., 2000).
Fig. 3-5. The effect of C48 and C48α1 on Cdk5 kinase activity. In pre-incubation assay, GST-Cdk5 (0.6 mg/ml) and GST-p25nck5a (1.0 mg/ml) were incubated for ½ h, increasing amount of GST-C48 and GST-C48α1 were then added separately and reconstituted for another ½ h (C48-Pre and C48α1-Pre). In co-incubation assay, GST-Cdk5 (0.6 mg/ml) and GST-p25nck5a (1.0 mg/ml) was incubated with increasing amount of GST-C48 and GST-C48α1 for 1h (C48-Co and C48α1-Co). The samples were then assayed for Cdk5 kinase activity. Equal amount of the GST protein was used as a control (GST-Pre and GST-Co).
Additional experiments were carried out to examine whether or not the smallest size C48 binding peptide, Nck5a(Gln145-Ser170) is sufficient to show simultaneous interactions with both Cdk5 and C48. These experiments involved incubation of GST-fused C48 or C48α1 with both (His)$_6$-tagged Nck5a(Gln145-Ser170) and Cdk5, followed by affinity precipitation of GST-C48 using GSH-agarose beads. The affinity precipitates were then analysed by Western blots for the existence of Nck5a(Gln145-Ser170) and Cdk5. Since Cdk5 is capable of associating with Nck5a(Gln145-Ser170) (Fig. 3-6A) but not directly with C48 (Fig. 3-6B) (In this experiment, trace amount of Cdk5 was pulled down by C48 and C48α1 due to the protein quality, whereas no Cdk5 was detected in our previous studies), the high amount of Cdk5 together with Nck5a(Gln145-Ser170) in the C48 affinity precipitates further confirms the suggestion that Nck5a can simultaneously associate with both Cdk5 and C48 to form ternary complex. Furthermore, the result supports the view that the 26-amino acid region of Nck5a is used in both free and Cdk5-bound Nck5a to bind C48.

3.4 C48 and Cdk5 Use Different Mechanisms to Bind Nck5a—

The observations that the minimal C48-binding peptide of Nck5a, Nck5a(Gln145-Ser170), can bind both Cdk5 and C48 to form a ternary complex and that C48 protein does not affect the Nck5a-activated Cdk5 kinase activity suggest that C48 and Cdk5 react with different residues in this small peptide protein. Structural analysis of the peptide Nck5a(Gln145-Ser170) has shown that this peptide contains an amphipathic α-helix (Chin et al., 1999). It is conceivable that the two sides of the
Fig. 3-6. The formation of the Cdk5, p25 and C48 ternary complex. Panel A shows that Cdk5 binds to both p25\textsubscript{nck5a} and p25\textsubscript{145-170} from the “pull-down” assay. The presence of His-p25\textsubscript{nck5a} and His-p25\textsubscript{145-170} in the GSH-agarose precipitated protein complexes were detected by monoclonal Anti-His antibody. Panel B shows that p25\textsubscript{nck5a} and Cdk5 form ternary complexes with C48 or C48\textsubscript{a1}. In this experiment, both untagged Cdk5 and (His)\textsubscript{6}-tagged p25\textsubscript{145-170} can be precipitated by GSH-agarose bound form of GSH-C48 (or GST-C48\textsubscript{a1}), whereas very little direct interactions between C48(C48\textsubscript{a1}) and Cdk5 were detected (last two lanes).
Fig. 3-7  Helical Wheel of C48α1 From 57 to 74. Red lines were drawn between two consecutive amino acids that both with hydrophilic side chains or from amino acid with hydrophilic side chains to amino acid with hydrophobic side chains. In contrast, black lines were drawn between two consecutive amino acids that both with hydrophobic side chains or from amino acid with hydrophobic side chains to amino acid with hydrophilic side chains. It is the same in Fig. 3-13 and Fig. 3-14.
amphipathic α-helix may be used to bind the two different proteins. On the basis of protein mutation analysis and the computer modeling of Nck5a-Cdk5 complex, it has been suggested that the association between this amphipathic α-helix and Cdk5 involves mainly hydrophobic interactions (Tang et al., 1997; Chou et al., 1999). Secondary structure prediction of C48α1 peptide also shows an amphipathic helix structure (Fig. 3-7). Thus, it is possible that the interaction between the two α-helices of Nck5a and C48 involves the hydrophilic faces of the two amphipathic α-helices.

Two types of experiments were carried out to test the hypothesis that the peptide Nck5a (Gln145-Ser170) uses different faces of its amphipathic α-helix to associate with Cdk5 and C48. One of these examines the effect of salt and non-ionic detergent on the interaction of the peptide with the two proteins. As shown in Fig. 3-8A, the association between C48α1 and Nck5a(Gln145-Ser170) decreased with an increase in NaCl concentration in the binding reaction buffer, and the binding of C48α1 to the peptide disappeared essentially completely when salt concentration reached 1 M. On the other hand, 1% Triton X-100 had no effect on the interaction between the peptide Nck5a(Gln145-Ser170) and C48α1. In striking contrast to the C48-Nck5a interaction, the association between C48α1 and Cdk5 was not weakened by high concentrations of NaCl but was completely abolished by 1% Triton X-100 (Fig. 3-8B). These results support the suggestion that, in the region spanning residues 145 to 170, Nck5a interacts with Cdk5 and C48 via the hydrophobic and hydrophilic faces of its amphipathic α-helix respectively.

Site-directed mutagenesis was carried out as the second approach to test this hypothesis. The hydrophilic faces of the amphipathic α-helices of both Nck5a
### Fig. 3-8. Characterization of the interaction of p25^{145-170} with C48α1 and Cdk5. The interaction of p25^{145-170} with C48α1(A) and Cdk5(B) under different concentrations of NaCl and in the presence of 1% Triton X-100. In this experiment, GST-C48α1 was bound onto GSH beads, subsequently about 15 μg (His)_6-tagged p25^{145-170} were added to GST-C48α1 sample in the presence of various concentration of NaCl, or 1% Triton X-100. The C48α1-bound form of p25^{145-170} was precipitated by GSH-agarose beads, and detected using Western Blot. The concentration of NaCl and Triton X-100 indicated in the figure represent the final concentration of the assay mixture. In this experiment, the weaker signal of C48 α1-binding with (His)_6-tagged p25^{145-170} in penal A line 3 compare with panel A line 4 samples containing 0.1 M NaCl is due to some experimental error.
Fig. 3-9. The effect of Glu157 to Gln mutation of p25\(^{145-170}\) on its binding to p25\(^{145-170}\) with C48α1 and Cdk5. The binding assay was carried out under identical conditions described in Fig. 3-2. Panel A shows the binding of p25\(^{145-170}\) and p25\(^{145-170(E157Q)}\) with (His)\(_6\)-tagged C48α1. Panel B shows the binding of p25\(^{145-170}\) and p25\(^{145-170(E157Q)}\) with GST free Cdk5.
(Gln145-Ser170) and C48α1 are rich in charged amino acid side chains (Chin et al., 1999; also see Fig. 3-7). Thus, it is possible that ionic interactions play important roles in the association between the two proteins, and mutations involving certain charged residues may therefore adversely effect this protein-protein interaction. Although attempts were made to produce mutations at two individual charge residues, only one protein mutant was successfully expressed in *E.coli*. Fig. 3-9A shows that substitution of Glu157 by glutamine completely abolished the ability of the peptide to bind C48α1. Since Glu 157 is located at the centre of the hydrophilic face of the α-helix, the result strongly implicates the hydrophilic face of the α-helix in the association between C48 and Nck5a(Gln145-Ser170). In contrast, the association between the mutant peptide and Cdk5 was not adversely effected by the amino acid substitution (Fig. 3-9B). In a previous study, we showed that a dual substitution mutant of Nck5a with both Leu151 and Leu152 substituted by asparagine lost most of its activity due to severely diminished affinity for Cdk5. This mutant protein was found to bind C48 as well as the wild type protein (results not shown). The differential effects of protein mutation on the interaction of Nck5a with Cdk5 and C48 have provided additional strong support for the suggestion that the binding of Cdk5 and C48 to Nck5a(Gln145-Ser170) involves the hydrophobic and hydrophilic face of the amphipathic α-helix respectively.

3.5 Residues 145-170 Is The Common Binding Region of Several p35Nck5a Associated Proteins—
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<th>Beads</th>
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**Fig. 3-10.** The p25\textsuperscript{nck5a} associated proteins share the same binding site in Nck5a. The binding assay was carried out under an identical condition as described in Fig. 3-2 (see Procedures for more details). The MW indicated in this figure is the MW of the gene product plus the MW of thiooredoxin from the vector.
To test whether the C48-binding site of Nck5a is specific to C48, or shared by many of the Nck5a-binding proteins, C42 and C53 were tested for their abilities to bind Nck5a(Gln145-Ser170). Fig. 10 shows that both C42 and C53 showed strong Nck5a(Gln145-Ser170) as well as Nck5a binding. This observation suggests that the three novel Nck5a-binding proteins may share the same binding site on p35\textsuperscript{nck5a}.

To further confirm this hypothesis, a competitive Nck5a-binding assay of these p35\textsuperscript{nck5a} associated proteins with respect to C48 was carried out. The GSH-beads were first pre-coated with the competing Nck5a-binding protein (C42 or C53) in its GST-fused form and then incubated with a constant amount of (His)\textsubscript{6}-tagged p25\textsuperscript{nck5a} and increasing concentrations of (His)\textsubscript{6}-tagged C48. The samples were then affinity precipitated and the precipitates were analysed by Western blotting for the presence of (His)\textsubscript{6}-tagged p25\textsuperscript{nck5a}. Fig.3-11A shows that as the amount of C48 increased in the binding reaction, the amount of (His)\textsubscript{6}-tagged p25\textsuperscript{nck5a} in the affinity precipitates was reduced, and that at the highest concentrations of the competing Nck5a-binding proteins used, little or no p25\textsuperscript{nck5a} could be demonstrated in the precipitates. Similar results could be obtained if C48\textsubscript{α1} instead of the full length C48 or Nck5a(Gln145-Ser170) instead of p25\textsuperscript{nck5a} was used in the competitive binding assay (Fig. 3-11B and C). These results further support the suggestion that both C42 and C53 compete with C48 for Nck5a binding at site Nck5a(Gln145-Ser170). Although the competitive binding between two binding proteins seems to suggest that the binding proteins share a common binding site, the possibility that they have overlapping binding sites cannot be excluded.
Like C48, C42 and C53 bind strongly to Nck5a(Gln145-Ser170) but when the residue Glu157 was substituted by Gln, the binding activity of the peptide for these two proteins was mostly eliminated (Fig. 3-12). Structure prediction of C42 and C53 helical region also shows an amphipathic character (Fig. 3-13, 3-14). The result suggests that the mechanisms used by C48, C42 and C53 for binding to Nck5a are the same, all through the hydrophilic side of Nck5a, further supporting that these three proteins may bind to the same region of Nck5a.
Fig. 3-11. p25^{nck5a} associated proteins compete with each other for Nck5a. A, C48 competes with C42 and C53 for p25^{nck5a}. About 30 of µg GST-fusion C42 and C53 proteins were pre-bound to GSH-agarose beads, 15 µg of (His)\textsubscript{6}-tagged p25^{nck5a} together with increasing amount of (His)\textsubscript{6}-tagged C48 (150 µg, 300 µg, 750 µg) were subsequently mixed with the GST-fused proteins. The amount of (His)\textsubscript{6}-tagged p25^{nck5a} pulled down by GST-fusion proteins was analyzed by p35^{nck5a}-specific antibody (C-19). GSH bead and GST protein were used as the negative controls and GST-Cdk5 was used as the positive control. B, C, C48α1 competes with C42 and C53 for p25^{nck5a} and p25^{145-170}, respectively. The experimental conditions were identical to that described in Panel A.
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<thead>
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<th>Beads</th>
<th>GST</th>
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Fig. 3-12. The effect of Glu157 to Gln mutation of p25^{145-170} on its binding to C42 and C53. GST-fused C42 and C53 were used to interact with (His)$_6$-tagged p25^{145-170} or (His)$_6$-tagged p25^{145-170(E157Q)}. The experiment was performed with an identical procedure as described in Fig. 3-9.
Fig. 3-13 Helical Wheel of C53 From 102 to 119.
Fig. 3-14  Helical Wheel of C42 From 4 to 21.
Discussion

A variety of cellular proteins have been found during the last few years to undergo specific and high affinity association with Nck5a (Qi et al., 1998; Lee et al., 1997; Kwon et al., 2000; Nikolic et al., 1998; Ching et al., 2000). Characterizations of some of these protein-protein interactions have shed important light on the function, regulation and the mechanism of action of Nck5a (Nikolic et al., 1998; Moorthamer et al., 1999). However, there is no study on the biochemical mechanisms of interactions between Nck5a and its binding proteins. The present study is concerned mainly with the molecular basis of the interactions between Nck5a and three novel Nck5a binding proteins, designated as C42, C48 and C53 (Ching et al., 2000). Upon analyses of binding characteristics of a large number of Nck5a deletion mutants, a region of 26 amino acid residues proximal to the N-terminal boundary of the kinase activation domain of Nck5a is identified to contain the binding site(s) of these Nck5a-binding proteins. The peptide corresponding to this region of Nck5a, a 26-residue peptide designated Nck5a(Gln145-Ser170), can associate with these Nck5a-binding proteins with affinities in the same order of magnitude as those of the full length Nck5a. Interestingly, this region of Nck5a has also been suggested in a previous structure-function study of Nck5a and from the computer modeling of Cdk5-Nck5a complex to contain structural elements essential for the kinase activation (Tang, et al., 1997; Chou et al., 1999). This suggestion is substantiated in the present study by the observation that Nck5a(Gln145-Ser170) binds Cdk5 with high affinity.
Due to its small size and availability as purified bacterially expressed samples, C48 was used initially as the model Nck5a-binding protein for the study. When the full length C48 was used to examine the binding properties of the various Nck5a deletion mutants, the results suggested the existence of two binding sites, one within the 26-residue region, called a-binding site, and another in the region of residues 214 to 240, b-binding site. The Nck5a binding site was also mapped to within the first α-helix of C48 and a C48 deletion mutant corresponding to this α-helix region, C48α1, could bind only to the a-binding site. This observation suggests that probably only the a-binding site can bind C48 in intact p25\textsuperscript{nck5a} or p35\textsuperscript{nck5a}. The result obtained from the later binding competitive assay, that C48α1 can interact with p25\textsuperscript{nck5a} thus block the binding of p25\textsuperscript{nck5a} with C42 and C53 further supported this hypothesis. Attempt has been made to mutate Glu157 in the full length p25\textsuperscript{nck5a} protein, but I never succeed in that. It would be more confident to announce that b-binding site is not functional if the mutated p25\textsuperscript{nck5a} lose its binding ability with C48. It is a common practice to use deletion protein mutants to map specific binding sites on a protein molecule. The observation that a binding site demonstrated on deletion mutants may not function in the intact protein suggests caution against such artifacts.

All three Nck5a-binding proteins used in this study to characterize interactions between Nck5a and the binding proteins show common binding characteristics. They all display high affinity and specific binding to the peptide Nck5a(Gln145-Ser170) and they compete with each other in their bindings to the peptide or the intact Nck5a thus suggesting that they bind to Nck5a at a common site. The suggestion is further supported by the observation that the interactions of Nck5a with the three binding proteins are similarly effected by the reaction conditions such as ionic strength and
detergent contents of the reaction medium, as well as by site-directed mutation of Nck5a (see later section for details). However, the possibility that they have distinctive but overlapping sites in this region cannot be completely excluded.

In an earlier study (Chin et al., 1999), we showed that a 29-residue peptide derived from Nck5a displayed potent inhibitory activity towards Cdk5 and Cdk2. This Nck5a-derived inhibitor that spans residues Gln145 to Asp173 can be seen to correspond closely to the peptide Nck5a(Gln145-Ser170). Secondary structure prediction and analysis of the Cdk inhibitory peptide by circular dichroism and two-dimensional $^1$H NMR spectroscopy have identified an amphipathic $\alpha$-helix that spans amino acid residues Ser149 to Arg162. A number of amino acid side chains at the hydrophobic face of this amphipathic $\alpha$-helix have been suggested in a number of studies to play important roles in the interaction between Nck5a and Cdk5 (Tang et al., 1997; Chou et al., 1999), a suggestion supported by the observation in this study that 1% Triton X-100 essentially completely abolishes the binding of the peptide Nck5a(Gln145-Ser170) to Cdk5 (Fig. 3-8B). While the interaction between Cdk5 and Nck5a is dominated by hydrophobic interactions, several lines of evidence suggest that the hydrophilic face of the amphipathic $\alpha$-helix plays a major role in the association of Nck5a with the Nck5a-binding proteins. First, Cdk5 and the Nck5a-binding proteins do not compete in their interactions with Nck5a(Gln145-Ser170), and Nck5a binding proteins can bind to both monomeric Nck5a and Nck5a in the heterodimer Cdk5-Nck5a (Ching et al., 1997). These observations indicate that Nck5a uses distinct binding sites to interact with Nck5a-binding proteins and with Cdk5. Second, Nck5a(Gln145-Ser170) loses its ability to associate with the Nck5a-binding proteins in high concentrations of NaCl, whereas the interaction between the peptide
and Cdk5 is not adversely effected. On the other hand, while the interactions of the peptide with the Nck5a-binding proteins are totally refractory to the presence of 1% Triton X-100, the peptide-Cdk5 interaction is negated in the presence of the nonionic detergent. These results suggest that, in contrast to the Nck5a-Cdk5 interaction that depends on hydrophobic interactions, the association of Nck5a-binding proteins with Nck5a(Gln145-Ser170) is dependent of interactions of hydrophilic nature. Lastly, substitution of a glutamate, Glu157, that is situated at the center of the hydrophilic face of the α-helix, by glutamine results in almost complete elimination of the interaction of Nck5a(Gln145-Ser170) peptide with the Nck5a-binding proteins, without interfering with the binding of the peptide to Cdk5. On the other hand, substitution of two leucine residues, Leu151 and Leu152, in the hydrophobic face of the amphipathic α-helix by asparagines, previously shown to significantly decrease the interaction between Nck5a and Cdk5, has little effect on the association of Nck5a with the binding proteins.

As the three Nck5a-binding proteins appear to bind a common site on Nck5a, structural comparison of the three proteins may be expected to reveal a structural motif that is specific for the Nck5a binding site. Amino acid sequence alignment however, has failed to reveal such a structural motif. Perhaps the binding motif depends on structural features other than those in the primary structure. The smallest C48 fragment displaying high affinity binding to Nck5a, C48α1, contains an amphipathic α-helix. It is possible that the binding of C48 to Nck5a involves the hydrophilic face of this protein. Work is in progress to isolate the smallest Nck5a-binding fragments of the three binding proteins so as to define the binding motif.
Cyclin-dependent kinase 5 has many distinct functional and regulatory properties among members of Cdk family. It has been suggested that many of the distinct properties of Cdk5 arise from the unique structure of Nck5a. While all the other known activators of Cdk5 belong to the cyclin protein family, Nck5a does not contain in its structure a conserved cyclin-box characteristic of cyclins. Structural and functional analysis of Nck5a, however, has localized the kinase activation domain of Nck5a to a region of 142 residues, Glu150 to Asn291, similar in size to active domains of other cyclins, and this region of Nck5a appears to assume a cyclin-like folded structure (Chou et al., 1999). The result appears to suggest that the unique structure of Nck5a is evolved to support mainly functions other than the kinase activation. Presumably, some of these functions are manifested in the specific interactions of Nck5a with the various cellular proteins. The identification of a specific protein binding site in Nck5a represents the first attempt in the elucidation of the structural basis of interactions between Nck5a and Nck5a-binding proteins. The binding site is within a sub-domain of Nck5a that is important for Cdk5 activation. An amphipathic α-helix in this domain uses its hydrophobic and hydrophilic phase to interact with Cdk5 and the Nck5a-binding proteins respectively. It should be reiterated that although all three binding proteins studied in the present work appear to bind to this site, it does not indicate that this is a common binding site for all Nck5a-binding proteins. Preliminary results have shown that certain other Nck5a-binding proteins bind to Nck5a at distinct sites (Qi et al, unpublished results).
CONCLUSION

In this MPhil thesis research, a 26-amino acid region of Nck5a was found as a common region which is capable of interacting with three novel Nck5a-associated proteins—C42, C48 and C53. The binding of C48 has no effect on Cdk5 kinase activity. In addition, the 26-amino acid binding region of Nck5a can also interact with Cdk5 with high binding affinity, thus form ternary complex with Cdk5 and C48. Unlike the binding between Cdk5 and Nck5a which is via hydrophobic-hydrophobic association, the binding between Nck5a and its associated proteins was proved to be charge-charge interaction. Residue Glu157 of Nck5a was essential for such interaction, mutation of Glu157 to Gln totally abolished the binding of Nck5a and these Nck5a–associated proteins.

Many questions should be asked and addressed from current result. For example, what are the physiological roles of these three novel proteins and what are the effects of their association with Nck5a? Cell transfection work is carrying in our lab to study the physiological functions of these three novel proteins. Glu157 to Gln mutation on the full-length p25<sub>Nck5a</sub> protein is still needed to conform that the second C48 binding site of Nck5a, residues 214-240, is not functional in the intact Nck5a protein. Co-transfection of such mutated p25<sub>Nck5a</sub> together with C48 would be very helpful on C48 function study. Over-expression of peptide Nck5a(145-170) within cells would block the association of C48 with full-length Nck5a (if the b-binding site, residues 214-240, is not functional in vivo), provide clues for C48 function. We can also use C42, C48 or C53 as the bite to search for there association proteins in yeast.
two-hybrid system, to check whether they are related to any protein involved in signal transduction pathways. The reveal of physiological functions of these three novel Nck5a-associated proteins—C42, C48 and C53, might shed light on the study of biological roles of Cdk5 and Nck5a.
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