Pctaire1 phosphorylates N-ethylmaleimide
sensitive fusion protein and regulates exocytosis

by

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by

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This is to certify that I have examined the above PhD thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

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# Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>Ad</td>
<td>adult</td>
</tr>
<tr>
<td>AdB</td>
<td>adult brain</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AD</td>
<td>activation domain</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ions</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CAK</td>
<td>Cdk-activated kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>Cdk(s)</td>
<td>cyclin-dependent kinase (s)</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CKIs</td>
<td>Cdk-inhibitory subunits</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>con</td>
<td>control</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>a dopamine and cyclic AMP-regulated phosphoprotein</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's minimal essential medium</td>
</tr>
</tbody>
</table>
DMSO  dimethylsulfoxide
DNA   deoxyribonucleic acid
DNCdk5 dominant negative cyclin-dependent kinase 5
dNTP deoxyribonucleotide triphosphate
DPBS Dublecco's phosphate buffered saline
DTT  Dithiothreitol
E18  Embryonic day 18
e.g. example(s)
EDTA ethylenediamine tetraacetic acid
ErbB a family of receptor tyrosine kinase highly related to epidermal growth factor
ERK extracellular signal-regulated kinase
EtBr ethidium bromide
FBS fetal bovine serum
FITC fluorescein-5-isothiocyanate
FL  full length
 g  gram
GABP GA-binding protein
GFP green fluorescent protein
H1  histone H1
HCl hydrochloric acid
hr  hour
HRP horasidase peroxidase
HS  horse serum
IP  immunoprecipitation
IPTG isopropylthio-β-D-galactoside
JNK c-jun N-terminal kinase
Kb  kilobase pair
KD  kinase dead
kDa kilo-Dalton
L  liter
LiCl lithium chloride
LTP  long term potentiation
M  molarity
m micro- \((10^{-6})\)
MAPK mitogen activated protein kinase
MBP myelin basic protein
MEM Eagle’s minimal essential medium
MgCl₂ magnesium chloride
Min minute(s)
MOPS 3’-[morpholono] propanesulfonic acid
Mr molecular weight
mRNA messenger ribonucleic acid
MuSK muscle-specific kinase
n nano- \((10^{-9})\)
Na⁺ sodium ions
NaCl sodium chloride
NaOH sodium hydroxide
NaOV sodium orthovanadate
N-CAM neural cell adhesion molecules
NCLK neuronal cdc2-like kinase
NGF nerve growth factor
NMDA \(N\)-methyl-\(D\)-Aspartate
NMJ(s) neuromuscular junction(s)
NP40 Nonidet P40
NRG Neuregulin
NSF N-ethylmaleimide sensitive fusion protein
ONPG o-nitrophenyl-b-D-galactopyranoside
P5 postnatal day 5
PBS phosphate buffered saline
PCNA proliferation cell nuclear protein
PCR polymerase chain reaction
Pct1 Pctaire1
PFA paraformaldehyde
PKA protein kinase A
PMSF phenylmethylsulfonyl fluoride
PNS peripheral nervous system
PSD95 a protein enriched in the postsynaptic density
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RACE</td>
<td>rapid amplification of cDNA end</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>Ros</td>
<td>roscovitine</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNAP</td>
<td>soluble NSF attachment protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP receptor</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride/sodium citrate solution</td>
</tr>
<tr>
<td>SV</td>
<td>Synaptic vesicle</td>
</tr>
<tr>
<td>SV2</td>
<td>a protein present in varicosity-like structure</td>
</tr>
<tr>
<td>Tau</td>
<td>Tau isoform 1 and 2</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-boric acid-EDTA solution</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA solution</td>
</tr>
<tr>
<td>Tris</td>
<td>hydroxymethyl aminomethane</td>
</tr>
<tr>
<td>Trk</td>
<td>trypomyosin receptor kinase</td>
</tr>
<tr>
<td>U373</td>
<td>human astrocytoma cell line</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-Chloro-3-Indoly1-β-D-Galactosidase</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
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Pctaire1 phosphorylates N-ethylmaleimide sensitive fusion protein and regulates exocytosis

by

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The Hong Kong University of Science and Technology

Abstract

Pctaire1, a member of the cyclin-dependent kinase (Cdk)-related kinase family, has recently been identified as a substrate of Cdk5, where Cdk5-mediated phosphorylation enhances Pctaire1 activity. Although Pctaire1 is expressed in both neuronal and non-neuronal cells, its precise functions remain elusive. To examine the potential functions of Pctaire 1, we performed a yeast two-hybrid screen and mass spectrometry to identify proteins that interact with Pctaire1. Interestingly, Pctaire1 was observed to interact with several synaptic vesicle-associated proteins including syntaxin 1, synaptotagmin and synapsin I. In particular, N-ethylmaleimide sensitive fusion protein (NSF), a crucial factor in vesicular transport and membrane fusion, was also identified as one of the Pctaire1-interacting proteins. Further studies revealed that the D2 domain of NSF, which is required for the oligomerization of NSF subunits, bind directly to, and was phosphorylated by Pctaire1 at serine-569. Mutation of this phosphorylation site on NSF (S569A) augmented its ability to oligomerize. Moreover, inhibition of Pctaire1 activity by transfecting its kinase-dead
(KD) mutant into COS-7 cells enhanced the self-association of NSF. These observations collectively suggest that phosphorylation of NSF by Pctaire1 may negatively regulate oligomerization of NSF, thereby affecting NSF-mediated membrane fusion events in exocytosis. To investigate whether NSF phosphorylation by Pctaire1 is involved in the regulation of Ca\textsuperscript{2+}-dependent exocytosis, we examined the effect of expressing Pctaire1 or NSF phosphorylation mutants on the regulated secretion of growth hormone from PC12 cells. Importantly, expression of either Pctaire1-KD or NSF-S569A in PC12 cells significantly increased high K\textsuperscript{+}-stimulated growth hormone release, indicating that Pctaire1 phosphorylation of NSF suppressed high K\textsuperscript{+}-stimulated growth hormone release. Taken together, our findings provide the first demonstration that phosphorylation of NSF by Pctaire1 regulates its oligomerization, which may result in the suppression of Ca\textsuperscript{2+}-dependent exocytosis. More importantly, the concomitant identification of multiple synaptic vesicle-associated proteins as Pctaire1-interacting protein implicates a functional role of Pctaire1 in the regulation of synaptic vesicle physiology. Further studies on the potential interaction of these synaptic vesicle-associated proteins with Pctaire1 will help unravel the functional roles of Pctaire1 in the nervous systems.
Chapter 1 Introduction

As a Cdk-related kinase, Pctaire1 is identified as a p35-interacting protein and a substrate of Cdk5 (Cheng et al., 2002). Previous study from our laboratory demonstrated that Pctaire1 activity is elevated upon phosphorylation by Cdk5. In addition, Pctaire 1 kinase activity is significantly reduced in Cdk5/−/− brain, suggesting that Cdk5 may function as a crucial regulator of Pctaire1 activity in vivo (Cheng et al., 2002). Understanding Cdk5 functions may therefore provide essential insights on unraveling the biological functions of Pctaire1.

1.1 Cyclin dependent kinase 5

Cyclin-dependent kinase 5 (Cdk5), a serine/threonine kinase also known as neuronal Cde-2 like kinase (NCLK), was initially purified from bovine brain (Lew et al., 1992). Although Cdk5 shows high degree of sequence homology to Cdc2 and Cdk2, 58% and 59% respectively in mammalian system, there are fascinating differences between Cdk5 and other Cdk5s. Cdk5s are activated upon association with cyclins and are involved in the regulation of cell cycle progression (Morgan, 1995). However, activation of Cdk5 does not require the association of cyclins, but two non-cyclin activators, p35 and p39. Furthermore, Cdk5 does not participate in cell cycle regulation, but is involved in various biological functions in the nervous systems including neuronal migration, neuronal survival and regulation of synaptic functions.
1.1.1 Activators of Cdk5

Similar to other Cdns, monomeric Cdk5 displays no kinase activity and requires association with regulatory partners for activation. Nonetheless, despite the demonstrated association between Cdk5 and cyclin D and cyclin E, interaction with these two cyclins does not result in Cdk5 activation. Rather, two non-cyclin activators, p35 and p39, were identified as activators of Cdk5. p35, also known as neuronal non-cyclin activator, was first identified as Cdk5 binding partner in brain extract by two different groups (Lew et al., 1994; Tsai et al., 1994). Another activator of Cdk5, p39, was identified by its sequence homology to p35 (Tang et al., 1995). Neither p35 nor p39 shows sequence similarity to cyclins. However, structural studies show that p35 might use a cyclin-like structure during the association and activation of Cdk5 (Brown et al., 1995; Tang et al., 1997).

Although Cdk5 is expressed in all tissues, its kinase activity is restricted to the nervous system (Hellmich et al., 1992; Tsai et al., 1993; Ino et al., 1994). This is attributed to the localized expression of p35 and p39, whose expression is found prominently in the postmitotic neurons of both CNS (central nervous system) and PNS (peripheral nervous system), but not in the proliferating neuronal precursors (Zheng et al., 1998; Delalle et al., 1997; Tsai et al., 1994). The localized Cdk5 activity suggests that these two regulatory proteins are essential for the activation of Cdk5. Indeed, this hypothesis is supported by transgenic mice studies, where mice deficient in Cdk5 display strikingly similar phenotypic abnormalities compared to
mice lacking both p35 and p39. Cdk5 knockout mice display severe cortical lamination defects and perinatal death (Ohshima et al., 1996). Although mortality of p35 knockout mice is reduced to 15%, deletion of p35 results in similar abnormality in cortical lamination (Chae et al., 1997). More importantly, simultaneous deletion of p35 and p39 results in the same phenotype as Cdk5−/− mice, although p39−/− mice display no overt phenotypic abnormality compared to wild type (Ko et al., 2001). These studies therefore suggest that p35 and p39 are the major activators of Cdk5 in the nervous system, where p35 is more important for the early neurodevelopment. In addition, these observations reveal that p39 only partially compensates for some functions of p35 (Ko et al., 2001). Recent studies find that p35 and p39 may confer different substrate specificity to Cdk5, eg. Cdk5/p39, but not Cdk5/p35, preferentially phosphorylates Tau (Takahashi et al., 2003).

1.1.2 Regulation of Cdk5 activity

The activity of Cdks is regulated in three ways in proliferating cells: association with cyclins, binding to inhibitor proteins or through phosphorylation/dephosphorylation of the kinase (Reviewed in Obaya and Sedivy, 2002). Similar to other Cdks, Cdk5 activity is also stringently regulated in neurons. First of all, Cdk5 activity is regulated by the levels of Cdk5 or its activators. Extracellular signals have been observed to regulate Cdk5 activity through modulating the expression of Cdk5 or p35 (Bibb et al., 2001; Harada et al., 2001; Chen et al., 2000; Li et al., 2000; Tokuoka et al., 2000; Paglini et al., 1998; Pigino et
Moreover, Cdk5 activity is controlled by the degradation of its activators in neuron after phosphorylation by Cdk5 (Patrick et al., 1998; Patrick et al., 1999; Patzke and Tsai, 2002). On the other hand, Cdk5 activity is also modulated by phosphorylation/dephosphorylation regulatory mechanisms (Qu et al., 2002; Zukerberg et al., 2000; Shetty et al., 1995). For example, an inhibitory kinase from bovine thymus has been observed to inactivate Cdk5 \textit{in vitro} through phosphorylation at threonine-14 (Matsuura and Wang, 1996). In addition, c-Abl and Fyn phosphorylates Cdk5 at tyrosine-15 to enhance its activity (Zukerberg et al., 2000; Sasaki et al., 2002). Unlike other Cdns, nonetheless, no inhibitor proteins have been identified for Cdk5 in nervous system.

\textit{1.1.3 Substrates for Cdk5/p35}

Up to date, more than thirty substrates for Cdk5 have been reported. Cdk5 has a phosphorylation consensus sequence similar to other Cdns, which phosphorylates proline (+1) directed serine or threonine residues (S/T-P). In addition, Cdk5 has a higher preference for the phosphorylation site when a basic residue is localized at the +3 position of the phosphorylation site. The preferential consensus phosphorylation sequence for Cdk5 is therefore (S/T)PX(K/H/R), where S or T is the phosphorylatable serine or threonine, P is the proline residue in the +1 position, X is any amino acid except aspartate (D) and glutamate (E), and K/H/R are the basic residues lysine/histidine/arginine (Lew et al., 1995).
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Putative function of phosphorylation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulating of Cdk5 kinase activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p35 and p39</td>
<td>Promotes ubiquitin-mediated proteolysis</td>
<td>(Patrick et al., 1999) (Patzke and Tsai, 2002)</td>
</tr>
<tr>
<td><strong>Modulating of axonal transport and microtubule dynamics</strong></td>
<td></td>
<td></td>
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<tr>
<td>Nudel</td>
<td>Modulates dynein-mediated axonal transport</td>
<td>(Niethammer et al., 2000) (Sasaki et al., 2000)</td>
</tr>
<tr>
<td>Adenomatous polyposis coli (Tumor suppressor protein, APC)</td>
<td>Affects microtubule binding proteins that are responsible for fast axonal transport</td>
<td>(Rainer et al., 1998)</td>
</tr>
<tr>
<td>Tau</td>
<td>Decreases binding to microtubules, and inhibits microtubule nucleation</td>
<td>(Baumann et al., 1993) (Ishiguro et al., 1994)</td>
</tr>
<tr>
<td>Microtubule associated protein (MAP1B)</td>
<td>Affects microtubule stability for neurite outgrowth</td>
<td>(Pigino et al., 1997) (Paglini et al., 1998)</td>
</tr>
<tr>
<td>Neurofilament (NF160-P and NF200-P)</td>
<td>Modulates axonal structure and radial growth and also transport</td>
<td>(Grant et al., 2001) (Lew et al., 1992)</td>
</tr>
<tr>
<td><strong>Regulating neurite outgrowth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pak1</td>
<td>Inhibits Pak1 kinase activity, regulation of actin-cytoskeleton dynamics</td>
<td>(Nikolic et al., 1998)</td>
</tr>
<tr>
<td>Src</td>
<td>Affects cell adhesion, actin dynamics and integrin signaling</td>
<td>(Kato and Maeda, 1999)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Decreases the interaction with presenilin 1 that attenuates cell adhesion Regulates the N-cadherin mediated homophilic cell adhesion</td>
<td>(Kesavapany et al., 2001) (Kwon et al., 2000)</td>
</tr>
<tr>
<td>Cables</td>
<td>An adaptor protein that mediates the interaction of Cdk5 kinase with c-Abl</td>
<td>(Zukerberg et al., 2000)</td>
</tr>
<tr>
<td><strong>Modulating synaptic functions</strong></td>
<td></td>
<td></td>
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<tr>
<td>Synapsin I</td>
<td>Inhibits synaptic transmission</td>
<td>(Matsubara et al., 1996)</td>
</tr>
<tr>
<td>Munc-18</td>
<td>Disrupts the Munc-18/syntaxin 1A complex that attenuates neurosecretion</td>
<td>(Shuang et al., 1998) (Fletcher et al., 1999)</td>
</tr>
<tr>
<td>P/Q-type Voltage-dependent calcium channel</td>
<td>Inhibits neurotransmitter release in the presynaptic terminal</td>
<td>(Tomizawa et al., 2002)</td>
</tr>
<tr>
<td>Dynamin I</td>
<td>Modulates synaptic vesicle endocytosis</td>
<td>(Tan et al., 2003) (Tomizawa et al., 2003)</td>
</tr>
<tr>
<td>Amphiphysin I</td>
<td>Modulates synaptic vesicle endocytosis</td>
<td>(Floyd et al., 2001) (Rosales et al., 2000)</td>
</tr>
<tr>
<td>Protein</td>
<td>Function</td>
<td>Reference</td>
</tr>
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<td>---------</td>
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<tr>
<td>Synaptojanin 1</td>
<td>Inhibits its phosphatase activity and hinders clathrin uncoating</td>
<td>(Tomizawa et al., 2003)</td>
</tr>
<tr>
<td>NR2A (NMDA receptor)</td>
<td>Cdk5 activity is required for LTP and NMDA-evoked current</td>
<td>(Li et al., 2001)</td>
</tr>
<tr>
<td>ErbB</td>
<td>Regulates signaling at the neuromuscular junction</td>
<td>(Fu et al., 2001)</td>
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**Modulating the amplitude of signaling transduction**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DARPP32</td>
<td>Downregulates the efficacy of dopamine signaling pathway</td>
<td>(Bibb et al., 1999)</td>
</tr>
<tr>
<td>Protein phosphatase inhibitor 1 and 2 (PP1/2-inhibitor)</td>
<td>Modulates the cAMP-dependent signaling amplitude</td>
<td>(Agarwal-Mawal and Paudel, 2001)</td>
</tr>
<tr>
<td>MAP kinase kinase (MEK1)</td>
<td>Regulates neuronal differentiation through inhibiting MEK1 activity</td>
<td>(Bibb et al., 2001)</td>
</tr>
<tr>
<td>c-Jun N-terminal kinase 3 (JNK)</td>
<td>Inhibits JNK3 leading to reduced c-Jun phosphorylation, thereby preventing neuronal apoptosis</td>
<td>(Li et al., 2002)</td>
</tr>
</tbody>
</table>

**Modulating gene transcription**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3</td>
<td>Regulates STAT3 phosphorylation and its transcriptional activity</td>
<td>(Fu et al., 2004)</td>
</tr>
<tr>
<td>m-Sds3</td>
<td>Controls the mSds3-HDAC transcriptional repressor activity</td>
<td>(Li et al., 2004)</td>
</tr>
</tbody>
</table>

**Others**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pctaire1</td>
<td>Enhances Pctaire1 activity</td>
<td>(Cheng et al., 2002)</td>
</tr>
<tr>
<td>Retinoblastoma (Fernandez-Chacon et al.)</td>
<td>Affects neuronal differentiation and apoptosis</td>
<td>(Lee et al., 1997)</td>
</tr>
<tr>
<td>MEF2</td>
<td>Inhibits MEF2 activity and induces neuronal apoptosis</td>
<td>(Gong et al., 2003)</td>
</tr>
<tr>
<td>Canoe</td>
<td>Affects Drosophila morphogenesis during development</td>
<td>(Takahashi et al., 2002)</td>
</tr>
<tr>
<td>Pgamma (PDE regulator)</td>
<td>Modulates retinal phototransduction</td>
<td>(Hayashi et al., 1994)</td>
</tr>
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</table>

Table 1.1 Substrates of Cdk5 and their putative functions after phosphorylation.
Since the purification of Cdk5, identification of Cdk5 substrates and the explication of their interactions have shed light on understanding the biological roles of Cdk5. These substrates are summarized in Table 1.1 and will be discussed in the next sections. The diversity of these substrates implicates a physiological functional role of Cdk5 in a wide range of cellular processes (Cruz and Tsai, 2004; Dhavan and Tsai, 2001).

1.1.4 Physiological functions of Cdk5/p35 in synapse

During the last decade, a number of proteins have been identified to associate with Cdk5/p35 and most of them are substrates of active Cdk5. The involvement of these proteins in multiple biological processes suggests that Cdk5 is extensively involved in the regulation of various cellular functions such as neuronal migration, synaptic transmission and neuronal degeneration, etc (Fig. 1.1; Cruz and Tsai, 2004; Dhavan and Tsai, 2001). Interestingly, accumulating evidence indicates that Cdk5 is an emerging key player at synapses, both at the central synapse and at the neuromuscular junctions (NMJs).

Initial evidence on a potential role of Cdk5 in synapse formation and functions come from the observation that Cdk5 and p35 are expressed at the growth cone of neurons during axon guidance (Nikolic et al., 1998). In addition, Cdk5 is localized to both pre- and postsynaptic terminals of the neurons (Niethammer et al., 2000). Furthermore, subcellular fractionation experiments showed that Cdk5, p35 and p39 are enriched in the synaptosome and postsynaptic densities,
Figure 1.1 Cellular processes regulated by Cdk5. The multiple functional roles of Cdk5 are evident in its involvement in various cellular processes.

(Adapted from Dhavan and Tsai, 2001)
indicating that Cdk5 may be involved in synaptic functions (Humbert et al., 2000).

1.1.4.1 Modulation of synapse formation

Synapse formation begins when axonal growth cones contact target cells. This process involves specializations in both the pre- and postsynaptic compartments. At the NMJs, one of the postsynaptic changes is the induction of acetylcholine receptors (AChRs) by neuregulin, a secreted factor from motor neurons that interacts with the ErbB receptor. It has been demonstrated that Cdk5 may play a role in regulating the induction of AChRs (Fu et al., 2001). Neuregulin increases Cdk5 expression and activity in cultured myotubes, where it phosphorylates ErbB, resulting in ErbB activation and increased AChR expression in nuclei near the nascent synaptic site. Inhibition of Cdk5 activity blocks neuregulin-induced activation of ErbB and a downstream MAP/ERK kinase cascade, inhibiting increased AChR expression in myotubes. These observations suggest that Cdk5 may function as part of a positive-feedback loop that reinforces ErbB activation in developing synapses.

In agreement with the phosphorylation of ErbB2/3 by Cdk5 in myotubes, ErbB2/3 phosphorylation and the ErbB2 kinase activity are reduced in Cdk5 deficient muscle, indicating that Cdk5 may also phosphorylate ErbB2/3 in vivo. Furthermore, Cdk5 null mice display morphological abnormalities at the NMJ pre- and postsynaptically. The agrin-induced formation of large AChR clusters is significantly increased in primary muscle cultures prepared from Cdk5 null mice and
in C2C12 myotubes when Cdk5 activity is suppressed. These observations revealed an unexpected role of Cdk5 in the regulation of AChR clusters in vivo. Taken together, Cdk5 is found to have an essential role in regulating the development of NMJ (Fu et al., 2005).

1.1.4.2 Modulation of neurotransmitter release

Efficient neurotransmission is dependent on two presynaptic cycles, the neurotransmitter cycle and the synaptic vesicle cycle. The neurotransmitter cycle involves transmitter biosynthesis, storage, reuptake and degradation; while the synaptic vesicle cycle consists of targeting of synaptic vesicles to the nerve terminal, docking, fusion, endocytosis and recycling (Fon and Edwards, 2001). Emerging evidence demonstrates that Cdk5 plays an important role in regulating neurotransmitter release at synapse (Fig. 1.2, Review in Smith and Tsai, 2002; Lai and Ip, 2003; Cheng and Ip, 2003; Cheung and Ip, 2004).

In particular, Cdk5 has been observed to phosphorylate pre-synaptic protein Munc-18. The presynaptic terminal is accumulated with numerous synaptic vesicles containing neurotransmitters. Upon influx of Ca\(^{2+}\), synaptic vesicles located in the active zone fuse with presynaptic membrane and release the neurotransmitter into the synaptic cleft. This process of membrane fusion is regulated by Munc-18. In addition, v- and t-SNAREs (soluble NSF attachment protein receptors) are also key components for vesicle transport and membrane fusion events. Munc-18 interacts with syntaxin 1A, which is one of the t-SNAREs, and prevents the association of
Figure 1.2 Regulation of synaptic vesicle cycles by Cdk5. Substrates for Cdk5 consist of three proteins involved in synaptic vesicle exocytosis and three ones in endocytosis. SV, synaptic vesicle.
syntaxin 1A with v-SNAREs. It has been reported that phosphorylation of Munc-18 at threonine-574 by active Cdk5 disassembles Munc-18/syntaxin 1A complex, thus allowing the association of syntaxin 1A and v-SNAREs, facilitating fusion of synaptic vesicles. Therefore, Cdk5 phosphorylates Munc-18 and leads to enhanced synaptic vesicle fusion to the presynaptic membrane and neurotransmitter release (Fletcher et al., 1999; Shuang et al., 1998).

On the contrary, Cdk5 activity can negatively regulate the efficiency of neurotransmitter release by phosphorylating the α1A isoform of P/Q type voltage-dependent calcium channel (VDCC). It is demonstrated that phosphorylation of VDCC by Cdk5 results in dissociation of VDCC from SNAREs, leading to reduced activity of VDCC and Ca\(^{2+}\) influx, thereby attenuating neurotransmitter release (Tomizawa et al., 2002).

Another synaptic vesicle-associated protein, synapsin I, is also identified as a substrate of active Cdk5. Synapsin I mediates the tethering of synaptic vesicles with the cytoskeleton. It is demonstrated to be phosphorylated by Cdk5 at serine-551 and serine-553 (Matsubara et al., 1996). However, the functional effect of synapsin I upon phosphorylation remains unclear. Taken together, these studies suggest that Cdk5 may positively or negatively regulate neurotransmitter release through phosphorylation of different substrates.

**1.1.4.3 Modulation of synaptic vesicle endocytosis**

Neurotransmitter release is followed rapidly by endocytosis of synaptic
vesicles, which is necessary for recycling synaptic vesicles at the nerve terminals. During the high rates of neurotransmitter release, the nerve terminals maintain a relatively constant surface area by endocytosis (Fon and Edwards, 2001). Synaptic vesicle endocytosis is required for maintaining the small pool of synaptic vesicles within nerve terminals after exocytosis and is thus important for sustaining synaptic transmission. It is activated by a calcineurin-mediated dephosphorylation event that is stimulated by depolarization-dependent calcium influx. In resting nerve terminal, dephosphins, a family of pre-synaptic proteins whose dephosphorylation is essential for synaptic vesicle endocytosis, are constitutively phosphorylated. Upon stimulation, dephosphins are dephosphorylated and activated by calcineurin upon each round of calcium influx. Dephosphins are subsequently rephosphorylated by protein kinases to maintain continual synaptic vesicles endocytosis (Liu et al., 1994; Marks and McMahon, 1998; Cousin, 2000; Cousin and Robinson, 2000; Cousin et al., 2001; Cousin and Robinson, 2001; Tan et al., 2003). Dephosphins involved in the process of synaptic vesicle endocytosis include dynamin I, amphiphysin I, synaptopjanin I and endophilin (Cremona and De Camilli, 1997).

Some dephosphins are recently shown to be phosphorylated by Cdk5. Amphiphysin I, for example, interacts with p35 and can be phosphorylated by Cdk5 at serine-272, serine-276 and serine-285 (Floyd et al., 2001; Rosales et al., 2000). Dynamin I, on the other hand, is phosphorylated by Cdk5 at serine-774 and serine-778. Inhibition of Cdk5 by pharmacological inhibitor or expression of dominant negative Cdk5 blocks both rephosphorylation of dynamin I and repetitive
rounds of synaptic vesicle endocytosis in nerve terminals, suggesting that Cdk5 functions as a dephosphin kinase and has essential role in synaptic vesicle endocytosis (Tan et al., 2003). However, another report claims that Cdk5 negatively regulates synaptic vesicle endocytosis via cophosphorylation of amphiphysin I and dynamin I (Tomizawa et al., 2003). The two apparently contradictory findings were later reconciled by a model proposed by Nguyen and Bibb, who hypothesized that Cdk5 may inhibit the first round of endocytosis, but is necessary for the second round (Nguyen and Bibb, 2003). On the other hand, Cdk5 has also been observed to regulate clathrin-uncoating of synaptic vesicles. The inositol 5-phosphatase activity of a dephosphin synaptojanin 1 is crucial for clathrin uncoating of synaptic vesicles. Cdk5 has been observed to phosphorylate synaptojanin 1 at serine-1144 to inhibit the interaction of synaptojanin 1 with endophilin 1, thereby inhibiting the phosphatase activity of synaptojanin 1 and hinders clathrin uncoating (Lee et al., 2004).

1.1.4.4 Modulation of receptor signaling at synapse

Reception of information across synapses relies on the precise clustering of different receptors on the postsynaptic neurons for ligand activation and signal transduction. Accumulating evidence reveals that Cdk5 also modulates the signaling of various receptor including the NMDA receptor, dopamine receptor, ErbB receptor and cadherin-mediated signaling at synapse (Li et al., 2001; Bibb et al., 1999; Liu et al., 2001; Nishi et al., 2001; Fu et al., 2001; Fu et al., 2004).

For example, Cdk5 has been observed to phosphorylate NR2A subunit of
NMDA receptors at serine-1232 both in vitro and in vivo. Cdk5 associates with NR2A and PSD-95 in the brain, and phosphorylation of NR2A is reduced in Cdk5 null mice. In addition, the induction of LTP (long-term potentiation) in the CA1 of hippocampus is abolished upon inhibition of Cdk5 by roscovitine which is an inhibitor of Cdk5 (Li et al., 2001). These observations collectively implicate Cdk5 in the regulation of NMDA receptors activation and signal transduction.

In addition to directly phosphorylating neurotransmitter receptors, Cdk5 has also been demonstrated to modulate clustering of postsynaptic receptors through phosphorylation of scaffold proteins. PSD-95, a key scaffold protein in CNS synapses, plays crucial role in the organization and localization of postsynaptic proteins in the CNS. Interestingly, Cdk5 was recently demonstrated to phosphorylate PSD-95. Phosphorylation of PSD-95 by Cdk5 was found to suppress multimerization of PSD-95, thereby reducing the ability of PSD-95 to cluster NMDA receptor and shaker type K⁺ channels. In accordance with this observation, synaptic PSD-95 clusters are enlarged in Cdk5⁻/⁻ cortical neurons, indicating that Cdk5 is involved in the regulation of PSD-95 clustering in vivo (Morabito et al., 2004).

DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein with molecular mass 32 kDa) is a phosphoprotein critical in modulating neurotransmission and dopamine signaling in the striatum. Stimulation of dopamine receptors leads to activation of PKA (cAMP-dependent protein kinase), which phosphorylates DAPRR-32 at serine-34, turning DAPRR-32 into an inhibitor of PP1 to enable downstream activation of PKA signaling pathways. Interestingly, Cdk5 was
recently found to phosphorylate DARPP-32 at threonine-75, turning DARPP-32 into an inhibitor of PKA, which in turn attenuates the phosphorylation of PKA substrates such as PP1 (protein phosphatase-1) to dampen dopamine signaling (Bibb et al., 1999; Liu et al., 2001; Nishi et al., 2002). It is therefore possible that Cdk5 is involved in the modulation of synaptic transmission through the phosphorylation of DARPP-32 and the subsequent regulation of PKA activity (Greengard et al., 1999; Hemmings et al., 1984).

1.1.4.5 Modulation of gene transcription

Finally, Cdk5 has also been suggested to regulate gene transcription at the NMJ via phosphorylation of transcription factor STAT3 and mSin3-histone deacetylase (HDAC) complex component mSds3. In addition to the modulation of ErbB receptor, Cdk5 regulates the activity of a transcription factor activated downstream of NRG stimulation, STAT3 (signal transducer and activator of transcription 3). Cdk5/p35 complex associates with STAT3 and phosphorylates STAT3 on serine-727 in vitro and in vivo. The serine phosphorylation of STAT3 is absent in embryonic brain and muscle of Cdk5-deficient mice. Interestingly, treatment of cultured myotubes with neuregulin enhances the serine phosphorylation of STAT3 and transcription of STAT3 target genes, such as c-fos and junB, in a Cdk5-dependent manner. However, both the DNA-binding activity of STAT3 and the transcription of specific target genes are reduced in Cdk5-deficient muscle. Therefore, Cdk5 also plays a physiological role in regulating STAT3 phosphorylation and
modulation of its transcriptional activity (Fu et al., 2004).

mSds3, an essential component of the mSin3-histone deacetylase (HDAC) co-repressor complex, is also identified as a substrate of the Cdk5/p35 complex and represents a new regulatory mechanism through which Cdk5 affects gene transcription. mSds3 binds to p35 both in vitro and in vivo, enabling active Cdk5 to phosphorylate mSds3 at serine-228. Notably, overexpression of p35 enhances the mSds3-mediated transcriptional repression in vitro. The ability of exogenous mSds3 to rescue cell growth and viability in mSds3 null mice correlates with its ability to be phosphorylated by Cdk5 (Li et al., 2004).

1.1.5 Regulation of kinase activity by Cdk5

In addition to the multiple functions discussed above, Cdk5 also regulates the activity of some kinases. A good example is the PI3K/Akt kinase signaling pathway. It is reported that Cdk5 regulates Akt activity through phosphorylation of the neuregulin receptors (ErbB2/ErbB3) and regulation of PI3K/Akt kinase signaling pathways. In the brain extracts and cortical neurons of Cdk5 knockout mice, reduced PI3K/Akt activity is detected (Li et al., 2003).

Another example is Pctaire1 which is a cdk-related kinase, as mentioned previously. It has been recently reported that Pctaire1 can be phosphorylated by Cdk5/p25 complex, and serine-95 is the major phosphorylation site. In brain and muscle of Cdk5 null mice, Pctaire1 activity is significantly reduced. Moreover, Pctaire1 activity is enhanced after phosphorylation by Cdk5/p25 complex (Cheng et
1.2 Pctaire1

1.2.1 A member of the Cdk-related kinase family

Pctaire1 was originally identified as a Cdc2-like kinase (Meyerson et al., 1992; Okuda et al., 1992). Previous work in our laboratory identifies Pctaire1 as a p35-interacting protein and a substrate of Cdk5. Although it has been previously reported that Cdk5-mediated phosphorylation of Pctaire1 enhances the kinase activity of Pctaire1, the precise functional significance of this interaction, and the biological function of Pctaire1 remains unknown (Cheng et al., 2002).

Pctaire1 and its related proteins, Pctaire2 and 3, are 50-60 kDa proteins consisting of a core kinase domain flanked by unique N-terminal and C-terminal domains. The catalytic domain of Pctaire proteins contains the motifs and amino acid residues that are conserved in the Cdk family (Meyerson et al., 1992). It has been suggested that the N-terminal region of Pctaire defines the specific function of the particular Pctaire family member (Hirose et al., 2000). Pctaire family is designated based on the presence of a cysteine-for-serine substitution in the PSTAIR E amino acid sequence that is conserved in the Cdks identified earlier. Three distinct Pctaire isoforms, Pctaire1, 2 and 3, exist in higher organisms, each of which is characterized by the PCTAIR E sequence (Okuda et al., 1992). Two of the isoforms are also present in rodents, and one in Dictyostelium (Okuda et al., 1992; Gao et al., 1996; Michaelis and Weeks, 1993). Each isoform shows high homology over the
protein kinase domain with less homology in N- and C-terminal extensions. They have not been found to bind with any cyclin but associate with other cellular factors which regulate Pctaire activity (Graeser et al., 2002; Charrasse et al., 1999).

The best characterized Pctaire family member is Pctaire1. The Pctaire1 gene (PCTK1) has been mapped to human chromosome Xp, close to the ubiquitin-activating enzyme E1 gene (UBE1; Knight et al., 1995; Okuda et al., 1994). UBE1 and Pctaire1 escape X inactivation in human but not in mouse (Carrel et al., 1996). Moreover, UBE1 and Pctaire1 are mapped to Xp21.2-p11.2, a region known to be involved in several retinal and X-linked mental retardation diseases (Lubs et al., 1996; Rosenfeld et al., 1994).

The primary structure of human Pctaire1 contains three regions, a 295-amino acid core kinase region with 52% sequence identity to that of Cdc2, a 161-amino acid N-terminal extension, and a 40-amino acid C-terminal extension (Meyerson et al., 1992). The amino acids involved in the regulation of Cdk activity by phosphorylation are highly conserved in Pctaire1 (Le Bouffant et al., 2000).

### 1.2.2 Pctaire1 expression

Pctaire1 is ubiquitously expressed in various tissues. Two transcripts, 3.0 kb and 2.2 kb respectively, can be identified by Northern blot analysis in human and murine tissues as well as a number of cell lines (Meyerson et al., 1992; Okuda et al., 1992; Gao et al., 1996). Prominent expression of Pctaire1 is found in highly differentiated tissues including brain, muscle, heart, testis and ovary. The 3.0 kb
transcript corresponds to the predominantly expressed transcript, except in the testis, while the 2.2 kb transcript represents the minor form (Meyerson et al., 1992; Okuda et al., 1992). *In vitro* translated Pctaire1 yields two polypeptide products of 60 and 65 kDa (Okuda et al., 1992), while in rodent tissue, two protein bands with very similar molecular masses (62 and 68 kDa) for Pctaire1 immunoreactivity are reported (Besset et al., 1999; Le Bouffant et al., 1998; Rhee and Wolgemuth, 1995). Pctaire1 immunoreactivity and mRNA are present at high levels in terminally differentiated germ cells (Rhee and Wolgemuth, 1995). In addition, Pctaire1 protein is present in the cytoplasm of Swiss 3T3 cells transfected with Pctiare1 throughout the cell cycle (Charrasse et al., 1999). Interestingly, with the use of immunohistochemical staining, two distinct staining patterns are detected for Pctaire1 in the brain. Firstly, a diffuse labelling in almost all of the brain regions, particularly intense in the molecular layer of cerebellum and the mossy fiber region of hippocampus is detected. Secondly, a spot-like localization is also evident in the nuclei of large neurons such as cerebellar Purkinje cells and the pyramidal cells of hippocampus (Le Bouffant et al., 2000).

### 1.2.3 Pctaire1 activity

The regulation of Pctaire1 remains a mystery. Conflicting data exist as to whether Pctaire activity is regulated in a cell cycle-dependent manner. Although one report indicates that Pctaire1 activity is cell cycle-dependent and displays a peak in the S and G2 phases (Charrasse et al., 1999), other studies suggest that Pctaire1 is not involved in the regulation of cell cycle control (Meyerson et al., 1992; Rhee and
Wolgemuth, 1995). Large scale array-based screening of cell cycle regulated genes reveals no change in Pctaire expression, although it is unknown whether there is regulation of its activity (Whitfield et al., 2002).

Purified recombinant protein Pctaire1 does not exhibit any kinase activity, but it gains activity after preincubation with fibroblast extract (Charrasse et al., 1999), indicating the presence of Pctaire1-activating machinery in the fibroblast extract. Pctaire1 activity can be detected in adult testis and brain, and the testicular Pctaire1 activity is reduced in high-salt conditions, suggesting that it may require a regulatory partner for its activation (Besson et al., 1999; Le Bouffant et al., 1998). It is suggested that Pctaire1 has an activator, like p35 for Cdk5 and cyclins for other Cdns, but the activator has not been identified to date. A recent report raises the possibility that Pctaire1 activation requires phosphorylation rather than association with an activator, based on the evidence that monomeric Pctaire1 exhibits activity (Graeser et al., 2002). In addition to the lack of activator binding, interaction of Pctaire1 with cdk inhibitors has not been reported, suggesting that the mechanisms by which Pctaire1 activity is regulated may differ from other Cdns. Whether the N-terminal and/or C-terminal extension of Pctaire1 may substitute for the effect of regulatory proteins on the activity of the kinase activity remains to be explored (Charrasse et al., 1999).

Although the mechanisms by which Pctaire1 is activated await further investigation, recent studies revealed that the activity of Pctaire1 could be modified through phosphorylation by different kinases (Fig. 1.3).
Figure 1.3 Regulation of Pctaire1 activity by phosphorylation. Phosphorylation of serine-153 on Pctaire1 by PKA results in an inhibition of its activity. Active Cdk5 phosphorylates Pctaire1 on serine-95 enhances its kinase activity.
It has been recently reported that Ptaire1 is phosphorylated by Cdk5/p25 complex, and serine-95 is the major phosphorylation site. In brain and muscle of Cdk5 null mice, Ptaire1 activity is significantly reduced. Moreover, Ptaire1 activity is enhanced after phosphorylation by Cdk5/p25 complex (Cheng et al., 2002). In another study, Ptaire1 was identified as a substrate of PKA. Phosphorylation of Ptaire1 at serine-153 by PKA inhibits the kinase activity of Ptaire1 (Graeser et al., 2002). These observations highlight phosphorylation of Ptaire1 as an important regulatory mechanism of its kinase activity.

1.2.4 Ptaire1 function

Although Ptaire1 has been identified for a decade, precise functions of this enzyme remain enigmatic. Majority of the studies for Ptaire1 have focused on its spatial and temporal expressions, which helps to speculate the potential functional roles of this enzyme. For example, Ptaire1 was detected in differentiated cells such as post-mitotic neurons and spermatogenic cells (Besset et al., 1999). In addition, Ptaire1 was expressed in the nucleus of large neurons and also in regions containing important granule cell projections (Le Bouffant et al., 2000). The localization of Ptaire1 to these regions suggests that Ptaire1 may exhibit a functional role in these cells or subcellular compartments.

Despite the scarcity of studies on the functions of Ptaire1, recent reports have associated Ptaire1 activity to several biological functions. For example, an earlier report revealed that the kinase activity of Ptaire1 is cell cycle dependent,
where maximal activity is detected when cells transit through S phase, indicating that Pctaire1 might be related to S phase transition (Charrasse et al., 1999). In addition, recent evidence reveals that Pctaire1 is potentially involved in neurite outgrowth and membrane traffic (Graeser et al., 2002; Palmer et al., 2005). Neuro-2A cells overexpressing the kinase-dead mutant of Pctaire1 (K194R) was observed to exhibit longer neurites compared to those overexpressing wild type enzyme, suggesting that Pctaire1 is potentially involved in controlling neurite outgrowth (Graeser et al., 2002). Furthermore, a recent study by Palmer and his colleagues identified Pctaire3, as well as Pctaire1, as human sec23Ap interacting protein using yeast two-hybrid screening (Palmer et al., 2005). Sec23Ap is one of the subunits of the COPII complex, which mediates the accumulation of secretory cargo into ER exit sites (Barlowe et al., 2002; Barlowe et al., 1994). The process of cargo export from the ER is largely constitutive but there is now increasing evidence for regulation of this process by protein phosphorylation. Inhibition of Pctaire1 activity by overexpression of a kinase dead mutant, or specific depletion of Pctaire1 with RNAi, results in defects in early secretory pathway function including cargo transport, vesicle-tubular transport carrier and Golgi localization. These observations suggest a functional role of Pctaire1 in membrane traffic through the early secretory pathway (Palmer et al., 2005).

1.2.5 Pctaire1-interacting proteins

To explore the potential role of Pctaire1, different groups have attempted to
identify Pctaire1-interacting proteins. Screening of a mouse brain cDNA library with
the yeast two-hybrid system leads to the identification of the annexin II-interacting
protein p11 and three isoforms of 14-3-3 proteins as Pctaire1-interacting proteins
(Sladeczek et al., 1997). Full interaction of Pctaire1 with p11 and 14-3-3 proteins
requires both the N-terminal and C-terminal ends of Pctaire1 (Le Bouffant et al.,
1998). However, the highly conserved kinase region of Pctaire1 is unable to interact
with 14-3-3 proteins in yeast system. In accordance with this, no direct association
between 14-3-3 and classical Cdk5 has been reported. 14-3-3 proteins are known to
associate with many other proteins, often kinases and have been suggested to play
important roles in the regulation of signal transduction (Ichimura et al., 1997; Aitken
1996; Liu et al., 1996; Zha et al., 1996; Crapo et al., 1997). For example, the
binding of 14-3-3 with Cdc25 phosphatase and Wee1 kinase is suggested to affect the
phosphorylation state of tyrosine-15 in Cdc2 and other Cdk5 (Conklin et al., 1995;
Honda et al., 1997). Although 14-3-3 protein can be used to purify Pctaire1 protein
by affinity chromatography, the physiological relevance of the interaction remained
to be studied (Le Bouffant et al., 1998). p11 proteins, on the other hand, belongs to a
family of small dimeric proteins that share sequence similarities with S100 (Kligman
and Hilt, 1988). Despite the observed interaction between Pctaire1 and p11 in yeast,
the functional significance of their association is nonetheless far from clear. Further
studies will be required to characterize the potential function of this interaction.

By screening the brain library with the N-terminus of Pctaire2 as bait, Trap
(tudor repeat associator with Pctaire2) has also been identified to interact with
Pctaire2, as well as Pctaire1, but not Pctaire3. Trap is abundantly expressed in brain and testis, where active Pctaire1 exists. Therefore, it is likely that Trap can act as a partner of Pctaire1 in brain as well as in testis (Hirose et al., 2000). However, the physiological significance of this interaction remains to be explored.

As described above, previous findings from our laboratory identify Pctaire1 as a p35-interacting protein in muscle using yeast two-hybrid screen, and the interaction has been confirmed both in vitro and in vivo. Pctaire1 is associated with p35 in cultured myotubes and skeletal muscle, and is concentrated at the neuromuscular junction (Cheng et al., 2002).

Finally, Palmer et al. have recently found that the human sec23p subunit of COPII interacts with Pctaire3 and Pctaire1 in yeast two-hybrid screen. This interaction is also confirmed by direct binding assay and immunoprecipitation (Palmer et al., 2005).

1.3 The molecular machinery of exocytosis

Although the precise biological roles of Pctaire1 is far from clear, recent identification of Pctaire1 as an interacting partner of the human sec23Ap of COPII suggests that Pctaire1 may be involved in membrane traffic through the early secretory pathway (Palmer et al., 2005). In light of the increasing implication of Cdk5 in the regulation of neurotransmitter release, and the importance of Cdk5 in the regulation of Pctaire1 activity in vivo, it is therefore tempting to speculate that Pctaire1 may play a functional role in membrane traffic or exocytosis during
secretion.

The highly organized eukaryotic cells contain many membrane-enclosed intracellular organelles/compartment which require precise mechanisms to govern protein trafficking between different organelles, particularly in the secretory and endocytic pathways. Small shuttling transport vesicles or larger transport containers are the major intermediates in anterograde or retrograde transfer of cargo molecules between various organelles of the secretory and endocytic pathways. According to the vesicular transport hypothesis, vesicles bud from a donor compartment (vesicle budding) by a process that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment (protein sorting). The vesicles are subsequently targeted to a specific acceptor compartment (vesicle targeting), which then unload their cargo upon docking and fusion with the target compartment. All of these steps are tightly organized and regulated. In this way, a large quantity of cargo can smoothly flow through the secretory pathway (Reviewed in Bonifacino and Glick, 2004).

It is interesting to note that all intracellular membrane fusion events use the same basic protein machinery that is highly conserved in organisms ranging from yeast to human (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). NSF (N-ethylmaleimide sensitive fusion protein), a soluble protein required for membrane fusion in the mammalian Golgi in vitro, is the ortholog of Sec18 which is a budding yeast protein essential for membrane fusion in vivo (Wilson et al., 1989). The synaptic SNAREs (soluble NSF attachment protein receptors) and yeast proteins are
highly homologous and are involved in various membrane trafficking steps. In addition, α-SNAP (soluble NSF attachment protein) is the ortholog of yeast Sec17 (Rothman, 1994). The presence of highly homologous components in the membrane fusion machinery in yeast and mammalian cells further strengthens the idea that the same basic membrane fusion machinery is conserved through evolution.

At the plasma membrane, vesicular transport either occurs constitutively or in a regulated manner, known as constitutive exocytosis and regulated exocytosis respectively. Both constitutive and regulated exocytosis utilize the same basic core components for membrane fusion. Identification of different proteins involved in membrane fusion events leads to the SNARE hypothesis (Rothman and Warren, 1994; Scheller, 1995; Sudhof, 1995), which identifies four key components in exocytosis: a vesicle membrane protein named v-SNARE, a target membrane protein named t-SNARE, a cytosolic protein NSF required for membrane fusion, and finally, adaptors for NSF termed SNAPs.

1.3.1 Constitutive exocytosis

In all eukaryotic cells, vesicles are constitutively budding from the trans Golgi network, which eventually fuse with the plasma membrane. The constitutive exocytosis operates continually and supplies newly made lipids and proteins to the plasma membrane. This is the mechanism by which the plasma membrane increases in length, which is essential for the enlargement of cells before dividing. Moreover, some proteins are carried to the cell surface and released from the cell via the
constitutive exocytosis route.

During constitutive exocytosis, no signals are required for the entry into this nonselective secretion pathway. It is therefore sometimes referred to as the default pathway. Since secreted proteins do not aggregate in the cytosol or at the plasma membrane during constitutive secretion, proteins secreted in this manner are carried without delay by the transport vesicles to the plasma membrane. Thus, the release of secretory products via the constitutive exocytosis route occurs at a constant rate. Among the constitutively secreted pathway, newly synthesized soluble proteins and lipids adhere to the cell surface and become part of the plasma membrane. Some of the released proteins are incorporated into the extracellular matrix; while others diffuse into the extracellular compartment to signal other cells (Alberts et al., 2004).

1.3.2 Regulated exocytosis

Regulated exocytosis, on the other hand, is tightly controlled by specific signals and occurs in a spatially restricted manner only at distinct areas of the plasma membrane (Burgess and Kelly, 1987). Under resting condition, specialized secretory cells produce large quantities of products stored in secretory vesicles which are accumulated in the cytosol or dock at the plasma membrane or both. These secretory vesicles are highly enriched in one or a few distinct classes of cargo molecules, such as neurotransmitters, insulin, digestive enzymes, cell surface receptors or specific transporters. The accumulation of secretory vesicles enables the release of high amounts of cargo molecules in a relatively short time upon stimulation of specific
signals. The release of these secretory molecules permits intercellular communication at a distance by endocrine hormones, locally by paracrine mediators, or at a short range by neurotransmitters. Thus, regulated exocytosis is crucial in the regulation of development, maintenance of tissues, signals transmission through the organism and information processing and storage (Reviewed in Sollner, 2003).

Regulated exocytosis is relatively complex and requires multiple steps including recruitment of secretory vesicle to the plasma membrane, specific tethering at the plasma membrane, fusion machinery priming, and triggered membrane fusion (Reviewed in Sollner, 2003). In the case of synaptic vesicle, a pre-docked pool of synaptic vesicles in close proximity to calcium channels and a pre-assembled fusion machinery ensure a rapid response time. According to the “kiss-and-run” model of exocytosis and endocytosis, the transient opening and closure of the fusion pore enables the synaptic vesicles to undergo fast and efficient recycling following fusion with the plasmalemma, without intermixing with the plasma membranes (Valtorta et al., 2001). A good example of regulated exocytosis present at the synapse is the release of neurotransmitters.

1.3.3 Neurotransmitter release

The synapse has served as an excellent model for the study of regulated exocytosis due to the presence of large number of homogenous vesicels that can be signaled to undergo exocytosis in a synchronized fashion. Communication among different neurons and the target cells is mediated by the regulated secretion of
neurotransmitters. Indeed, precise neurotransmission underlies almost all functions of the nervous system. From sensory perception to learning and memory, regulation of neurotransmitter release has monumental role on the preservation of proper functioning of the nervous systems.

Signals in the nervous system are propagated from the presynaptic neuron to the postsynaptic cells. At the resting state, some synaptic vesicles are docked at the active zone. When an impulse reaches the presynaptic nerve terminal, the action potential induces the opening of Ca\(^{2+}\) channels. The resulting Ca\(^{2+}\) influx triggers the stored synaptic vesicles to move to the plasma membrane, fuse with it, and release the luminal neurotransmitter into the synaptic cleft. Such Ca\(^{2+}\) regulated neurotransmitter release requires specific modification of the basic exocytosis machinery to accommodate the unique features of synaptic vesicle fusion: docked vesicles must be prevented from undergoing immediate fusion and the fusion machinery must respond very rapidly to the Ca\(^{2+}\) influx. Subsequent to exocytosis of the synaptic vesicles, the neurotransmitters act on specific receptors at the postsynaptic membrane. The synaptic vesicle proteins are then retrieved from the presynaptic membrane via endocytosis, where the endocytotic vesicles are refilled with neurotransmitters by associated membrane channels and pathway (Goda and Sudhof, 1997).

Neurotransmitter release and vesicle pool dynamics are highly regulated by a series of molecular interactions among vesicular, plasma membrane and cytosolic proteins (Sudhof, 1995). As shown in Fig. 1.4, in synaptic vesicle exocytosis,
Figure 1.4 Model for regulated exocytosis in the neuronal synapse. Major steps and the participating components are indicated in the figure.
the three SNAREs VAMP2 (synaptobrevin), synaptatin 1 and SNAP-25 form a four helical bundle aligned in a parallel fashion which pulls the synaptic vesicle membrane and target membrane together in a "zipper"-like fashion (Hanson et al., 1997a; Lin and Scheller, 1997; Sutton et al., 1998). Elevated Ca\(^{2+}\) then triggers the conformational or electrostatic change in synaptotagmins which act as the exocytotic Ca\(^{2+}\) sensor and then complete the fusion events (Geppert et al., 1994; Fernandez-Chacon et al., 2001; Chapman, 2002). After fusion, α-SNAP and NSF are recruited from the cytoplasm and subsequent ATP hydrolysis by NSF causes dissociation of the SNARE complex. In this way, individual SNAREs are ready for recycling and a new round of transport (Chen et al., 2001; Chen and Scheller, 2001; Leenders and Sheng, 2005).

1.3.4 Regulation of neurotransmitter release

Over the past decade, significant progress has been made in identifying protein-protein interactions of presynaptic proteins and their contribution to the modulation and regulation of vesicular release (Reviewed in Sudhof, 1995; Chen and Scheller, 2001; Li and Chin, 2003; Murthy and De Camilli, 2003; Leenders and Sheng, 2005; Li and Chin, 2003). Except for the highly conserved components of release machinery, synaptic vesicular release requires several unique components, including synaptotagmin, Munc 13, RIM and complexin (Li and Chin, 2003). A good example is depicted in Fig. 1.4. Active zone proteins Munc 13, Munc 18 and RIM (Rab 3 interacting molecule) have been identified as important factors in synaptic
vesicle priming steps (Augustin et al., 1999; Verhage et al., 2000; Schoch et al., 2002).

The alteration of the intracellular level of second messenger is required for stimulation and regulation of synaptic vesicles release (Bliss and Collingridge, 1993; Sudhof, 1995; Martinez and Derrick, 1996). Short-term synaptic plasticity is due to changes in intracellular calcium concentration (Zucker and Regehr, 2002). The short-term process occurs very rapidly and is probably achieved by calcium binding to a high-affinity calcium-binding protein. In contrast, slower changes lasting from seconds to minutes are achieved by activation of Ca\(^{2+}\)-dependent kinases. For instance, CaMKII is able to phosphorylate synapsin and thereby regulates availability of synaptic vesicles for release (De Camilli and Jahn, 1990; Leenders et al, 2005).

Many studies indicate that the regulation of synaptic secretion is controlled by protein phosphorylation (Micheau and Riedel, 1999). Protein kinases identified in modulation of synaptic secretion include CaMKII, CKII, MAPK, PKA, PKC and Cdk5 (Blanquet, 2000; Fischer et al., 2003; Lisman et al., 2002; Nguyen and Woo, 2003; Thomas and Huganir, 2004; Van der Zee and Douma, 1997). All of the protein kinases are localized in the presynaptic compartment. Interestingly, a number of presynaptic proteins involved in synaptic vesicle release and recycling are reported as *in vitro* substrates of the above kinases (Greengard et al., 1993; Turner et al., 1999; Leenders and Sheng, 2005), implicating them as pivotal factors in synaptic secretion.

1.4 *N-ethylmaleimide sensitive fusion protein*
As an attempt to explicate the functions of Pctaire1, a yeast two-hybrid screen was performed to identify interacting protein for Pctaire1. Interestingly, NSF was identified as a novel interacting partner for Pctaire1. As a crucial factor in exocytosis machinery and the target protein in our proteomic study, NSF and its regulation become the focus of our study.

1.4.1 Introduction

N-ethylmaleimide sensitive fusion protein, also known as N-ethylmaleimide sensitive factor (NSF), is a member of AAA (ATPase associated with cellular activities) family and was initially identified by Block and collaborators in James Rothman’s group at Stanford University (Beckers et al., 1989; Block et al., 1988). It was originally found to be the protein that can restore the ability of Golgi membranes to engage in vesicular transport, following inactivation by sulphydryl modifying reagent, NEM (Glick and Rothman, 1987).

NSF is the first protein isolated as a crucial factor in intracellular membrane-fusion events, such as the fusion of synaptic vesicles with the presynaptic membrane during neurotransmission. The first model of NSF action is that it would act in concert with “soluble NSF attachment proteins” (SNAPs) on transmembrane SNAP receptors (SNAREs) to form a “20S fusion complex”, bridging the membranes destined to fuse (Wilson et al., 1992). The 20S complex would be dissociated by ATP hydrolysis via NSF, and the hydrolysis energy would also drive membrane fusion (Sollner et al., 1993b). Later, it became clear that, at least in some
systems, NSF could act long before the actual fusion takes place (Mayer et al., 1996; Colombo et al., 1996), raising the question of whether NSF is really a protein directly involved in membrane fusion. Instead, it is suggested that NSF and SNAP act as molecular chaperones that change the conformation of SNAREs at the expense of ATP so that they can facilitate subsequent membrane fusion events (Morgan and Burgoyne, 1995). This process would dissociate SNAREs that are tightly bound to each other on the same membrane (Unger mann et al., 1998a; Unger mann et al., 1998b) so that they can bind to different SNAREs on the partner membrane and promote fusion (Gotte and von Mollard, 1998). However, the mechanism by which NSF uses ATP hydrolysis to disassemble the stable coiled-coil interactions of the SNARE complex remains enigmatic.

### 1.4.2 Functional domains of NSF

NSF was previously suggested to contain four 76-kDa subunits (Block et al., 1988). Recently, NSF was demonstrated to exist as a hexameric complex (Fleming et al., 1998; Lenzen et al., 1998; Yu et al., 1998). Analysis of NSF structure by electron microscopy suggests that NSF is a hexagonal cylinder similar in size to related ATPase thought to be hexamers in solution (Hanson et al., 1997b; Parsell et al., 1994; Peters et al., 1992; Beyer, 1997; Kessel et al., 1995; Zhang et al., 1994; Pamnani et al., 1997). Furthermore, using a variety of quantitative analytical methods, active NSF was clearly demonstrated to exist in a hexameric conformation (Fleming et al., 1998). Sedimentation equilibrium and sedimentation velocity analytical
ultracentrifugation, transmission electron microscopy with rotational image analysis, scanning transmission electron microscopy, and multiangle light scattering all demonstrate that, in the presence of nucleotide, NSF is predominantly a hexamer. This finding highlights structural similarities among NSF and several related ATPases which act by switching the conformational states of substrate proteins in order to activate them for subsequent reactions (Fleming et al., 1998).

Sequence analysis and proteolytic dissection reveal that each protomer of NSF contains three domains: N-terminal domain (residues 1-205), and two homologous ATP-binding domains NSF-D1 (residues 206-488) and NSF-D2 (residues 489-744) (Tagaya et al., 1993). Each domain is connected by a protease sensitive linker region and makes a unique contribution to the overall activity of NSF (Tagaya et al., 1993).

NSF-N is responsible for the interaction with α-SNAP-SNARE complex (Tagaya et al., 1993). Truncation mutant of NSF lacking the N-terminal domain are hexameric and exhibit ATPase activity, but could not bind to the SNAP-SNARE complex and fail to promote vesicular transport in vitro (Nagiec et al., 1995). Nonetheless, the monomeric N domain is not sufficient for binding to the SNAP-SNARE complex. Binding only occurs when monomeric N domain is adjacent to the D1 domain or when it is in a trimeric form (Nagiec et al., 1995).

The two ATP-binding domains contain a 230-250 amino acids motif that is the hallmark of the AAA family (Patel and Latterich, 1998). NSF-D1 is an active ATPase that provides the driving force for SNARE complex disassembly. Indeed,
compared with the D2 domain, D1 domain accounts for the majority of the basal ATPase activity of NSF. Point mutations showed that K266A, a mutation in the P-loop lysine, not only eliminates nucleotide binding to D1 domain but also disrupts NSF activity. In addition, while hydrolysis defective mutant E329Q binds to the SNAP-SNARE complex, it fails to mediate their disassembly (Nagiec et al., 1995; Whiteheart et al., 1994; Whiteheart and Matveeva, 2004). These observations indicate that association of NSF with α-SNAP-SNARE complex via the D1 domain and the hydrolysis activity of NSF are both crucial for its disassembly of the SNAP-SNARE complex. In addition, it was recently observed that NSF-D1 must first binds ATP in order to interact with the α-SNAP-SNARE complex. Association of NSF with the complex will then stimulates the ATPase activity of NSF to trigger disassembly of the α–SNAP-SNARE complex (Steel and Morgan, 1998).

NSF-D2, on the other hand, is responsible for maintaining NSF as a hexamer (Fleming et al., 1998; Nagiec et al., 1995). It has a higher affinity to ATP than NSF-D1 but contains no significant ATPase activity (Matveeva et al., 1997; Nagiec et al., 1995; Whiteheart et al., 1994). K549A, a point mutatation of a D2 P-loop lysine, decreases the vesicular transport activity of NSF, but does not completely eliminate it (Nagiec et al., 1995). The nucleotide binding by NSF-D2 is, however, important for hexamerization. Moreover, the D2 structure helps us to understand the chaperone function of NSF in that this module is related to ATPase modules present in many other chaperones and chaperone-like proteins. These include members of AAA family whose activities are related to transcription, signal transduction, cytoskeletal
interactions, cell cycle control, mitotic spindle formation, assembly of mitochondrial membrane proteins, peroxisome biogenesis and protein degradation by the 26S proteasome. The NSF-D2 module is also related to DNA polymerase clamp-loader subunits, as demonstrated both by structural comparison of D2 with the E.coli DNA polymerase III δsubunit and by sequence analysis (Lenzen et al., 1998; Guenther et al., 1997; Neuwald, 1999).

1.4.3 Multiple NSF-interacting proteins

It is reported that the proposed stoichiometry of the NSF-SNAP-SNARE complex shows a ratio of 6:3:1 in a neuronal 20S particles with a mass of 788 ± 122 kDa judging by scanning transmission electron microscopy (Fig. 1.5, Wimmer et al., 2001). Although monomeric α-SNAP only binds to NSF when associated with SNAREs (Whiteheart et al., 1992), trimerized α-SNAP has been observed to bind to NSF even in the absence of SNARE complexes in vitro (Wimmer et al., 2001). The different ability of monomeric and trimeric α-SNAP to bind to NSF suggests that the spatial arrangement and perhaps the local density of α-SNAP are critical for stable NSF binding. It has been reported that the C-terminal 45 amino acids of α-SNAP are required for binding and stimulation of NSF ATPase activity (Barnard et al., 1996; Barnard et al., 1997; Morgan et al., 1994). Furthermore, oligomeric NSF N-terminals are able to bind α-SNAP in complex with SNAREs, whereas monomeric NSF N-terminal does not. Therefore, oligomerization of both NSF N-terminal
Figure 1.5 Model of the protein interaction within 20S complex. A SNARE complex binds three α-SNAPs, and this trimerization is sufficient to recruit the NSF hexamer.
and α-SNAP provides a critical driving force for their interaction and the assembly of
the 20S particles (Wimmer et al., 2001).

In addition to its binding to the SNAP-SNARE complexes, NSF also
associates with other proteins and complexes. For example, NSF interacts with both
Rabs and Rab-containing complexes. The Rab family is a large family of small
GTP-binding proteins involved in a number of membrane trafficking events (Zerial
and McBride, 2001). NSF binds Rab6 in the presence of ATP/Mg\(^{2+}\) (hydrolysable
ATP), but not when ATP/EDTA (non-hydrolysable ATP) is added. Interestingly, Rab6
stimulates the ATPase activity of NSF by two folds and co-localizes with NSF on
Golgi complexes in L6 myoblasts. Rab3 and 4, on the other hand, also bind NSF in
the presence of ATP/Mg\(^{2+}\) and stimulate NSF ATPase (Han et al., 2000). In addition,
NSF also binds to a Rab11-containing complex made up of γ-SNAP and Rip11/Gaf-a
(Tani et al., 2003), and complexes that are associated with Rab5 effectors such as
early endosomal autoantigen 1 (EEA-1), Rabaptin-5, Rabex-5, and syntaxin13
(McBride et al., 1999). It therefore appears that NSF may function as a
"Rab-complex chaperone".

In addition to the Rab family, Golgi-associated ATPase enhancer of 16kDa
(GATE-16) also binds to NSF and stimulates ATPase activity of NSF by 3.5-fold
(Sagiv et al., 2000). Finally, low molecular weight activity 1 (LMA1) is proposed to
serve as a SNARE regulator by initially binding to Sec18p/NSF and then being
passed to the vacuolar SNARE, Vam3p, just prior to membrane fusion (Xu et al.,
1998).
NSF was also found to be involved in receptor recycling in the post-synaptic cells. NSF was demonstrated to bind to the C-terminus of GluR2 (GluR2C, Glu834-Ile883) using yeast two-hybrid screening by three different groups. These initial studies indicated that NSF binds to GluR2 and weakly associates with GluR3, while it does not bind to GluR1, 4, 6 or the NMDAR1 subunit. Stable binding is nucleotide-dependent and only occurs in the presence of ATP/EDTA or ATPγS (non-hydrolysable ATP), but not in the presence of ATP. This suggests that NSF may regulate the function of the AMPA receptor at the synapse (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998).

An interaction between NSF and the terminal cytoplasmic domain of the β2-adrenergic receptor (β2AR) is identified by Cong et al. using a similar yeast two-hybrid approach. In this case, the NSF binding site overlaps with the binding site for Na⁺/H⁺ exchanger regulatory factor. This interaction may play a role in receptor recycling (Cong et al., 2001). Interestingly, NSF is also identified as a β-Arrestin-interacting protein. β-Arrestins play important roles in the desensitization of many G-protein coupled receptors. Moreover, NSF appears to play a role in β-arrestin 1-mediated receptor clearance (McDonald et al., 1999).

A recent study revealed that in addition to AMPA receptor subunits and β2-adrenergic receptor, NSF also directly interacts with GABA(A) receptor beta subunit (Goto et al., 2005). The association is confirmed by in vitro protein binding and coimmunoprecipitation assays. Furthermore, these two proteins were observed to co-localize in hippocampal neurons. Findings from this study indicate that NSF can
regulate GABA(A) receptor cell surface expression through binding to the residues 395-415 in the β3 subunit of GABA(A) receptor (Goto et al., 2005).

At present there is no obvious sequence that could be considered as a NSF binding motif. The wide variety of NSF substrates raises many questions as to how these multiple interacting partners are specifically recognized (Reviewed in Whiteheart and Matveeva, 2004). Further explication on the mechanisms by which NSF interacts with these proteins may help provide some much needed answers.

1.4.4 Modification of NSF

Recent studies showed that NSF can be reversibly inactivated by both phosphorylation and S-nitrosylation (Fig. 1.6). Different cell types use distinct post-translational modifications of NSF for localized regulation of membrane fusion (Reviewed in Morgan and Burgoyne, 2004).

The classic example of protein post-translational modification is phosphorylation which controls cellular and intercellular communication. It has been demonstrated that a depolarization-induced, calcium-dependent phosphorylation of NSF is concomitant with neurotransmitter release and requires an influx of external calcium. Indeed, in vitro phosphorylation of NSF by PKC at serine-237 has been demonstrated. Molecular modeling studies suggest that serine-237 is adjacent to an inter-subunit interface at a position where its phosphorylation affects NSF activity.
Figure 1.6 Post-translational modification of NSF. The residues targeted by S-nitrosylation and phosphorylation are indicated.
Consistently, mutation of serine-237 to glutamic acid to mimic phosphorylation leads to a hexameric form of NSF that does not bind to SNAP-SNARE complex (Matveeva et al., 2001).

Besides the serine/threonine phosphorylation, phosphorylation of NSF in vitro by the tyrosine kinase Fes increases intrinsic ATPase activity and prevents α-SNAP binding. Tyrosine-83 is located at the junction between the two N-terminal domains of NSF, and phosphorylation of NSF at tyrosine-83 disrupts the interface between the sub-domains and hence affect α-SNAP binding. The tyrosine phosphatase PTP-MEG2, on the other hand, reduces the phosphotyrosine content of NSF and colocalizes with NSF and syntaxin 6 in intact cell. PTP-MEG2 promotes secretory vesicle fusion through the local release of NSF from a tyrosine-phosphorylated inactive state, indicating that tyrosine phosphorylation/dephosphorylation regulates a dynamic cycle of vesicle fusion (Huynh et al., 2004).

NSF can also be inactivated via S-nitrosylation. The discovery of the gaseous modulator nitric oxide (NO) and NO synthases has revealed an independent, chemically and functionally distinct regulatory system. Redox-based regulation by NO reversibly targets metal-coordinating centers (direct reaction) and thiol-containing proteins (S-nitrosylation) with signature allosteric requirements (Sollner and Sequeira, 2003; Stamler et al., 2001). Reversible inactivation of NSF by S-nitrosylation in vitro as well as in vivo as a major regulator of exocytosis is reported recently (Matsushita et al., 2003). In this study, Weibel-Palade bodies are
rapidly released from endothelial cells that mediate vascular thrombosis and inflammation (Wagner et al., 1993). Exocytosis of Weibel-Palade bodies leads to the release of vWF, which promotes platelet adhesion and aggregation (Ruggeri et al., 1997). Mutagenesis of the nine cysteines in NSF implicated C21, 91 and 264 as likely sites of S-nitrosylation in vitro. C91 and C264 are required for NSF to disassemble v-/t-SNARE complexes, but not necessary for the SNAP-dependent interaction with SNARE complexes. In contrast to the alanine mutants, S-nitrosylation does not affect the basal ATPase activity. Interestingly, the addition of recombinant NSF but not S-nitrosylated NSF to NO-pretreated, permeabilized endothelial cells restores vWF release. This suggests that NO inhibits NSF and disassembly of SNARE complexes by nitrosylating critical cysteine residues of NSF (Matsushita et al., 2003). S-nitrosylation of NSF may therefore serve as an alternative mechanism for the regulation of exocytosis independently of the initial stimulus and signal transduction pathway triggering membrane fusion.

1.4.5 Function of NSF

1.4.5.1 Exocytosis

As mentioned briefly in previous section, sequences analysis reveals that NSF contains two tandem “ATP domains” which places NSF in the AAA superfamily (Confalonieri and Duguet, 1995; Neuwald, 1999). AAA proteins, which contain at least one copy of a conserved ~230-amino acid cassette, are involved in a wide variety of cellular functions, including membrane fusion, proteosome regulation,
transcription, organelle biogenesis, and microtubule transport and regulation (Neuwald, 1999; Vale, 2000). Despite the apparent functional diversity, the ability to assemble or disassemble multisubunit macromolecular complexes, or to fold or unfold polypeptides as molecular chaperones, appears to be common to the family.

Numerous experiments have shown that NSF is involved in almost all heterotypic membrane fusion events that occur in a cell (Reviewed in Whiteheart et al., 2001). Experiments with conditional mutations of the yeast and fly orthologues of NSF (Sec18p and comatose, respectively) demonstrated that NSF is required for each of the transit steps in the yeast secretory pathway and for regulated exocytosis of neurotransmitter (Eakle et al., 1988; Graham and Emr, 1991; Littleton et al., 1998; Pallanck et al., 1995a; Pallanck et al., 1995b). For example, conditional inactivation of the Sec18p or comatose protein leads to an accumulation of SNARE complexes (Littleton et al., 1998; Sogaard et al., 1994; Reviewed in Whiteheart and Matveeva, 2004). These studies therefore confirm the pivotal role of NSF in SNAP-SNARE complex disassembly in both yeast and mammalian cells.

One of the main models used to investigate the role of NSF in intracellular trafficking is the fusion of synaptic vesicles with the presynaptic membrane during neurotransmission. The hexameric ATPase NSF uses energy from ATP hydrolysis to dissociate SNARE complexes after membrane fusion, thus allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion (Jahn, 1999). NSF binds to and dissociates SNARE complexes only in the presence of adaptor protein, α-SNAP. α-SNAP interacts directly with the SNARE complex and with ATP-bound
NSF to form the so-called "20S particle" (Tagaya et al., 1993; Wilson et al., 1992). In the 20S particle, α-SNAP stimulates the ATPase activity of NSF, leading to SNARE complex disassembly (Matveeva and Whiteheart, 1998; Morgan et al., 1994). Specific v- and t-SNAREs are associated with each intercompartmental transport step, but NSF and α-SNAP are general cytosolic factors that can disassemble the SNARE complexes from most, if not all, intracellular transport steps (Jahn, 1999; Jahn and Sudhof, 1999).

1.4.5.2 Modulation of synaptic plasticity

With the recent identification of novel interacting partners for NSF as described in 1.4.3, it has become increasingly apparent that NSF may exhibit very diverse functions. In addition to a presynaptic role in SNARE complex disassembly, other lines of evidence support the idea that NSF may also reside in the postsynaptic density where it plays an important role. First, NSF is enriched within isolated postsynaptic density material, particularly after transient cerebral ischemia (Hu et al., 1998; Song et al., 1998). Second, long-term potentiation is inhibited by N-ethylmaleimide, which could inhibit NSF, while addition of recombinant SNAP increases excitatory postsynaptic currents (Lledo et al., 1998).

Interestingly, NSF and GluR2 was observed to colocalize in dendrites (Nishimune et al., 1998; Osten et al., 1998). More importantly, a GluR2-NSF-α/β-SNAP complex was isolated from a hippocampal detergent extract by Osten, while neither of the major nerve terminal SNAREs, synaptobrevin and
syntaxin, was coprecipitated with GluR2 (Osten et al., 1998). This complex is stable in the presence of nonhydrolyzable ATPγS but not in the presence of Mg²⁺-ATP (Osten et al., 1998). These findings suggest that the GluR2-NSF-α/β-SNAP complex resembles the NSF-SNAP-SNARE complex in that it is also disassembled by ATP hydrolysis.

In addition to the biochemical data, Nishimune and Song also provided functional data to support the NSF-dependent modulation of AMPA receptor function (Nishimune et al., 1998; Song et al., 1998). Blocking NSF with anti-NSF monoclonal antibody in rat hippocampal CA1 pyramidal neurons results in a marked, progressive decrement of AMPA receptor-mediated synaptic transmission (Nishimune et al., 1998). Competing the association of NSF and GluR2 with peptide rapidly decreases the amplitude of mEPSCs (miniature excitatory postsynaptic currents) (Song et al., 1998). Given that NSF is a chaperone that mediates the unfolding and refolding of proteins, it is conceivable that NSF may regulate the folding of the AMPA receptor complex independently of any action on vesicle fusion, thereby influencing postsynaptic AMPA receptor activity (Lin and Sheng, 1998). GluR2, in addition to its binding with NSF-α/β-SNAP, associates with GRIP which contains seven PDZ domains. Although the precise function of GRIP is not clear, it may serve as a molecular scaffold for the large complex of proteins including AMPA receptors. ATP hydrolysis by NSF may in turn disassemble the interaction between AMPA receptor and GRIP, resulting in mobilization, insertion or internalization of the receptor (Dong et al., 1997; Lin and Sheng, 1998).
Finally, NSF also interacts weakly with GluR3 and GluR4c (Osten et al., 1998; Song et al., 1998). None of these subunits is absolutely required for AMPA receptor function (Song et al., 1998), so their interaction with NSF is likely involved in regulation of AMPA receptors but the precise significance of this interaction remains to be elucidated. Taken together, in addition to the modulation of exocytosis, NSF may also take part in the modulation of synaptic transmission and mediation of specific forms of synaptic plasticity (Reviewed in Haas, 1998).

1.5  *Xenopus laevis* as an animal model

1.5.1  Introduction

*Xenopus laevis* is a gentle, freshwater animal that can be induced by simple hormone injection to lay eggs repeatedly. These features, coupled with the large size of the embryos, which allows micromanipulation and microinjection, and their rapid rate of development, make *Xenopus* an excellent animal for analyzing early vertebrate development. It has therefore been adopted in our study to examine the potential functional role of Pctaire1 during development (Sive et al., 2000).

The experiments using *Xenopus laevis* embryos have led the way in understanding signaling events in the early development of vertebrates. Over the years, *Xenopus laevis* has come to dominate experimental embryology. Many new techniques have helped to understand early *Xenopus* development and continue to ensure *Xenopus* a prominent position in the group of “model organisms”.

50
1.5.2 Morphology of *Xenopus laevis* embryos

The morphology of *Xenopus laevis* has been well studied. The stages of embryonic development are described by Nieuwkoop and Faber (1994). Shortly after fertilization, the first three cleavages occur. Subsequent rapid cleavages result in the formation of the blastula, and gastrulation commences at 9 hpf (hours postfertilization, at 22 °C) when the blastopore is first discernible.

The process of gastrulation occurs over a period of several hours (beginning at stage 10 and ending at stage 12) and entails an extremely complex set of movements. The first sign of gastrulation is the appearance of a condensed area of pigmentation on the dorsal side of the embryo where endodermally derived bottle cells elongate. Subsequently, involution of dorsal marginal zone tissue begins. The dorsal marginal zone includes both dorsal mesoderm and endodermal cells. Involution then progresses to lateral tissues and finally to the ventral side of the embryo. The end result of this extensive rearrangement is to move the mesoderm and endoderm inside the embryo, displacing the blastocoel and forming a new body cavity, the archenteron. Tissues along the anteroposterior embryonic axis become elongated due to movements of cells from ventral and lateral regions of the embryo toward the dorsal midline (Sive et al., 2000).

The gastrulation process requires tight regulation of cell migration, mesodermal gene transcription and cell division. The movements during gastrulation include both epiboly of ectoderm (ectoderm expands to cover the entire embryo) and convergent-extension of mesodermal tissue. Recent studies have shown the
importance of cell cycle regulation in gastrulation. Moreover, mesodermal gene \textit{Xbra} and \textit{chordin} transcription have been studied extensively.

Shortly after the end of gastrulation, the neural plate becomes more prominent. Neural plate is formed by extensive cell rearrangements within the ectoderm, resulting in narrowing (convergence) and elongation (extension) of dorsal ectoderm during gastrulation. During the process of neurulation, the neural plate folds to become the neural tube (Sive et al., 2000). Following closure of the neural tube, the period of organogenesis commences. Adult morphology and physiology are thoroughly described (Deuchar et al., 1975).

1.6 Aims of study

As mentioned above, little is known concerning the Cdk-related kinase \textit{Pctaire1}. It has been reported that \textit{Pctaire1} interacts with 14-3-3, p11 and Trap (Sladeczek et al., 1997; Hirose et al., 2000), but no functional significance has been found. We have previously shown that \textit{Pctaire1} associates with Cdk5/p35 complex and its activity is enhanced by Cdk5-mediated phosphorylation (Cheng et al., 2002). According to Graeser's report, it is probably that \textit{Pctaire1} is potentially involved in the control of neurite outgrowth (Graeser et al., 2002). To further study the functional roles of \textit{Pctaire1}, especially in brain, it is critical to identify the interacting proteins of \textit{Pctaire1}. To address this question, a yeast two-hybrid screening was carried out. The full length \textit{Pctaire1} was used as bait to screen an adult mouse brain cDNA library. Meanwhile, flag-tagged \textit{Pctaire1} was used to pull down the interacting
proteins in adult rat brain membrane fraction. The potential interacting proteins were then analyzed by mass spectrometry. The aim of this study is to identify Pctaire1-interacting proteins in brain and to further study the potential functions of the interactions.

To further dissect the involvement of Pctaire1 in neural development, intact embryos of *Xenopus laevis* will be used as the model system. The advantage of this system is the rapid external development of embryos. The expression profile of Pctaire1 in *Xenopus laevis* embryos during development will be examined. The synthesized mRNA encoding *Xenopus laevis* Pctaire1 wild type or its kinase dead mutant will be microinjected into the embryos at 2 or 4-cell stage. The morphological change will be observed in microinjected embryos and the functional role of Pctaire1 is likely to be revealed.
Chapter 2  Materials and Methods

2.1  Materials

**Materials**

**Animals:**
- Rat Embryo, Pup & Adult
- *Xenopus laevis*

**Suppliers**
- Animal Care Centre, HKUST, HK.
- Carolina Biological Supply, Burlington, NC, USA

**Antibodies:**
- Anti-p35 (C19) pAb
- Anti-p35 (C-terminal) pAb (EDKKRLLGLDR, a.a.296-307)
- Anti-Cdk5 (C8) pAb
- Anti-Cdk5 (DC17) mAb
- Anti-Cdk5 (DC34) mAb
- Anti-Pctaire1 (C16) pAb
- Anti-Pctaire1 (N-terminal) pAb (GEAPTRVAPGELSIR, a.a.45-60)
- Anti-total-S95-Pctaire1 pAb
- Anti-NSF (H-300) pAb
- Anti-phospho-Ser/thr-Pro (MPM-2) mAb
- Anti-NSF mAb
- Anti-HA mAb
- Anti-His pAb
- Anti-Syntaxin-1 mAb (HPC-1)
- Anti-synaptotagmin mAb

- Santa Cruz Biotech., Inc, CA, USA.
- HKUST, HK
- Santa Cruz Biotech., Inc, CA, USA.
- NeoMarkers, CA, USA.
- Santa Cruz Biotech., Inc, CA, USA.
- Animal Care Centre, HKUST, HK.
- Animal Care Centre, HKUST, HK.
- Santa Cruz Biotechnology, Santa Cruz, CA, USA.
- Upstate Biotechnology, Lake Placid, NY
- Calbiochem, San Diego, CA, USA
- Santa Cruz Biotechnology, Santa Cruz, CA, USA
- Santa Cruz Biotechnology, Santa Cruz, CA, USA
- Santa Cruz Biotechnology, Santa Cruz, CA, USA
- Prof. Benjamin Peng, HKUST, HK
Anti-synapsin I pAb
Anti-synaptophysin mAb
Anti-PSD95 mAb
Anti-SV2 mAb
Anti-actin pAb
Anti-β-tubulin mAb
Anti-cyclin I pAb
Anti-MAGI-3 pAb (RLNRTELPTRSAPQES, a.a.835-850)
Fluorescein-5-Isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit secondary Abs
Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rat and anti-rabbit secondary Abs
Rhodamine (Rh)-conjugated anti-mouse and anti-rabbit secondary Abs

**Chemicals:**
Adenosine triphosphate (ATP)  
Agarose  
All-trans retinoic acid (RA)  
Ampicillin  
Antipain  
Aprotinin

Cell Signaling Technology, Beverly, MA, USA
Chemicon, Temecula, CA, USA
Upstate Biotechnology, VA, USA.
Sigma, St. Louis, MO, USA.
Sigma, St. Louis, MO, USA.
Santa Cruz Biotechnology, Santa Cruz, CA, USA
Dr. Elior Peles, the Weizmann Institute of Science, Israel
Cappel, Durham, NC, USA.
Amersham–Biosciences, Sweden.
Cappel, Durham, NC, USA.
Roche Molecular Biochemicals, Germany.
InvitrogenTM Life Technologies, CA, USA.
Sigma, St. Louis, MO, USA.
Amresco, Solon, Ohio, USA.
Roche Molecular Biochemicals, Germany.
Roche Molecular Biochemicals,
Bacto-agar
Bacto-typtone
Bacto-Yeast Extract
Benzamidine

Bovine serum albumin (BSA)
Bromophenol blue
Calcium chloride (CaCl2)
Cytosine arabinoside
di-sodium ethylenediaminetetraacetatic acid (EDTA)
di-sodium hydrogen phosphate
Dithiothreitol (DTT)
Dublecco’s modified Eagle’s medium (DMEM)
Dublecco’s phosphate buffered saline (DPBS)
Eagle's minimal essential medium (MEM)
Ethylenebis(oxyethylenenitrilo)tetra-acetic acid (EGTA)
Formaldehyde
Formamide

Glucose
Guanidium thiocyanate

HEPES

Isopropylthio-β-D-galactoside (IPTG)

Germany.

Oxoid, Unipath Ltd., UK.
Oxoid, Unipath Ltd., UK.
Oxoid, Unipath Ltd., UK.
Roche Molecular Biochemicals, Germany.

Sigma, St. Louis, MO, USA.
Riedel-de Haën, Seelze, Germany.
Amresco, Solon, Ohio, USA.
Sigma, St. Louis, MO, USA.
BDH, Poole, UK.

BDH, Poole, UK.
Sigma, St. Louis, MO, USA.
InvitrogenTM Life Technologies, CA, USA.
InvitrogenTM Life Technologies, CA, USA.
InvitrogenTM Life Technologies, CA, USA.
InvitrogenTM Life Technologies, CA, USA.
Sigma, St. Louis, MO, USA.

Merck & Co., Inc., NJ, USA.
InvitrogenTM Life Technologies, CA, USA.
Fisher Scientific, NJ, USA.
InvitrogenTM Life Technologies, CA, USA.
InvitrogenTM Life Technologies, CA, USA.
Sigma, St. Louis, MO, USA.
<table>
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<tr>
<th>Chemical</th>
<th>Source</th>
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<tr>
<td>Laminin</td>
<td>Invitrogen™ Life Technologies, CA, USA.</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Roche Molecular Biochemicals, Germany.</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>Sigma, St. Louis, MO, USA.</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>Sigma, St. Louis, MO, USA.</td>
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<tr>
<td>Magnesium sulphate</td>
<td>Invitrogen™ Life Technologies, CA, USA.</td>
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<tr>
<td>Maltose</td>
<td>USB Amersham Biosciences, Sweden.</td>
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<tr>
<td>MOPS</td>
<td>Sigma, St. Louis, MO, USA.</td>
</tr>
<tr>
<td>Propane-sulfonic acid</td>
<td>Calbiochem-Novabiochem CN Biosciences, Inc., Germany.</td>
</tr>
<tr>
<td>Mowiol® 4-88</td>
<td>Riedel-de Haën, Seelze, Germany.</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma, St. Louis, MO, USA.</td>
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<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
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<tr>
<td>Poly-D-Lysine</td>
<td>Sigma, St. Louis, MO, USA.</td>
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<tr>
<td>Potassium di-hydrogen phosphate</td>
<td>Riedel-de Haën, Seelze, Germany.</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>Merck &amp; Co., Inc., NJ, USA.</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Riedel-de Haën, Seelze, Germany.</td>
</tr>
<tr>
<td>Roscovitine (Ros)</td>
<td>Calbiochem-Novabiochem CN Biosciences, Inc., Germany.</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>Sigma, St. Louis, MO, USA.</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Merck &amp; Co., Inc., NJ, USA.</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>USB Amersham Biosciences, Sweden.</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>USB Amersham Biosciences, Sweden.</td>
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<tr>
<td>Sodium dihydrogen phosphate</td>
<td>Merck &amp; Co., Inc., NJ, USA.</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>USB Amersham Biosciences, Sweden.</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>Sigma, St. Louis, MO, USA.</td>
</tr>
</tbody>
</table>
Sodium hydroxide  
Sigma, St. Louis, MO, USA.

Sodium Orthovanadate (Na3OV4)  
Sigma, St. Louis, MO, USA.

Sucrose  
Sigma, St. Louis, MO, USA.

Tetramethylrhodamine-conjugated  
Molecular Probes, Oregon, USA.

β-bungarotoxin (BTX)  
Sigma, St. Louis, MO, USA.

Tetrodotoxin (TTX)  
USB Amersham Biosciences, Sweden.

Tris-HCl  
CalBiochem, CA, USA.

Tryphostin AG1478  
InvitrogenTM Life Technologies, CA, USA.

Trypsin  
Roche Molecular Biochemicals, Germany.

Trypsin inhibitor  
Oxoid, Unipath Ltd., UK.

Tryptone  
USB Amersham Biosciences, Sweden.

Urea  
Promega, Madison, WI, USA.

X-Gal  
Oxoid, Unipath Ltd., UK.

(5-Bromo-4-Chloro-3-Indoly1-β-D-Galactoside  

Yeast Extract  

Solutions and Reagents:

Acetic acid  
Merck & Co., Inc., NJ, USA.

Acrylamide:bis-acrylamide solution  
Amresco, Solon, Ohio, USA.

Diethyl pyrocarbonate (DEPC)  
Sigma, St. Louis, MO, USA.

Dimethylsulfoxide (DMSO)  
InvitrogenTM Life Technologies, CA, USA.

Ethidium bromide (EtBr)  
InvitrogenTM Life Technologies, CA, USA.

Fetal bovine serum (FBS)  
InvitrogenTM Life Technologies, CA, USA.

Glycerol  
USB Amersham Biosciences,
Goat serum (GS) Invitrogen™ Life Technologies, CA, USA.
Hank's buffered saline solution Invitrogen™ Life Technologies, CA, USA.
Horse serum (Cleveland and Rothstein) Invitrogen™ Life Technologies, CA, USA.
Human chorionic gonadotropin (HCG) Sigma, MO, USA
Hydrochloric acid (HCl) Merck & Co., Inc., NJ, USA.
Isoamylalcohol Riedel-de Haën, Seelze, Germany.
Isopropanol Merck & Co., Inc., NJ, USA.
Nonidet P40 (NP40) BDH, Poole, UK.
Penicillin/Streptomycin Invitrogen™ Life Technologies, CA, USA.
Pentobarbital Sigma, St. Louis, MO, USA.
Phenol Invitrogen™ Life Technologies, CA, USA.
Puck’s solution Invitrogen™ Life Technologies, CA, USA.
Triton-X 100 Sigma, St. Louis, MO, USA.

Kits:
GENECLEAN III Kit BIO 101, La Jolla, CA, USA
Lipofectamine plus transfection kit Invitrogen™ Life Technologies, CA, USA.
Luminescent β-galactosidase enzyme kit Clontech, Palo Alto, CA, USA
T7 DNA Polymerase Sequencing Kit Amersham Biosciences, Sweden.
hGH ELISA kit Roche Molecular Biochemicals, Mannheim, Germany
Qiaex II Gel Extraction Kit Qiagen Inc., CA, USA
Qiagen Plasmid Preparation Kit Qiagen Inc., Chatsworth, CA, USA
T7 Cap-Scribe kit
Roche. Molecular Biochemicals, Mannheim, Germany

Subcellular Proteome Extraction Kit
Calbiochem, San Diego, CA, USA

Others:
Prestained protein marker, broad range
Cell signaling Technology, Inc, MA, USA.
Prestained protein molecular weight standard, high range
Invitrogen™ Life Technologies, CA, USA.
Kinase substrates [histone H1 (H1) and myelin basic protein (MBP)]
Upstate Biotechnology, VA, USA.
Molecular weight marker (Lambda DNA/Hind III fragments, 100 bp ladder DNA)
Invitrogen™ Life Technologies, CA, USA.
NICK column
Amersham Biosciences, Sweden.
Nitrocellulose membrane
Micron Separations Inc., Westboro, MA, USA.
Nylon membrane
Micron Separations Inc., Westboro, MA, USA.
Osmotic minipump
Alzet 2002, Alza Corp., CA, USA.
Phosphorothioate oligonucleotides (S-modified):
Genset Oligos, CA, USA.
Protein-A/G sepharose
Amersham Biosciences, Sweden.
Protein peptides
ResGen™ Invitrogen Corporation, CA, USA.
Radioactive nucleotide:
NEN™ Life Science Products, Inc.
MA, USA.
[γ-32P]ATP
Dow Corning Cooperation, Midland, Michigan, USA.
Silastic tubing
Genset Oligos, CA, USA.
Synthetic nucleotides
Amersham Biosciences, Sweden.
Terminator Ready Reaction Mix
Applied Biosystems
2.2 *Yeast two-hybrid screen*

Yeast two-hybrid screen was performed following the Matchmaker Two-hybrid Screen protocol (Clontech, Palo Alto, CA). Pctaire1 was used as a bait to screen an adult mouse brain cDNA library that has been constructed in the GAL4 transcriptional activation vector (pACT2). Yeast strain Y190 was transformed with the bait and the library plasmids and transformants were selected on SD-Trp-Leu-His plates. β-galactosidase activity of His+ colonies was assayed by filter assay. The NSF clone isolated from the two-hybrid screen encodes the protein that lacked the five amino acids at the N-terminus. Subsequent two-hybrid interaction analyses were carried out by co-transformation of plasmids containing the GAL4 DNA-binding (pAS2–1) and activation (pACT2) domains into yeast.

ONPG (O-nitrophenyl-β-D-galactopyranoside) based β-galactosidase activity assay was performed according to the Yeast Protocols Handbook (Clontech, Palo Alto, CA).

2.3 *Construction of expression plasmids in different systems*

Complementary DNA (cDNA) encoding full-length mouse Pctaire1 was subcloned into the yeast GAL4 DNA-binding vector, pAS2–1 (Clontech, Palo Alto, CA), to construct GAL4bd-Pctaire1 which was used as a bait in the yeast two-hybrid screen. Similarly, the cDNAs encoding Cdk5 and p35 were subcloned into pAS2–1.
Full-length and partial cDNA fragments of mouse NSF (encoding amino acids 1–205, 206-477, or 478-744) were amplified by PCR and subcloned into different expression vectors including pGEX-6P-2, pETH-32, pcDNA3-His6 and pcDNA3-3HA. Point mutations [e.g. serine-569 to alanine (S569A)] were generated by PCR using complementary primers containing the mutations and subcloned into pGEX-6P-2. All of the constructs were confirmed by sequencing. pXGH5 containing the human GH gene was a gift from Prof. Masami Takahashi (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan).

Expression plasmids were prepared by cutting the empty vector and the DNA inserts by the desired restriction enzymes. The linearized DNAs were electrophoresed on a 1% agarose gel and purified by QIAquick® gel extraction kit according to the manufacturer’s protocol. Quality of the DNA was confirmed by agarose gel electrophoresis. The quantity of DNA was estimated by comparing with the intensity of Hind III digested lambda DNA marker (Invitrogen). The DNA inserts were ligated to vectors in a 3 to 1 ratio using T4 ligase (New England Biolabs Inc., MA, USA). The ligation product was then transformed into TG1 competent cells prepared by CaCl2 method.

2.4 Preparation of DNA

Minipreparation of DNA plasmid was prepared by alkaline lysis method (Sambrook et al., 1989). A single E.coli bacterial colony was picked from a culture plate and inoculated into 5 ml of LB medium with appropriate antibiotics. The medium was incubated with shaking at 37 °C overnight. Bacteria were then
centrifuged at 3000 g for 10 min and the cell pellet was harvested by alkaline lysis method. The pelleted bacteria were resuspended in 100 µl of Solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 9% glucose). The cells were subsequently lysed with 100 µl of Solution II (1% SDS, 200 mM NaOH), and neutralized with 150 µl of Solution III (30 mM potassium acetate, pH 8.0). The mixture was subjected to centrifugation at 10000 g for 20 min. The supernatant was manipulated with phenol/chloroform/isoamylalcohol (25:24:1) and the plasmid DNA was purified by ethanol precipitation. The DNA pellet was resuspended in 30 µl of Tris-EDTA (TE) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and kept at −20°C before use.

For large scale preparation, 5 ml bacterial culture was inoculated in a 500 ml LB medium supplemented with the appropriate antibiotics for overnight culture at 37°C. The bacteria were pelleted and the DNA was purified by the Qiagen plasmid maxi kit. The concentration of DNA was determined by measuring the optical density at 260 nm. Quality of the DNA was confirmed by agarose gel electrophoresis.

2.5 Agarose gel electrophoresis

DNA was electrophoresed on 1% agarose gel in 1X TBE running buffer with 0.5 µg/ml EtBr. DNA sample was mixed with 10X sample buffer (0.25% bromophenol blue, 0.25% cyanol FF, 15% Ficoll). Gel electrophoresis was performed at 6 V/cm. A photograph of the gel was then taken under UV illumination for visualization of the separated DNA.
2.6 Primary hippocampal neuron culture

Rat hippocampal neuron cultures were prepared from E18 rat cortex. After digestion with trypsin in Hanks' Balanced Salt Solution without Ca\(^{2+}\) and Mg\(^{2+}\) (Invitrogen) at 37°C for 2-3 min, the reaction was stopped by adding 10% heat inactivated horse serum. The cells were centrifuged at 1000 rpm for 5 min and the pellet was resuspended in DMEM and triturated 10 times to obtain single cells. The cells were then seeded in culture plates coated with a 0.1 mg/mL solution of poly-L-lysine (Sigma). The medium is replaced by NEUROBASAL medium with 1 mM L-glutamine and 2% B27 supplement 1 hr after cell seeding.

2.7 Cell lines

All of the cell lines were obtained from American Type Culture Collection (ATCC). COS-7 cells and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics. Both COS-7 and 293T cells were cultured in 37 °C incubator supplied with 5% CO\(_2\) and medium was changed every 3 days.

PC12 cells were cultured in DMEM supplemented with heat-inactivated horse serum (6%, v/v), heat-inactivated FBS (6%, v/v) plus antibiotics. These cells were normally maintained at 37 °C in a humidified atmosphere with 7.5% CO\(_2\) and medium was changed every 3 days.
2.8 Transient transfection

COS-7 cells, 293T cells or PC12 cells in 100 mm culture dishes were transiently transfected with different combinations of the following plasmids, including wild-type Pctaire1 (WT), Pctaire1 kinase-dead mutant K194A (KD; by point mutating the key residue lysine-194 involved in the catalytic process of phosphorylation to alanine), NSF, different domain of NSF, etc, using Lipofectamine plus reagent according to the protocol supplied. Control cells (mocks) were transfected with the expression vector only. The efficiency for transient transfection of COS-7 cells or 293T cells was estimated to be 80%. The efficiency for transient transfection of PC12 cells was estimated to be 30%. The transfection efficiency was estimated by counting the number of cells emitting green fluorescence, one day after transfection of GFP construct (Quantum, USA) under a fluorescence microscope equipped with a selective filter for fluorescein.

2.9 Protein preparation and immunoprecipitation

COS-7 cells were lysed in lysis buffer A (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 0.5% Nonidet P-40). Brain tissues were homogenized in lysis buffer B (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with various protease inhibitors. 293T cells were lysed in lysis buffer C (25 mM Tris, pH 7.5, 50 mM NaCl, 20 mM beta-glycerophosphate, 0.1% Nonidet P-40, 1 mM EDTA, dithiothreitol) supplemented with various protease inhibitors.
After transfection, COS-7 cell lysates were extracted using lysis buffer A. Two hundred μg of COS-7 cell lysates were incubated with the corresponding antibody (2 μg) at 4 °C overnight and then incubated with 40 μl of protein G-Sepharose at 4 °C for 1 hr. The samples were washed with buffer A and resuspended in SDS sample buffer, and co-immunoprecipitated proteins were detected using Western blot analysis.

In vivo co-immunoprecipitation studies using brain lysates were performed as described previously (Cheng et al., 2002). Two mg adult brain lysates were incubated with the corresponding antibody (2 μg) in lysis buffer B at 4 °C overnight. To examine the ATP-dependence of the interaction, adult rat brain were prepared, immunoprecipitated and washed with lysis buffer B supplemented with 1 mM ATP/0.5 mM MgCl₂, 1 mM ATP-γ-S/0.5 mM MgCl₂, or 2 mM EDTA (Sollner et al., 1993; Osten et al., 1998; Han et al., 2000).

2.10 Size exclusion chromatography

Analytical size exclusion chromatography was performed using a Superdex 200 HR 10/30 gel filtration column (GE healthcare; Amersham Biosciences, Piscataway, NJ) at 4°C with a flow rate of 0.5ml/min. Prior to the size exclusion chromatography, the column was first equilibrated with 10 mM HEPES at pH 7.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 0.5 mM MgCl₂, 5% glycerol, 0.1% Nonidet P-40 and various proteinase inhibitors.
2.11 Preparation of recombinant proteins

For preparation of recombinant proteins, 50 ml of overnight bacterial culture was subcultured to 1 L of LB medium supplemented with the appropriate antibiotics. Protein expression was induced by Isopropylthio-β-D-galactoside (IPTG) in a final concentration of 0.5 mM when the OD_{600nm} of the culture reached 0.6-0.8. Cells were centrifuged at 4,000 rpm at 4 °C for 10 min and the pellet was resuspended by 30 ml of cold resuspension buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 2 mM PMSF, 2 mM DTT, 2 mM antipain and 2 mM leupeptin. The mixture was sonicated at 70 kHz per min for 10 min. The broken cell lysates were then spun down at 18,000 rpm at 4°C for 30 min. The supernatant was incubated with either 5 ml of glutathione agarose or Ni-NTA agarose depending on the type of the recombinant proteins. GST-Pctaire1 was expressed in BL21 strain and purified using a glutathione-Sepharose 4B column following the instruction of manufacturer (GE Healthcare; Qiagen, Valencia, CA). His-NSF was purified using a Ni-NTA column according to the protocol of the manufacturer (GE Healthcare; Qiagen, Valencia, CA).

2.12 In vitro pull-down and mass spectrometric analysis

The pull-down assay was carried out as described previously (Cheng et al., 2002). Briefly, the pull down was performed in PBS buffer supplemented with 5% BSA (137 mM NaCl and 2.7 mM KCl in PBS buffer with no Mg^{2+}, EDTA and ATP).

To identify the proteins that interact with Pctaire1 using mass spectrometry, adult brain membrane fraction was prepared as described previously (Lai et al., 2004). 293T cells were transfected with flag-tagged Pctaire1. Ten mg lysates were
collected after twenty-four hours transfection, immunoprecipitated using anti-flag antibody which was immobilized on agarose beads at 4 °C for 2-3 hr (Sigma, St. Louis, MO). The immunoprecipitates were then incubated with adult rat brain lysate for 1 hr. The beads were washed extensively with buffer C plus 0.1% Nonidet P-40. The precipitates were subjected to the SDS-PAGE and the gel was stained by Gel Code blue stain reagent (Pierce, Rockford, IL). Protein bands were excised and subjected to in-gel digestion with trypsin. Recovered peptides were analyzed by nanoelectrospray tandem mass spectrometry using a quadrupole/time-of-flight hybrid mass spectrometer (QSTAR-Pulsar, Applied Biosystems/Sciex). Protein identity was revealed by searching sequence databases (National Center for Biotechnology Information, NCBI) with peptide sequence tags generated from tandem mass spectra.

For in vitro pull down analysis for Pctaire1 or Cdk5, 200 µg lysate was similarly pulled down by the anti-flag antibody immobilized on agarose beads and extensively washed with buffer C with 100 mM NaCl and then incubated with adult brain membrane fraction at 4 °C for 3 hr. The samples were washed and resuspended in SDS sample buffer. Proteins co-immunoprecipitated with flag-tagged Pctaire1 and Cdk5 were detected by Western blot analysis.

2.13 Subcellular Fractionations

Subcellular fractions including membrane, cytosol and nucleus fraction of adult rat brain were prepared according to the protocol of Dr. L.H. Tsai (Zukerberg et al., 2000). Briefly, the whole brain of adult rat was lysed in STM buffer. Extracts
were spun at 600 g and then at 100,000 g to obtain cytoplasmic fractions. Membranes were extracted from the 600 g spin by STM lysis containing 0.5% NP-40. Nuclear fractions were the final 600 g pellet lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM DTT, protease and phosphatase inhibitors).

Subcellular fractions including cytosol, membrane/organelle, nucleus and cytoskeleton fraction of hippocampal neuron were generated using the subcellular proteome extraction kit (Calbiochem, San Diego, CA, USA). During the extraction, S-PEK takes advantage of the differential solubility of certain subcellular compartments in special reagent mixtures, thus preserving the structural integrity of the subcellular structures.

A third fractionation protocol was adopted that yielded ten subcellular fractions of adult rat brain as previously described (Hutner et al., 1983; Niethammer et al., 2000). Briefly, rat brain was homogenized in 0.32 M sucrose buffer (fraction H) and centrifuged at 800 g to pellet nuclei and large cellular debris (P1). The supernatant (S1) was subjected to 10,000 g centrifugation to yield the crude synaptosomal fraction (P2). The supernatant (S2) was centrifuged at 165,000 g for 2 hr, resulting in cytosol (S3) and light membrane and Golgi (P3) fractions. The synaptosomal fraction P2 was lysed hypo-osmotically and spun at 25,000 g for 20 min to obtain the synaptosomal membrane fraction LP1. The supernatant, LS1, was centrifuged at 165,000 g for 2 hr, resulting in a synaptic vesicle enriched fraction (LP2) polyacryl and a supernatant (LS2).
2.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a 15%, 12.5%, 10%, 7.5% or 6% acrylamide resolver gel and 4% stacker gel according to Laemmli et al. (1970). The stacker and resolver gels were prepared according to the protocol supplied with the electrophoresis apparatus, Mini-Protein II (Hoefer, Amersham Biosciences). The samples were loaded into the wells of a 1.0 mm thick gel and electrophoresed at 20 mA through the stacker gel and 30 mA after entering the resolver gel for 3 hr in 25 mM Tris-HCl and 192 mM glycine (pH 8.3), containing 0.1% SDS. Prestained molecular weight markers were run alongside the samples.

2.15 Western blot analysis

The proteins on the polyacrylamide gel were transferred onto a nitrocellulose membrane in 1X transfer buffer using a Trans-Blot electrophoretic transfer cell (Bio-Rad, CA, USA) at 100 V for 1 hr at 4 °C. The membrane was washed with Tris-buffered saline with 0.1% Tween-20 (TBS-T). The membrane was blocked with 5% non-fat dry milk in TBS-T for 1 hr at room temperature. The membrane was then incubated with primary antibodies in 1X TBS-T with 5% BSA at 4 °C overnight, followed with HRP-conjugated secondary Ab incubation. The immunoreactive proteins were detected by Enhanced Chemiluminescence (ECL) Western Blot System (Amersham Biosciences) or Pical detection system (Pierce,
Rockford, USA) according to the supplier's instruction.

2.16 **CIP assay**

100 µg of protein samples from xenopus embryos were incubated with 1 µl/30 U of CIP (Calf Intestinal Phosphatase) at 37 °C for 30 min. The control samples were incubated without addition of CIP. The reactions were stopped by adding sample buffer.

2.17 **In vitro kinase assay**

Active Pctaire1 was immunoprecipitated from adult rat brain lysates with Pctaire1 antibody in RIPA buffer and then extensively washed with RIPA buffer for 5 times. The kinase assay was performed at 30 °C for 30 min in kinase buffer (20 mM MOPS, pH 7.4, 15 mM MgCl₂, 100 µM ATP) containing 1 µCi of [γ³²P] ATP. Two hundred ng of proteins (His-NSF full length, His-NSF-S569A, His-NSF D1, His-NSF D2, GST-NSF N-terminus and GST-NSF D2S569A) were utilized as the substrates in the kinase assay. The phosphorylated proteins were separated on a SDS-PAGE and visualized by autoradiography.

2.18 **GH release assay in PC12 cells**

hGH release assays in PC12 cells were carried out at 37 °C as previously described (Hibino et al., 2002; Ohnishi et al., 2001). Forty-eight hours after transfection, PC12 cells were washed with a low K⁺ solution (20 mM Hepes/NaOH
(pH 7.4), 128 mM NaCl, 5.6 mM KCl, 2.7 mM CaCl₂, 10 mM Glucose, 1 mM MgCl₂) and maintained in the low K⁺ solution for 30 min. To measure the low or high K⁺-evoked hGH secretion, the cells were then incubated either in the same solution (low K⁺) or in the high K⁺ solution (20 mM Hepes/NaOH (pH 7.4), 78 mM NaCl, 56 mM KCl, 2.7 mM CaCl₂, 10 mM Glucose, 1 mM MgCl₂) for another 10 min. The culture medium was collected and the amount of hGH was measured using the hGH ELISA kit (Roche Molecular Biochemicals). Student’s t test was used for statistical analysis, and the results are presented as mean±SEM.

2.19 Xenopus Embryos

Xenopus laevis were purchased from Carolina Biological Supply (Burlington, NC, USA). They were raised and kept under standard condition at about 22 °C. They were induced to spawn by injection with human chorionic gonadotropin (Sigma, MO, USA). Embryos were obtained by artificial fertilization and were staged according to Nieuwkoop and Faber (1994).

2.20 Expression constructs and in vitro synthesis of mRNA

The cDNA encoding full length Xenopus Ptega1 was generously provided by Dr. Randy Poon (HKUST, HK). Point mutation of kinase-dead mutant was generated by PCR using complementary primers containing the mutation. The coding sequences of HA tagged Xenopus Ptega1 wild-type (WT), kinase-dead mutant (KD) and GFP control were individually amplified with addition of a Kozak sequence
(CACC) and cloned into the expression vector pXT7 that has 5' and 3' UTR from *Xenopus* β-globin gene (a gift from Prof. Anming Meng, Tsing Hua University, China). The cDNA constructs were then linearized with restriction enzymes ClaI digestion. Capped mRNAs were synthesized in vitro using linearized plasmids and the T7 Cap-Scirbe kit (Roche Molecular Biochemicals, Mannheim, Germany). To remove the EDTA, the mRNAs were precipitated overnight, washed by ethanol and dissolved in RNase-free water. The quality and size of synthesized mRNAs were confirmed by running a formaldehyde gel.

2.21 *Microinjection*

The 5'-capped mRNA was diluted in DEPC H$_2$O to an appropriate concentration before microinjection. About 2 nl mRNA was injected into the side of animal poles of two to four-cell stage embryos which were previously dejellied using 2% cysteine chloride. mRNAs were injected using a pressure-injection system (Medical Systems, PLI-188), together with a micromanipulator (Zeiss MMJ) and a bottom illumination stereomicroscope (Zeiss Stemi SV6). Injection pressure was maintained at 15 p.s.i. and the duration of pressure pulse ranged from 150 to 250 ms to allow a constant volume to be delivered for each injection. The injected embryos were transferred to 0.1 X MMR solution, allowed to develop at room temperature, and staged for subsequent analysis.

2.22 *Total RNA extraction*
Different tissues were dissected from rats at various developmental stages and immediately frozen in liquid nitrogen. Tissues were homogenized and the total RNAs were extracted by lithium chloride method as previously described (Heuer et al., 1990; Ip et al., 1995). Frozen tissues were weighted and placed into 1:10 (w/v) 3 M LiCl/6 M urea solution. The tissues were then homogenized at 2000 rpm for 1 min twice using a polytron. RNA precipitation was performed at 4 °C for more than 4 hr. The RNA was pelleted by centrifugation at 3000 g for 30 min. The RNA was then rinsed with LiCl/urea solution and dissolved in 0.5 volume of TE buffer with 0.5% SDS. The RNA was extracted with phenol/chloroform/isoamylalcohol. The aqueous phase was recovered by centrifugation at 3000 g for 30 min. The RNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol and pelleted by centrifugation at 12000 g for 15 min. The RNA was washed with 75% ethanol and resuspended in DEPC treated water and then stored at -80 °C.

Total RNAs obtained from small tissues and cell cultures were prepared by guanidinium thiocyanate extraction. Procedures for guanidinium thiocyanate extraction were performed as previously described (Chomczynski and Sacchi, 1987). Generally, 0.1 g tissue or 1x10^6 cells were lysed in 1 ml denaturing solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol and 0.5% sarcosyl). The tissues were homogenized in solution D as described above. The RNA in the solution was then extracted by 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of phenol and 0.2 volume of chloroform:isoamylalcohol
The aqueous phase was obtained by centrifugation at 3000 g for 30 min and RNA precipitation was performed at -20 °C for more than 2 hr after the addition of 2.5 volume of absolute ethanol and 8 μg/ml glycogen. RNA concentration was obtained by measuring the optical density of RNA at 260 nm using a spectrophotometer (GeneQuant DNA/RNA calculator; Amersham-Biosciences).

2.23 Formaldehyde-RNA gel electrophoresis

Total RNA was electrophoresed on 1% agarose-formaldehyde gel (6% formaldehyde in 1X MOPS running buffer with 0.2 μg/ml EtBr). RNA sample was mixed with 2X sample buffer (50% formamide, 7.3% formaldehyde/1X MOPS running buffer), incubated at 65 °C for 15 min and chilled on ice. The mixture was then centrifuged and 6X RNA loading buffer [0.25% bromophenol blue, 0.25% cyanol FF, 30% glycerol, 1 mM EDTA (pH 8)] was added. The gel was run in 1X MOPS running buffer at 0.8 V/cm overnight. A photograph of the gel was taken.

2.24 Morpholino antisense oligo

Two pieces of morpholino were designed to knockdown endogenous *Xenopus* Pctaire1: 5'-GAGCCTTCTCTGAATTTTTTCATA-3'  (XMO-1) and 5'-GTGTCTTTATGTGTTTCCAG-3' (XMO-2). They were purchased from Gene Tools (Philomath, OR).
Chapter 3 The cloning and functional study of Xenopus laevis

Pctaire1

Despite the identification of Pctaire1 more than a decade ago, little is known concerning its biological functions. To investigate if Pctaire1 exhibits a potential role in the regulation of development, in particular phenotypically, the effect of Pctaire1 on the development of Xenopus laevis was examined. Xenopus laevis, the South African clawed toad, is a primarily aquatic animal that offers several advantages for developmental studies. It is also hardy and disease resistant. Oocytes at different stages of differentiation are easily obtained from adult females and can be readily induced to spawn with a simple gonadotrophic hormone injection. Moreover, the entire process of embryonic development from the fertilized egg until the end of metamorphosis has been carefully documented. The ease with which the development of embryos can be manipulated and the versatility of neuromuscular cultures have made Xenopus laevis a popular tool for elucidating the molecular mechanisms involved in neuronal development.

3.1 The cloning of Xenopus Pctaire1 and its mutants

Since the knockout mice of Pctaire1 have not been generated, Xenopus laevis embryos were selected as a model to examine the functional roles of Pctaire1 during development. First, the phenotype of Xenopus laevis embryos microinjected with Xenopus laevis Pctaire1 was examined. Xenopus laevis Pctaire1 gene was cloned by
Dr. Randy Poon in pcDNA3.1 vector. The full length *Xenopus* Pctaire1 has an open reading frame of 1566 bp that encodes a polypeptide of 522 amino acids. DNA sequencing confirmed that it contained a highly conserved Cdk kinase domain, an N-terminal and C-terminal extension. The functional domains and motifs were shown in Fig. 3.1. The amino acid sequence was then aligned to Pctaires of different species, such as *Zebrafish, Xenopus tropicalis*, mouse and human (Fig. 3.2). By comparing the sequence identity of *Xenopus* Pctaire1 with *Zebrafish, Xenopus tropicalis*, mouse and human Pctaires, we found that Pctaire1 is highly conserved in these different species.

According to the previous studies (Charrasse et al., 1999; Cheng et al., 2002), kinase dead mutant of mouse Pctaire1 (K194A) was generated by mutating the key residue involved in the catalytic process of phosphorylation using site-directed mutagenesis. We have therefore generated the kinase dead mutant (K221A, KD) of *Xenopus* Pctaire1 using a similar approach by mutating K221 of *Xenopus* Pctaire1 to alanine, which corresponds to K194 of mouse Pctaire1. *Xenopus* Pctaire1, as well as GFP control, was digested with *Xho* I and *Spe* I, and then subcloned into pXT7 vector with HA tag (Fig. 3.3). Linearized *Xenopus* Pctaire1 was transcribed into 5'-capped mRNA *in vitro* and microinjected into *Xenopus* embryos later. The size and quality of synthesized mRNAs were confirmed by running a formaldehyde gel (Fig. 3.4).
Figure 3.1 Sequence of Xenopus Pctarel. Translated amino acid sequence of Xenopus Pctarel was shown. The highly conserved Cdk kinase domain was indicated in bold. The kinase motif was denoted with emphasis marker in red. The ATP binding motif was underlined in blue. The amino acid sequence recognized by anti-S95 antibody was labeled with underline _ _ _.
Figure 3.2 Comparison of the amino acid sequence between Xenopus Pctaire1 and that of other species. The amino acid sequence of the Xenopus Pctaire1 was shown as single-letter codes. Amino acid differences between Xenopus Pctaire1 and Pctaire1 from other species were indicated. zPct2, Zebradish Pctaire2; xPct1, Xenopus laevis Pctaire1 from Dr. Randy Poon; xPct, Xenopus laevis Pctaire from gene bank of NCBI; xtPct1, Xenopus tropicalis Pctaire1; mPct3, mouse Pctaire3; mPct2, mouse Pctaire2; mPct1, mouse Pctaire1; hPct3, human Pctaire3; hPct2, human Pctaire2; hPct1, human Pctaire1.
Figure 3.3 Expression construct of full length *Xenopus Pctaire1*. The full length cDNA encoding for *Xenopus Pctaire1* followed by a HA tag was subcloned into pXT7 vector. Relevant restriction enzyme sites for cloning and verification of the constructs are indicated. * represents stop codon. The pXT7 vector contains a T7 bacteriophage promoter for *in vitro* transcription, globin 5' and 3' UTR sequences for *in vivo* translation. The sizes of the *Xenopus Pctaire1* and pXT7 vector were 1566 bp and 3.1 kb, respectively.
Figure 3.4 5'-capped mRNA of GFP, *Xenopus* Pctaire1 wild type and kinase dead mutant in pXT7. Two micrograms of 5'-capped mRNA of GFP, *Xenopus* Pctaire1 WT and KD were loaded onto an RNA gel. Position of size 4 kb and 1.8 kb were indicated by 28S and 18S rRNA, respectively.
3.2 Characterization of anti-S95 antibody for *Xenopus* Pctaire1 for immunoblotting

According to the sequence of *Xenopus* Pctaire1, custom polyclonal Pctaire1 antibody against serine-95 is likely to recognize *Xenopus* Pctaire1. The specificity of the antibody was therefore analyzed. Lysates of COS-7 cells overexpressing HA-tagged *Xenopus* Pctaire1 was subjected to SDS-PAGE and Western blot analysis (Fig. 3.5, left panel). We found that the anti-S95 antibody detected doublet bands of *Xenopus* Pctaire1 around 62-70 kDa. A doublet of Pctaire1 proteins is also recognized in adult rat brain (Fig. 3.5, left panel) as a positive control. Similar bands were detected in the lysate of *Xenopus* embryos at stage 40 (Fig. 3.5, left panel). Interestingly, the bands corresponding to *Xenopus* Pctaire1 could be blocked after the antibody was preincubated with specific blocking peptide (Fig. 3.5, right panel), indicating that the antibody was sufficiently specific for the detection of *Xenopus* Pctaire1.

The titer of the anti-S95 antibody was tested using the lysates of *Xenopus* embryos at stage 40. Western blot analysis was performed using anti-S95 antibody at various dilution (1:5,000, 1:10,000, 1:20,000 and 1:50,000) and incubated at room temperature for one hour (Fig. 3.6). The sensitivity of the antibody was high and the optimal concentration used was 1:10,000 dilution. Subsequent immunoblotting will therefore be performed at 1:10,000 dilution.

To explore whether *Xenopus* Pctaire1 is phosphorylated *in vivo*, we performed CIP assay to check whether Pctaire1 is phosphorylated endogenously in
*Xenopus* embryos. CIP (calf intestine phosphotase) was used to dephosphorylate the phosphoprotein present and this would result in a change in mobility, allowing us to determine if the protein was phosphorylated prior to CIP assay. Interestingly, following CIP treatment, a downshift in mobility was observed for the slower migrating form of *Xenopus* Pctaire1 in extracts of *Xenopus* embryos at stage 40. A downshift in Pctaire1 mobility was also observed in adult rat brain lysates after CIP treatment (Fig. 3.7). The presence of the slower migrating band following CIP treatment suggested that *Xenopus* Pctaire1 existed as a phosphoprotein *in vivo*.

### 3.3 Temporal profile of *Xenopus* Pctaire1

The protein expression of *Xenopus* Pctaire1 during development was examined by Western blot analysis. Multiple bands were identified (Fig. 3.8). The protein expression of *Xenopus* Pctaire1 increased during development. As shown in Fig. 3.5, *Xenopus* Pctaire1 could be detected as a doublet in Western blotting. During the embryo development, majority of *Xenopus* Pctaire1 existed as a lower molecular weight form before stage 20. However, slower migrating forms of *Xenopus* pctaire1 were apparent starting after stage 20. By stage 40, the most abundantly expressed form of *Xenopus* Pctaire1 was identified as a higher molecular weight form that was absent prior to stage 20 (Fig. 3.8).
Figure 3.5 Characterization of anti-S95 antibody for immunoblotting. *Xenopus*

Pctaire1 was transfected into COS-7 cells, and the lysates were subjected to Western blot analysis. Lysates of adult rat brain and *Xenopus* embryos at stage 40 were detected by anti-S95 antibody. Preincubation of the antibody with blocking peptide abolished the signal in Western blot analysis, indicating that the antibody could specifically recognize *Xenopus* Pctaire1. Actin was used as a control for peptide blocking assay.
Figure 3.6 Dilution of anti-S95 antibody for immunoblotting. Lysates of Xenopus embryos at stage 40 were detected by anti-S95 antibody incubation at various dilution (1:5,000, 1:10,000, 1:20,000 and 1:50,000). The secondary antibody was rabbit-HRP of 1:10,000, incubated for one hour at room temperature.
Figure 3.7 Pctaire1 was a phosphoprotein in *Xenopus*. *Xenopus* embryos extracts and adult rat brain extracts were incubated with (+) or without (-) calf intestinal phosphatase (CIP) at 37 °C for 30 min. CIP incubation caused a downshift in mobility for slower migrating forms of *Xenopus* Pctaire1 as well as Pctaire1 in adult rat brain.
Figure 3.8 Developmental expression profile of *Xenopus* ptairel in embryos.

Extracts of whole *Xenopus* embryos were prepared from stage 4 to 45. Western blot analysis of *Xenopus* Ptairel was performed using anti-S95 antibody (upper panel). The nitrocellulose membrane was then stripped and blotted with Actin antibody (bottom panel).
3.4 Microinjection of Xenopus Pctaire1 mRNA

To investigate the effect of Pctaire1 on the development of Xenopus, synthesized 5'-capped mRNAs of GFP, Xenopus Pctaire1 WT and KD were adjusted to the concentration of 400 pg/nl, 100 pg/nl or 25 pg/nl prior to microinjection into the Xenopus embryos. Two nl mRNA was injected into Xenopus embryos at 2 or 4-cell stage. Subsequent to the injection of GFP, green fluorescence was evident starting from stage 8, and the fluorescence became stronger through development. GFP fluorescence was distributed evenly in the embryos even through stage 33/34 (data not shown). This indicated that the expression of GFP remained until stage 33/34 with no major degradation of the injected mRNA throughout development.

Injected and uninjected embryos were collected at stage 8 and 12, which corresponded to the blastulation and gastrulation stage, respectively. Proteins were extracted and the expression of Xenopus Pctaire1 WT was detected by Western blot using anti-S95 antibody. In Fig. 3.9, anti-S95 antibody recognized a lower band that was present in all samples, suggesting that this band corresponded to the endogenous Xenopus Pctaire1. HA-tagged exogenous Xenopus Pctaire1 was expressed as a higher molecular weight form due to the presence of the HA tag. Our results showed that Pctaire1 was successfully expressed in these Xenopus embryos, with expression of exogenous Xenopus Pctaire1 dependent on the dosage of microinjected RNA.

3.5 Pctaire1 was involved in the regulation of gastrulation

To examine if Pctaire1 affects embryo phenotypes, Xenopus embryos were
microinjected with 2 nl mRNA of GFP, Pctaire1 WT and KD at different concentrations (400 pg/nl, 100 pg/nl and 25 pg/nl). Because of the limited number of viable Xenopus embryos, the number of embryos injected with 25 pg/nl of mRNA was relatively less and hence not included in the statistical analysis. Embryos injected with GFP showed normal phenotype similar to the uninjected controls (Fig. 3.10A-F). Interestingly, embryos injected with Xenopus Pctaire1 WT grew more slowly and showed some morphological defects such as developmental retardation, winding axis, small head, reduced pigment, decreased mobility and abnormal gastrulation change (Fig. 3.10G-I). It should be noted that, nonetheless, embryos with opened back, termed as abnormal gastrulation, could only be observed in embryos injected with 400 pg/nl of WT mRNA (Fig. 3.10I). Although injection of Xenopus Pctaire1 KD resulted in a lower incidence of developmental defects compared to embryos injected with Xenopus Pctaire1 WT, similar morphological abnormality could be detected in the embryos injected with KD (Fig. 3.10J-L).

Three trials of microinjection were performed. The results were summarized in Table 3.1. The percentage of developmental retardation (delayed growth and maturation of the embryos), morphological abnormality (including all abnormal phenotypes except abnormal gastrulation) and abnormal gastrulation were analyzed (Fig. 3.11A-C). Developmental retardation was evident in embryos injected with Pctaire1 WT and KD, with the percentage of embryos exhibiting developmental retardation reduced when lower dosage of mRNA was injected (Fig. 3.11A). For the morphological abnormality index, all groups injected with different dosage of
Pctaire1 WT or KD showed similar ratio (Fig. 3.11B). A possible explanation for the results is that a very little change of Pctaire1 expression in embryos could cause a wide range of "morphological abnormality". The abnormal gastrulation was specific in the group injected with high dosage of Pctaire1 WT mRNA (Fig. 3.11C).

To confirm that there is no degradation of the exogenous Pctaire1 during and after microinjection, Western blot analysis was carried out to examine the expression of exogenous *Xenopus* Pctaire1 at early stages. As shown in Fig. 3.12, equal amount of *Xenopus* Pctaire1 WT and KD was detected with anti-S95 antibody at stage 8 and 12. Taken together, our observations suggested that the abnormal gastrulation was specific for high dosage microinjection of *Xenopus* Pctaire1 WT, but not the KD.

### 3.6 Preparation of antisense oligo for *Xenopus* Pctaire1

Morpholino antisense oligos are the premier tool for gene knockdown in developmental biology and are excellent gene-silencing reagent for cells in culture. In order to block the expression of endogenous *Xenopus* Pctaire1, two morpholino antisense oligos were designed according to the protocol of Gene Tools (Philomath, OR) by targeting the 5’ UTR through the first twenty-five bases of coding sequence.

Two nl morpholino antisense oligo against *Xenopus* Pctaire1 (XMO-1, XMO-2) was microinjected into *Xenopus* embryos at 2 or 4-cell stage with variant concentration: 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, 5 ng/ml, 10 ng/ml and up to 20 ng/ml.
Figure 3.9 Protein expression of microinjected *Xenopus* Pctaire1 WT. Embryos were injected with GFP control or *Xenopus* Pctaire1 WT. Protein was extracted from the whole embryos at stage 8 and 12. Western blot analysis of *Xenopus* Pctaire1 was performed using anti-S95 antibody.
Figure 3.10 Morphology of embryos at stage 33/34. (A-C) Uninjected embryos and (D-F) embryos injected with mRNA of GFP showed normal phenotype. (G-I) Embryos injected with Pctaire1 WT mRNA at 400 pg/ml and embryos injected with Pctaire1 KD mRNA at 400 pg/ml showed abnormal morphology. G, shortened body and two tails; H, small head and winding axis; I, abnormal gastrulation; J, apparently normal; K, winding axis; L, decreased pigmentation, shortened body and two tails.
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<th>mRNA pg/nl</th>
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<th>Petaire1 WT 400</th>
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Table 3.1 Number of embryos in different groups for phenotype study. Number of uninjected embryos and embryos microinjected with GFP, *Xenopus* Petaire1 WT or KD mRNA in three independent trials was listed.
Figure 3.11 Phenotype analysis of *Xenopus* embryos. Seven groups of *Xenopus* embryos were included: uninjected control; GFP400, WT400, KD400: embryos microinjected with GFP, *Xenopus* Pctaire1 WT or *Xenopus* Pctaire1 KD mRNA at concentration of 400 pg/ml, respectively; GFP100, WT100, KD100: embryos microinjected with GFP, *Xenopus* Pctaire1 WT or *Xenopus* Pctaire1 KD mRNA at concentration of 100 pg/ml, respectively. All embryos were injected with 2 nl of mRNA. The percentage of each phenotype in each group was shown. The mean of three independent experiments performed was depicted. Error bars represented the SEM. (A) The ratio of developmental retardation in each group. (B) The ratio of abnormal morphology in each group. (C) Abnormal gastrulation was specific in the embryos microinjected with high dosage of *Xenopus* Pctaire1 WT mRNA.
**Figure 3.12** Protein expression of microinjected *Xenopus* Pctaire1. Uninjected embryos or embryos injected with GFP control, *Xenopus* Pctaire1 WT or KD mRNA were collected at stage 8 and 12. Protein was extracted from the whole embryos. Western blot analysis of *Xenopus* Pctaire1 was performed using anti-S95 antibody.
To verify the efficiency of gene knockdown, the endogenous *Xenopus* Pctaire1 expression for embryos injected with the antisense oligos (XMO-1 and XMO-2) was examined by Western blot analysis at different stages, such as stage 8 (blastulation), 12 (gastrulation), 16 (neurulation) and 28. Compared with the uninjected embryos and embryos injected with FITC control, neither XMO-1 nor XMO-2 reduced the endogenous *Xenopus* Pctaire1 expression. In accordance with the lack of gene knockdown, no obvious morphology change was observed during development (Fig. 3.13).
Figure 3.13 XMO-1 and XMO-2 could not inhibit endogenous Xenopus Pctaire1 expression. XMO-1 (A) and XMO-2 (B) of different concentration were microinjected into embryos at 2 to 4-cell stage. Protein lysates were collected at stage 8, 12, 16 and 28 and subjected to SDS-PAGE. Western blot analysis of Pctaire1 was performed using anti-S95 antibody.
Chapter 4   Identification of Pctaire1-interacting proteins in yeast two-hybrid screen and mass spectrometry

*Xenopus laevis* development study suggested that Pctaire1 activity may be involved in the gastrulation process. Nonetheless, to study the functional roles of Pctaire1 in the brain at a molecular level, it is important to identify the interacting proteins of Pctaire1. Two proteomics approaches have therefore been employed to identify Pctaire1-interacting protein: one is the yeast two-hybrid screen and another one is mass spectrometry.

4.1 Identification of Pctaire1-interacting proteins using yeast two-hybrid screen

First of all, a yeast two-hybrid screen was performed using full length wild type Pctaire1 as bait to screen an adult mouse brain cDNA library. The yeast two-hybrid system used was the MATCHMAKER GAL4 system 2.

The yeast two-hybrid assay is based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site (UAS). One or more activation domains (AD) direct the RNA polymerase II complex to transcribe the gene downstream of the UAS. Since both DNA-BD and AD are required for the activation of gene transcription, physically separated DNA-BD and AD peptides, cloned by
recombinant DNA technology and expressed in the same host cells, cannot directly interact with each other and therefore cannot activate the reporter genes. However, if the DNA-BD and AD are brought to close physical proximity in the promoter region, the transcriptional activation function will be restored.

In the MATCHMAKER two-hybrid system 2, DNA-BD and AD are derived from the same yeast GAL4 protein (a.a. 1-147 and 768-881, respectively). Two different vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other. The bait gene, Pctaire1, was subcloned into pAS2-1 vector, fused with DNA-BD, whereas the library was constructed into pACT2 vector, fused with AD. Both plasmids were cotransformed into yeast for overexpression. An interaction between the bait protein and the library protein will create a novel transcriptional activator that activates the transcription of the reporter genes, HIS3 and lacZ, whose expressions make this interaction phenotypically detectable (Fig. 4.1).

To screen for potential Pctaire1 interacting protein using the yeast two-hybrid system, the intracellular region of mouse Pctaire1 (1-496 a.a.) was subcloned into the pAS2-1 vector containing the DNA-BD (Fig. 4.2) with Nco I and Sal I sites, whereas the cDNA library was constructed into the pACT2 vector containing the AD by EcoR I and Xho I sites (Fig. 4.3). The reading frame of the bait construct was confirmed by auto-sequencing. In the yeast two-hybrid screen, a total of 3.4x10^6 clones were screened and nine positive clones were identified, among which six were known genes and three were novel genes (Table 4.1).
Figure 4.1 Principle of MATCHMAKER GAL4 yeast two-hybrid system.

DNA-BD, the GAL4 DNA-binding domain; AD, the GAL4 activation domain.
Figure 4.2 Construction of the bait plasmid. Full length Pctaire1 wild type was amplified by PCR, and the product was subcloned into the pAS2-1 vector using NcoI and SalI sites. The reading frame of the bait and the DNA Binding Domain (DNA-BD) was confirmed by auto-sequencing.
Figure 4.3 Information on the cDNA library. The adult mouse brain cDNA library was subcloned into the pACT2 vector by EcoR I and Xho I sites.
<table>
<thead>
<tr>
<th>Identity</th>
<th>Size</th>
<th>Number of clones</th>
<th>Full length / C-terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Known proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin I</td>
<td>377 a.a.</td>
<td>1</td>
<td>Full Length</td>
</tr>
<tr>
<td>N-ethylmaleimide sensitive fusion protein (NSF)</td>
<td>744 a.a.</td>
<td>5</td>
<td>C-terminal (739 a.a.)</td>
</tr>
<tr>
<td>Membrane-associated guanylate kinase-related MAGI-3</td>
<td>1126 a.a.</td>
<td>1</td>
<td>C-terminal (249 a.a.)</td>
</tr>
<tr>
<td>mago-nashi</td>
<td>146 a.a.</td>
<td>1</td>
<td>Full Length</td>
</tr>
<tr>
<td>TAFI68</td>
<td>586 a.a.</td>
<td>4</td>
<td>Full Length</td>
</tr>
<tr>
<td>Spectrin beta chain, brain</td>
<td>2363 a.a.</td>
<td>5</td>
<td>C-terminal (623 a.a.)</td>
</tr>
<tr>
<td><strong>Novel clones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLJ36090</td>
<td>929 a.a.</td>
<td>6</td>
<td>C-terminal (909 a.a.)</td>
</tr>
<tr>
<td>RIKEN cDNA 5730467H21</td>
<td>760 a.a.</td>
<td>4</td>
<td>C-terminal (633 a.a.)</td>
</tr>
<tr>
<td>Similar to hypothetical protein</td>
<td>94 a.a.</td>
<td>1</td>
<td>Full Length</td>
</tr>
</tbody>
</table>

Table 4.1 Positive clones for Peta1-interacting proteins. a.a., amino acids.
4.1.1 NSF

Of the nine positive clones, one of the Pctaire1-interacting proteins has been identified as N-ethylmaleimide sensitive fusion protein (NSF), which is a crucial factor involved in vesicular transport and membrane fusion events. The clone we obtained is about 2.2kb, encoding the full length of NSF lacking of the first five amino acids (a.a.) at the N-terminus. Firstly, the cDNA clone from the library was subcloned into mammalian expression vector pcDNA3-3HA so that the expressed protein could be detected by HA antibody. COS-7 cells were transiently transfected with Pctaire1 and HA tagged C-terminal of NSF, which lacks the first five a.a. at the N-terminus. Pctaire1 was immunoprecipitated from the cell lysates using its specific antibody, and the presence of NSF in the complex was detected by immunoblotting with HA antibody. It is shown that Pctaire1 antibody, but not normal IgG, co-immunoprecipitated NSF from the cell extracts (Fig. 4.4). Stripping and re-blotting of the membrane with Pctaire1 antibody revealed that Pctaire1 was effectively immunoprecipitated (Fig. 4.4, lower panel), suggesting that Pctaire1 similarly interacted with the C-terminal of NSF in mammalian cells. Interaction between full length NSF and Pctaire1 has also been verified, and it will be described in later sections.

4.1.2 Cyclin I

Cyclin I was identified as a Pctaire1-interacting protein. Cyclin I is initially isolated from an equalized cDNA library derived from human forebrain cortex.
(Nakamura et al., 1995). It contains a typical cyclin box near the N-terminus and a
PEST sequence near the C-terminus. Cyclin I may share similar functional roles with
cyclin G1 and G2 based on their high structural similarity among deduced amino
acid sequences (Jensen et al., 2000). Cyclin I mRNA is expressed in postmitotic
tissues at high levels, including skeletal muscle, heart, and brain, and was expressed
constantly during cell cycle progression, indicating a novel function independently of
the cell cycle control (Nakamura et al., 1995). Moreover, transient expression of
cyclin I-GFP fusion proteins in cell line shows that cyclin I is distributed throughout
the cell in contrast with the restricted cytoplasmic localization of cyclin G2 and
nuclear localization of cyclin G1. It indicates that despite the close structural
similarity among cyclin G1, G2 and I, these three proteins are likely to have distinct
biological roles (Jensen et al., 2000). Interestingly, a new study reported that cyclin I
protein expression in 144 invasive human breast cancers was correlated with cell
cycle and angiogenesis-related proteins and clinico-pathological data (Landberg et al.,
2005).

To verify the interaction between cyclin I and Pctaire1 in mammalian cells,
the full length cyclin I from the library was subcloned into mammalian expression
vector pcDNA3 with 3HA tag. COS-7 cells were transiently transfected with Pctaire1
and HA tagged cyclin I. Pctaire1 was immunoprecipitated from the cell lysates using
Pctaire1 specific antibody, and the presence of cyclin I in the complex was detected
by immunoblotting with HA antibody. As shown in Fig 4.4, Pctaire1 antibody, but
not normal IgG co-immunoprecipitated cyclin I from the cell extracts of COS-7
overexpressing Pctaire1 and cyclin I, verifying the interaction between Pctaire1 and cyclin I in mammalian cells.

The interaction between cyclin I and Pctaire1 was further examined in rat tissues. However, direct interaction between Pctaire1 and cyclin I could not be detected by pull down assay using adult rat brain or muscle lysates (data not shown). Therefore, whether Pctaire1 and cyclin I interacted endogenously remained unknown. Nonetheless, since three proline-directed serine phosphorylation sites and one proline-directed threonine site are detected in the amino acid sequence of cyclin I, we are interested to examine whether Cyclin I is a substrate of Pctaire1. *In vitro* kinase assay was performed using His-tagged cyclin I recombinant protein as a substrate. However, active Pctaire1 could not phosphorylate cyclin I *in vitro* (Fig. 4.5A). Finally, whether cyclin I serves as an activator of Pctaire1 as other cyclins can activate Cdns was explored. Nonetheless, we found that cyclin I alone was insufficient to activate Pctaire1 in *in vitro* kinase assay (Fig. 4.5B). Taken together, our findings suggest that cyclin I was not likely to be a Pctaire1-interacting protein *in vivo*. In addition, cyclin I could not activate Pctaire1, nor could cyclin I be phosphorylated by Pctaire1.

### 4.1.3 MAGI-3

Another Pctaire1-interacting protein identified through the yeast-two hybrid screen was MAGI-3. The clone we obtained was about 0.8 kb, encoding the C-terminal (249 a.a.) of MAGI-3.
Figure 4.4 Pctaire1 interacted with positive clones in COS-7 cells. HA tagged constructs encoding positive clones were transiently transfected into COS-7 cells with Pctaire1. The cell extracts were immunoprecipitated using Pctaire1 antibody and immunobotted with HA antibody. The membrane was then stripped and re-blotted with antibody against Pctaire1 (lowest panel).
Figure 4.5 Cyclin I was not likely to be substrate or activator of Pctaire1. (A) Pctaire1 could not phosphorylate cyclin I in vitro. Active Pctaire1 immunoprecipitated from adult rat brain extracts phosphorylated histone H1 but not purified His-cyclin I in vitro. (B) Cyclin I alone could not activate Pctaire1. Purified GST-Pctaire1, or GST-Pctaire1 and His-cyclin I together could not phosphorylate histone H1 in vitro.
MAGI (membrane-associated guanylate kinase with inverted orientation) proteins are a distinct subgroup of the MAGUKs (membrane-associated-guanylate kinase) which include MAGI-1 (Dobrosotskaya et al., 1997), MAGI-2 and MAGI-3 (Hibino et al., 2002). It is a family of scaffold proteins containing several PDZ domains, SH3 domains and a catalytically inactive guanylate kinase domain, all of which mediate protein-protein interactions (Harris and Lim, 2001).

Several studies on MAGI-3 indicates that MAGI-3 functions as a scaffold protein for frizzled-4 and Ltap and regulates the JNK signalling cascade; and links receptor tyrosine phosphatase with its substrates at the plasma membrane (Adamsky et al., 2003; Yao et al., 2004).

To verify the interaction between MAGI-3 and Pctaire1 in mammalian cells, the C-terminal fragment (249 a.a.) of MAGI-3 was obtained from the screening and subcloned into pcDNA3 vector with 3HA tag. COS-7 cells were transiently transfected with Pctaire1 and HA tagged C-terminal of MAGI-3. Pctaire1 antibody, but not normal IgG, co-immunoprecipitated Pctaire1 with the C-terminal of MAGI-3 from the cell extracts (Fig. 4.4).

To further explore if Pctaire1 interacted with full length MAGI-3, full length MAGI-3 was subcloned into pcDNA3 vector with 3HA tag and then transiently transfected into COS-7 cells with Pctaire1. Full length MAGI-3 cDNA construct and polyclonal antibody against MAGI-3 are gifts generously provided by Dr. Elior Peles (the Weizmann Institute of Science, Israel). However, no association was detected between full length MAGI-3 and Pctaire1 (data not shown). Therefore, it appeared
that full length MAGI-3 might not interact with Pctaire1.

4.1.4 mago-nashi

The full length mago-nashi was obtained as a Pctaire1-interacting protein in the yeast-two-hybrid screen. The interaction was also detected in COS-7 cells overexpressing HA-tagged mago-nashi and Pctaire1 (Fig. 4.4). Nonetheless, whether Pctaire1 and mago-nashi interact endogenously and the functional significance of this interaction remained to be explored.

Mago-nashi was first identified as a strict maternal effect, grandchildless-like, gene in Drosophila melanogaster. It is a component of the posterior determinative system, required during oogenesis, both for germ cell determination and delineation of the longitudinal axis of the embryo (Boswell et al., 1991). In Drosophila melanogaster, mago-nashi protein interprets the posterior follicle cell-to-oocyte signal to establish the major axes and to determine the fate of the primordial germ cells. It is demonstrated that the gene mago-nashi encodes an evolutionarily conserved protein that must be localized within the posterior pole plasm for germ-plasm assembly and Caenorhabditis elegans mago is a functional homologue of Drosophila mago. In the absence of mago function during oogenesis, the anteroposterior and dorsoventral coordinates of the oocyte are not specified and the germ plasm fails to assemble (Newmark et al., 1997).

Magoh, a human homolog of the Drosophila mago nashi gene product is identified as a novel component of the multiprotein complex that assembles
approximately twenty nucleotides upstream of exon-exon junctions. Magoh binds avidly and directly to RNA-binding protein Y14 and TAP, but not to other known components of the complex. Importantly, magoh also binds to mRNAs produced by splicing upstream of exon-exon junctions and its binding to mRNA persists after export. It suggests an important role for the highly evolutionarily conserved magoh protein in this complex (Kataoka et al., 2001). Meanwhile, the identification and a detailed gene expression analysis of zebrafish mago-nashi during development is reported recently (Pozzoli et al., 2004).

4.1.5 TAFI68

Another Pctaire1-interacting protein was TAFI68, which is one of the TATA-binding protein (TBP)-associated factors (TAFs). Up to date, little is known about TAFI68. The clone we obtained is about 1.8 kb encoding the full length of TAFI68. The association between Pctaire1 and TAFI68 was also observed in COS-7 cells (Fig. 4.4). The association of these two proteins is to be confirmed and the function of the potential association is still unknown.

4.1.6 Spectrin beta chain, brain

Spectrin beta chain was also identified as a Pctaire1-interacting protein in the yeast-two-hybrid screen. Brain spectrin (fodrin) is a major cytoskeletal protein underlying the plasma membrane that is involved in establishing and stabilizing regional membrane specializations (Willard, 1977; Marchesi, 1985; Srinivasan et al.,
1988; Goodman et al., 1988; Bennett, 1990). Spectrin αβ heterodimers self-associate head-to-head into stable tetramers, and ankyrin binds to the 15th repeat unit of β spectrin (Kennedy et al., 1991) and to integral proteins, joining fodrin to the membrane (Bennett, 1990). The fodrin tetramer binds protein 4.1 and actin near each end (Tyler et al., 1980), linking the spectrin tetramers into a hexagonal lattice (Liu et al., 1987). Brain spectrin is also linked to the plasma membrane by proteins other than ankyrin because brain spectrin binds with high affinity to brain membranes that have been depleted of spectrin and ankyrin (Steiner and Bennett, 1988). Brain spectrin α and β chains also bind $^{45}\text{Ca}^{2+}$ (Wallis et al., 1992). Since we are more interested in the potential role of Pctaire1 in synapse, this clone has not been examined in our study.

4.2 Identification of Pctaire1-interacting proteins by mass spectrometry

In addition to yeast two-hybrid screen, mass spectrometry was also used to identify Pctaire1-interacting proteins in the membrane fraction of adult rat brain.

4.2.1 Identification of some presynaptic proteins as Pctaire1-interacting proteins

To identify the proteins that interact with Pctaire1, flag-tagged Pctaire1, Cdk5, or vector (Mock) expression constructs were transfected into 293T cells. The cell lysates were immunoprecipitated using anti-flag antibody and then incubated with or
without adult rat brain membrane fraction. The precipitates were analysed by SDS-PAGE and the gel was stained by Gel Code blue stain reagent. Protein bands indicated in Fig. 4.6 were excised and subjected to trypsin digestion and analyzed by nanoelectrospray tandem mass spectrometry. Interestingly, based on mass spectrometry analysis, we have identified a number of synaptic vesicle-associated proteins as Pctaire1-interacting proteins (Table 4.2). Importantly, NSF was identified as a Pctaire1 interacting protein in both the yeast two-hybrid screen and the mass spectrometry. In addition to NSF, other Pctaire1-interacting proteins identified using mass spectrometry included synaptotagmin, a core component of SNAREs, and Munc18-3, a SNARE regulator, and synaptic vesicle associated proteins such as synapsin IIa, SV2 related protein and presynaptic cytomatrix protein piccolo. Moreover, synaptojanin 2B1, syndapin1 and clathrin-associated protein complex 2, which are involved in endocytosis were also shown to interact with Pctaire1. These results suggest that Pctaire1 is potentially involved in membrane trafficking events, in accordance with the recently demonstrated involvement of Pctaire1 in the early secretory pathways (Palmer et al., 2005).

4.2.2 Pctaire1 was associated with some presynaptic proteins in adult brain

To confirm the association of Pctaire1 with some presynaptic proteins identified by mass spectrometry, co-immunoprecipitation experiment in adult rat brain was performed. Cdk5, one of the Cdks, which is well established to be involved
in neurotransmission was used as control. As predicted, NSF and p35 were found to
interact with Pctaire1 (Fig 4.7). Core components of SNARE, syntaxin 1 and
synaptotagmin were also co-immunoprecipitated with Pctaire1 in adult brain lysates
(Fig 4.7). In addition, some of synaptic vesicle markers, such as synapsin I,
synaptophysin and SV2 were also co-immunoprecipitated with Pctaire1 (Fig. 4.7).
Although the direct association between Pctaire1 and these presynaptic proteins need
to be further verified, these observations suggest that Pctaire1 is potentially involved
in the synaptic vesicle associated proteins related cellular processes. These findings
open a new avenue of research in studing the functional roles of Pctaire1 in the
nervous system.
Figure 4.6 Gel code blue staining of SDS-PAGE showing immunoprecipitates that interacted with Pctaire1. Flag-tagged Pctaire1 as well as Cdk5, vector (Mock control) cDNA were transfected into 293T cells. The cell lysates were immunoprecipitated using anti-flag antibody and then incubated with or without adult rat brain membrane fraction. The precipitates were analysed by SDS-PAGE and the gel was stained by Gel Code blue stain reagent. Indicated protein bands were excised and subjected to trypsin digestion. +, incubated with adult rat brain membrane fraction; -, without incubation of adult rat brain membrane fraction. The input of adult rat brain membrane fraction was shown in the left panel.
<table>
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</tr>
<tr>
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<td>piccolo</td>
<td>VDKAK</td>
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<td>Clathrin-associated protein complex 2, beta chain minor component</td>
<td>SLPPR</td>
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</tr>
</tbody>
</table>

Table 4.2 Identification of presynaptic proteins that interacted with Petaire1 by mass spectrometry.
Figure 4.7 Pctaire1 was associated with some presynaptic proteins in adult brain.

Adult rat brain lysate was immunoprecipitated by Pctaire1 or Cdk5 (as control) antibody and Western blot analysis was performed. The panels depict Western blot analysis for NSF, syntaxin-1, synaptotagmin, synapsin I, synaptophysin, SV2 and p35 (upper panels). Rabbit normal IgG was used as the negative control. The nitrocellulose membrane was stripped and blotted with Pctaire1 or Cdk5 (Bottom panels).
Chapter 5  Characterization of the association between NSF and Pctaire1 in vitro and in vivo

5.1  Pctaire1 interacted with full length NSF in yeast

Since NSF was identified as a Pctaire1-interacting protein in both the yeast two-hybrid screen and pull down analysis, detailed analysis of their interaction was performed. The ability of the full length NSF to interact with Pctaire1 in yeast was first examined using β-galactosidase filter assay. Only the combination of full length NSF and Pctaire1, but not Pctaire1 or NSF alone, showed positive result in the filter assay (Table 5.1). Interstringly, the interaction of Pctaire1 with NSF was even stronger than that observed with p35 which was previously reported to associate with Pctaire1 (Cheng et al., 2002).

To examine the specificity of the interaction between Pctaire1 and NSF, the interaction between NSF and Cdk5 or p35 was also examined in yeast using β-galactosidase filter assay. Neither Cdk5 nor p35 interacted with full length NSF in yeast (Table 5.1), indicating that the interaction between Pctaire1 and NSF was specific.

To compare the interaction in yeast more quantitatively, ONPG based β-galactosidase activity assay was performed. Consistent with the result of the β-galactosidase filter assay, the association between Pctaire1 and NSF was stronger than the known association of Pctaire1 and p35 (Fig. 5.1).
<table>
<thead>
<tr>
<th>BD</th>
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<th>β-galactosidase filter assay</th>
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<tbody>
<tr>
<td>Pctaire1</td>
<td>NSF</td>
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</tr>
<tr>
<td>Pctaire1</td>
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<td>++</td>
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<td>-</td>
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<tr>
<td>-</td>
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</tr>
<tr>
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</tr>
<tr>
<td>P35</td>
<td>NSF</td>
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</tbody>
</table>

**Table 5.1 Association of NSF with Pctaire1 in yeast.** NSF was cloned into pACT2 vector and cotransformed into yeast Y190 with Pctaire1, Cdk5 or p35 construct individually in pAS2 vector. The interaction of Pctaire1 and NSF was examined by filter assay and the strength of interaction was determined as compared to other groups in the table. +++, strong interaction; ++, mild interaction; -, no interaction.
Figure 5.1 β-galactosidase activity of Pctaire1 and NSF in yeast. Indicated constructs were cotransformed into yeast Y190. The interaction was measured by ONPG based β-galactosidase activity assay. Data of three independent trials were shown.
5.2 Pctaire1 interacted with D2 domain of NSF directly

The interaction between Pctaire1 and NSF has to be further confirmed by biochemical approaches since the yeast two-hybrid system might give rise to false positive clones in screening. Various approaches were employed to confirm the interaction as below.

First of all, to validate if direct interaction occurred between pctaire1 and NSF, in vitro binding assay was performed. GST-Pctaire1 fusion protein was purified using glutathione sepharose column chromatography. The GST or GST-Pctaire1 protein was immobilized on glutathione-agarose beads and incubated with recombinant His-NSF. His-NSF bound specifically to GST-Pctaire1, but not the GST control (Fig.5.2A, upper panel). This observation demonstrated that Pctaire1 and NSF interacted directly in vitro.

The NSF monomer is comprised of three domains: an amino (N)-terminal domain (a.a. 1-205) and two homologous ATP-binding domains, NSF-D1 (a.a. 206-477) and NSF-D2 (a.a. 478-744; Fig. 5.2B). Different domains are responsible for binding with different proteins and play different functional roles. We are thus interested in identifying the NSF domain involved in the binding of full length Pctaire1. Since the three-dimensional structure of Pctaire1 is important for its association with p35, p11 and 14-3-3 (Cheng et al., 2002; Sladeczek et al., 1997; Le Bouffant et al., 1998), the full length Pctaire1 was used in the binding study.

To map the specific regions of NSF that interacted with Pctaire1, recombinant proteins encoding various domain of NSF were generated and their ability to interact
with Pctaire1 was analyzed using pull-down assays. Our results indicated that the D2 domain of NSF (NSF-D2) strongly interacted with Pctaire1 (Fig. 5.2A, middle panel), but the N-terminal or D1 domain failed to bind to GST-Pctaire1 (Fig. 5.2A, middle panel). The nitrocellulose membrane was then stripped and blotted with anti-GST antibody (Fig. 5.2A, bottom panel) to verify the efficiency of the immunoprecipitation.

5.3 *Pctaire1 interacted with NSF in COS-7 cells*

The interaction between Pctaire1 and NSF was examined in mammalian cells. COS-7 cells were transiently transfected with Pctaire1 and NSF cDNA expression constructs. Pctaire1 was immunoprecipitated from the cell lysates using Pctaire1 antibody, and the presence of NSF in the immuno-complex was detected by immunoblotting with NSF antibody. We found that Pctaire1 antibody, but not the normal IgG, co-immunoprecipitated Pctaire1 with NSF from the total lysates of COS-7 cells overexpressing Pctaire1 and NSF. On the other hand, Pctaire1 antibody could not immunoprecipitate NSF from the COS-7 cells that were transfected with NSF alone. Similarly, NSF antibody co-immunoprecipitated NSF with Pctaire1 from the COS-7 cells co-transfected with both cDNA constructs (Fig. 5.3).

5.4 *Expression profile of Pctaire1 and NSF*

5.4.1 *Protein expression of Pctaire1 and NSF in brain and muscle during development*
A

pull down

<table>
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<tr>
<th>Input</th>
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</table>

B

AA 1-205  206-477  478-744

N   D1   D2
Figure 5.2 Pctaire1 interacted with the D2 domain of NSF in vitro. (A) Pctaire1 associated with NSF in vitro. Recombinant His-tagged proteins encoding for NSF or different domains (NSF-N, NSF-D1, NSF-D2) were incubated with purified GST-Pctaire1 protein or GST immobilized on glutathione-agarose beads. The bound proteins were eluted and analyzed by SDS-PAGE and immunoblotted using NSF or His antibody (upper panels). The input of NSF or NSF domain proteins was indicated on the left lane. The nitrocellulose membrane was then stripped and probed with anti-GST antibody (bottom panel). (B) Schematic representation of NSF. N-terminal region (a.a. 1-205), required for binding to SNAP-SNARE complex; D1 domain (a.a. 206-477), the major ATP-binding domain required for the binding and hydrolysis of ATP; D2 domain (a.a. 478-744), the ATP-binding domain required for oligomerization of the NSF subunits.
Figure 5.3 Association of Pctaire1 with NSF in COS-7 cells. Pctaire1 and NSF were co-transfected into COS-7 cells and cell lysates were immunoprecipitated using NSF antibody and immunoblotted with Pctaire1 antibody, or vice versa. The rabbit normal IgG was used as a negative control.
To examine the temporal profiles of Pctaire1 and NSF expression in rat tissues, Western blot analysis was performed to check the protein expression of Pctaire1 and NSF during development in rat brain and muscle. The expression level of Pctaire1 protein during development was similar to that previously reported (Fig. 5.4 upper panel; Cheng et al., 2002). Briefly, Pctaire1 was expressed throughout development in brain and muscle, but the highest expression was detected in P5 brain and muscle. On the other hand, while the expression of NSF protein increased in rat brain during development, NSF protein was expressed at high level in embryonic stage but decreased during development in muscle (Fig. 5.4 lower panel).

5.4.2 Protein expression of Pctaire1 and NSF in PC12 cells during NGF-induced differentiation

PC12 cell is a neuroendocrine cell line that has been used widely for the study of exocytosis (Khvotchev et al., 2003; Machado et al., 2004; Hibino et al., 2002; Ohnishi et al., 2001). To exam the expression profile of Pctaire1 and NSF in PC12 cells during differentiation, western blot analysis was performed to examine the protein expression of Pctaire1 and NSF in PC12 cells during NGF treatment. On day 0, PC12 cells were switched from medium supplemented with high serum to growth medium supplemented with low serum which was conducive for neuronal differentiation. Western blot showed that the NSF expression remained relatively unchanged in PC12 cells even after treatment with NGF for 8 days. Nonetheless,
Pctaire1 expression was observed to increase slightly in PC12 cells at day 8 after NGF treatment compared to that of day 0 or day 4 (Fig. 5.5).

5.4.3 Subcellular expression of Pctaire1 and NSF in 14 DIV hippocampal neuron

Although Pctaire1 protein is diffusely expressed in almost all brain regions, the protein is found to be concentrated in the nuclei of large neurons (Le Bouffant et al., 2000). To further examine the subcellular localization of Pctaire1 and NSF in neuron, cultured hippocampal neurons at 14 DIV were collected. Different subcellular fractions including membrane, cytosol, nuclear and cytoskeleton fractions were obtained. As shown in Fig. 5.6, Pctaire1 as well as NSF was concentrated in the cytosol fraction but was also detected in the membrane fraction. Nonetheless, both Pctaire1 and NSF were barely detected in the nuclear and cytoskeleton fractions. These observations indicated that both Pctaire1 and NSF were enriched in the cytosol of differentiated hippocampal neuron.

5.4.4 Subcellular expression of NSF in adult brain

The highest expression level of NSF was detected in adult rat brain lysate. To understand the subcellular localization of NSF in adult brain, membrane, cytosol and nuclear fractions of adult brain were obtained by a different centrifugation and extraction method. As shown in Fig. 5.7,
Figure 5.4 Protein expression of Pctaire1 and NSF during development in brain and muscle. Rat brain and muscle tissues were collected at different stages and subjected to Western blot analysis using specific antibodies against Pctaire1 or NSF.

E18, embryonic day 18; P5, postnatal day 5, Ad, adult.
Figure 5.5 Protein expression of Pctaire1 and NSF in PC12 cells upon differentiation. PC12 cells were treated with 50 ng/ml NGF in low serum for the indicated time periods. Cell extracts were subjected to SDS-PAGE and Western blot to examine the protein expression of Pctaire1 or NSF using specific antibody. D0, day 0 treated with NGF; D4, day 4 treated with NGF; D8, day 8 treated with NGF.
Figure 5.6 Subcellular expression of Pctaire1 and NSF in 14 DIV hippocampal neurons. Subcellular fractions of cultured hippocampal neurons at day 14 after differentiation were obtained by extraction and centrifugation. Western blot was performed to detect the expression of Pctaire1 and NSF in the fractions as above. CS, cytoskeleton fraction; C, cytosol fraction; M, membrane fraction; N, nuclear fraction; HN, hippocampal neuron.
Figure 5.7 Subcellular expression of NSF in adult brain. Subcellular fractions of adult rat brain or muscle lysates were obtained by extraction and centrifugation. Western blot was performed to detect the expression of NSF in the fractions. N, nuclear fraction; M, membrane fraction; C, cytosol fraction.
NSF was mainly detected in the cytosol fraction. Some NSF was also detected in the membrane fraction but no NSF was detected in the nuclear fraction of adult brain.

5.5  *Pctaire1* associated with NSF in adult rat brain

Based on the developmental expression profile, highest level of NSF and Pctaire1 expression was detected in adult rat brain. We were thus interested to further demonstrate the association of these proteins in adult rat brain. Whole brain lysate was immunoprecipitated using anti-Pctaire1 antibody and the resolved immunoprecipitates were probed with anti-NSF antibody. As shown in Fig. 5.8, NSF was precipitated by the anti-Pctaire1 antibody. In contrast, NSF was not detected in the immunocomplex precipitated by normal IgG. Similarly, Pctaire1 was precipitated by the anti-NSF antibody, but not the IgG. Thus, the *in vivo* interaction between Pctaire1 and NSF has been confirmed in adult rat brain.

5.6  *ATP-hydrolysable condition had no effect on the interaction between Pctaire1 and NSF*

It has been reported that the NSF-SNAP complex exists in a stable association only during inhibition of ATP hydrolysis (Sollner et al., 1993). It is also shown that GluR2 and β-arrestin1 are preferentially immunoprecipitated with NSF in the presence of ATP/EDTA (Osten et al., 1998; McDonald et al., 1999). To investigate whether the interaction of Pctaire1 and NSF is influenced by the nucleotide-binding status of NSF, the immunoprecipitation was carried out in
different conditions: in the presence of ATP/MgCl$_2$ (ATP hydrolysable condition), ATP-γ-S/MgCl$_2$ (ATP non-hydrolysable condition) or EDTA (Mg$^{2+}$ chelating condition). Unexpectedly, the association between Pctaire1 and NSF apparently remained stable under these three conditions (Fig. 5.9), suggesting that the interaction observed between Pctaire1 and NSF was independent of the ATP hydrolysable condition. These results showed that the property of the interaction between Pctaire1 and NSF was different from those between NSF and its previously identified binding proteins, such as SNAP, GluR2 and Rab6 (Sollner et al., 1993; Osten et al., 1998; Han et al., 2000).
Figure 5.8 Petaire1 associated with NSF in adult brain. Adult rat brain lysates were immunoprecipitated using monoclonal NSF antibody and immunoblotted with Petaire1 antibody (left), or vice versa (right). The normal IgG was used as a negative control.
Figure 5.9 ATP-hydrolysable condition had no effect on the association between NSF and Pctaire1 in adult rat brain. Adult rat brain extracts prepared either in the presence of ATP/MgCl$_2$ or ATP-$\gamma$-S/Mg, EDTA (ATP hydrolysable and non-hydrolysable condition respectively) were immunoprecipitated with Pctaire1 antibody and immunoblotted with NSF antibody (upper panel). The nitrocellulose membrane was then stripped and blotted with Pctaire1 antibody (bottom panel).
Chapter 6  Pctaire1 phosphorylates NSF and is involved in the regulation of exocytosis

6.1 Phosphorylation of NSF by Pctaire1

6.1.1 NSF had two potential phosphorylation sites for Pctaire1

To date, the identification of physiological substrates for Pctaire1 remains elusive. Pctaire1 is a Cdk-related serine/threonine kinase containing 295-amino acid core kinase region with 52% sequence identity to Cdc2 (Meyerson et al., 1992). Similar to Cdk5, but not other members of Cdk family, Pctaire1 most likely is not related to the regulation of cell cycle progression. Purified Pctaire1 has no kinase activity. Moreover, no physiological substrate of Pctaire1 has been reported.

Cdk5 is a proline-directed serine/threonine kinase and phosphorylates the consensus sequence S/T-P-X-K/H/R, where S or T is the serine or threonine, X refers to any amino acid, and P is the proline residue at the +1 position (Beaudette et al., 1993; Songyang et al., 1996). Since Pctaire1 shows a higher similarity to Cdk5 when compared to other Cdks, we speculated that Pctaire1 might utilize preferential phosphorylation consensus sequence (S/T-P-X-K/H/R) similar to that of Cdk5. Two proline-directed serine/threonine residues can be identified in NSF, at serine-40 and serine-569 (Fig. 6.1A).

6.1.2 Pctaire1 phosphorylated NSF-D2 domain in vitro

Since there are two potential Pctaire1 phosphorylation sites and other serine
or threonine sites in NSF, the ability of active Pctaire1 to phosphorylate NSF was examined by \textit{in vitro} kinase assay. Active Pctaire1 can only be detected in brain and testis (Bessel et al., 1999; Le Bouffant et al., 1998). Active Pctaire1 protein was therefore immunoprecipitated from adult rat brain lysates. Rabbit IgG was used as a control for the immunoprecipitation assay. Interestingly, we observed that active Pctaire1 immunoprecipitated from adult rat brain lysates phosphorylates D2 domain of NSF in a dosage-dependent manner, but not the N or D1 domain (Fig. 6.1B & C).

To determine the phosphorylation site in the D2 domain, we generated a serine to alanine mutant for the potential phosphorylation site, SPDK. His-D2 recombinant protein containing S569A mutant was purified and subjected to the \textit{in vitro} kinase assay. As expected, mutation of S569 to alanine completely abolished the phosphorylation of D2 domain by kinase active Pctaire1 (Fig. 6.1C).

We have previously shown that Pctaire1 associates with p35 and is phosphorylated by Cdk5 (Cheng et al., 2002). It is therefore of interest to examine whether active Cdk5 could phosphorylate NSF. Purified His-NSF-D2 was subjected to \textit{in vitro} kinase assay using Cdk5 immunoprecipitated from adult rat brain. Histone H1, a Cdk substrate, was used as a positive control. We found that while histone H1 was effectively phosphorylated by Cdk5 \textit{in vitro}, Cdk5 failed to phosphorylate NSF, indicating that Pctaire1-mediated phosphorylation of NSF was specific (Fig. 6.1C).

These findings, together with the localization of serine-40 to the N-terminal domain, suggest that serine-569 within the D2 domain might be a potential phosphorylation site for Pctaire1.
Two proline-directed serine phosphorylation sites: N----MVRTSPNHKYIF

D2-----IKICSPDKMIGF

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Figure 6.1 Pctaire1 phosphorylated NSF-D2 domain *in vitro* at S569. (A) Sequence of two potential proline-directed serine phosphorylation sites in NSF N-terminal fragment and D2 domain. (B) Pctaire1 phosphorylated NSF-D2 domain at serine-569 *in vitro*. Purified His-NSF-D2 fusion protein was phosphorylated by active Pctaire1 immunoprecipitated from adult rat brain extracts (of different amounts from 0.1 mg to 2 mg) using *in vitro* kinase assay (left panel). The ratio of the NSF phosphorylation (right panel) was shown as the mean of three trials. The rabbit normal IgG was used as a negative control. (C) Pctaire1 phosphorylated NSF-D2 domain at serine-569. His-tagged NSF N-terminal, D1, D2 and its specific alanine mutant (NSF-D2-S569A) were subjected to the Pctaire1 kinase assay (left panel). Histone H1, a Cdk substrate, was used as a positive control. Cdk5 immunoprecipitated from the brain lysate (2 mg) could not phosphorylate serine-569 of NSF *in vitro* (right panel).
6.1.3 Pctaire1 phosphorylated full length NSF recombinant protein

_in vitro_

To provide additional evidence that Pctaire1 phosphorylates hexameric NSF, recombinant His6-tagged NSF protein which was previously shown to exist as hexamer (Fleming et al., 1998) was subjected to _in vitro_ phosphorylation assay. Whereas the recombinant wild type NSF protein could be phosphorylated by active Pctaire1, the phosphorylation was not observed with the NSF-S569A mutant protein (Fig. 6.2). This suggested that the hexameric NSF was likely to be phosphorylated by active Pctaire1 _in vitro_.

6.1.4 Pctaire1 phosphorylated NSF _in vivo_

To provide evidence that NSF is phosphorylated by Pctaire1 _in vivo_, NSF was co-transfected with either Pctaire1-WT or its kinase-dead (Pctaire1-KD) mutant [K194A; generated by mutating the key residue involved in the catalytic process of phosphorylation (Charrasse et al., 1999)] into HEK 293T cells. NSF protein was co-immunoprecipitated and its phosphorylation status was examined by Western blot analysis using proline-directed phospho-serine/threonine antibodies. NSF was found to be phosphorylated in cells that were co-transfected with Pctaire1-WT (Fig. 6.3), while the specific phosphorylation was not observed in the cells co-transfected with Pctaire1-KD. Taken together with the results of _in vitro_ kinase assay, NSF was likely to be a physiological substrate of Pctaire1.
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Figure 6.2 Petaire1 phosphorylated the full length NSF protein. His6-tagged recombinant NSF and NSF-S569A mutant proteins were purified and subjected to in vitro phosphorylation assay. Histone H1 was used as a positive control.
Figure 6.3 Pctaire1 phosphorylated NSF in HEK 293T cells. His-NSF was co-transfected with FLAG-tagged Pctaire1-WT or Pctaire1-KD into HEK 293T cells. Cell lysates were immunoprecipitated with anti-His antibody and blotted with proline directed phospho-serine/threonine antibody or NSF antibody. A protein doublet with a slower migrating form of NSF was detected in the cells co-transfected with Pctaire1-WT when compared to that of mock and Pctaire1-KD transfected cells, showing that Pctaire1 phosphorylated NSF in vivo.
6.2 Protein structure of NSF-D2 and mutants

According to the crystal structural characteristics of NSF-D2 domain (Fig. 6.4A, Yu et al., 1998), the phosphorylation site serine-569 is likely to be exposed and located on the interface of NSF monomers in the hexameric complex. It is therefore reasonable to speculate that phosphorylation of S569 might result in some conformational change that would affect the self-association of NSF.

Two phosphorylation mutants of NSF: the phosphorylation-deficient mutant (serine to alanine, S569A) and the phosphomimetic mutant (serine to glutamic acid, S569E) were generated. NSF D2 domain and D2-S569A, D2-S569E mutants were purified in GST fusion protein and the GST tag was cleaved by Precission protease (Fig. 6.4B). The secondary structure of these purified proteins was then analyzed by Circular Dichroism Spectroscopy (CD). All three proteins showed an α helix dominant structure. The difference between each other was in the normal range of secondary structure (Fig. 6.4C). It therefore appears that CD was insufficient in elucidating the structural difference between NSF D2 domain and its point mutants. The physiological significance of these point mutants need to be further examined by self association assay.

6.3 Phosphorylation of NSF by Pctaire1 reduced its oligomerization

The D2 domain of NSF, which interacts directly with Pctaire1, has been reported to be required for the oligomerization of NSF (Lenzen et al., 1998).
(Yu et al., 1998)
Figure 6.4 Protein structures of NSF-D2 and mutants. (A) Crystal Structure of D2 hexamer. SPDK phosphorylation motif was indicated in dark blue. (B) Coomassie blue staining of D2 domain and D2-S569A, D2-S569E mutants. NSF D2 domain and D2-S569A, D2-S569E mutants were purified as GST fusion protein, cleaved from GST, then analyzed by SDS-PAGE and stained by Coomassie blue staining. (C) Secondary structure of D2 domain and its mutants analyzed by CD.
Thus, we proceeded to examine whether the interaction of NSF-D2 with Pctaire1 and/or phosphorylation by Pctaire1 could regulate the oligomerization of NSF. HA-tagged full length NSF and His-tagged D2 domain of NSF were transfected into COS-7 cells with Pctaire1 wild-type (WT) or its K194A kinase-dead mutant (KD). Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA antibody. In the presence of Pctaire1-KD, there was an increase in HA-tagged full length NSF proteins detected in the co-immunoprecipitated complex compared to that of control. Conversely, in the presence of Pctaire1-WT, reduced level of HA-tagged full length NSF was detected in the co-immunoprecipitated complex (Fig. 6.5, upper panel). This finding suggested that alteration of Pctaire1 activity might regulate the oligomerization of NSF.

We further examined the possibility that the dynamic phosphorylation at serine-569 of NSF could regulate its ability to oligomerize. Two phosphorylation mutants of NSF, i.e. the phosphorylation-deficient mutant (S569A) or the phosphomimetic mutant (S569E) were generated. COS-7 cells were transiently transfected with HA-tagged and His-tagged (wild-type, S569A or S569E mutant) of NSF. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA antibody. As predicted, the anti-His antibody could co-immunoprecipitate the HA-tagged NSF protein. The self-association of NSF-S569A increased compared to that of the wild-type NSF, albeit only weakly elevated (Fig. 6.6). However, in the presence of S569E, the oligomerization of NSF was significantly reduced (Fig. 6.6), suggesting that phosphorylation of NSF by Pctaire1 at serine-569 inhibited its
oligomerization.

Similar results on the regulation of NSF oligomerization were observed when the individual mutants were expressed together with the wild-type NSF (Fig. 6.7). The self-association of NSF was attenuated in cells co-transfected with NSF-S569E and NSF-WT, similar to the results obtained with NSF-S569E alone. Interestingly, the reduced self-association of NSF was not observed when the cells were co-transfected with both S569E and S569A mutants, suggesting that the effect of overexpressing NSF-S569A mutant was dominant compared to that of NSF-S569E (Fig. 6.7). Taken together, these findings suggested that the oligomerization of NSF might be regulated by the phosphorylation status of NSF at serine-569.

We next examined whether the oligomerization of NSF regulated by the serine-569 phosphorylation could be reflected by the molecular size of NSF protein complex when expressed in HEK 293T cells. Following transient transfection of the cells with NSF or its phosphorylation-mutants, S569A or S569E, the cell lysates were subjected to size exclusion chromatography, followed by SDS-PAGE and Western blot analysis for NSF. Whereas NSF-WT and NSF-S569A were both eluted in the fractions corresponding to higher molecular weights of >440 kDa, NSF-S569E was eluted in the subsequent fractions (corresponding to lower molecular weights of between 232-158 kDa). These findings suggested that NSF-WT or NSF-S569A formed a higher molecular weight protein complex compared to that of the NSF-S569E mutant. It is tempting to speculate that NSF-WT and NSF-S569A both existed as a hexamer (the predicted molecular weight of the hexameric complex of
NSF is 510 kDa), whereas the phosphomimetic mutant of NSF (NSF-S569E) prevented NSF from conglomering as a higher molecular weight protein complex (Fig. 6.8).

To demonstrate that the assembled holoenzyme NSF could be converted to monomeric subunits via phosphorylation by Pctaire1, His<sub>6</sub>-tagged recombinant proteins of NSF-WT or its phosphorylation mutants were purified by Ni-NTA column. Additional separation of recombinant proteins by size exclusion chromatography was performed subsequently. Each fraction of size exclusion chromatography was analyzed by SDS-PAGE and Western blot. Similar observations were obtained as NSF and its mutants overexpressed in 293T cells (Fig. 6.9). While NSF-WT and NSF-S569A were both eluted in the fractions corresponding to higher molecular weights of 440-669 kDa, NSF-S569E was eluted in the subsequent fractions (corresponding to lower molecular weights between 67-158 kDa). The size of the recombinant protein complex is slightly smaller than that of the proteins overexpressed in 293T cells when estimated by the size exclusion chromatography. The possible explanation might be that NSF hexamer associated with the proteins in 293T cells. The NSF recombinant protein is comparatively pure and is therefore free from association with other proteins after its purification using Ni-NTA column.

Taken together with the results from the phosphorylation assay, these findings suggested that Pctaire1 phosphorylated hexameric holoenzyme NSF and the phosphorylation would prevent NSF to exist as a hexamer.
Figure 6.5 *Pctaire1-KD enhanced NSF oligomerization.* COS-7 cells were transiently transfected with combinations of HA-tagged full length NSF, His-tagged D2 domain of NSF and *Pctaire1-WT* or KD. Cell lysates were immunoprecipitated using anti-His antibody and blotted with antibodies against HA, His or Flag. The mean of three trials were shown in bottom. Error bars represented the SEM. *p<0.05, **p<0.01, significantly different from that of Mock.
Figure 6.6 Phosphorylation of NSF at serine-569 regulated its oligomerization.

Oligomerization of NSF-WT with its phosphorylation mutants, NSF-S569A or NSF-S569E. COS-7 cells were transiently transfected with HA-tagged and His-tagged of NSF (wild-type, S569A or S569E) as illustrated. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA or anti-His antibody. The mean of three trials were shown. Error bars represented the SEM. * $p<0.05$, significantly different from that of NSF-WT control.
Figure 6.7 Phosphorylation of NSF at serine-569 regulated its oligomerization. Oligomerization of NSF-WT with its phosphorylation mutants, NSF-S569A or NSF-S569E. COS-7 cells were transiently transfected with HA-tagged and His-tagged of NSF (wild-type, S569A or S569E) as illustrated. Cell lysates were immunoprecipitated using anti-His antibody and blotted with anti-HA or anti-His antibody. The mean of three trials were shown. Error bars represented the SEM. * $p<0.05$, significantly different from that of NSF-WT control.
Figure 6.8 Size exclusion chromatography analysis of NSF and its phosphorylation mutants, NSF-S569A or NSF-S569E. Expression constructs encoding NSF and its phosphorylation mutants were overexpressed in 293T cells, and the cell lysates were separated by size exclusion chromatography, followed by Western blot analysis for NSF. The molecular size of corresponding fractions was calibrated by specific protein marker as indicated.
Figure 6.9 Size exclusion chromatography analysis of recombinant protein encoding His<sub>6</sub>-NSF and its phosphorylation mutants, NSF-S569A or NSF-S569E. His<sub>6</sub>-tagged recombinant protein of NSF and its phosphorylation mutants S569A and S569E were purified and separated by size exclusion chromatography. The amount of NSF eluted in different fractions was detected by Western blot analysis. The molecular size of corresponding fractions was calibrated by specific protein marker as indicated.
6.4 Binding of Pctaire1 to SNARE complexes and other synaptic vesicle-associated proteins

6.4.1 Pctaire1 and NSF were concentrated in the synaptosome-enriched fraction in adult brain

To further characterize the interaction between Pctaire1 and NSF, we examined the subcellular localization of these proteins in the brain. Biochemical analysis of adult rat brain revealed that Pctaire1 and NSF were expressed in overlapping fractions. Pctaire1, as well as NSF, was concentrated in synaptosomal membrane fraction (LP1) and synaptic vesicle enriched fraction (LP2), similar to that of the SNAREs, such as syntaxin-1, synaptotagmin, or synaptic vesicle-associated proteins including synapsin I and synaptophysin (Fig. 6.10). The localization of Pctaire1, NSF and these pre-synaptic proteins to similar subcellular fractions suggested that these proteins might function coherently to modulate synaptic functions in the synaptosomal membrane and synaptic vesicles fractions. Thus, Pctaire1 might regulate the mobilization of vesicles through the phosphorylation of NSF.

6.4.2 Pctaire1 was associated with synaptic vesicle-associated proteins

To further explore the involvement of Pctaire1 in synaptic vesicle functions, we performed a pull-down analysis and found that Pctaire1 interacted with a number of synaptic vesicle-associated proteins in vivo. For example, components of SNAREs,
such as syntaxin-1 and synaptotagmin, could be pulled down from rat brain
membrane fraction by FLAG-tagged Pctaire1 (Fig. 6.11). Synapsin I and p35, a Cdk5
activator, which was previously demonstrated to interact with Pctaire1 in vivo, could
also be pulled down by Pctaire1 (Fig. 6.10; Cheng et al., 2002). The
immunoprecipitations were specific because other synaptic vesicle proteins such as
synaptophysin as well as SV2 could not be detected in the co-immunoprecipitated
complex (Fig. 6.11). It should be noted that whether Pctaire1 interacted directly with
these synaptic-vesicle proteins or if they were pulled down indirectly through the
interaction between NSF and Pctaire1 remained to be explored. On the other hand,
consistent with our previous observation that Pctaire1 activity can be regulated by
Cdk5/p35, majority of the synaptic proteins that interacted with Pctaire1 (Fig. 6.11)
could also be pulled down by FLAG-tagged Cdk5. These results suggested that
Pctaire1 was potentially involved in membrane trafficking events.

6.5 Effect of NSF phosphorylation by Pctaire1 on Ca^{2+}-dependent
exocytosis

NSF plays a critical role in the process of exocytosis. According to the
NSF-SNAP-SNARE hypothesis, the hexameric NSF uses energy from ATP
hydrolysis to dissociate SNARE complexes after membrane fusion, separating
syntaxin, SNAP-25 and VAMP and allowing the individual SNARE proteins to be
recycled for subsequent rounds of fusion (Jahn and Sudhof, 1999). Our findings on
the association of Pctaire1 with NSF and SNARE complexes, together with its
enriched expression in the synaptosomal fractions, raised an intriguing possibility that Petaire1 is a component of the NSF-SNAP-SNARE complex and plays a regulatory role in Ca^{2+}-dependent exocytosis through the phosphorylation of NSF.

To determine whether Petaire1 regulates exocytosis, we examined the effect of overexpressing Petaire1 and NSF-phosphorylation mutants on exocytosis in PC12 cells using a growth hormone (GH) release assay which has been widely adapted as a functional system to monitor exocytosis. PC12 is a well characterized neuroendocrine cell line that shares many characteristics with sympathetic neurons, such as secretion of neurotransmitters. Human GH was expressed in PC12 cells as a reporter to monitor the exocytosis.

In the current study, PC12 cells were transiently transfected with plasmid pXGH5 encoding hGH and different expression constructs. In transfected PC12 cells, hGH is concentrated in large dense core vesicles, and upon depolarization in the presence of high K^+, cells undergo Ca^{2+}-dependent exocytosis. The release of dense core vesicles in PC12 cells under basal and stimulated conditions was determined by measuring the amount of hGH upon exposure to low and high K^+ conditions, respectively. GFP transfected cells yielded similar results as mock control, and were therefore used as the control. As shown in Fig. 6.12A & B, over-expression of Petaire1-WT reduced the GH release compared to that of the GFP control significantly whereas inhibition of Petaire1 activity by overexpression of Petaire1-KD increased the hGH secretion significantly in both low and high K^+-stimulated conditions.
Moreover, over-expression of NSF phosphorylation deficient mutant (S569A) also significantly enhanced the hGH release in both conditions (low and high K⁺) compared to that observed with NSF-WT or the NSF-S569E (Fig. 6.13A & B).

Together, our findings suggested that Pctaire1 activity down-regulated constitutive and regulated exocytosis in PC12 cells through the phosphorylation of NSF.
Figure 6.10 Localization of Pctaire1 and NSF to the synaptosomal fraction.

Subcellular fractions of adult rat brain were obtained by differential centrifugation, extraction and subjected to Western blot analysis using various markers of SNARE and synaptic vesicle-associated proteins (including syntaxin-1, synaptotagmin, synapsin I, synaptophysin and SV2). P1, nuclear pellet and debris; P3, light membranes (Golgi, ER); S3, cytosolic fraction; S2, cytosol plus light membranes; H, whole brain; P2, crude synaptosomal fraction; LS1, synaptosomal cytosol; LP1, synaptosomal membrane fraction; LP2, synaptic-vesicle enriched fraction; LS2, LS1 minus LP2.
Figure 6.11 Association of Pctaire1 with synaptic vesicle-associated proteins.

Overexpressed FLAG-tagged Pctaire1 or Cdk5 in 293T cells were immunoprecipitated and then incubated with adult brain membrane fraction. The panels depict Western blot analysis for NSF, syntaxin-1, synaptotagmin, synapsin I, synaptophysin, SV2 and p35 (as control). Immunoblotting with anti-FLAG antibody detected the overexpressed Pctaire1 and Cdk5 proteins. Input, adult brain membrane fraction.
Figure 6.12 Pctaire1 activity regulated both low and high K⁺-evoked hGH release in PC12 cells. Expression constructs encoding hGH and Pctaire1-WT or KD were transfected into PC12 cells. Culture medium and cell lysates were collected and the hGH in culture medium was assayed by hGH ELISA. The ratio of hGH release in PC12 cells expressing Pctaire1-WT or Pctaire1-KD was compared to that of GFP control under low K⁺ (A) and high K⁺ (B) conditions. The mean of five experiments performed in duplicates was depicted. Error bars represented the SEM. *p<0.05, **p<0.01, compared to GFP using paired Student’s t test.
Figure 6.13 Phosphorylation of NSF regulated both low and high K⁺-evoked hGH release in PC12 cells. PC12 cells were transfected with various NSF expression constructs (WT, S569A or S569E). The ratio of hGH release in PC12 cells expressing S569A or S569E was compared to that of NSF wild-type under both low K⁺ (C) and high K⁺ (D) conditions. Shown were the mean of five experiments performed in duplicate. Error bars represented the standard error mean for each point. * p<0.05, ** p<0.01, was significantly different from that of NSF-WT using paired Student’s t test.
Chapter 7 Discussion

As a member of Cdk-related serine/threonine kinases, Pctaire1 harbors a kinase domain which is conserved among Cdns (Le Bouffant et al., 1998). Nonetheless, Pctaire1 exhibits two features that are distinct from other members of the Cdk family. First, the molecular size of Pctaire1 protein is almost twice of other Cdns due to the presence of the N-terminal and C-terminal fragments. Second, similar to Cdk5, Pctaire1 is probably not involved in the regulation of cell cycle progression (Meyerson et al., 1992; Rhee and Wolgemuth, 1995; Whitfield et al., 2002). Despite these observations, our understanding on the biological functions of Pctaire1 and its mechanisms of activation remained rudimentary.

To explore the potential functional roles of Pctaire1, several approaches were adopted. On one hand, making use of Xenopus laevis as a model, we examined the involvement of Pctaire1 in the early steps of embryogenesis. Our results revealed that Pctaire1 is involved in the progress of gastrulation. On the other hand, the potential functions of Pctaire1 were examined at a molecular level through the identification of Pctaire1-interacting proteins using yeast two-hybrid screen and mass spectrometry. Interestingly, Pctaire1 was observed to interact with a number of synaptic vesicle-associated proteins. In particular, NSF was identified as a Pctaire1-interacting protein in both the yeast two-hybrid screen and mass spectrometry, thus prompting us to further examine the interplay between NSF and Pctaire1. We found that Pctaire1 associated with NSF in adult brain, and that the D2 domain of NSF is required for its
interaction with Pctaire1 *in vitro*. More importantly, we observed that in addition to acting as a Pctaire1-interacting protein, NSF also serves as a novel substrate of Pctaire1. Phosphorylation of NSF by Pctaire1 at serine-569 in the D2 domain reduced the oligomerization of NSF. Since the oligomerization of NSF N-terminal appears to be crucial for the binding of NSF to the SNAP-SNARE complex (Tagaya et al., 1993; Nagiec et al., 1995), and hence facilitation of exocytosis, we went on to examine if Pctaire1 may affect Ca\(^{2+}\)-dependent exocytosis through its inhibition of NSF oligomerization. Subsequent study revealed that while overexpression of Pctaire1 reduced Ca\(^{2+}\)-dependent exocytosis, overexpression of dominant negative Pctaire1 enhanced exocytosis. In addition, overexpression of NSF mutant lacking the Pctaire1 phosphorylation site similarly resulted in an elevation of Ca\(^{2+}\)-dependent exocytosis. Our findings therefore demonstrated for the first time that Pctaire1 functions as a negative regulator of Ca\(^{2+}\)-dependent exocytosis, possibly through its inhibition of NSF oligomerization by phosphorylating NSF at serine-569. Together with our novel observation on the involvement of Pctaire1 in the regulation of *Xenopus* phenotype during development, our study reveals that Pctiare1 may exhibit crucial roles during early embryogenesis and also in the regulation of synapse functions through the modulation of regulated secretion.

### 7.1 Identification of NSF as a novel substrate of Pctaire1

Based on the findings of both the yeast two-hybrid screen and mass spectrometry, NSF was identified as a novel interacting protein of Pctaire1. We
further demonstrated that Pctaire1 bind to NSF endogenously in adult rat brain. More importantly, NSF is the first physiological substrate of Pctaire1 identified. Although several groups have carried out yeast two-hybrid screening in an attempt to identify interacting proteins of Pctaire1, no physiological substrate of this enigmatic kinase was found (Cheng et al., 2002; Sladeczek et al., 1997; Le Bouffant et al., 1998; Hirose et al., 2000; Palmer et al., 2005). Palmer and his colleague demonstrated that, as a human sec23p-interacting protein, Pctaire1 kinase activity is involved in early secretory pathway function (Palmer et al., 2005). Graeser and his colleague also suggested a possible role of Pctaire1 kinase activity in neurite outgrowth (Graeser et al., 2002). However, neither groups identified the physiological substrate for Pctaire1 in their studies. Here, we demonstrated that Pctaire1 phosphorylated NSF both in vitro and in vivo, thus rendering NSF a likely substrate of Pctaire1 in physiological condition.

It should be noted that some of the proteins previously demonstrated to function as Pctaire1-interacting proteins such as p35, p11, 14-3-3, Trap and Sec23p were not identified as positive clones in the current study (Cheng et al., 2002; Sladeczek et al., 1997; Le Bouffant et al., 1998; Hirose et al., 2000; Palmer et al., 2005). The apparent discrepancy is attributed to the use of different cDNA library.

Although the N-terminal domain of Pctaire1 is required for the association between Pctaire1 and p35, p11 or 14-3-3, the three-dimensional structure of Pctaire1 is critical for maximal association of Pctaire1 with these interacting proteins (Cheng et al., 2002; Sladeczek et al., 1997; Le Bouffant et al., 1998). Therefore, full length
Pctaire1 was used as the bait to screen different cDNA library in present study and in other groups.

7.2 *Pctaire1 affects NSF function through modulation of its oligomerization*

Having identified NSF as a novel substrate of Pctaire1, we were interested to further explore the physiological significance of this phosphorylation and their interaction. Several mechanisms have been demonstrated to regulate the downstream functions of NSF. On one hand, previous study revealed that several NSF-interacting proteins affect its ATPase activity upon association. A good example is α-SNAP, the most important partner of NSF, which binds to the N-terminal of NSF to mediate formation of the 20S complex, thereby stimulating the ATPase activity of NSF (Barnard et al., 1996; Barnard et al., 1997; Hayashi et al., 1994; Morgan et al., 1994; Wimmer et al., 2001). The observed interaction between NSF and Pctaire1 prompted us to first explore if binding of Pctaire1 similarly regulates the ATPase activity of NSF. However, purified Pctaire1 was not observed to result in any changes in the ATPase activity of NSF using *in vitro* ATPase activity assay (data not shown). It therefore appeared that direct binding of purified Petiare1 failed to modulate the ATPase activity of NSF.

In addition, ATPase activity of NSF in turn appeared to have no effect on the association between NSF and Pctaire1. Nucleotide-binding status of NSF has been reported to affect the association of NSF with other proteins. For example, binding of
NSF with SNAP, GluR2 or β-arrestin1 were stabilized when ATP hydrolysis is inhibited (Sollner et al., 1993a; Sollner et al., 1993b; McDonald et al., 1999; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Alternatively, association between NSF and Rab6 is impaired when ATP hydrolysis is hindered (Han et al., 2000). However, the association between NSF and Pctaire1 was independent of the nucleotide-binding status of NSF. This suggests that the ATPase activity of NSF has no effect on the association of these two proteins.

Aside from direct association, phosphorylation of NSF has been suggested to affect NSF activity. The post-translational modification of NSF through phosphorylation was summarized in Fig. 7.1. For example, tyrosine phosphorylation of NSF was observed to regulate its ATPase activity as well as its affinity for α-SNAP (Huynh et al., 2004). Furthermore, Phosphorylation of NSF by PKC affects the ability of NSF to interact with the SNAP-SNARE complex (Matveeva et al., 2001). Since Pctaire1 did not appear to affect NSF functions through direct interaction, we went on to examine the effect of Pctaire1-mediated NSF phosphorylation on NSF function. The D2 domain of NSF, which is phosphorylated by Pctaire1 at Ser-569, was reported to be required for NSF oligomerization (Fleming et al., 1998; Nagiec et al., 1995). We were therefore interested to examine if Pctaire1 affects the oligomerization of NSF. It has been demonstrated that NSF exists predominantly as a hexamer in the presence of nucleotide (Fleming et al., 1998; Lenzen et al., 1998; Yu et al., 1998). The hexameric status of NSF is crucial for its function as an ATPase, and its ability to drive dissociation of the 20S complex.
Figure 7.1 Schematic diagram illustrates post-translational modification of NSF. Activity of NSF is regulated through multiple post-translational modifications, such as S-nitrosylation and phosphorylation. The residues targeted by phosphorylation are indicated. Besides the previously reported phosphorylation sites of Y83 and S237, S569 in D2 domain is indicated as the phosphorylation site for Pctaire1.
Previous studies aimed to examine the regulation of 20S fusion complex formation and subsequent dissociation have focused mostly on elucidating mechanisms implicated in modulating the affinity of NSF to SNAP-SNARE complex and the regulation of NSF ATPase activity (Matveeva et al., 2001; Nagiec et al., 1995; Steel and Morgan, 1998; Whiteheart et al., 1994). Remarkably, through characterizing the functional significance of Pctaire1-mediated phosphorylation, we unexpectedly revealed that Pctaire1 negatively regulated the oligomerization of NSF via phosphorylation. This is the first report demonstrating that phosphorylation of NSF, in addition to affecting its ATPase activity or association with the SNAP-SNARE complex, can also modulate the hexamerization status of NSF.

In order to support the conclusion that Pctaire1 regulated the oligomeric status of NSF, we provided several lines of evidence to demonstrate that Pctaire1 phosphorylated NSF at serine-569 to reduce its oligomerization. Firstly, the wild type Pctaire1 reduced the oligomerization of NSF through its D2 domain. Secondly, while overexpression of the phosphorylation deficient mutant S569A enhanced its oligomerization, phosphomimetic mutant S569E overexpression led to reduced oligomerization when compared with that of the wild type NSF. Thirdly, our findings demonstrated the importance of the phosphorylation status of NSF on the oligomerization of NSF by size exclusion chromatography. These observations collectively suggested that Pctaire1 inhibits the oligomerization of NSF via the phosphorylation of NSF at serine-569. These findings are further supported by the information obtained from the crystal structure of NSF. According to the structural
analysis, serine-569 is located on the interface of D2 domain of NSF subunit in the hexameric complex (Yu et al., 1998). It suggests that if the specific serine site is phosphorylated, the change of the amino acid charge might lead to dissociation of the NSF hexamer. This hypothesis is supported by our results showing that mutation of serine-569 of NSF to glutamic acid resulted in the monomeric form of NSF while mutation of the same site to alanine allowed the proteins to remain as a hexameric form. Taken together, our findings demonstrated that Pctaire1 negatively regulates the oligomerization of NSF through phosphorylation of NSF at serine-569.

7.3 Roles of Pctaire1 in regulated secretion

A study has been revealed that oligomerization of NSF N-terminals are crucial for binding of α-SNAP in complex with SNAREs in vitro (Nagiec et al., 1995). While the importance of NSF oligomerization in regulated exocytosis remained to be solidly demonstrated, observation from this study suggested that disruption of NSF oligomerization hinders formation of the 20S complex, which is a pivotal component in regulated exocytosis. The observed inhibitory effect of Pctaire1 exhibiting on NSF oligomerization therefore suggested that Pctaire1 may also affect regulated exocytosis. Indeed, several lines of evidence support a potential role of Pctaire1 in the regulation of membrane fusion events at the synapse. First of all, Pctaire1 was localized to the synaptosomal membrane and synaptic vesicle enriched fraction. In addition, Pctaire1 was demonstrated to interact with a number of synaptic proteins, including SNAREs such as syntaxin1 and synaptophysin. More importantly,
a recent study revealed that Pctaire1 is functionally involved in membrane traffic during the early secretory pathway (Palmer et al., 2005). Furthermore, Cdk5, an important kinase implicated in the enhancement of Pctaire1 activity in vivo, is implicated in the regulation of neurotransmitter release. It is thus tempting to speculate that Pctaire1 may function as a downstream kinase of Cdk5 to mediate the effect of Cdk5 on neurotransmitter. These mounting evidence all points to a potential involvement of Pctaire1 in regulated exocytosis at the synapse.

We have thus proceeded to examine if Pctaire1 affects Ca$^{2+}$-dependent exocytosis. PC12 cell was chosen as a model system because it is a well established neuroendocrine cell line and widely used for exocytosis studies (Hibino et al., 2002; Khvotchev et al., 2003; Machado et al., 2004; Ohnishi et al., 2001). It shares many characteristics with sympathetic neurons especially in the secretion of neurotransmitter. By examining the effect of Pctaire1 on Ca$^{2+}$-dependent exocytosis in PC12 cells, we showed that while Pctaire1 overexpression suppressed regulated exocytosis, overexpression of dominant negative Pctaire1 markedly enhanced growth hormone release. This suggests that Pctaire1 is involved in vesicular transport events especially in regulated secretion in PC12 cells, in addition to its involvement in the membrane traffic of the early secretory pathway.

While our observations indicated that Pctaire1 negatively regulated Ca$^{2+}$-dependent exocytosis, whether this is directly related to its inhibitory effect on NSF oligomerization remained to be explored. By overexpressing phosphorylation mutants of NSF, we demonstrated that phosphorylation of NSF by Pctaire1
contributed at least partially to the observed regulation of Ca\textsuperscript{2+}-dependent exocytosis by Pctaire1. Nonetheless, whether this is preceded by disruption of the 20S complex requires further characterization.

It should be noted that data from the in vitro kinase assay showed that serine-569 of NSF could be phosphorylated by active Pctaire1 but not by Cdk5. It is interesting to observe that despite the highly conserved kinase domain between Cdk5 and Pctaire1, the two kinases exhibit a certain extent of substrate specificity. Since Cdk5 has been observed to regulate neurotransmission and synaptic vesicle cycle through the phosphorylation of various synaptic proteins, such as Munc18, synapsin I, amphiphysin I, dynamin I and synaptotagmin I (Fletcher et al., 1999; Matsubara et al., 1996; Tan et al., 2003; Tomizawa et al., 2003; Lee et al., 2004). The demonstrated involvement of Pctaire1 in the regulation of regulated exocytosis suggests that Pctaire1 may function downstream of Cdk5, or in parallel with Cdk5 to modulate presynaptic functions such as release of neurotransmitter. Given the known association between Pctaire1 and Cdk5/p35, it would also be interesting to examine if Pctaire1 and Cdk5 exist in a large protein complex at the synapse to act on various substrate proteins. Further identification of specific substrate for Pctaire1 might shed light on the functional role of this kinase. The presynaptic functions of Cdk5 and Pctaire1 were summarized in Fig. 7.2.
Figure 7.2 Targets of Cdk5 and Pctaire1 involved in neurotransmission and synaptic vesicle cycle in presynaptic terminal. Cdk5 phosphorylates synapsin I, Munc18, P/Q-type VDCC and therefore attenuates neurotransmitter release. Phosphorylation of Pctaire1 by Cdk5/p25 enhances its kinase activity. Pctaire1 functions as a negative regulator of $\text{Ca}^{2+}$-dependent exocytosis, possibly through its inhibition of NSF oligomerization by phosphorylating NSF. Dynamin I, Amphiphysin I and synaptojanin I are substrates of Cdk5. In this way, Cdk5 plays a regulatory role in synaptic vesicle recycle.
7.4 *Pctaire1* modulated early embryogenesis in *Xenopus*

In addition to demonstrating that Pctaire1 may regulate Ca\(^{2+}\)-dependent exocytosis through phosphorylation of NSF, phenotypic study of *Xenopus* suggested a potential functional role of Pctaire1 in gastrulation during early embryogenesis. The control of the gastrulation probably depended on precise regulation of Pctaire1 activity in that abnormal gastrulation was only observed in the embryos microinjected with the wild type Pctaire1. It is known that the gastrulation process requires tight regulation of cell migration, mesodermal gene transcription and cell division. Recent studies have shown the importance of cell cycle regulation in gastrulation (Hashiguchi et al., 2004). It is thus of interest to further investigate whether Pctaire1 is involved in cell cycle regulation *in vivo*.

As Pctaire1 protein could be detected in *Xenopus* embryos as early as stage 4, when the cells are undergoing rapid division, it is possible that Pctaire1 may contribute to the onset of abnormal gastrulation in early embryogenesis via regulation of cell cycle. Pctaire1 protein was found to be increased upon development. Similar regulation of Pctaire1 expression was observed in rat tissues. It is therefore tempting to speculate that Pctaire1 plays a functional role during early development since its protein expression is prominently detected in animals during the early stages of development.

As a Cdk-related kinase, there is only one study reporting that Pctaire1 possesses cell cycle dependent activity (Charrasse et al., 1999). Other studies showed that Pctaire1 is not involved in the cell cycle progression (Meyerson et al., 1992;
Rhee and Wolgemuth, 1995). In addition, no change in Pctaire1 expression was found during cell cycle through large scale array-based screening (Whitfield et al., 2002). Therefore, whether Pctaire1 acts as a typical Cdk in cell cycle control remains unknown. Since only the protein expression of *Xenopus* Pctaire1 was examined during development in the current study, whether the expression and activity of *Xenopus* Pctaire1 change during cell cycle in *Xenopus* awaits further investigation. More thorough explication on the role of *Xenopus* Pctaire1 in cell cycle progression during gastrulation will provide essential insight on the mechanisms by which Pctaire1 affects gastrulation.

The possible functional role of *Xenopus* Pctaire1 in regulation of cell migration and mesodermal gene transcription has not been explored. There are no reports on the role of *Xenopus* Pctaire1 or its ortholog on cell migration or mesodermal gene transcription. Therefore, the molecular mechanism underlying the phenotype change of *Xenopus* during early embryogenesis remains to be investigated.

7.5 Other potential functional roles of Pctaire1 in *Xenopus* development

In the current study, we observed that phospho-Pctaire1 was detected in *Xenopus* embryos in later development stages, suggesting that Pctaire1 was regulated by other kinases in later development and might function differently. Phosphorylation of *Xenopus* Pctaire1 first appeared in the animals when
organogenesis started (after stage 20) and was prominently detected in embryos at stage 40. Meanwhile, phosphorylation of Pctaire1 was also peaked in rat brain and muscle at adult stage. The phosphorylation of Pctaire1 occurred at later developmental stages suggested a potential functional role of Pctaire1, although further characterization will be needed. Does the phospho-Pctaire1 have a regulatory role in early secretory pathway or regulated secretion as we discussed? Could phospho-Pctaire1 be involved in neurite outgrowth as suggested by Graeser and his colleague (Graeser et al., 2002)? And does it have a novel function? All of questions need to be answered by further studies.

The presence of phospho-Pctaire1 suggested that it was regulated by other kinases. It has been reported that Pctaire1 could be phosphorylated by different kinases including PKA and Cdk5, where Pctaire1 activity is reported to be regulated by phosphorylation at different sites (Cheng et al., 2002; Graeser et al., 2002). PKA phosphorylation of Pctaire1 results in the reduction of Pctaire1 activity, while its phosphorylation by Cdk5 enhanced Pctaire1 activity. In later developmental stages of Xenopus, whether Pctaire1 is phosphorylated by PKA and/or Cdk5, or unknown kinases still remains far from clear. Further experiments on the regulation of Pctaire1 phosphorylation should be carried out in Xenopus model.

7.6 Summary and future perspective

In the present study, we identified a number of Pctaire1-interacting proteins in adult brain. The identification of these candidates enabled exploration of the
functional roles of Pctaire1 in nervous system. Two proteomic methods, yeast two-hybrid screening and mass spectrometry, were used to identify the proteins that interacted with Pctaire1 in adult brain. Among several candidates including some synaptic vesicle-associated proteins, one of the Pctaire1-interacting proteins, NSF, was identified in both screenings. The association between Pctaire1 and NSF was confirmed both \textit{in vitro} and \textit{in vivo}. The D2 domain of NSF, which is required for the oligomerization of NSF subunits, bind directly to, and was phosphorylated by Pctaire1 at serine-569. We also demonstrated that NSF was a novel substrate of Pctaire1 \textit{in vitro} as well as \textit{in vivo}. The significance of this specific phosphorylation was later revealed as a negative regulation of NSF hexamerization, as demonstrated by the self-association assay in 293T cells and size exclusion chromatography. More interestingly, Pctaire1 activity inhibited the constitutive and Ca^{2+}-dependent exocytosis in PC12 cells. Taken together, our findings provided the first demonstration that phosphorylation of NSF by Pctaire1 regulated its oligomerization, implicating an unexpected role of Pctaire1 in modulating exocytosis.

Our study has therefore provided novel insights on the mechanisms implicated in the regulation of Ca^{2+}-dependent exocytosis, or more specifically, regulated neurotransmitter release. NSF acts as a crucial factor in vesicular transport and membrane fusion events. Phosphorylation of NSF by the Cdk-related kinase Pctaire1 provided a new mechanism through which NSF activity is regulated. The potential regulatory role of Pctaire1 in neurotransmitter release upon phosphorylation of NSF should be examined in neuron culture. Meanwhile, the novel function of
Pctaire1 suggested in regulated secretion also prompts us to explore the potential relationship between Pctaire1 and Cdk5 in the regulation of neurotransmitter release in the presynaptic cells. As a substrate of Cdk5/p35, Pctaire1 activity increases after phosphorylation by Cdk5. The potential regulatory role of Cdk5 in Pctaire1-mediated modulation of NSF remains to be unraveled. In addition, the possible cooperative or competitive relationship between Pctaire1 and Cdk5 in synaptic vesicle cycle is also due to be explored.
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