Regulation of cell survival by CIB1, a new modulator of 3-phosphoinositide-dependent protein kinase 1 (PDK1)

By Bin Zhao

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Approved by:

Dr. Leslie V. Parise (Advisor)

Dr. Lee M. Graves (Chair)

Dr. Klaus M. Hahn

Dr. Gary L. Johnson

Dr. Rudolph L. Juliao

ABSTRACT

Bin Zhao. Regulation of Cell Survival by CIB1, a New Modulator of 3-Phosphoinositide-dependent Protein Kinase-1 (PDK1)

(Under the direction of Dr. Leslie V. Parise)

CIB1, a calcium and integrin binding protein, was first identified by its specific binding to the cytoplasmic tail of the platelet-specific integrin αIIb. Later experiments showed that CIB1 is widely expressed and binds to a variety of proteins including many serine/threonine kinases such as PAK1, suggesting that it acts as a regulator of kinase function. The overall goal of this work has been to further identify new CIB1-binding proteins, and therefore to better understand the role of CIB1 in human diseases. Using a high stringency *in silico* analysis of the CIB1 protein sequence, we determined that CIB1 contains a consensus PDK1 hydrophobic binding motif. Further detailed experiments confirmed that CIB1 binds PDK1 both *in vitro* and in cells. PDK1, 3-phosphoinositide-dependent protein kinase 1, is a member of the AGC protein kinase family and plays an important role in regulating cell growth, survival, and cell cycle progression by activating other AGC kinases such as Akt, S6K, or RSK. However, how PDK1 itself is regulated is less clear. We demonstrated that the binding of CIB1 to PDK1 upregulates PDK1 kinase activity and promotes cell survival. We found that CIB1 depletion significantly enhances

cell apoptosis in response to a variety of apoptotic stimuli, a response significantly rescued by PDK1 overexpression, whereas CIB1 overexpression protects cells from apoptotic stimuli in a PDK1-dependent manner. The ability of CIB1 to promote cell survival appears to require both membrane localization and PDK1 binding, since CIB1 mutants that either do not bind PDK1 or do not localize to membranes induce a marked redistribution of PDK1, and no longer protect cells from apoptosis. Moreover, depletion of CIB1 reduces Akt and RSK3 phosphorylation by PDK1, suggesting a CIB1/PDK1/Akt or RSK3 dependent pathway. Taken together, our results indicate that CIB1 is an important upstream activator of PDK1, thereby resisting apoptosis and promoting cell survival.

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ABBREVIATIONS

AGC kinases	PKA, PKG and PKC related kinase
CIB1	Calcium and integrin binding protein 1
EBs	embryoid bodies
ECs	endothelial cells
EGFR	epidermal growth factor receptor
GLUT4	Glucose transporter 4
GST	glutathione S-transferase
HM	hydrophobic motif
IGF1	insulin-like growth factor-1
mAKAPα	muscle A-kinase anchoring protein mAKAPα
MEFs	mouse embryonic fibroblasts
MMP	matrix metalloproteases
mTOR	Mammalian target of rapamycin
NF-kB	nuclear factor kB
NHE1	Na^+/H^+ exchanger NHE1 isoform
NHERF2	Na^+/H^+ exchanger regulating factor 2
PAK1	p21-activated kinase
PDGF	platelet derived growth factor
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C

PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology
PI3 kinase	phosphoinositide 3-kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PRK	PKC-related protein kinase 1 and 2
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten
PIF	PDK1 interacting fragment
PyMT	polyoma middle T antigen
ROS	reactive oxygen species
RSK	p90 ribosomal S6 kinase
RTKs	Receptor tyrosine kinases
S6K	p70 ribosomal S6 kinase
SGK	serum- and glucocorticoid-induced protein kinase
TRAIL	Tumor necrosis (TNF)-related apoptosis-inducing ligand
VSMC	vascular smooth muscle cells
VEGF	vascular endothelial growth factor-A

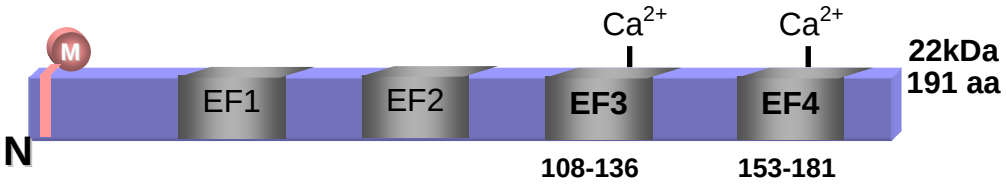
Chapter I

An Introduction to CIB1

1. Characterization of CIB1 -- a Ca^{2+} and integrin binding protein

Integrins are transmembrane α/β heterodimers that serve as links between the extracellular matrix (ECM) and intracellular signals and regulate a variety of important cellular functions including cell migration and cell survival. Each integrin subunit is composed of a large extracellular domain, a single transmembrane (TM) domain and a small cytoplasmic domain. The transmembrane and cytoplasmic domains are believed to play an important role in integrin inside-out and outside-in signaling events. The $\alpha IIb\beta_3$ is a platelet specific integrin and plays a critical role in thrombosis and other hemostatic events (Parise, 1999). Therefore, identification of proteins that bind to the $\alpha IIb\beta_3$ cytoplasmic tail has been intensely studied. CIB1 is one example--it was first identified as a protein that specifically binds to the platelet integrin αIIb cytoplasmic tail through a yeast two hybrid screen (Naik et al., 1997). Cloning and sequence analysis reveals that CIB1 is a 191 amino acid protein that shares significant homology with two well-known Ca^{2+}-binding regulatory proteins, calmodulin (27% identity, 55% similarity) and calcineurin B (28% identity, 58% similarity) (Naik et al., 1997). Sequence analysis also reveals an N-terminal myristoylation site and several consensus phosphorylation sites for PKC and casein kinase II. However, CIB1 has not been shown to be regulated by phosphorylation so far (Tina Leisner, personal communication). CIB1 contains four EF-hand motifs and the two C-terminal EF-hands 3 and 4 bind Ca^{2+} (Naik *et al.*, 1997;Gentry et al., 2005) (Fig. 1-1).

A



B

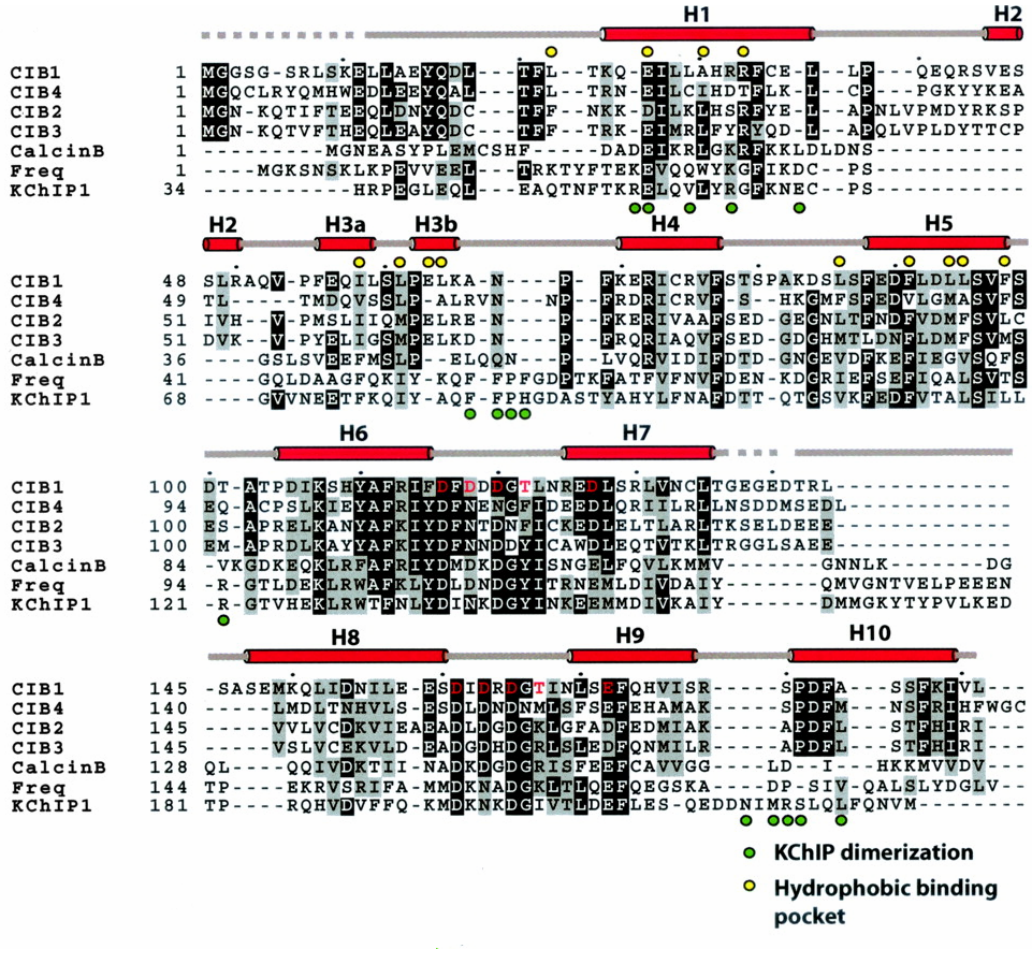
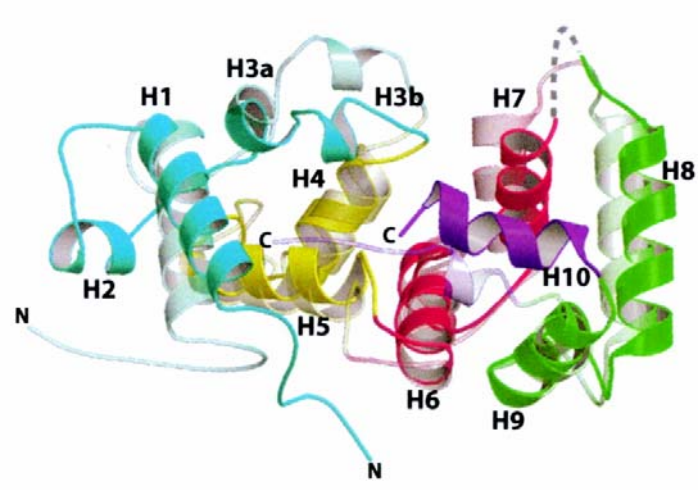


Figure 1-1 Secondary structure and sequence alignment of CIB1. **(A)** Secondary structure of CIB1 shows an N-terminal myristoyl group shown in pink box, four EF-hand domains in grey boxes, with EF3 and EF4 binding Ca^{2+}. **(B)** Sequence alignment of CIB1 with its three human homologues (CIB2, CIB3, and CIB4), as well as calcineurin B and two neuronal calcium sensor proteins (NCS) Frequenin (Freq) and KCHIP1. Residues in CIB1 predicted to bind Ca^{2+} are in red and residues contributing to the hydrophobic binding pocket are highlighted by yellow circles. Reprinted from Gentry et al 2005, with permission from the Journal of Biological Chemistry 280(9), 8407-8415, 2005.

2. CIB1 crystal structure

The crystal structure for Ca^{2+}-bound CIB1 has been solved at 2.0 Å resolution and reveals significant structural similarity to some other EF-hand containing proteins such as calcineurin B and the neuronal calcium sensor (NCS) (Gentry et al., 2005) (Fig. 1-2). In addition, sequence analysis of CIB1 indicates that it has four homologues (CIB1-4) and they form a distinct subfamily of EF hand-containing proteins. CIB2 (KIP2), CIB3 (KIP3), and CIB4 share 59%, 62%, and 64% sequence similarity to CIB1, respectively (Fig. 1-1 B). No functional data about CIB2 and CIB3 have been reported, and CIB4 is named based on a sequence from computational analysis of the human genome (NCBI accession # XP_059399) (Gentry et al., 2005). The crystal structure reveals a hydrophobic binding pocket in CIB1 (Fig. 1-2 B) and is conserved in its homologues (Gentry et al., 2005), as well as other EF-hand-containing proteins such as calcineurin B. However, the CIB1 binding pocket appears to be narrower than that of calcineurin B. Some proposed integrin αIIb binding residues within CIB1 include Phe 115, Leu 131 and Phe 173, based on the finding from chemical shift perturbation assays (Yamniuk et al., 2006). However, the crystal structure study shows that these residues are buried under the C-terminal H10 helix and are therefore not surface-accessible. A recent study shows that αIIb binding to CIB1 displaces the H10 helix of CIB1, whereby it effectively increases the size of the hydrophobic binding pocket and makes these residues surface-accessible for binding (Yamniuk et al., 2006) (Fig. 1-2).

A



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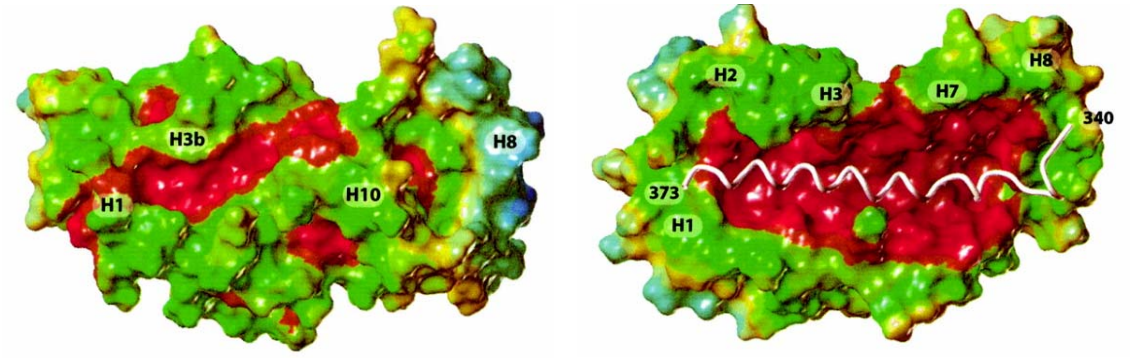


Figure 1-2 CIB1 crystal structure. (A) Superposition of CIB1 crystal structure with its closest structural homolog calcineurin B. CIB1 ribbons are in color and calcineurin B ribbons are semi-transparent. The N-terminal region and EF1 hand are in blue, EF2 hand is yellow, EF3 hand is red, EF4 hand is green, and the C-terminal H10 helix is purple. **(B)** CIB1 (left) contains a hydrophobic pocket (red color) that is preserved in other EF-hand-containing proteins such as calcineurin B (right), but is narrower than that of calcineurin B, partly due to the fold-back of the H10 helix. Reprinted from Gentry et al 2005, with permission from the Journal of Biological Chemistry 280(9), 8407-8415, 2005.

3. CIB1 binding proteins and biological functions

1) Integrin $\alpha IIb\beta_3$

CIB1 directly binds the integrin αIIb cytoplasmic tail *in vitro* with an affinity of approximate 0.3 μM, as demonstrated by a variety of approaches, including solid-phase binding (Naik et al., 1997), isothermal titration calorimetry (ITC) (Shock et al., 1999), intrinsic tryptophan fluorescence binding (Barry et al., 2002), and GST pulldown (Tsuboi, 2002). Endogenous CIB1 and $\alpha IIb\beta_3$ also coimmunoprecipitate with each other in both resting and agonist-activated platelets (Yuan et al., 2006b), but it remains unclear whether the CIB1/$\alpha IIb\beta_3$ interaction is Ca^{2+}-dependent. Experiments using megakaryocytes (platelet precursor cells) indicates that CIB1 functions as an endogenous inhibitor of agonist-induced $\alpha IIb\beta_3$ activation (Yuan et al., 2006b). In this study, overexpression of CIB1 markedly inhibits agonist-induced $\alpha IIb\beta_3$ activation, as indicated by fibrinogen binding to megakaryocytes, whereas knockdown of endogenous CIB1 by siRNA significantly increases fibrinogen binding to megakaryocytes (Yuan et al., 2006b). The mechanism of CIB1 inhibition of integrin $\alpha IIb\beta_3$ activation has been hypothesized to occur via the competition of CIB1 with talin for binding to $\alpha IIb\beta_3$ (Yuan et al., 2006b).

Although first identified in platelets, CIB1 was later found to be widely expressed in many other tissues, such as heart, pancreas, liver, muscle, brain, and kidney (Stabler et al., 1999;Shock *et al.*, 1999), suggesting it has biological functions in other cell types. Consistent with this, some recent *in vitro* binding assays including NMR and ITC data show that in addition to αIIb, CIB1 binds other integrin cytoplasmic tails including α5, α2, α4, αL, and αM (Gentry H, unpublished data), demonstrating that CIB1 has platelet-

independent functions. However, detailed *in vivo* interaction studies are needed to investigate the *in vivo* biological significance of these interactions. In addition, CIB1 has been reported to bind a wide variety of proteins that include a number of serine/threonine kinases, which are discussed below.

2) PAK1

P21-activated kinase 1 (PAK1) is a serine/threonine kinase that is a major effector of the Rho GTPases Cdc42 and Rac (Manser et al., 1994). It plays a central role in regulating cell spreading and migration by promoting cytoskeletal rearrangement and actin turnover (Kiosses et al., 1999;Sells et al., 1999). In addition to being regulated by small GTPases, other molecular interactions also regulate PAK1, such as the phospholipid sphingosine (Bokoch et al., 1998;Lian et al., 1998), PI3K (Papakonstanti and Stournaras, 2002), PDK1 (King et al., 2000a), actin-binding protein filamin A (FLNa) (Vadlamudi et al., 2002), the SH2/SH3-containing adaptor protein Nck (Lu and Mayer, 1999), and PAK-interacting exchange factors Cool/PIX (Bagrodia et al., 1998;Daniels et al., 1999).

CIB1 was shown to directly bind PAK1 both *in vitro* and in cells (Leisner et al., 2005). CIB1 binding to PAK1 was found to be Ca^{2+}-dependent and effectively stimulated PAK1 kinase activity. The CIB1 binding site in PAK1 was mapped to two discrete regions flanking the p21-binding domain (PBD) that are referred to as Site I and Site II (Leisner et al., 2005). Site II overlaps the PAK1 autoinhibitory domain and it has been hypothesized that CIB1 binding to this site may relieve the autoinhibitory constraints and promote PAK1 activation. The biological significance of CIB1/PAK1 binding has been underscored by the finding that CIB1 negatively regulates cell migration on fibronectin

and this event appears to involve a PAK1-LIM kinase-phospho-cofilin-dependent signaling pathway. Another important finding of this study is that PAK1 is regulated in a distinct temporal manner by Rac/Cdc42 and CIB1; it appears that CIB1 is required for adhesion-induced PAK1 activation at early time points, whereas Rac/Cdc42 becomes a more important regulator at later time points (Leisner et al., 2005).

3) Polo-like kinases (Fnk, Snk)

Polo-like kinases (plks) are a family of serine/threonine kinases containing three members in mammalian cells, including Plk1, Snk (Plk2), and Fnk (Plk3). This family of kinases possesses a conserved C-terminal polo box, but the function of this domain has not been characterized (Glover et al., 1998;Kauselmann et al., 1999). All members of this family of kinases associate with spindle poles early in mitosis and are implicated in regulating cell cycle control. Kauslemann et al., found that CIB1 localized to the neuronal somata and dendrites of rat hippocampal nerurons and that this expression pattern was similar to that of Snk. In addition, they showed that overexpression of CIB1 and Snk colocalize in the cytoplasm. Both yeast two-hybrid and GST pull down assays demonstrate that CIB1 binds Fnk and Snk *in vitro*. However, the functional significance of CIB1 binding to these polo-like kinases has yet to be determined (Kauselmann et al., 1999).

4) DNA-dependent protein kinase

Another serine/threonine kinase that binds to CIB1 is DNA-dependent kinase (DNA-PKcs), a eukaryotic kinase activated by DNA ends, which is believed to play a role in

repairing DNA double-strand breaks. DNA-PKcs interacts specifically with CIB1 [also known as KIP for kinase-interacting protein (Wu and Lieber, 1997)] in a yeast two-hybrid screen and GST pull down *in vitro* binding assays, but no *in vivo* functional association of DNA-PKcs and CIB1 was reported in this study (Wu *et al.*, 1997). A recent study reported that CIB1 may form a complex with the catalytic subunit of telomerase (hTERT) and DNA-PKcs in the nucleus (Lee et al., 2004). This study also showed that overexpression of CIB1 stimulated endogenous telomerase activity and increased telomere length, suggesting a role for CIB1 in cell replication and senescence (Lee et al., 2004). However, the binding of CIB1 to hTERT could not be independently verified (Tracy Bryan, personal communication).

5) FAK

CIB1 was reported to bind the tyrosine kinase focal adhesion kinase (FAK) both in CHO cells and in platelets (Naik and Naik, 2003). In this study, endogenous CIB1 localized within focal adhesions and exogenous CIB1 promoted focal adhesion complex formation. The binding was reported to stimulate FAK activity and increase platelet filipodia formation and spreading (Naik *et al.*, 2003). However, the direct or indirect interaction of CIB1 with FAK also could not be independently verified (MD Schaller and LV. Parise, unpublished data).

6) Presenilin 2

Presenilins (PS1 and PS2) are integral membrane protein components of γ-secretase complexes that contribute to the generation of β-amyloid peptides (Aβ) in neurons. The

extracellular deposit of insoluble β-amyloid peptides referred to as senile plaques is a major pathological characteristic of Alzheimer's disease (AD). In a yeast two-hybrid screen, CIB1 [also known as calmyrin in this study (Stabler et al., 1999)] bound presenilin, and the interaction was confirmed by several binding assays including affinity chromatography and coimmunoprecipitation experiments, indicating that the CIB1/presenilin interaction plays a role in the pathogenesis of AD (Stabler et al., 1999). These observations and speculations are challenged by a recent study showing that although CIB1 is present in a subset of forebrain neurons and accumulates in senile plaques, which is a hallmark of AD, its expression levels in brain areas associated with the onset of AD are very low (Blazejczyk et al., 2006). Immunostaining and subcellular fractionation studies also showed a very limited overlap between CIB1 and PS2 at neuronal membranes (Blazejczyk et al., 2006). Another feature of AD is the dysregulation of intracellular Ca^{2+}-signaling events. However, CIB1 binding to PS2 appeared to be Ca^{2+}-independent and CIB1 did not exhibit a Ca^{2+}-dependent translocation to intracellular membranes. Based on these observations, it was concluded that CIB1 may not contribute significantly as a Ca^{2+}-sensor that transduces Ca^{2+}-signaling events to AD-related PS2 in forebrain (Blazejczyk et al., 2006).

7) IP3R

Binding of extracellular ligands to G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) at the plasma membrane activates phospholipase C (PLC), which converts phosphoinositol-4,5-bisphosphate (PIP_2) to the second messenger inositol 1,4,5-trisphosphate (IP_3) (Patterson et al., 2004). IP_3 binds to its receptor, IP_3R, a Ca^{2+} release

channel localized to the endoplasmic reticulum (ER), and rapidly releases Ca^{2+} from intracellular Ca^{2+} pools within the ER and other cellular membranes into the cytoplasm, where it transduces cellular signals and regulates numerous cellular events such as contraction/excitation, secretion, and gene expression (Mak et al., 1998;Patterson et al., 2004). IP_3R functions are regulated by a variety of factors, including Ca^{2+} binding, phosphorylation, nucleotides such as ATP, and protein-protein interactions (Patterson et al., 2004). A number of Ca^{2+}-binding proteins have been shown to associate with the IP_3R and regulate its function. These proteins include calmodulin, CaBP/caldendrin, chromogranins, FKBP12, and the recently identified binding ligand CIB1 (Mak et al., 1998;Patterson et al., 2004;White et al., 2006). In this study, CIB1 was shown to bind to all mammalian IP_3R isoforms in a Ca^{2+}-dependent manner within EF3 and EF4. In addition, the CIB1 binding site on IP_3R overlaps the IP_3-binding region of the IP_3R (White et al., 2006). Therefore, in the absence of IP_3, CIB1 acts as a direct activating ligand of the IP_3R channel. By contrast, pre-exposure of the IP_3 receptor to CIB1 decreases the number of channels available for subsequent activation by IP_3, suggesting that CIB1 functions as both an activating and inhibitory protein ligand of the IP_3R.

3. CIB1 knockout mice

In order to understand the *in vivo* function of CIB1, CIB knockout (KO) mice were generated via homologous recombination in embryonic stem (ES) cells (Yuan et al., 2006a). The phenotypes associated with the CIB1 knockout mice are described below

1) Male sterility:

CIB1-KO mice are viable, reach all developmental milestones, grow normally, and display no obvious detrimental phenotype, except that adult male mice are sterile due to a defect in the haploid phase of spermatogenesis (Yuan et al., 2006a). The phenotypes associated with this defect include smaller testis size, decreased numbers of germ cells in seminiferous tubules, increased spermatocyte and spermatid apoptosis, and loss of elongated spermatids and sperms in CIB1-KO mice. In addition, mouse embryonic fibroblasts (MEFs) derived from CIB1-KO mice proliferate at a significantly slower rate compared to wild-type MEFs. All these observations, together with the finding that testes from CIB1-KO mice have increased expression of the cell cycle regulatory protein Cdc2/Cdk1, suggest that CIB1 may play an important role in regulating cell cycle, differentiation and proliferation of spermatogenic germ cells, and is therefore essential for mouse spermatogenesis.

2) Defect in endothelial cell (EC) function

ECs play an important role in angiogenesis, which is a fundamental process to physiological events including embryonic development, tissue remodeling, and reproduction, but also contributes to pathological conditions such as inflammatory disorders and tumor development (Folkman, 1995;Risau, 1997). ECs derived from CIB1- KO mice display reduced migration, proliferation, and tubule formation, which is associated with impaired activation of PAK1, ERK1/2 and decreased expression of matrix metalloproteinase MMP-2, all of which contribute to angiogenesis (Zayed MA, manuscript in preparation). In addition, tissues derived from CIB1-KO mice have

decreased growth factor-induced microvessel sprouting, and CIB1-KO mice have a defect in ischemia-induced angiogenesis (Zayed MA, manuscript in preparation).

3) Platelet phenotype:

CIB1 has been shown to be an endogenous inhibitor of agonist-induced $\alpha IIb\beta_3$ activation in murine megakaryocytes (Yuan et al., 2006b), and therefore we would predict a hyperactive platelet phenotype in CIB1-KO mice. However, unexpectedly, CIB1-KO mice show normal bleeding time, thrombus formation, platelet aggregation and spreading, as well as normal $\alpha IIb\beta_3$ activation compared to CIB1 wild-type mice (DeNofrio J, unpublished data). The apparent discrepancy between the *in vivo* and the previous cellular data might be explained by the possibility that the megakaryocytes (platelet precursor cells) used in the previous cellular studies function differently from the platelets derived from the CIB1-KO mice. However, a recent experiment using megakaryocytes and platelets from CIB1-WT or CIB1-KO cells demonstrated that these two types of cells function the same (DeNofrio, personal communication). In addition, the acute removal of CIB1 protein (via siRNA) might not be functionally similar to the genetic deletion, which gives cells more time to compensate. In this regard, the lack of *in vivo* platelet phenotypes may also be due to compensation by other CIB1 families (CIB2-4) for the loss of CIB1 in CIB1-KO mice. Evidence for this possibility is supported by the finding that CIB3 and CIB4 mRNA levels are upregulated in CIB1-KO mice (DeNofrio J, unpublished data). It remains to be determined whether the protein levels of the CIB1 isforms are also upregulated in CIB1-KO mice, and whether they also bind αIIb cytoplasmic tail and inhibit $\alpha IIb\beta_3$ activation. Finally, it is likely that CIB1 plays a

supportive role in inhibiting platelet activation in animals, by raising the threshold of 'pro-activation' signaling required for induction of platelet activation pathways, and therefore a more dramatic phenotype might be seen after mice are challenged under stress conditions.

4) Other defects:

CIB1-KO mice show increased plasma P-selectin and von Willebrand factor (vWF) compared to the CIB1 wild-type mice, based on the screening of plasma samples using Luminex (DeNofrio, unpublished data).

P-selectin is a 140 kDa glycoprotein that is present in the platelet α-granules and rapidly translocates to the cell surface upon platelet activation (Hsu-Lin et al., 1984). P-selectin mediates platelet rolling on activated endothelial cells and the recruitment of leukocytes to activated platelets and endothelial cells by its high-affinity P-selectin glycoprotein ligand-1 (PSGL-1). It participates in platelet aggregation by stabilizing $\alpha IIb\beta_3$-fibrinogen interactions and facilitating the formation of large stable platelet aggregates (Merten and Thiagarajan, 2000;Merten et al., 2000). P-selectin has been found to be elevated in patients with congestive heart failure, stroke, and peripheral artery disease, highlighting an important role in arterial thrombosis (Minamino et al., 1998;O'Connor et al., 1999;Blann et al., 1999).

Von Willebrand factor (vWF) is an extracellular macromolecular ligand for integrin $\alpha IIb\beta_3$, and also plays an important role in platelet aggregation. Shear stress induces vWF binding to the GP Ib/IX/V complex on platelets, and this interaction transduces

signals into platelets, followed by activation of $\alpha IIb\beta_3$ (Blann et al., 1999). In addition to binding fibrinogen, activated $\alpha IIb\beta_3$ can bind vWF and functions as a bridge between the $\alpha IIb\beta_3$ on adjacent platelets. Elevated levels of vWF are also observed in patients with unstable angina and acute myocardial infarction (Eto et al., 1999). These findings in the CIB1-KO mice suggest that CIB1 may also mediate platelet activation and aggregation via these factors.

4. Overall goal and hypothesis of the study

Since the first discovery of CIB1 as a platelet integrin αIIb cytoplasmic tail binding protein about a decade ago, significant progress has been made in characterizing its structure, interacting proteins, and biological functions. In this Chapter, I have provided a summary of these findings. In brief, crystal structure studies of CIB1 have led to the determination of CIB1 as a new subfamily of EF hand-containing proteins with high homology to two well-known Ca^{2+}-binding regulatory molecules calmodulin and calcineurin B. *In vitro* and cell culture studies have identified a variety of CIB1 binding proteins and attributed CIB1 with various functions, such as inhibiting platelet αIIbβ3 activation, activating PAK1 kinase activity and inhibiting cell migration on fibronectin, as well as acing as an activating and inhibitory protein ligand of a Ca^{2+} release channel IP_3R. In addition, *in vivo* animal knockout studies demonstrated that CIB1 is essential for spermatogenesis and might be involved in cell cycle control.

Although CIB1 is widely expressed and binds to a variety of proteins, the biological significance of many of these interactions remains unclear. Therefore, the overall goal of

this study is to identify new CIB1 binding partners to further understand the role of CIB1 in human diseases. Given the fact that among the CIB1-binding proteins, a number of them are serine/threonine and tyrosine kinases, we hypothesize that CIB1 might function as an important kinase regulator. Therefore, we analyzed CIB1 protein sequence using a database of known protein-kinase recognition motifs. Here we identified and verified a new CIB1 binding protein, 3-phosphoinositide-dependent protein kinase 1 (PDK1), a critical signaling molecule in regulating diverse cellular functions, including cell proliferation and survival. The characteristics of PDK1, including its structure, biological functions and regulation, will be discussed in detail in Chapter II.

Chapter II

An introduction to PDK1 and AGC kinases

1. PDK1, a Link between PI3 Kinase and AGC Kinase Family

Extracellular signals, such as growth factors, hormones, cytokines, and matrix interact with their cell-surface receptors to control intracellular events and cell functions. One of the most important signal transduction pathways is the phosphoinositide 3-kinase (PI3K) pathway that regulates diverse fundamental cellular functions such as cell growth, survival, proliferation, differentiation and motility [review in (Cantley, 2002)]. Growth factor receptor tyrosine kinases, integrin-mediated cell adhesion, and G protein coupled receptors can all stimulate PI3K. Once activated, PI3K phosphorylates the plasma membrane-bound phosphatidylinositol 4,5-bisphosphate [(PI(4,5)P2] to generate the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits specific phospholipid-binding proteins, including the pleckstrin homology (PH) domain-containing proteins to the membrane, where they can be activated. PI3K can be negatively regulated by two types of phosphatases, the Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) that dephosphorylate PIP3 to PI(3,4)P2, and PTEN phosphatase that catalyzes PIP3 to PI(4,5)P2. The PI3K pathway has attracted tremendous research interest because of its frequent activation in cancers via a variety of mechanisms. Aberrant activation of the PI3K pathway includes amplified or activated receptor tyrosine kinases (RTKs), amplification of genes encoding PI3K itself (especially its catalytic domains) or its downstream effectors, and loss or inactivation of PIP3 phosphatase, PTEN (Cantley, 2002;Sulis and Parsons, 2003;Luo et al., 2003). It is now well established that the diverse biological functions of PI3K are accomplished by its ability to activate multiple distinct signaling cascades. Among them, one key mediator of PI3K signaling is the AGC family (i.e. homologous to protein kinases A, G and C) of

serine/threonine protein kinases, including isoforms of protein kinase B (PKB)/Akt, p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), various protein kinase C (PKC) isoforms, PKC-related protein kinase 1 and 2 (PRK), and serum- and glucocorticoid-induced protein kinase (SGK) (Alessi, 2001). The 3-phosphoinositide-dependent protein kinase 1 (PDK1), another member of the AGC family, was found to be the link that is upstream of the AGC kinases and downstream of the PI3 kinase pathway (Alessi et al., 1997b) (Fig. 2-1)

2. Characterization of PDK1

1) Domain and Motif Structures of PDK1

PDK1 was originally purified from tissue extracts based on its ability to phosphorylate PKB/Akt in a PIP3-dependent manner *in vitro* (Stokoe et al., 1997;Alessi *et al.*, 1997b). It was later demonstrated to phosphorylate and activate other memembers of the AGC family of protein kinases. Cloning and sequencing of PDK1 demonstrate that it is a 556 amino acid protein with an N-terminal catalytic domain (residues 70–359) that closely resembles the AGC subfamily of protein kinases and a C-terminal pleckstrin homology (PH) domain (residues 459–550) (Alessi et al., 1997a;Stephens et al., 1998). The PH domain interacts with high affinity with the second messengers PIP3 and PIP2 *in vitro*, which contributes significantly to its regulation by PI3K (Currie et al., 1999) (Fig. 2-2).

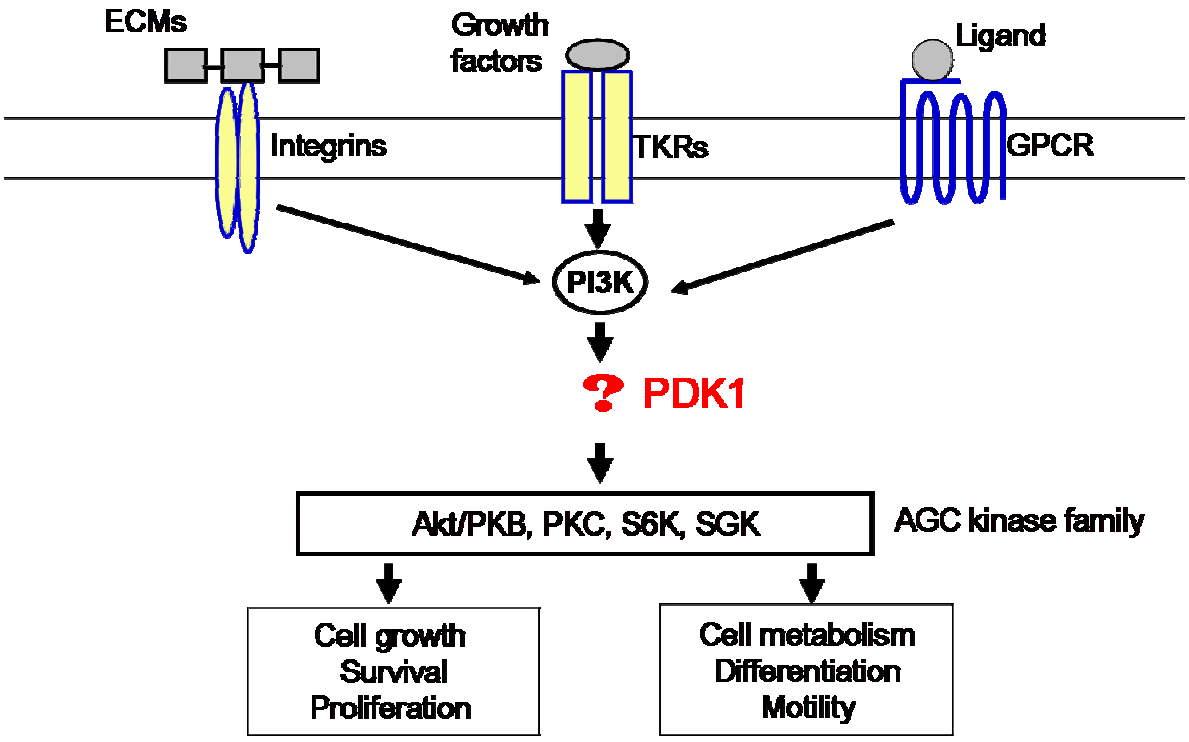


Figure 2-1 Identification of PDK1 as a link between PI3K and AGC kinase family. PI3K can be activated by growth factor receptor tyrosine kinases (TKRs), integrin-mediated cell adhesion to extracelluar matrix (ECMs), and G protein coupled receptors (GPCRs). AGC family kinases are one of the key mediators of PI3K signaling pathway. PDK1 was identified to be the upstream of the AGC kinases and downstream of the PI3 kinase pathway.

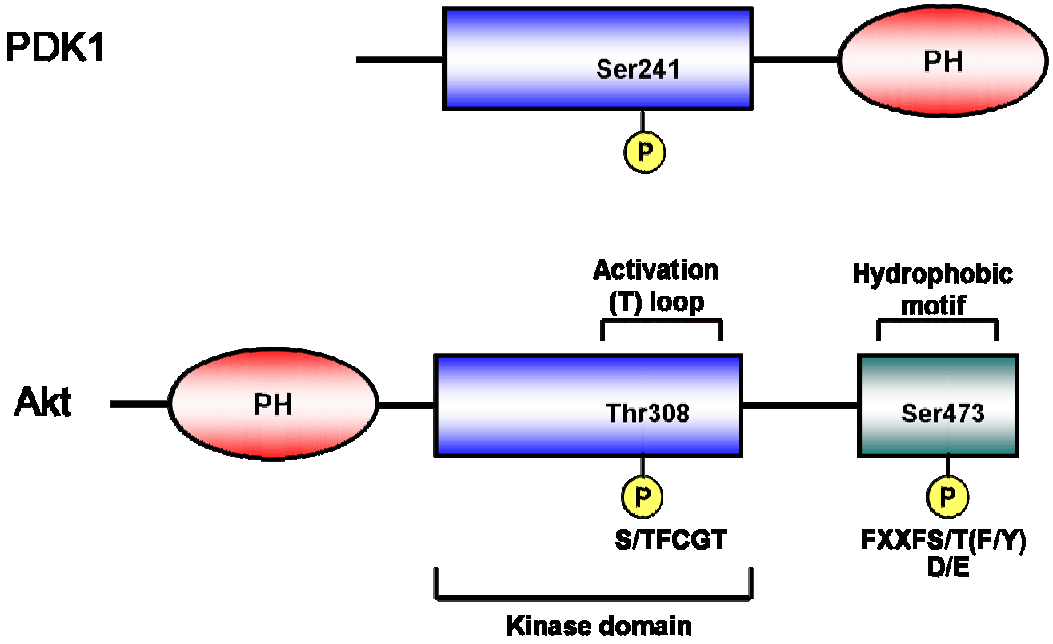


Figure 2-2 Domain and motif structures of PDK1 and PKB/Akt. PDK1 contains an N-terminal catalytic domain (residues 70-359) and a C-terminal pleckstrin homology (PH) domain (residues 459-550). PKB contains an N-terminal PH domain (residues 1-109) and a C-terminal kinase domain (residues 148-411). Most AGC kinases have two highly conserved Ser/Thr residues. One is located in the activation-loop (also known as T-loop; in PKB, this residue corresponds to Thr308), and the other is C-terminal to the catalytic domain in a region termed the hydrophobic motif (in PKB, this residue corresponds to Ser473). Phosphorylation of both residues is required for the maximal activation of these enzymes. PDK1 phosphorylates AGC kinases on the conserved Ser/Thr residue in the T loop. Kinases responsible for the phosphorylation of the hydrophobic motif Ser/Thr of most AGC kinases have not been identified. Atypical PKCs and PRK1/2 have an acidic residue instead of the conserved Ser/Thr phosphorylation site. Adapted from Stokoe et al, 1997.

All AGC kinases need to be phosphorylated at two highly conserved Ser/Thr residues to be fully activated. One residue is located in the activation-loop (T-loop) within the core of the catalytic domain (in Akt, this residue is Thr308), and the other is located C-terminal to the catalytic domain in a region called the hydrophobic motif (HM) (in Akt, this residue is Ser473) (Fig. 2-3). PDK1 functions as a 'master kinase' to phosphorylate the T-loop residue of these AGC kinases (Niederberger and Schweingruber, 1999;Toker and Newton, 2000b;Alessi, 2001). Kinases responsible for phosphorylation of the hydrophobic motif Ser/Thr residues for most AGC kinases have not been completely identified. The T-loop phosphorylation residue in PDK1 corresponds to Ser241, which undergoes autophosphorylation. Unlike other AGC kinases, PDK1 does not have a C-terminal HM (Fig. 2-2 and Fig. 2-3).

Akt also contains an N-terminal PH domain (residues 1-109) and a C-terminal catalytic domain (residues 148-411) (Fig. 2-1). One proposed model of Akt activation may be through the binding of PIP3 which provides a docking site for the PH domains of PDK1 and Akt, and therefore directs the localization of Akt and possibly PDK1 to the membrane. PIP3 binding to PDK1 and Akt also induces a conformational change that increases the accesssibility of Akt to PDK1, which phosphorylates Akt at Thr308 in the T-loop leading to Akt activation (Andjelkovic et al., 1997;Alessi *et al.*, 1997a;Stephens *et al.*, 1998;Currie *et al.*, 1999;Biondi et al., 2001) (Fig. 2-4).

Unlike Akt, other PDK1 substrates described above do not have a PH domain and therefore do not bind PIP3. Instead, the current model for their activation indicates that

the prior phosphorylation of these kinases at the Ser/Thr residue within the HM appears to facilitate their binding to PDK1, which allows PDK1 phophorylation within the T-loop and further activate their kinase activity (Collins et al., 2003) (Fig. 2-3).

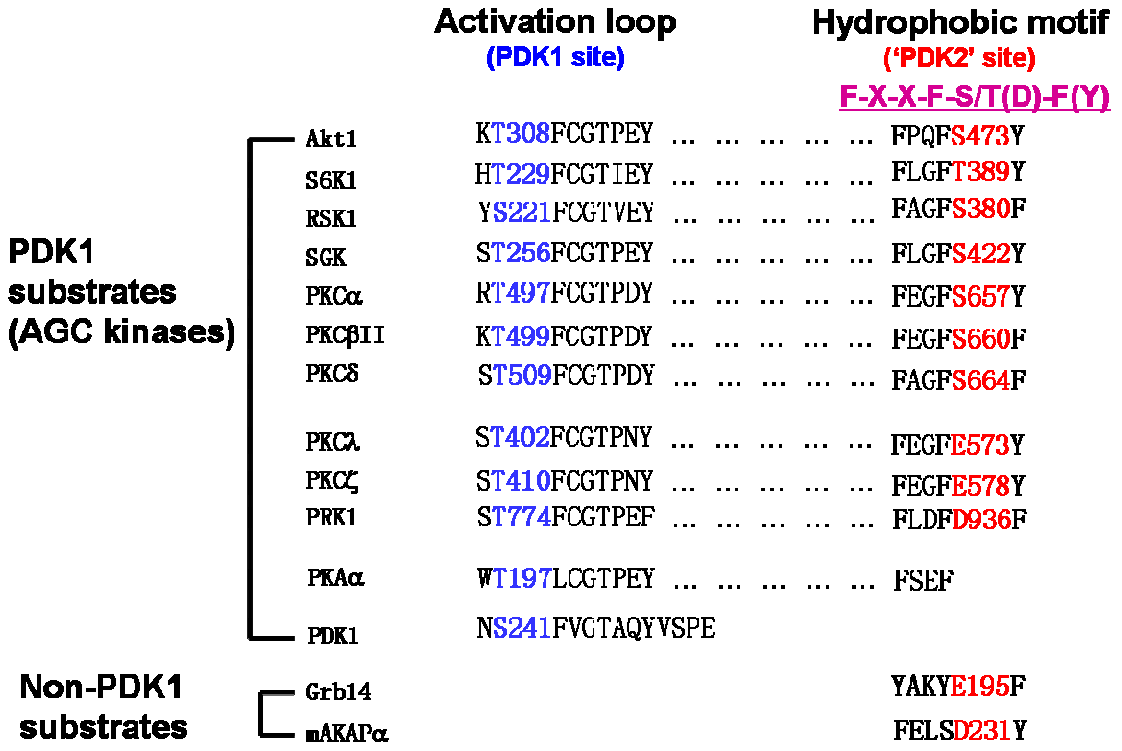


Figure 2-3 Sequence alignment of the activation loop (T-loop) motif and the hydrophobic motif (HM) of AGC family of protein kinases. The AGC family includes the well-characterized Akt, SGK, RSK, S6K and conventional PKC isoforms; novel and atypical PKC isoforms, PKA, and PDK1 itself. AGC kinases require phosphorylation of two consensus Ser/Thr residues to be fully activated. One Ser/Thr residue in the T-loop is phosphorylated by PDK1, the other in the HM is phosphorylated by other kinases. Some kinases contain acidic residues substituting the Ser/Thr residues in the HM (PKCλ, PKCζ, and PRK1). PKA lacks the HM Ser/Thr residue and PDK1 lacks the HM motif. Adapted from Storz and Toker 2002, and Peterson 1999.

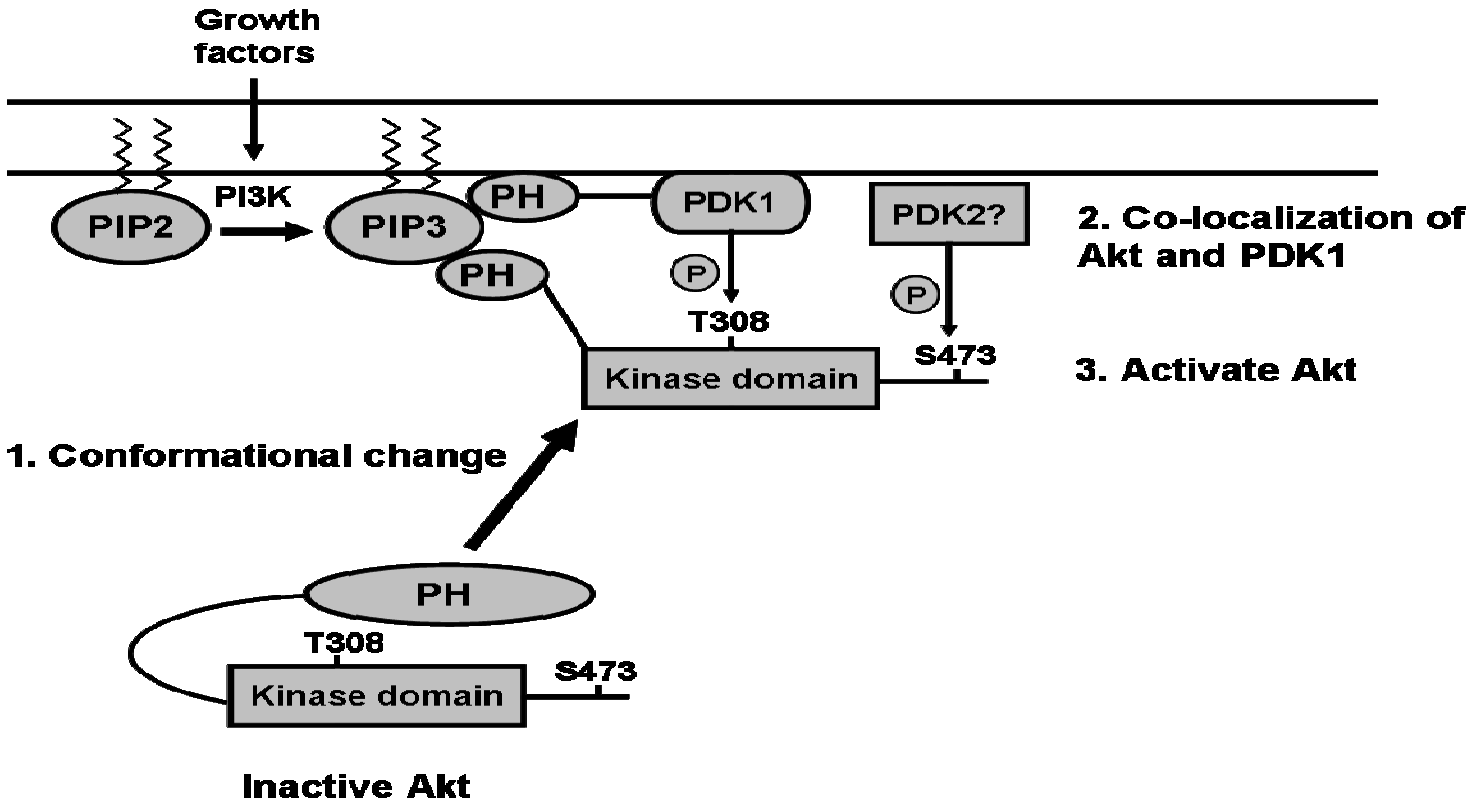


Figure 2-4 Schematic description of PKB/Akt activation. Growth factors such as insulin activate PI3K through receptor tyrosine kinases. PI3K then phosphorylates PIP2 to form second message PIP3, which provides a docking site for the PH domains of PDK1 and Akt, and therefore directs the localization of Akt and possibly PDK1 to the membrane. Binding of PDK1 and Akt with PIP3 also induces a conformational change that increases the accessibility of Akt to PDK1, which phosphorylates Akt at the T loop T308 residue. The kinase responsible for phosphorylation of the hydrophobic residue (S473) has been elusive (termed 'PDK2'), and many potential candidates have been suggested.

2) Crystal Structure of PDK1

The crystal structure of the kinase domain of PDK1 has been solved at 2Å resolution, and it resembles the crystal structure of its related AGC kinase member PKA (Biondi et al., 2002). Like PKA, the PDK1 catalytic domain also contains a β-sheet N-terminal lobe and an α-helical C- terminal lobe. The ATP-binding site is located between these two lobes (Taylor et al., 1992;Huse and Kuriyan, 2002). PKA contains a hydrophobic motif FXXF at the C-terminus, which resembles the hydrophobic motif of PDK1 substrates such as S6K and RSK (FXXFS/TF/Y) where the S/T is the phosphorylation residue. According to the crystal structure of PKA, the FXXF sequence folds back onto its catalytic domain and binds to a hydrophobic pocket located within its catalytic domain. Mutation of the Phe (F) residue dramatically decreases PKA kinase activity (Edwards and Newton, 1997;Batkin et al., 2000). Most AGC kinases but not PDK1 contain the C-terminal hydrophobic motif FXXF (Fig. 2-3). The crystal structure of PDK1 reveals that like PKA, it also contains a hydrophobic pocket within its catalytic domain termed 'PIF (PDK1-interacting fragment)-pocket,' and an adjacent phosphate-binding site. However, unlike PKA, the hydrophobic pocket of PDK1 is unoccupied, which may mediate the docking interaction of PDK1 with the phosphorylated hydrophobic motif of its substrates such as S6K and SGK (Biondi et al., 2002). The information obtained from the crystal structure also provides a potential mechanism for the design of specific PDK1 inhibitors, which will be discussed in the 'PDK1 as a drug target' section.

The crystal structure of the PDK1 PH domain demonstrates that it possesses a unique feature, that is, it has a much more spacious ligand-binding site that enables PDK1 to

interact with cytosolic soluble inositol phosphate Ins(1,3,4,5,6)P_5 (IP5) and IP6 with high affinity (Komander et al., 2004). Binding of PDK1 to these cytosolic inositol phosphates has great functional significance, since a portion of PDK1 can be anchored to the cytosol, where it can activate its cytosolic substrates such as SGK and S6K that do not have PH domains and therefore do not interact with phosphoinositides on the membrane. This will be further discussed in the 'regulation of PDK1--PH domain' section.

The information obtained from the crystal structure of both the PDK1 kinase domain and PH domain provides a potential mechanism for the CIB1/PDK1 binding and CIB1 activation of PDK1, which will be described in detail in Chapter III 'CIB1 Binds PDK1 and Activates PDK1'.

3) PDK1 functions, downstream effectors and upstream regulators

In order to determine the biological significance of the CIB1/PDK1 interaction, which will be discussed in Chapter IV 'CIB1 Protects Cells from Apoptosis via Signaling to PDK1', it is essential to first discuss the important biological functions of PDK1, both in normal and tumorigenic contexts. A variety of genetic models have been generated to study the *in vivo* functions of PDK1 and will also be discussed in the following section. In addition, PDK1 is a unique kinase in that it transduces upstream signals to diverse downstream effectors. These distinct downstream effectors of PDK1 contribute to its different functions in a stimuli and cell context-specific manner. To dissect the CIB1-mediated PDK1 downstream signaling pathways, we will discuss several well-characterized downstream effectors of PDK1 and how they are regulated to contribute to

PDK1-mediated functions. Finally, although considerable progress has been made towards understanding its biological functions and downstream effectors, how PDK1 itself is regulated is not well-understood. Therefore, in this section, we will also discuss the current models for regulation of PDK1, thereby facilitating our understanding of how CIB1 contributes to regulate PDK1 to obtain its biological functions.

(1) Biological Functions of PDK1

A. PDK1 Regulates Insulin Function

Insulin functions to stimulate the uptake of nutrients such as glucose, amino acids, and fatty acids, and subsequently converts and stores them as macromolecules such as glycogen, proteins, and lipids in skeletal muscle, adipose tissue and liver. Glucose transporter 4 (GLUT4)-dependent glucose transport is the rate-limiting step in insulin-induced glucose uptake in adipocytes, which is achieved by translocation of GLUT4 to the plasma membrane in a PI3K-dependent manner (Li et al., 2000).

Mixed data exist regarding whether PDK1 plays a role in insulin signaling. Several studies report that the insulin-stimulated GLUT4 translocation to the membrane is PDK1-dependent since overexpression of PDK1 stimulates translocation of GLUT4 to the plasma membrane of isolated rat adipocytes, whereas the kinase-dead mutant of PDK1 and ΔPH-PDK1 inhibit this event (Bandyopadhyay et al., 1999;Grillo et al., 1999). However, other studies show that PDK1 might not be involved in this function, since membrane-bound PDK1 mutant that increases the Akt phosphorylation has no effect on insulin-induced glucose uptake in cultured adipocytes (Egawa et al., 2002). A more

recent genetic study using a conditional gene-targeting approach to delete PDK1 in a brown preadipocyte cell line demonstrates that PDK1 is required for insulin-stimulated glucose uptake, since insulin-induced activation of Akt and S6K as well as glucose uptake and translocation of the GLUT4 to the plasma membrane is dramatically inhibited in the adipocytes lacking PDK1 (Sakaue et al., 2003).

B. PDK1 Promotes Cell Survival and Growth

PDK1 is critical for cell growth and survival in both normal and tumorigenic contexts. Overexpression of PDK1 transforms mouse mammary epithelial cells *in vitro*, as determined by anchorage-independent growth in soft agar, and results in tumorigenesis *in vivo*, as demonstrated by forming poorly differentiated mammary carcinomas when cells retrovirally expressing PDK1 were injected into the mouse mammary fat pad (Zeng et al., 2002). PDK1 transforming and tumorigenesis ability is due to PDK1-mediated upregulation of PKCα, increased β-catenin activation, and downregulation of the breast tumor suppressor caveolin-1, but not due to PDK1-mediated Akt activation (Zeng *et al.*, 2002;Xie et al., 2003). These results indicate that PDK1 may be an important target in human breast cancer. Conversely, blocking PDK1 expression with antisense oligonucleotides in a glioblastoma cell line (U-87) decreases the activity of PDK1 substrates, Akt and S6K, inhibits cell proliferation and induces apoptosis (Flynn et al., 2000). In addition, small molecule inhibitors of PDK1 increase cytotoxicity (Zhu et al., 2004;Kucab et al., 2005;Crowder and Ellis, 2005). Depletion of PDK1 by small interfering RNA oligonucleotides sensitizes MCF7 human breast cancer cells to chemotherapeutic agents, which is more effective than knockdown of Akt1 (Liang et al.,

2006). Depletion of PDK1 also attenuates NF-κB activity and increases TRAIL-mediated cytotoxicity, and increases the anti-tumor effects of the EGFR inhibitor erlotinib (Zhang et al., 2006). All these data demonstrate that PDK1 is an important regulator in mediating tumorigenesis and a potential anti-cancer drug target. The data also indicate that distinct downstream effectors of PDK1 contribute to its cell survival and growth functions depending on different stimuli and cell contexts.

C. PDK1 Regulates Cell Migration

Overexpression of a kinase-dead version of PDK1 in PTEN -/- MEF cells reduces cell migration; however, overexpression of a constitutively active PDK1 alone is not sufficient to promote cell migration in PTEN wild-type MEF cells (Lim et al., 2004). Although this study shed some light on PDK1 function in cell migration and the authors speculate that the observed migration is through the PDK1/Akt signaling pathway, the mechanism by which PDK1 affects cell migration in these fibroblasts still requires further investigation. A recent study shows that PDK1 is essential for the migration of vascular endothelial cells (ECs) (Primo et al., 2007). This is supported by the findings that ECs differentiated from PDK1 -/- mouse embryonic stem cells completely lose the ability to migrate *in vitro* in response to vascular endothelial growth factor-A (VEGF-A). In addition, embryoid bodies (EBs) derived from PDK1-/- ES cells exhibit developmental and vascular defects that result from reduced cell migration. Overexpression of PDK1 increases EC migration induced by VEGF-A, and both PDK1 and Akt translocate to the plasma membrane and localize at the leading edge of migrating cells upon cell stimulation by VEGF-A. Therefore, the authors proposed a model of spatial distribution

of PDK1 and Akt to the leading edge of polarized ECs upon PI3K activation to regulate directional cell migration and chemotaxis.

D. PDK1 Increases Tumor Cell Invasion

In addition to its oncogenic role and anti-apoptotic function, PDK1 also causes a highly invasive phenotype when overexpressed in mammary epithelial cells grown on Matrigel (Xie et al., 2006). These observations are associated with increased matrix metalloprotease MMP-2 activity and MT1-MMP expression. Consistent with this, *in vivo* invasive studies show that injection of the cells retrovirally transduced with PDK1 into the mammary fat pad produces invasive and poorly differentiated adenocarcinomas (Xie et al., 2006). These data, together with the finding that PDK1 has increased expression in the majority of invasive breast cancers suggests that it may play an important role in tumor metastasis.

E. PDK1 Regulates T-cell Function

Mice with a conditional PDK1 gene deletion in T cells have been generated to study PDK1 function in T cell development (Hinton et al., 2004). These mice are viable, fertile and exhibit no obvious defect. They are normal in size and produce offspring at the expected Mendelian frequency. However, the size of their thymi and the number of thymocytes are greatly reduced. Complete loss of PDK1 in T cells blocks T cell differentiation in the thymus.

In addition, mice with hypomorphic PDK1 alleles that express about ten percent of the normal level of PDK1 in all tissues including lymphoid organs (Lawlor et al., 2002) (see

'PDK1 animal model' section below) have also been used to test PDK1 function in T cells. These PDK1 hypomorphic mice are viable and fertile, and display no severe defect except that they are about half the size of control animals. Unlike the PDK1-/- T cells, decreased PDK1 expression allows T cell differentiation in the thymus but blocks thymic expansion. Therefore, these studies suggest that PDK1 is essential for T cell development, and indicate that PDK1 might be a valuable target for immunotherapy of T cell-mediated diseases.

(2) PDK1 Downstream Signals

A. AGC Family Substrates

PDK1 phosphorylates and activates a variety of AGC protein kinases, which mediate many physiological and pathological functions triggered by growth factors and hormones. These kinases include Akt isoforms (Vanhaesebroeck and Alessi, 2000;Brazil and Hemmings, 2001;Scheid and Woodgett, 2001), S6K (Dufner and Thomas, 1999a;Avruch et al., 2001;Volarevic and Thomas, 2001a), RSK (Frodin and Gammeltoft, 1999;Dufner *et al.*, 1999a), SGK (Park et al., 1999;Kobayashi and Cohen, 1999a;Kobayashi et al., 1999b;Lang and Cohen, 2001), and PKC (Le Good et al., 1998;Dutil et al., 1998;Egawa et al., 2002), etc. (Fig. 2-2). Many of these kinases play important roles in anti-apoptosis, promoting cell growth and proliferation, as well as cell cycle control by converging on one or more of the known survival signals (Fig. 2-5). PDK1 also regulates non-AGC protein kinases, such as PAK1 (King et al., 2000) and Ral-GEF (Tian et al., 2002).

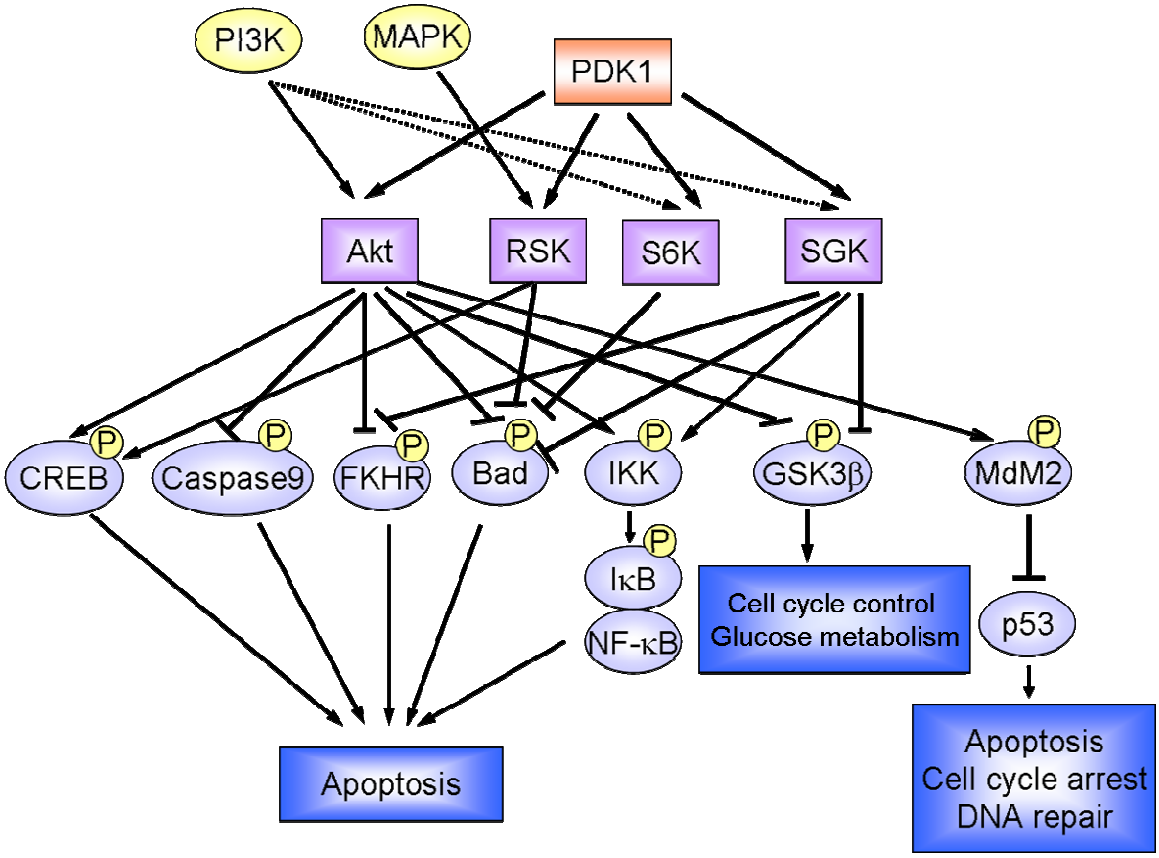


Figure 2-5 PDK1 downstream effectors converge on one or more survival signals to regulate cell survival function. The downstream effectors of PDK1, which include Akt, S6K, RSK and SGK, play important roles in anti-apoptosis, cell survival and proliferation, as well as cell cycle control. These functions are achieved by phosphorylation and inactivation of pro-apoptotic factors such as Bad, procaspase-9, p53, and Forkhead (FKHR) transcription factors, as well as GSK-3□, or phosphorylation and activation of anti-apoptotic factors such as cyclic-AMP response element-binding protein (CREB) and IkB kinase (IKK).

a. Akt

Akt regulation

There are three mammalian isoforms of Akt (Akt1, Akt2, and Akt3; also termed PKBα, -β, and -γ), which share significant sequence identity (Jones et al., 1991;Brodbeck et al., 1999;Konishi et al., 1995;Datta et al., 1999). Activation of Akt requires the phosphorylation of two highly conserved residues: Thr308 in the activation loop by PDK1 and Ser473 within the C-terminal hydrophobic motif (HM) by an uncharacterized kinase. This HM kinase was originally termed 'PDK2' (AGC HM kinase). Many kinases have been suggested to function as 'PDK2', including PDK1 (Balendran et al., 1999a), integrin-linked kinase (ILK) (Delcommenne et al., 1998;Troussard et al., 2003), PKC isoforms (Kawakami et al., 2004;Partovian and Simons, 2004), MAPKAP kinase-2 (MK2) (Alessi et al., 1996), p38 MAP kinase (Baudhuin et al., 2002;Gonzalez et al., 2004;Rane et al., 2001), DNA-dependent protein kinase (Hill et al., 2002), ATM (Viniegra et al., 2005), autophosphorylation by Akt itself (Toker and Newton, 2000), and a recently identified mTOR rictor complex, which is considered the major contributor to the 'PDK2' kinase activity (Sarbassov et al., 2005).

Despite the controversial data on the identity of 'PDK2', some basic characteristics of this kinase and the mechanisms for phosphorylation at the hydrophobic motif of Akt and other AGC kinase members have been identified. First, the function of 'PDK2' is PI3K dependent, since insulin and mitogen-mediated Akt Ser473 phosphorylation is largely blocked by PI3K inhibitors such as wortmannin or LY-394002 (Scheid and Woodgett, 2003). Second, 'PDK2' should be localized to the membrane since the 'PDK2' kinase

activity is found to be mostly associated with the membrane fractions of cells; (Hill et al., 2002;Hill et al., 2002;Hresko et al., 2003). Third, unlike PDK1, which is the 'master kinase' for phosphorylating the AGC kinases at the catalytic domain, it is likely that more than one 'PDK2' exist to phosphorylate the distinct AGC family of kinases at the HM site; their functions could be partially redundant; and they might be mediated by distinct mechanisms, depending on different cell types and specific upstream molecules and substrates.

The current model for Akt activation involves several steps (Fig. 2-4): First, binding of PIP3 and PIP2 following PI3K activation recruits Akt to the plasma membrane, but does not lead to Akt activation (Alessi et al., 1997;James et al., 1996); Second, binding to the PH domain relieves the proposed inhibitory effect of the PH domain that prevents the access to the activation loop site; Third, Akt is phosphorylated on Thr308 by PDK1 and Ser473 by 'PDK2'. In one study, PDK1-mediated Akt phosphorylation at Thr308 was shown to be dependent on prior phosphorylation at Ser473 (Scheid et al., 2002). However, other studies have shown that phosphorylation at Thr308 and Ser473 are completely independent events (Alessi et al., 1996); and finally, activated Akt phosphorylates substrates in the cytosol and nucleus.

Cellular functions and substrates of Akt

Akt is the first identified and best characterized PDK1 substrate (Alessi et al., 1997;Chan and Tsichlis, 2001). It is a key mediator of the physiological actions of insulin and several growth factors such as IGF-1, EGF and PDGF (Downward, 1998) and plays an important role in regulating a variety of cellular functions such as enhancing glucose

uptake and storage, inhibiting cell apoptosis and promoting cell proliferation. Akt is commonly activated in many tumors through multiple mechanisms, including mutation or inactivation of tumor suppressor PTEN, mutation or amplification of receptors that couple to PI3K, amplification of Akt itself, and activation of receptors or oncogenes upstream of PI3K/Akt signaling pathway (Brazil et al., 2004;Vivanco and Sawyers, 2002).

Many substrates contribute to Akt-mediated cell survival and cell proliferation functions (Review in (Testa and Bellacosa, 2001) (Fig. 2-5). Akt phosphorylation of the pro-apoptotic protein Bad prevents the interaction between Bad and the survival factors Bcl-2 and Bcl-xl, activates Bcl-2 and Bcl-xl, and thereby restores the anti-apoptotic function of Bcl2 and Bcl-xl. Akt also phosphorylates other important cellular factors involved in apoptosis, such as the pro-apoptotic protease caspase-9 (Cardone et al., 1998) and fork-head transcription factors such as FKHR (Brunet et al., 1999), which results in the inhibition of apoptosis.

Akt can also promote cell survival by indirect effects on two cell death regulators -- NF-κB and p53 (Review in (Downward, 2004). The NF-κB transcription factor complex promotes cell survival in response to several apoptotic stimuli. Akt phosphorylates and activates IκB kinase (IKK), a kinase that induces degradation of the NF-κB inhibitor, IκB. Degradation of IκB releases NF-κB from the cytoplasm, allowing its nuclear translocation and activation of target genes in the nucleus. Akt can also inhibit the activity of the pro-apoptotic tumor suppressor p53, by phosphorylation of the p53-binding protein Mdm2, a ubiquitin ligase for p53. Akt also promotes cell proliferation through signals to the cell-cycle regulatory machinery. By directly phosphorylating and

inhibiting glycogen synthase kinase-3β (GSK3), a cyclin D1 kinase, Akt prevents cyclin D1 degradation by the proteasome, whose levels are important in the G1/S phase transition.

Akt as anti-cancer drug target

The fact that the PI3K/Akt pathway is deregulated in various tumors and that the PH domain of Akt is important in transducing PI3K signals provides a potential strategy for developing targeted therapies. The best characterized inhibitor of Akt is the lipid-based inhibitor perifosine, which inhibits Akt translocation to the cell membrane. This results in decreased growth of melanoma, lung, prostate, colon, and breast cancer cells in patients and the outcome correlates with the inhibition of Akt activity (Crul et al., 2002;Kondapaka et al., 2003).

Phosphatidylinositol ether lipid analogues are another group of lipid-based Akt inhibitors. They were rationally designed to bind the PH domain of Akt, inhibit Akt translocation to the plasma membrane and activation, and therefore selectively increase apoptosis in cancer cells that express high levels of constitutively active Akt (Castillo et al., 2004;Martelli et al., 2003). Other approaches, such as high-throughput screening of small molecule libraries and kinase activity, rational design of adenoviruses expressing a mutant inhibitory form of Akt have all been developed and many show promising anti-apoptotic effects on certain human tumor cells.

b. p70 S6K/p90RSK/SGK

In contrast to Akt, S6K, RSK and SGK do not possess a PH domain and thus do not interact with PIP3/PIP2. One model describing the mechanism of how these 'non-PH domain PDK1 substrates' are activated by PDK1, is that prior phosphorylation of S6K1 (by mTOR), RSK (by ERK1/2 MAP kinases) and SGK1 (upstream not identified yet) within their hydrophobic motif promotes their interaction with PDK1 (Biondi et al., 2001;Vanhaesebroeck and Alessi, 2000). The interaction site in PDK1 has been identified as a hydrophobic pocket in the kinase domain of PDK1 named 'PIF'-binding pocket ('PIF' for PDK1 interacting fragment). This 'PIF-binding pocket' in PDK1 plays an important role in binding and phosphorylating S6K, RSK, and SGK within their T-loop, and ultimately leading to the maximal activation. Therefore, the phosphorylation of the hydrophobic motif of S6K, RSK and SGK serves as a substrate-docking site for PDK1 by converting them into substrates that can be further activated by PDK1.

<u>S6K</u>

There are two genes encoding p70 ribosomal S6 kinases in mammalian cells: S6K1 and S6K2 (Gout et al., 1998;Shima et al., 1998). Activation of S6K1 requires coordinated input from both the target of rapamycin (mTOR) and the PDK1 pathway. mTOR phosphorylates Thr389 in the C-terminus of S6K1 (Burnett et al., 1998;Volarevic and Thomas, 2001;Isotani et al., 1999) and PDK1 phosphorylates Thr229 in the catalytic domain of S6K1 (Alessi et al., 1998;Balendran et al., 1999b;Pullen et al., 1998). The phosphorylation of both residues is required for activation of S6Ks *in vivo*.

S6Ks play a critical role in regulating cell growth and proliferation by controlling the synthesis of ribosomal proteins (40S ribosomal subunit protein S6) and increasing the cellular translational capacity (Dufner and Thomas, 1999;Lane et al., 1993). S6K1 knockout embryonic stem cells proliferate at a slower rate than wild-type cells, indicating that S6K1 promotes cell proliferation. S6K1 or S6K2 knockout mice are viable and fertile, but they are significantly smaller in body size. However, S6K1/2 double knockout mice show a sharp reduction in viability (Pende et al., 2004).

S6K1 plays a role in cell-cycle progression through the G1 phase (Lane et al., 1993) and may also inhibit apoptosis through inactivating the pro-apoptotic molecule Bad (Harada et al., 2001;Hosoi et al., 1999) (Fig. 2-5). In addition, S6K1 is amplified in breast cancer, indicating a potential oncogenic function (Balendran et al., 1999b). In the $PTEN^{+/-}$ mouse tumor model system, phosphorylated Akt and activated S6K1 levels are increased, which is associated with an increase in cell proliferation. Inhibiting mTOR in these mice reduces tumor proliferation, tumor size, and S6K activity without affecting the phosphorylation status of Akt, suggesting that S6K1 contributes significantly to $PTEN^{+/-}$ tumor development and that S6K1 is a potential cancer therapeutic target (Podsypanina et al., 2001).

RSK

The p90 ribosomal S6 kinases (RSK1-4) are a family of broadly expressed serine/threonine kinases that are activated by many growth factors, polypeptide hormones, and neurotransmitters (Roux and Blenis, 2004). Like Akt and S6K, these kinases contain two distinct and functional catalytic domains and require at least two input signals to be

fully activated: ERK1/2 phosphorylates RSKs within the C-terminal kinase domain (Ser 380), and PDK1 phosphorylates RSKs within the N-terminal kinase domain (Ser 227). RSK1 activity is also negatively regulated through its interaction with 14-3-3β (Cavet et al., 2003).

Aside from being regulated by phosphorylation, the activity of RSKs can also be regulated by their subcellular localization. RSKs have protein substrates in all cellular compartments, including the cytosol, nucleus, and plasma membrane, and their localization is regulated by multiple mechanisms. For example, RSKs are usually present in the cytoplasm of quiescent cells, but upon stimulation, a significant portion of these proteins translocate to the nucleus (Chen et al., 1992;Vaidyanathan and Ramos, 2003;Zhao et al., 1995). Endogenous RSK is also localized to the membrane fraction of HeLa cells, and upon serum stimulation, increased membrane localization was observed (Chen et al., 1992). Myristoylation of RSK results in constitutive RSK activation, and RSK undergoes relocalization from the cytoplasm to the cell membrane in mitogen stimulated cells (Richards et al., 2001). This model suggests that upon mitogen stimulation, RSK undergoes a rapid translocation to the plasma membrane. PDK1 signaling as well as ERK- and PDK1-independent input leads to full activation of RSK at the plasma membrane (Richards et al., 2001), where it can phosphorylate its plasma membrane substrates such as the Na^+/H^+ exchanger NHE1 isoform (NHE1) or dissociate from the membrane and translocate to the nucleus to regulate transcriptional factors (Cavet et al., 2003).

The RSKs are involved in multiple cellular processes, including protein synthesis, transcriptional regulation, cell cycle control, and cell survival and proliferation (reviewed in (Roux and Blenis, 2004). RSKs phosphorylate a variety of transcription factors such as CREB (cAMP response element-binding protein) (Pende et al., 1997;Xing et al., 1996), c-fos (Chen et al., 1993;Chen et al., 1996) and estrogen receptor (Joel et al., 1998). RSK1 and RSK2 may promote G_1 progression through the phosphorylation of the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ and inhibition of its nuclear translocation (Fujita et al., 2003). In addition, RSK promotes cell survival through phosphorylation and inactivation of the pro-apoptotic molecule Bad (Bonni et al., 1999;Shimamura et al., 2000;Tan et al., 1999). RSK1 also phosphorylates and promotes the degradation of IκB, allowing NF-κB to translocate from cytoplasm to the nucleus, where it activates the transcription of many target genes (Ghoda et al., 1997) (Fig. 2-5).

SGK

Three genes encode the serum- and glucocorticoid-inducible kinase (SGK1-3), with 80% amino acid sequence identity in their catalytic domain (Firestone et al., 2003;Webster et al., 1993). In addition to serum and glucocorticoids, SGK1 transcription is also stimulated by high concentrations of glucose (Lang et al., 2000), heat shock, ultraviolet (UV) radiation, and oxidative stress (Leong et al., 2003).

Full activation of SGK1 requires PDK1-dependent phosphorylation at Thr256 within the activation loop and phosphorylation at Ser422 in the hydrophobic motif by an unidentified PI3K-dependent kinase (HM kinase, PDK2) (Kobayashi and Cohen, 1999). Prior phosphorylation of Ser422 promotes SGK1 binding to the PDK1 PIF-binding

pocket and facilitates PDK1 phosphorylation (Biondi et al., 2001) of SGK1. Another model for PDK1 activation of SGK1 involves the scaffold protein, Na^+/H^+ exchanger regulating factor 2 (NHERF2) (Chun et al., 2002). NHERF2 binds SGK1 at the PDZ binding motif via its PDZ domain and PDK1 at the PIF-binding pocket via its PIF sequence, thus assembling NHERF2, SGK1, and PDK1 into a ternary complex, which facilitates PDK1 phosphorylation of SGK1 on Thr256 within its activation loop (Chun et al., 2002).

SGKs activate ion channels (e.g., epithelial Na^+ channel ENaC and renal outer medullary K^+ channel ROMK1), carriers (e.g., Na^+/H^+ exchanger NHE3 and Glucose transporters GLUT1), and the Na^+-K^+-ATPase (review (Chun et al., 2002;Lang and Cohen, 2001). In addition, SGKs also play an important role in promoting cell proliferation and inhibiting apoptosis. The antiapoptotic effect of SGK1 is partially due to its phosphorylation of forkhead transcription factors such as FKRHL1 (Shelly and Herrera, 2002), as well as modulating NFκB in breast cancer cells (Zhang et al., 2005). Dominant negative IKKβ or knockout of IKKβ abolishes SGK1-mediated cell survival. Moreover, SGK3 inhibits apoptosis through phosphorylation and inactivation of the proapoptotic protein Bad (Liu et al., 2000;Xu et al., 2001).

B. Non-AGC Family Substrates

a. PAK1

PAK1 is a serine/threonine kinase that is a major effector of the small GTPases Cdc42 and Rac (Manser et al., 1994). PAK1 activity can also be increased by GTPase-independent mechanisms, including the SH2/SH3-containing adaptor protein Nck (Lu and Mayer, 1999), calcium and integrin binding protein CIB1 (Leisner et al., 2005), and activation by lipids such as sphingosine (Bokoch et al., 1998). PDK1 has been reported to interact with PAK1 both *in vitro* and *in vivo,* and PDK1 phosphorylation of PAK1 at Thr423 increases its kinase activity towards substrate, which is independent of PI3K activity (King *et al.*, 2000). PDK1 phosphorylates PAK1 in vascular smooth muscle cells (VSMC), and the Src/PDK1/PAK1 signaling pathway has been shown to be reactive oxygen species (ROS)-dependent and plays a crucial role in VSMC migration in response to platelet-derived growth factor (PDGF) (Weber et al., 2004). Since PAK1 was originally suggested to be autophosphorylated at Thr423, and it is not clear whether phosphorylation of PAK1 can still occur in PDK1 knockout cells, whether autophosphorylation or PDK1 phosphorylation is the dominant event in phosphorylating the PAK1 activation loop remains to be determined.

b. Ral-GEF

The Ras family of small GTPases transduces growth factor signals into cells through three major downstream pathways: Raf kinases (Robinson and Cobb, 1997;Moodie and Wolfman, 1994), PI3K (Rodriguez-Viciana et al., 1994), and Ral-GEF/Ral (Hofer et al., 1994;Kikuchi et al., 1994;Urano et al., 1996;Wolthuis et al., 1996). Studies have shown that EGF activates Ral-GEFs in part via a Ras-mediated relocalization of Ral-GEF to the

plasma membrane, where it can activate Ral-GTPases. Activated Ral binds to the exocyst complex and plays a critical role in cell secretion and endocytosis. The Ral pathway also plays an important role in Ras-induced transformation and tumorigenesis of human cells. A recent study reported that in addition to Ras-GTPase, Ral-GEF stimulation by EGF involves another input: PDK1, which binds the N-terminal region of Ral-GEF, Ral guanine nucleotide dissociation stimulator (RalGDS) (Tian et al., 2002). This binding relieves the auto-inhibitory effect of RalGDS on the catalytic domain, and increases its GEF activity. This also represents a kinase-independent function of PDK1.

(3) Genetic Models of PDK1

In order to explore the physiological roles of PDK1 in living system, a variety of *in vivo* models have been generated to answer this question. For a summary of these models, refer to Table 2-1.

A. Disruption of the PDK1 gene in Yeast and Drosophila

Deletion of the genes encoding PDK1 homologues in budding yeast (Casamayor et al., 1999;Inagaki et al., 1999) or fission yeast (Niederberger and Schweingruber, 1999) leads to a lethal phenotype. Similarly, in Drosophila disruption of the dPDK1 gene is lethal and shows an apoptotic phenotype in the embryonic stage (Cho et al., 2001). These observations suggest that PDK1 is essential for cell survival and regulates cell growth and apoptosis in yeast and Drosophila development.

Table 1-1 Summary of PDK1 genetic models

		Year	Phenotypes	Conclusions	Reference	Disadvantages
Knockout	**in yeast**	1999	lethal phenotype	PDK1 homologes are required for yeast cell growth	Casamayor, 1999; Inagaki et al., 1999 Niederberger, 1999	
	in drosophila	2001	lethal, apoptotic phenotype in the embryonic stage	dPDK-1 regulates cell growth and apoptosis during drosophila development via PI3K-dependent signaling	PNAS 2001, 98(11):6144 Cho KS, Chung J	
Conventional knockout mice	**ES cells**	2000	viable and proliferate normally Akt, S6K and RSK fail to be activated	PDK1 may not be intrinsically required for ES cell survival and proliferation	Curr Biol, 2000,10(8):439 Williams MR, Alessi DR	
	Embryos	2002	mouse embryos die at E 9.5 multiple abnormalities including lack of somites, forebrain and neural crest derived tissues	PDK1 is essential for normal embryo development in a mammalian system	EMBO J. 2002,21(14):3728 Lawlor MA, Alessi DR	may have embryonic lethality
Hypomorphic mice	**Express 10% of normal level of PDK1 in all tissues**	2002	viable and fertile half smaller than control animals. Cell size decreases, cell number and proliferation are normal. Insulin activates Akt, S6K and RSK normally	PDK1 regulates cell size,not cell number by unknown mechanism	EMBO J. 2002,21(14):3728 Lawlor MA, Alessi DR	

Table 1-2 Summary of PDK1 genetic models (continue 1)

		Year	Phenotypes	Conclusions	Reference	Disadvantages	Advantages
Conditional knockout	**Adipocytes**	2003	decreased Insulin-induced glucose uptake decreased insulin-induced phosphorylation of Akt (T308) and S6K (T389)	PDK1 is essential for insulin-induced glucose uptake in adipocytes	JBC. 2003, 278(40):38870 Sakaue H, Kasuga M		overcome embryonic lethal problem, useful for dissecting complex signaling networks when mutant proteins yield contradictory results
	Cardiac muscles	2003	develop dilated cardiomyopathy, heart failure, increased sensitivity to hypoxia Die in early adulthood (5-11 wks). Akt and S6K are not activated	PDK1 signalling is important in regulating cardiac viability and preventing heart failure	EMBO J. 2003, 22(18):4666 Mora A, Alessi DR	can not dissect the distinct physiological roles of PDK1 downstrems (Akt and S6K)	
Knockin PIF domain	**PIF pocket knockin ES cells**	2003	Akt, but not S6K and RSK can be activated by IGF1	confirmed the biochemical and structural studies that PIF pocket is required for PDK1 to phosphorylate S6K but not Akt	EMBO J. 2003, 22(16):4202 Collins BJ, Alessi DR		Help to explore the physiological roles of docking interactions in regulating specificity of signal transduction pathways. Provide the the first genetic evidence that the PIF-pocket of PDK1 is required for activation of S6K and RSK in vivo
	PIF pocket knockin mice	2004	embryonic lethal, die at E11, cause of death unknown no defect in blood vessel and placental development cell size is normal		EMBO J, 2004, 23(10): 2071 McManus EJ, Alessi DR	mutant gene being present in all cells may lead to pre-mature lethality or unwanted phenotypes	

Summary of PDK1 genetic models (continue 2)

		Year	Phenotypes	Conclusions	Reference	Advantages
Knockin PH domain	**PH domain knockin ES cells**	2004	RSK, not Akt activated by IGF1	PIP3 binding to PDK1 is required for Akt but not RSK activation	Same as above	Same as above, provide the first genetic evidence that the PH-domain of PDK1 is required for activating Akt, but not RSK in vivo
	PH domain knockin mice	2004	embryonic lethal, die at E11, probably due to blood vessel defects and lack of placental development; cell size is normal		Same as above	
Conditional knockin	**PIF pocket knockin in cardiac /skeletal muscles**	2006	animals viable, no phenotype no dilated cardiomyopathy Insulin induces activation of Akt but not S6K in muscle tissues insulin-stimulated glucose uptake normal in knock-in mice	Akt plays a key role in protecting mice from heart failure Consistent with the notion that Akt mediates glucose uptake	JBC 2006, 281(39): 28772, Bayascas JR And Alessi DR	Help to dissect distinct physiological roles of Akt and S6K

B. PDK1 Knockout Mouse ES Cells

In mammalian systems, PDK1 knockout mouse embryonic stem (ES) cells are viable and proliferate at the same rate as the control ES cells expressing wild-type PDK1. However, Akt, S6K and RSK can not be activated in these PDK1-/- ES cells, as opposed to their normal activation by extracellular stimuli in the PDK1 wild-type ES cells (Williams et al., 2000). This indicates that PDK1 is not required for ES cell survival and proliferation. In contrast, other AGC kinases such as PKA, MSK1 and AMPK can still be activated in PDK1-/- ES cells, indicating that PDK1 is not essential for the phosphorylation and activation of these AGC kinases in ES cells (Williams et al., 2000).

C. PDK1 Knockout Mice

In contrast to the ES cells, PDK1 knockout mouse embryos die at embryonic day 9.5, probably due to the lack of a functional circulatory system (Lawlor et al., 2002). These PDK1 knockout mouse embryos display multiple abnormalities including lack of somites, forebrain and neural crest derived tissues, suggesting that PDK1 is essential for normal embryo development in the mammalian system (Lawlor et al., 2002).

D. PDK1 Hypomorphic Mice

Mice that express only ten percent of the normal level of PDK1 in all tissues are viable and fertile; display no severe defect except that they are about half the size of control animals (Lawlor et al., 2002). Their organ volumes are proportionately smaller, which might be due to decreased cell size and not due to differences in cell number or proliferation. Although PDK1 kinase activity is greatly reduced, insulin injection induces

the normal activation of Akt, S6K and RSK in these PDK1 hypomorphic mice. This suggests that a small proportion of cellular PDK1 molecules might be sufficient for effective signal transmission to Akt, S6K and RSK.

E. PDK1 Conditional Knockout Mice

Since the PDK1 conventional knockout mice die at early embryonic stages, a variety of techniques have been used to generate conditional (tissue-specific) PDK1 gene-targeting mice to learn more about the *in vivo* functions of PDK1 after birth. Conditional PDK1 gene disruption using an adenoviral vector encoding Cre recombinase in immortalized brown adipocytes demonstrates that PDK1 is essential for insulin-induced glucose uptake in adipocytes (Sakaue et al., 2003). Deletion of the PDK1 gene in cardiac muscle using Cre/loxP technology results in cardiomyopathy, heart failure and increased sensitivity to hypoxia, which ultimately leads to death within a few weeks after birth (Mora et al., 2003). This suggests that PDK1 signaling is important in regulating cardiac viability and preventing heart failure. However, since both Akt and S6K are not activated in these heart muscle tissues, this study itself could not dissect the distinct physiological roles of Akt and S6K in protecting cardiac viability.

F. PDK1 Knock-in ES Cells and Mice

Different experimental strategies of silencing genes in mice give rise to very distinct phenotypes. Gene knockouts are not equivalent to gene knock-ins with an inactivating point mutation or with a defect in protein-protein interaction. The knock-in approach has significant advantages over the conventional knock-out approach and provides an

alternative way to analyze the function of genes and dissect distinct signaling pathways (Vanhaesebroeck et al., 2004). For example, kinase inhibitors do not abolish the expression of their target proteins, but instead competes with cellular ATP for binding to the target kinases. Based on this principle, gene knock-in that encodes a kinase with a mutation in the ATP binding site will abolish its catalytic activity while still maintaining the kinase-independent functions such as scaffolding. Such strategy mimics a small molecule inhibitors more accurately than gene knockout strategies (Patrucco et al., 2004;Vanhaesebroeck et al., 2004).

Therefore, to explore the physiological roles of the different regulatory domains of PDK1 in regulating the specific signal transduction pathways, knock-in mutations of PDK1 regulatory domains have been generated. To study the role of the PDK1 PIF pocket in regulating specific downstream pathways, Collins *et al*., generated an ES cell line expressing a PDK1 knock-in mutation within the PIF pocket (PDK1[L155E]) that abolishes its ability to bind S6K and SGK (Collins et al., 2003). Stimulation of the PDK1 PIF knock-in ES cells with IGF-1 induces normal activation of Akt and phosphorylation of the Akt substrates GSK-3 and the FOXO1 transcription factor but not S6K or SGK. These results demonstrate that the PDK1 PIF binding pocket promotes specific activation and signaling to S6K and SGK and is not required for Akt activation. However, ES cells expressing a PDK1 knock-in mutation within the PDK1 PH domain that abolishes its ability to bind PIP3, shows normal activation of RSK, but not Akt in response to IGF-1 (McManus et al., 2004). These experiments confirm the previous biochemical and structural studies that PDK1 recognizes Akt and S6K/SGK/RSK by

different mechanisms and emphasizes the physiological importance of the PDK1 PIF-pocket and the PH domain in regulating distinct signaling pathways. PDK1 is also important in regulating PKC phosphorylation and stability. Mice lacking PDK1 show decreased expression levels of PKC isoforms (Balendran et al., 2000). In addition, decreased expression of many PKC isoforms were observed in ES cells expressing the PDK1-L155E PIF binding pocket mutant but not the PH domain mutant, suggesting that the PDK1 PIF domain plays an important role in stabilizing protein kinase C isoforms (Collins et al., 2003). Both the PIF pocket knock-in and the PH domain knock-in mice are embryonic lethal (died between E10.5 and E11.5), but showed different phenotypes. The cause of the death of the PH domain knock-in mice might be due to blood vessel defects and lack of placental development; in contrast, the PIF pocket knock-in mice do not have these phenotypes and the cause of death is unknown.

G. Conditional PDK1 Knock-in Mice

Conditional knock-out mice lacking PDK1 in the heart muscle develop dilated cardiomyopathy and die of heart failure at 5–11 weeks of age (Mora et al., 2003). In these mice, both Akt and S6K fail to be activated. To further dissect the physiological roles of Akt and S6K in heart function, conditional PDK1 PIF pocket knock-in mice were generated in skeletal and cardiac muscle tissues to prevent activation of S6K, but not Akt (Bayascas et al., 2006). In contrast to the conditional PDK1 knock-out in cardiac muscle, these animals with knock-in mutations do not develop any symptoms of heart failure and survived normally for at least 1 year. These results clearly demonstrate that lack of Akt

activity, but not S6K activity, is the main cause of cardiomyophathy and heart failure in mice lacking cardiac PDK1.

H. Tumor Model

PTEN, the lipid phosphatase that dephosphorylates PIP3 to PIP2, is frequently mutated in human cancer (Sulis *et al.*, 2003). It is fundamental for embryonic development in mice since PTEN-/- mice die at embryo day 9.5. PTEN depleted cells possess higher levels of PIP3, Akt, and S6K activity (Maehama and Dixon, 1998;Stambolic et al., 1998;Haas-Kogan et al., 1998). PTEN +/- mice have an increased incidence of a variety of tumors (Suzuki et al., 1998;Di Cristofano et al., 1998;Podsypanina et al., 1999). Therefore, the PTEN mutant mice have been used as an experimental model for investigating the role of PTEN and its related signaling proteins in cancer progression. For example, depletion of Akt1 in PTEN-/- cells reduces aggressive growth and promotes cell apoptosis (Stiles et al., 2002), while treatment of PTEN+/- mice with the S6K inhibitor, rapamycin, reduces tumorigenesis (Neshat et al., 2001). In addition, reducing PDK1 expression by 80-90% in PTEN+/- mice significantly protects these mice from developing a variety of tumors (Bayascas et al., 2005). This indicates that PDK1 is a key regulator in mediating tumorigenesis resulting from loss of PTEN, and at the same time, it validates PDK1 as a potential anti-cancer drug target for tumors that have elevated Akt/S6K activity or PDK1 expression level.

(4) Regulation of PDK1

Despite its well-appreciated essential cellular roles, PDK1 regulation at the molecular level is less well understood, in contrast to its substrates. PDK1 was initially considered to be 'constitutively active' due to its high basal level of activity. However, with one kinase phosphorylating and activating abundant pathways, it is likely that PDK1 is involved in a more complex regulatory mechanism. Indeed, recent studies have clearly demonstrated that its function is critically regulated on multiple levels. The current proposed mechanisms of regulating PDK1 include subcellular localization (Anderson et al., 1998), substrate-directed targeting (Frodin et al., 2000), phosphorylation (Grillo et al., 2000;King et al., 2000b;Park et al., 2001), and protein-protein interactions. Therefore, understanding the current models by which PDK1 is regulated will facilitate our understanding of how CIB1 might regulate the specificity of PDK1 signaling and function, the details of which will be discussed in Chapter IV.

A. Subcellular Localization

The C-terminal PH domain of PDK1 facilitates its binding to the lipids PIP2 and PIP3 *in vitro* with high affinity. However, whether PI3K controls the spatial distribution of PDK1 in cells remains controversial. It is consistently observed that PDK1 is localized to the cytoplasm in quiescent cells. In contrast, there are mixed results on whether PDK1 translocates to the plasma membrane upon stimulation and if it does, whether this membrane redistribution is PI3K-dependent. One study shows that in porcine aortic endothelial cells stimulated with PDGF, overexpressed PDK1 translocated to the plasma membrane and activated Akt (Anderson et al., 1998). Kim *et al.* reported that in Rat2 cells stimulated with EGF, GFP-tagged PDK1 translocated to the plasma membrane (Kim

et al., 2001). However, opposite observations were also reported. For example, in porcine aortic endothelial or 293 cells stimulated with PDGF or IGF-1, a small proportion of overexpressed PDK1 localized to the plasma membrane, while most was present in the cytosol (Currie et al., 1999;Komander et al., 2004). The cytosolic portion of PDK1 did not translocate to the plasma membrane following IGF-1 stimulation, and the cytosolic PDK1 localization was not affected by the PI3K inhibitor wortmannin. King *et al.* recently observed that a scaffold protein, Grb14, facilitated membrane translocation of endogenous PDK1 in response to insulin but not PDGF stimulation. The authors also found that this recruitment promoted Akt phosphorylation and transduction of the insulin signal in a PH-domain independent manner (King and Newton, 2004). These mixed results indicate that subcellular localization and tranlocation of PDK1 might be dependent on the cell context and the presence of specific signaling pathways.

Both membrane-bound and cytosolic pools of PDK1 are able to regulate a wide range of substrates, due to its unique PH domain (Komander et al., 2004), which will be discussed in the 'lipid and PH domain' section. In addition to shuttling between the cytoplasm and membrane, nuclear localization of PDK1 has also been observed (Lim et al., 2003). Given the previous findings that the nucleus has a pool of PIP3 (Neri et al., 1999;Neri et al., 2002), nuclear PI3K is activated by a nucleus-specific GTPase PI3K enhancer (PIKE) (Ye et al., 2000), as well as the presence of PDK1 downstream effectors such as Akt, S6KβI, S6KβII and PKCζ in the nucleus (Minami et al., 2001;Kikani et al., 2005), it is highly possible that an independent and complete PI3K-PDK1-Akt/S6K signaling pathway exists in nucleus. Although physiological signals that stimulate PDK1 to

translocate to the nucleus are currently unknown, a significant increase in PDK1 nuclear localization in PTEN-/- cells as well as in insulin-treated cells have been observed (Lim et al., 2003). Nuclear localization of PDK1 does not affect its kinase activity *per se*, but decreases its tumorigenic ability such that cells show increased sensitivity to UV-induced apoptosis and decreased potential to induce anchorage-independent cell growth. These observations may be due to the inability of PDK1 to access its membrane-bound and cytosolic substrates, therefore acting as a negative regulatory mechanism for PDK1 signaling (Lim et al., 2003).

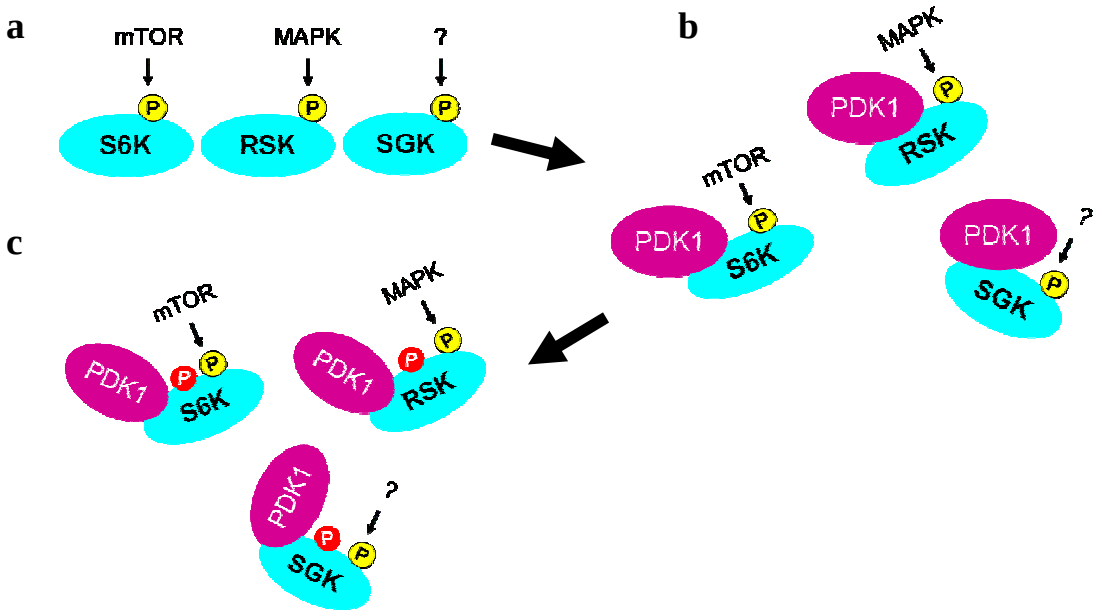
B. Substrate-directed Targeting

All members of the AGC family of kinases have two highly conserved Ser/Thr (S/T) residues. One S/T residue located in the activation loop is phosphorylated by PDK1, and the other is located in the hydrophobic motif that is C-terminal to the catalytic domain, with a conserved sequence FXXFS/TF/Y, where X is any amino acid and the S/T residue is the phosphorylation site. Phosphorylation of both residues is required for maximal activation of these kinases (Stokoe *et al.*, 1997;Alessi *et al.*, 1997b). Some members of the AGC family such as the atypical PKC isoforms and PRK (PKC-related kinase) isoforms have an acidic residue D/E in place of an S/T residue in their hydrophobic motif. It has been proposed that the phosphorylation of the hydrophobic motif of AGC kinases creates a PDK1-docking site and consequently promotes their activation by PDK1 (Fig. 2-6A). For example, Frodin *et al*, has proposed a model for the coordinated activation of RSK2 and PDK1 in which the C-terminal kinase of RSK2 is initially activated by MAPK, leading to autophosphorylation of RSK2 at Ser386 within its hydrophobic motif. This

phosphorylated residue serves as a docking site for PDK1 and stimulates PDK1 autophosphorylation and activity. Activated PDK1 then phosphorylates Ser227 in the N-terminal kinase domain of RSK2 resulting in complete activation of RSK2 (Frodin et al., 2000) (Fig. 2-6B). Therefore, Ser386 in the C-terminal hydrophobic motif of RSK2 acts as a phosphorylation-dependent docking site and activator of PDK1. Treatment of cells with growth factor has also been shown to induce the recruitment of PDK1 to the Ser386-phosphorylated hydrophobic motif and phosphorylation of RSK2 at Ser227 (Frodin et al., 2000).

This substrate-directed regulation model is also supported by the finding that mutation of the S/T residue in the hydrophobic motif of either S6K1 (Alessi et al., 1998;Pullen et al., 1998) or SGK1 (Park *et al.*, 1999;Kobayashi and Cohen, 1999a) to an acidic residue (D/E) to mimic phosphorylation, dramatically increases the phosphorylation of these kinases at the T-loop by PDK1 *in vitro*.

A



B

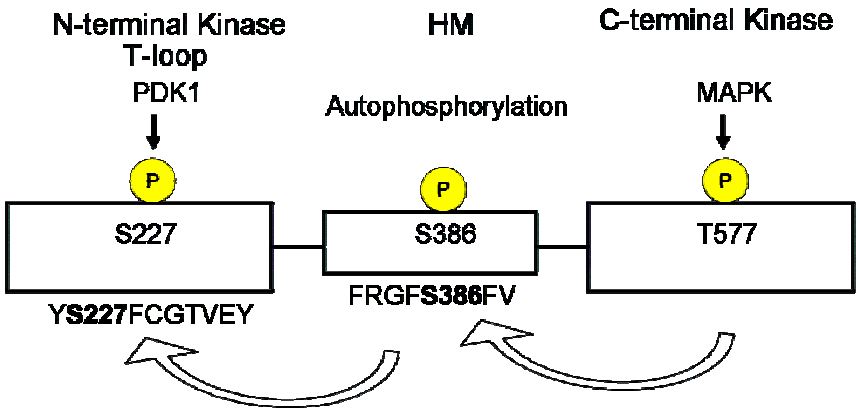


Figure 2-6 Substrate-directed targeting mechanism of regulating PDK1. **(A)** PDK1 substrates such as S6K, RSK, and SGK are first phosphorylated by mTOR, MAPK, and an unidentified kinase respectively on the HM motif (a). Phosphorylation of HM provides a docking site for binding PDK1 (b). Recruited PDK1 further phosphorylates these kinases on the T-loop (c) and fully activates them. **(B)** C-terminal kinase domain of RSK2 is phosphorylated by MAPK at T577, which leads to autophosphorylation of RSK2 at Ser386 in HM. The phosphorylated HM acts as a docking site to recruit PDK1 and facilitate PDK1 to phosphorylate RSK2 at S227 in T-loop.

C. Phosphorylation

Five phosphorylated serine residues have been identified in PDK1, and autophosphorylation of Ser241, which is located at the activation loop, appears to be critical for its kinase activity (Casamayor et al., 1999a). Lipids such as sphingosine also increase PDK1 autophosphorylation and significantly increase phosphate incorporation into PDK1 substrates such as Akt and PAK1 (King et al., 2000b). Recently, it has been reported that PDK1 can also be phosphorylated on tyrosine residues in response to pervanadate and oxidative stress (Grillo et al., 2000;Prasad et al., 2000). Tyrosine phosphorylation causes a modest (2-3 fold) increase in PDK1 activity, and enhances the membrane localization of PDK1. Protein kinases implicated in the tyrosine phosphorylation of PDK1 include Src, Abl, and Pyk2 (Grillo *et al.*, 2000;Park *et al.*, 2001;Taniyama et al., 2003). These data suggest that signaling through these tyrosine kinases can positively regulate PDK1 signaling. However, whether these pathways require PI3K for PDK1 activity toward substrates and the biological significance of the activation of PDK1 remains to be determined.

D. Protein-protein Interactions:

Several proteins and peptides have been shown to interact with and modulate the intrinsic kinase activity of PDK1. The kinase domain of PDK1 interacts with a region of protein kinase C related kinase 2 (PRK2), which is termed the PDK1-interacting fragment (PIF). The interaction of the PIF peptide with PDK1 converts PDK1 from a form that can not be directly activated by PIP3 to a form that is activated threefold by PIP3 *in vitro*, and a form having the ability to phosphorylate Akt on both Thr308 and Ser473 (Balendran et

al., 1999a). In addition, association of PDK1 with RSK2 stimulates PDK1 activation and autophosphorylation (Frodin et al., 2000). The scaffold protein 14-3-3 has also been shown to bind PDK1 via residues that surround the Ser241 autophosphorylation site and negatively regulates PDK1 activity (Sato et al., 2002b). Chaperon protein Hsp90 binds PDK1 and prevents PDK1 from proteasome-dependent degradation and helps to keep it in a soluble and a conformationally active state (Fujita et al., 2002). However, the biological significance of these interactions remains unclear. Recent studies have demonstrated that two adaptor proteins bind PDK1 through the consensus hydrophobic motif FXXFS/TF/Y and regulate its cellular functions. One is the adaptor protein Grb14, which contributes to the activation of PDK1 downstream of insulin signaling (King *et al.*, 2004). Another adaptor protein, the muscle A-kinase anchoring protein (mAKAPα), recruits PDK1, RSK, and ERK to the perinuclear membrane in cultured primary rat hippocampal neurons, allowing for RSK activation and signal transmission to the nucleus (Michel et al., 2005).

E. Lipids and PH domain

PIP3 stimulates PDK1 phosphorylation and activation of Akt and a PH domain deletion mutant of PDK1 prevents translocation of Akt to the membrane. In contrast, a PH domain deletion mutant of Akt efficiently translocates to the membrane in the presence of wild-type PDK1 (Filippa et al., 2000). These results indicate that the PH domain of PDK1, but not of Akt, is essential for Akt translocation to the membrane. In addition, the PDK1 PH domain has been suggested to act as a negative regulator of its activity, and PIP3 binding relieves this autoinhibition (Filippa et al., 2000).

PH domains of several proteins have been found to interact efficiently with not only the membrane-localized phosphoinositides, but also the soluble inositol phosphates naturally localized in the cytosol, and binding to these different inositol lipids and phosphates in different subcellular compartments seem to regulate distinct physiological functions (Kojima et al., 1997;Kavran et al., 1998;Hirose et al., 1999;Fleming et al., 2004). PDK1 has both membrane-bound and cytosolic substrates. One proposed mechanism of how PDK1 is regulated to activate a full range of substrates localized in different compartments is that the membrane-bound phosphoinositides PIP2 and PIP3 help to maintain the membrane pool of PDK1 via its PH domain, whereas the soluble inositol phosphate IP6 helps to maintain the cytosolic pool of PDK1 through binding to its PH domain (Komander et al., 2004;Downes et al., 2005). This hypothesized model enables PDK1 to be distributed in both the cytosol and plasma membrane, which allows PDK1 to achieve various cellular functions (Fig. 2-7). Therefore, instead of being constitutively active as previously thought, these studies clearly demonstrate that PDK1 activity is actually precisely regulated on multiple levels.

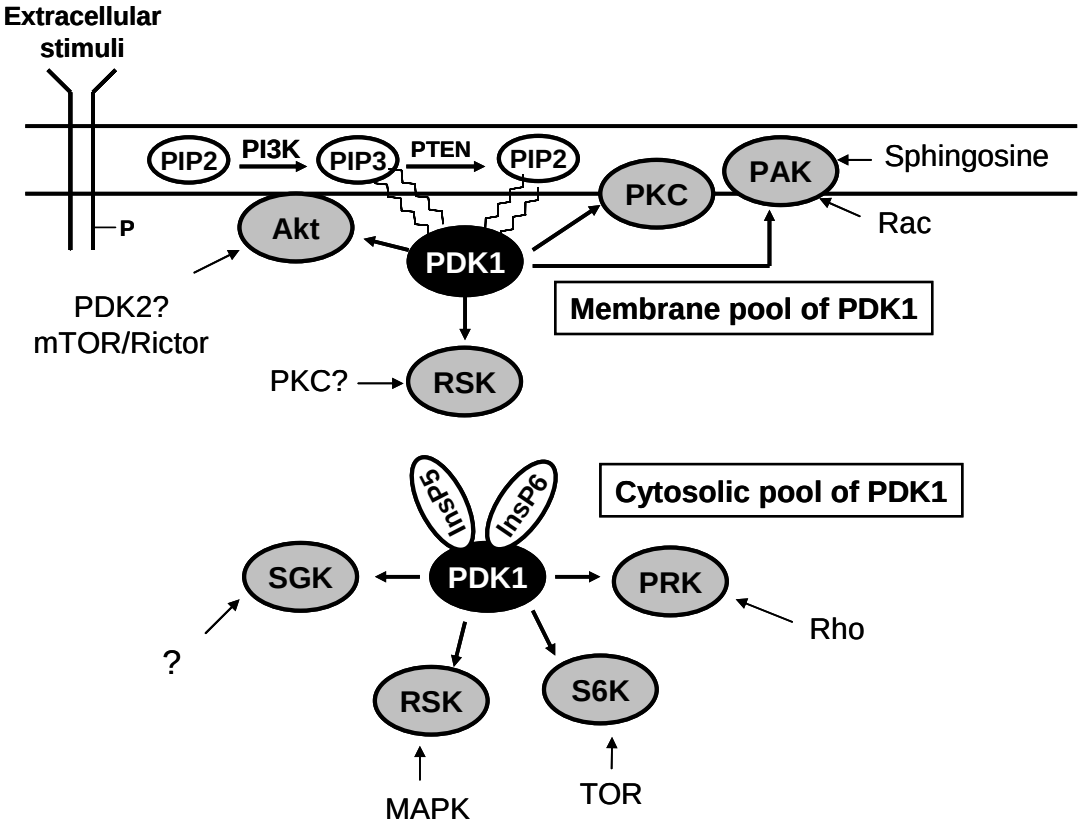


Figure 2-7 Membrane-bound inositol phospholipids and soluble inositol phosphates maintain distinct membrane-bound and cytosolic pools of PDK1. PIP2 and PIP3 binding to the PH domain of PDK1 may account for the membrane-associated pool of PDK1 upon growth factor stimulation, where PDK1 can phosphorylate and activate membrane-bound substrates such as Akt, PKC, PAK, and RSK. Soluble inositol polyphosphates InSP5 and InSP6 are potent ligands for the PH domain of PDK1, which may account for the cytosolic pool of PDK1, where it can phosphorylate and activate its cytosolic substrates such as SGK, S6K, RSK, and PRK.

F. PI3K -dependent and -independent Regulation

Not all PDK1 downstream effectors and biological functions are PI3K-dependent. The kinases that phosphorylate the hydrophobic motif of Akt are controversial although recent evidence favors rictor-mTOR as the upstream kinase (Sarbassov et al., 2005). Raptor-mTOR directly phosphorylates the hydrophobic motif of S6K (Hara et al., 2002;Kim et al., 2002). The kinases that phosphorylate the hydrophobic motif of SGK are unknown, but the phosphorylation of this residue *in vivo* for all these three kinases is PI3K-dependent (Scheid *et al.*, 2003). However, RSK and PRK2 are not activated by stimulation of cells with agonists that activate PI3K. Instead, RSK is phosphorylated by the Erk1/2 MAPK at the hydrophobic motif (Frodin *et al.*, 1999;Minami *et al.*, 2001), and PRK2 is regulated by the Rho family of GTPases (Flynn et al., 2000a). In addition, PDK1-mediated phosphorylation and activation of PAK1 within its activation loop is dependent on sphingosine but not PI3K activity (King et al., 2000a). Therefore, PDK1 transducing signals to downstream effectors can be both PI3K-dependent and -independent.

4) PDK1 as a Drug Target

Given the critical role of PI3K/PDK1/Akt signaling in cancer cell survival and proliferation, this pathway represents an attractive therapeutic target for developing anti-cancer drugs.

PDK1 is a very good anti-cancer drug target for several reasons. First, PDK1 phosphorylates and activates a variety of AGC kinases, and most of them contribute more

or less to cancer progression. Therefore, inhibitors that target PDK1 may indirectly inhibit most if not all of these downstream kinases for treatment of cancer cells possessing constitutively activated or upregulated AGC kinase(s). From a therapeutic point of view, direct inhibition of PDK1 may have significant advantages over targeting specific downstream signals, since distinct downstream signals are dominant in different cell types and different tumor tissues; moreover, it is very likely that PDK1 has other unidentified substrates. Because of these reasons, inhibiting individual downstream signal may miss the critical substrates that are actually responsible for PDK1-mediated certain cancer cell survival or proliferation. Therefore, inhibition of PDK1 might be more potent, although at the cost of potential greater toxicity. However, since PDK1 hypomorphic mice expressing only ten percent of the normal levels of PDK1 are viable, fertile and display no obvious defect (Lawlor et al., 2002), PDK1 inhibitor should not be highly toxic. Second, feedback activation of signaling pathways is a pitfall of inhibition of a distal component. For example, inhibition of mTOR by rapamycin causes a feedback activation of Akt that leads to increased tumor cell survival. Since PDK1 is a relatively proximal component in the PI3K/PDK1/AGC kinase(s) pathway, this feedback activation is less likely to happen. In addition, the fact that there is only one known isoform of PDK1 in human would facilitate the development of PDK1 inhibitors. Both PDK1 and Akt contain small-molecule regulatory binding sites close to the kinase active site, making them ideal targets for the development of strategies for structure-based small molecule inhibitors (Biondi et al., 2000). A number of current PDK1 inhibitors will be discussed in the following sections. In addition, since CIB1 binds PDK1 and regulates PDK1 function (Chapter III and IV), potential CIB1 and PDK1 interaction sites have

been suggested (Chapter III), and both CIB1 and PDK1 crystal structures have been solved (Chapter I and II), this information may be important in designing small molecule inhibitors that target PDK1 or CIB1.

(1) Lipid analogues that block membrane binding through PH domains

Ether lipid analogues of inositol phosphates (Qiao et al., 1998;Hu et al., 2000) and inositol polyphosphates (Razzini et al., 2000) inhibit PI3K signaling. These molecules bind to the PH domains of PDK1 and Akt, inhibiting their translocation to the plasma membrane and subsequent activation. Although a number of molecules of this type have been identified that inhibit PI3K, PDK1, and Akt activity and tumor cell growth *in vitro* in micromolar concentrations, the lack of target selectivity, together with the instability and toxicity have limited their clinical development as cancer therapeutics.

(2) Protein kinase inhibitor UCN-01, the 7-hydroxy-derivative of staurosporine

UCN-01, the 7-hydroxy-derivative of staurosporine and an ATP-competitive inhibitor has been shown to possess potent antitumor activity. UCN-01 directly inhibits PDK1 with a very low IC50 (< 33 nM) *in vitro* and *in vivo* and inhibits downstream signaling to Akt (Sato et al., 2002a). In addition to PDK1, UCN-01 is also found to be a potent inhibitor of many other serine/threonine kinases, including several PKC isoforms, cyclin-dependent kinases (CDK) 2, 4 and 6, and Chk1 kinase (Graves et al., 2000). UCN-01 has many potent effects such as inducing G1 cell cycle arrest, enhancing apoptosis, and facilitating DNA-directed cytotoxicity of chemotherapeutic cancer agents such as the anti-metabolites camptothecins and cisplatin. Given its potent antiproliferative activity *in*

vitro against several cell lines and its anti-neoplastic activity in several xenograft systems, UCN-01 was selected for clinical trials, and some promising results were achieved in certain cancer patients (Sausville et al., 2001). However, the ability of UCN-01 to inhibit many kinases makes it a less specific targeted pathway inhibitor. Therefore, whether the cell response to UCN-01 treatment is caused by inhibition of the targeted enzyme such as PDK1 or other targets will require further verification, and more detailed investigation into its critical targets in distinct cancer patients will greatly help its development and application.

(3) OSU-03012 and OSU-03013, derivatives of cyclooxygenase-2 (COX-2) inhibitor celecoxib

Celecoxib, a well-characterized COX-2 inhibitor, was found to potently induce apoptosis in the colon cancer cell line HT-29, which lacks COX-2 activity. This led to the finding that it specifically inhibits PDK1 activity (IC50 ≈ 50uM), suggesting that targeting the PDK1/Akt pathway is a major signaling mechanism by which celecoxib mediates apoptosis other than targeting COX-2 (Arico et al., 2002). This observation has prompted studies using structure-based optimization of celecoxib to develop PDK1 inhibitors with higher specificity and greater potency in growth inhibition. One study demonstrated that two derivatives, OSU-03012 and OSU-03013, also ATP-competitive inhibitors that possess no activity against COX-2, inhibit PDK1 activity and cell growth with a very low concentration (≈ 3 μM) in 60 different human tumor cell lines (Zhu et al., 2004). These two agents cause Akt dephosphorylation and inhibition of S6K activity. Overexpression of constitutively active PDK1 and Akt partially rescues inhibitor-induced apoptosis. However, these celecoxib derivatives also have other biological activities;

they induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9; they delay G2-M cell cycle progression independent of PDK1 inhibition (Ding et al., 2005). These data indicate that the anticancer activity of these inhibitors also has off-target effects.

(4) BX-795, BX-912, and BX-320

High-throughput screening of compound libraries and lead optimization have identified a group of potent PDK1 inhibitors BX-795, BX-912, and BX-320 with very low IC50s (nM) (Feldman et al., 2005;Ding *et al.*, 2005). Further biological characterization of these potent small molecule inhibitors indicates that they directly inhibit PDK1 activity as shown by their ability to block phosphorylation of Akt, S6K1, PKCδ and GSK3β. They block PDK1 signaling in tumor cells and inhibit the anchorage-dependent growth of a variety of tumor cell lines in culture and induce apoptosis. BX-320 inhibits the growth of LOX melanoma tumors in the lungs of nude mice after injection of tumor cells into the tail vein (Feldman et al., 2005). However, correlation of target inhibition with biological effects *in vivo* needs to be further validated (Granville et al., 2006).

In conclusion, although PDK1 is an ideal target for anti-cancer therapy, and a variety of small molecules have been identified to inhibit PDK1 activity and thus block its survival and proliferative function in tumors, specificity remains to be a challenging issue.

5) Specific aims and scope of this study

High stringency *in silico* analysis of the CIB1 protein sequence predicted a potential CIB1 binding protein, PDK1. Thus, the specific aims of my studies were:

First, using various, complementary binding assays, we determined whether CIB1 can indeed interact directly with PDK1 *in vitro* and in cells (Chapter III). Second, since PDK1 kinase activity can be either activated or inhibited by interacting proteins, we examined whether CIB1 binding to PDK1 can regulate PDK1 kinase activity *in vitro* and in cells. Further characterization of the motif/residues in CIB1 and PDK1 that contribute to the binding provided possible mechanisms by which CIB1 regulates PDK1 activity (Chapter III). Third, PDK1 is a master kinase of AGC family of kinases. Through phosphorylation and activation of one or more of its downstream effectors, PDK1 regulates diverse biological functions including promoting cell survival and proliferation. Our previous findings showed that MEFs derived from the CIB1-KO mice proliferate at a significantly slower rate compared to the wild-type MEFs by an uncharacterized mechanism. In addition, increased spermatocyte and spermatid apoptosis were also observed in CIB1-KO mice. Therefore, we examined the biological function in cell survival/apoptosis in cancer cells, and determined if CIB1 regulates cell survival/apoptosis via a PDK1 signaling pathway (Chapter IV). Fourth, although the biological functions and downstream effectors of PDK1 have been well-characterized, the mechanism(s) by which PDK1 is regulated is poorly understood. PDK1 has been shown to localize to different subcellular compartments under distinct extracellular stimuli and its subcellular localization appears to regulate its function. We therefore

determined if CIB1 can affect PDK1 subcellular localization and ultimately regulate PDK1 function (Chpater IV). And finally, PDK1 transduces signals via diverse downstream effectors. Therefore, we sought to dissect the potential CIB1-dependent pathway(s) downstream of PDK1, thereby achieving its specific functions (Chapter IV).

By assessing the binding and function of CIB1 and PDK1 in the context of cell survival/apoptosis and proliferation, my thesis will provide additional information into the novel functions of CIB1 and the mechanism of PDK1 regulation. This information may contribute to the identification of novel anti-cancer drugs that target signaling pathways mediated by CIB1 and PDK1.

Chapter III

CIB1 Binds PDK1 and Activates PDK1

1. Abstract

In order to identify new CIB1 binding proteins and therefore better understand its biological functions, we analyzed the CIB1 protein sequence using a database of known protein-kinase recognition motifs (www.scansite.mit.edu). A high stringency scan predicted a potential consensus PDK1 hydrophobic binding motif (FxxF[D/E/S][Y/F]) between CIB1 residues 112 and 117. Further pulldown assays demonstrated that these two recombinant proteins bind directly *in vitro*, and that the endogenous proteins bind in cells. Peptide competition assay and site-directed mutagenesis confirmed that this PDK1 hydrophobic binding motif within CIB1 contributes largely to the binding to PDK1, although there may be other unidentified sites involved. The PDK1 PH domain and the key residue Leu155 in the hydrophobic motif do not contribute to the binding, since a PH domain truncated form of PDK1 and the point mutation of the Leu155 to Glu do not inhibit binding. CIB1 binding to PDK1 further increases PDK1 autophosphorylation and kinase activity both *in vitro* and in cells. In summary, in this part of work I have identified a new CIB1 binding partner, PDK1, and have predicted an important new biological function for CIB1.

2. Introduction

PDK1 is a key upstream regulator of a variety of AGC superfamily protein kinases, including Akt (Alessi et al., 1997;Stephens et al., 1998;Williams et al., 2000), PKC isozymes (Dutil et al., 1998;Le Good et al., 1998), S6K (Alessi et al., 1998;Pullen et al., 1998), RSK (Jensen et al., 1999;Richards et al., 1999), and SGK (Kobayashi and Cohen, 1999;Park et al., 1999). Biochemical and genetic studies demonstrate that PDK1 plays critical roles in both normal and transformed cells by facilitating cell survival, proliferation, and cell cycle progression, via signaling to one or more of its downstream effectors. For example, overexpression of PDK1 transforms mouse mammary epithelial cells through PDK1-mediated activation of protein kinase Cα (Xie et al., 2003;Zeng et al., 2002). Conversely, blocking PDK1 expression in a glioblastoma cell line (U-87) decreases the activity of PDK1 substrates, Akt and S6K, and inhibits cell proliferation and induces apoptosis (Flynn et al., 2000). In addition, inhibition of PDK1 sensitizes certain cancer cells to chemotherapeutic agents (Liang et al., 2006) and increases cytotoxicity (Zhu et al., 2004a;Crowder and Ellis, 2005;Kucab et al., 2005). These data reinforce the idea that PDK1 is critical for cell growth and survival in both normal and tumorigenic contexts.

Despite its well-appreciated roles in cell growth and survival, PDK1 regulation at the molecular level is less well understood, especially compared to its more extensively studied downstream effectors, such as Akt. Although it was previously believed that PDK1 was constitutively active, more recent data indicate that PDK1 regulation occurs on many levels. Some proposed mechanisms for PDK1 regulation include subcellular

localization (Anderson et al., 1998b), substrate-directed targeting (Frodin et al., 2000), phosphorylation (Grillo et al., 2000;Park et al., 2001;King et al., 2000), and protein-protein interaction. A variety of proteins have been shown to interact with PDK1 and modulate its kinase activity, such as 14-3-3, PRK2, and Hsp90 (Sato et al., 2002b;Balendran et al., 1999b;Fujita et al., 2002), but the functional significance of these interactions remains unclear. Some adaptor proteins have also been demonstrated to regulate its subcellular localization. For example, Grb14 recruits PDK1 to the plasma membrane and contributes to the activation of PDK1 downstream of insulin signaling (King and Newton, 2004); Another adaptor protein, the muscle A-kinase anchoring protein mAKAPα, recruits PDK1 and ERK, allowing for p90RSK activation and signal transmission to the nucleus (Michel et al., 2005). All of these studies clearly demonstrate that PDK1 is subject to cellular regulation on multiple levels. Given the broad spectrum of the downstream effectors of PDK1 and its various cellular compartmentalizations, it is highly possible that other proteins will recruit and regulate PDK1 activity.

In the case of CIB1, it was first identified as a protein that binds to the cytoplasmic tail of the platelet-specific integrin αIIb (Naik et al., 1997). Later experiments found that CIB1 is widely expressed, suggesting it might be important in cell types other than platelets. Consistent with this, many CIB1-binding proteins have been identified and a lot of them are kinases, including the ser/thr kinases PAK1 (Leisner et al., 2005), DNA-dependent protein kinase (Wu and Lieber, 1997), the polo-like kinases Fnk and Snk (Kauselmann et al., 1999), and the tyrosine kinase FAK (Naik and Naik, 2003). This observation suggests that CIB1 may act as an important regulator of kinase function. Therefore, to

identify additional CIB1 binding partners and gain a better understanding of its cellular functions, we searched the CIB1 sequence for potential protein binding sites using a database of known protein-kinase recognition motifs (www.scansite.mit.edu). A high stringency scan identified a potential consensus PDK1 hydrophobic binding motif (FxxF[D/E/S][Y/F]) between CIB1 residues 112 and 117 (Fig. 3-1).

3. Results

1) CIB1 Directly Interacts with PDK1

To determine whether CIB1 interacts with PDK1, we used purified recombinant CIB1 and PDK1 in solid-phase binding assays and found that soluble CIB1 bound to immobilized PDK1 in a direct, saturable manner (Fig. 3-2 A, left panel). Conversely, we also observed a direct and saturable binding of soluble PDK1 to immobilized CIB1 (Fig. 3-2 A, right panel). In addition, purified recombinant GST-PDK1 but not GST alone specifically precipitated recombinant CIB1 (Fig. 3-2 B) and purified CIB1-GST was able to precipitate endogenous PDK1 from HeLa cell lysates (Fig. 3-2 C).

To confirm our *in vitro* binding data, co-immunoprecipitation assays were carried out using COS-7 cells overexpressing myc-PDK1 and CIB1 either alone or in combination.

A

Kinase binding site group (Kin_bind)				
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
D116	**0.1723**	0.014 %	**HYAFRIFDFDDDGTL**	0.561
			F-X-X-F-S/T(D)-F	

B

1 MGGSGSRLSK ELLAEYQDLT FLTKQEILLA HRRFCELLPQ EQRSVESSLR

51 AQVPFEQILS LPELKANPFK ERICRVFSTS PAKDSLSFED FLDLLSVFSD

101 TATPDIKS**HYA**FRIFDF**DDDGTL**NREDLSR LVNCLTGEGE DTRLSASEMK

151 QLIDNILEES DIDRDGTINL SEFQHVISRS PDFASSFKIV L

Figure 3-1 Scansite identifies PDK1 as a potential CIB1 binding protein. (A) A high stringency scan identifies that CIB1 contains a PDK1 consensus binding motif FXXFS/T(D/E)F between residues 112-117, with D116 preferred by PDK1. The FRIFDF motif in CIB is within the top 0.014% of all matching sequences potentially binding to PDK1. **(B)** CIB1 sequence with the consensus PDK1 binding sequence in red color and the synthetic peptide sequence for binding assay in bold.

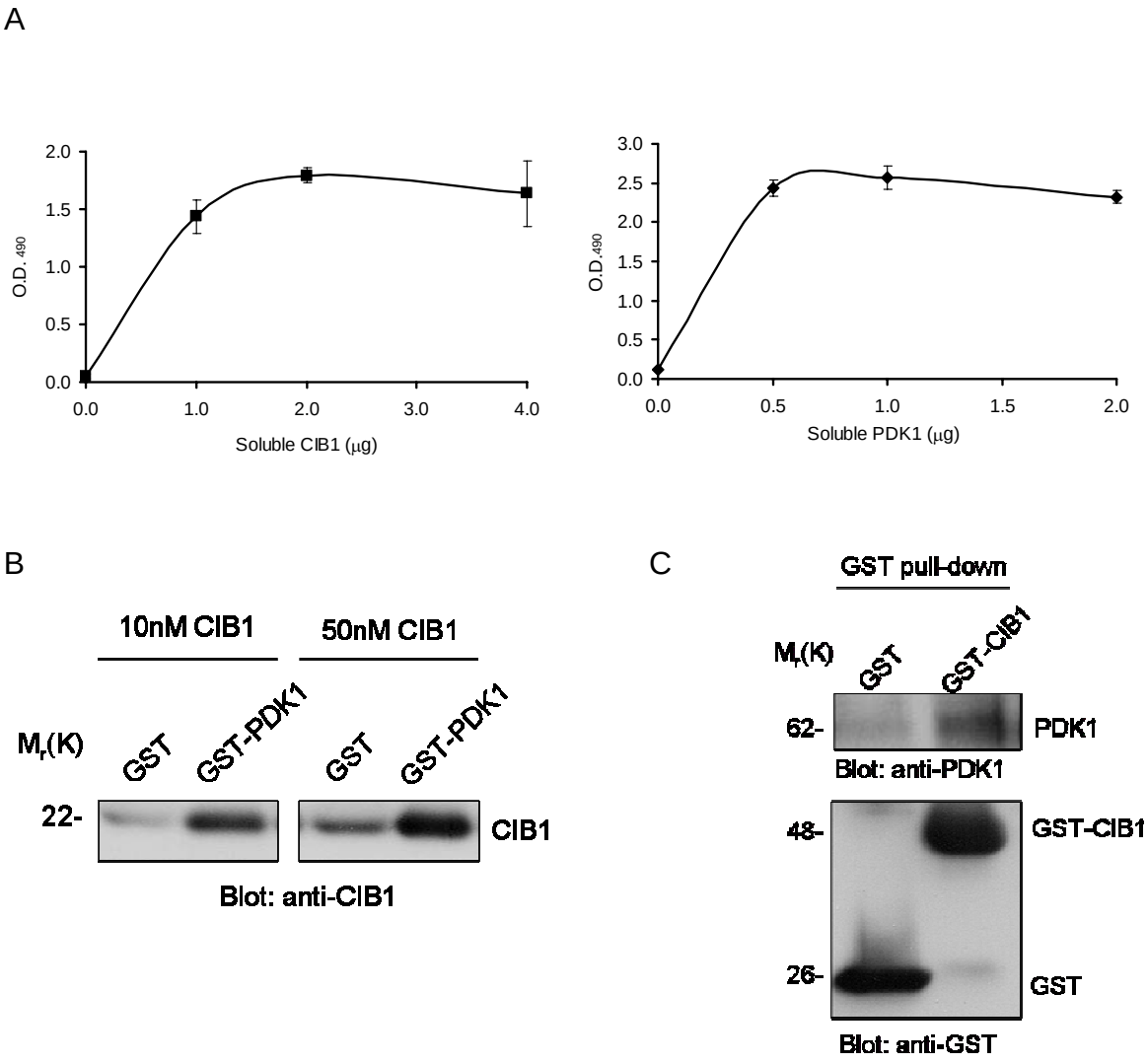


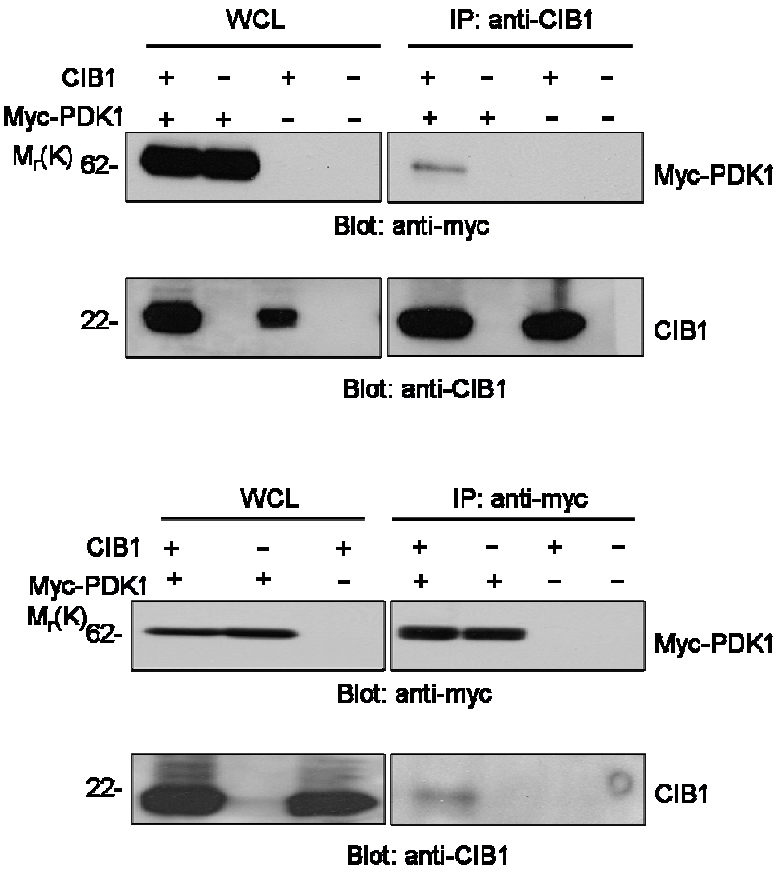
Figure 3-2 CIB1 directly interacts with PDK1 *in vitro.* (A) Solid-phase binding assays show a direct interaction between CIB1 and PDK1. Increasing concentrations of soluble CIB1 were added to immobilized PDK1 (left panel) and conversely, soluble PDK1 was added to wells coated with immobilized CIB1 (right panel). Binding of soluble PDK1 or CIB1 to immobilized proteins was detected using goat anti-PDK1 or chicken anti-CIB1 antibodies. **(B)** Pull-down of soluble recombinant CIB1 (10 nM left panel, 50 nM right panel) by GST-PDK1 immobilized on glutathione-Sepharose beads demonstrates a direct interaction between CIB1 and PDK1. Binding was detected by anti-CIB1 antibody. **(C)** HeLa cell lysates were precipitated with either GST or GST-CIB1 and analyzed by anti-PDK1 immunoblot (upper). GST-CIB1 and GST-fusion protein inputs (lower) were detected by anti-GST antibody.

When both PDK1 and CIB1 were overexpressed, we found that PDK1 coimmunoprecipitated with CIB1 (Fig. 3-3 A, upper panels) and conversely, CIB1 coimmunoprecipitated with PDK1 (Fig. 3-3 A, lower panels). To determine whether endogenous CIB1 and PDK1 interact, coimmunoprecipitation assays were performed using lysates prepared from platelets since these cells express high levels of both proteins (Fig. 3-3 B, right panel). As expected, PDK1 immunoprecipitated CIB1 with an anti-CIB1 antibody but not with a control IgY antibody (Fig. 3-3 B, left panel) further confirming the interaction between CIB1 and PDK1 in cells. Therefore, these data indicate that CIB1 interacts with PDK1 both *in vitro* and in cells.

2) Characterization of the PDK1 Binding Motif in CIB1

We next asked whether the predicted PDK1 binding sequence in CIB1 is actually required for the CIB1/PDK1 interaction. We performed solid-phase binding assays with a 15-residue synthetic peptide (HYAFRIFDFDDDGTL) containing the putative PDK1-binding sequence of CIB1 (FRIFDF). We tested whether this peptide could inhibit binding of soluble PDK1 to immobilized CIB1. Pre-incubation of soluble PDK1 with this peptide significantly inhibited PDK1 binding to immobilized CIB1. In contrast, corresponding scrambled peptides I (DDTIFLDHRGFYDFA) and II (RFFYIDHADGFTDLD) failed to inhibit PDK1 binding to immobilized CIB1 (Fig. 3-4 A). These results suggest that the PDK1 binding site in CIB1 is located within the HYAFRIFDFDDDGTL sequence.

A



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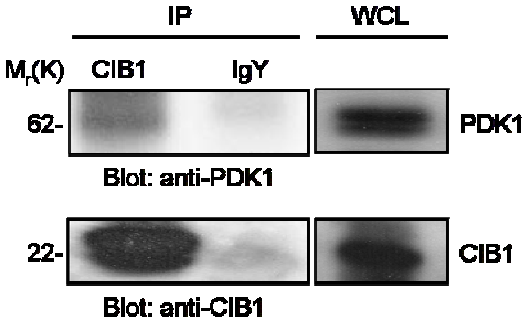
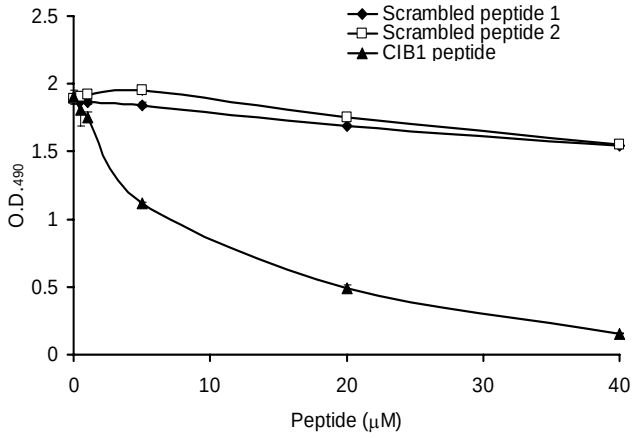


Figure 3-3 CIB1 interacts with PDK1 in cells. (A) Lysates from COS-7 cells expressing myc-PDK1 and CIB1 either alone or in combination were immunoprecipitated (IP) with antibodies to CIB1 (top) or myc (bottom) and the precipitates were immunoblotted with antibodies to myc or CIB1. Whole cell lysates (WCL) corresponding to 10% of the input for immunoprecipitation were analyzed by immunoblot. **(B)** Endogenous CIB1 co-immunoprecipitates with endogenous PDK1 in platelets. Platelet lysates were subjected to immunoprecipitation with either a chicken polyclonal anti-CIB1 or control IgY antibody and analyzed with anti-CIB1 and anti-PDK1 antibodies. WCL corresponding to 10% of the input for immunoprecipitation were also analyzed by immunoblot.

A



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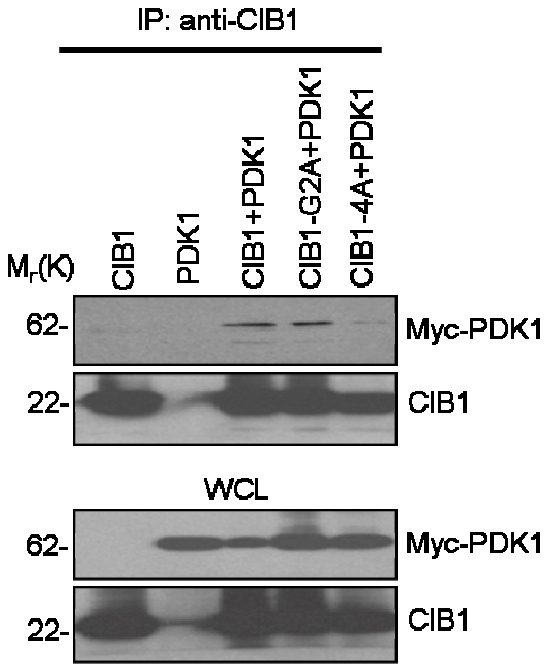


Figure 3-4 Identification of PDK1-binding sites within CIB1. (A) CIB1 binding to immobilized PDK1 was blocked with CIB1 peptides corresponding to the potential PDK1 consensus binding motif. Solid-phase binding assays were performed as in Fig 3-2, except that soluble GST-PDK1 was pre-incubated with WT CIB1 or scrambled CIB1 peptides I and II before adding to CIB1-coated wells. **(B)** PDK1 does not bind efficiently to mutant CIB1 "F112A/RIF115A/D116A/F117A (CIB1-4A)". CIB1 was immunoprecipitated from COS-7 cells transfected with PDK1 or wild-type CIB1 alone, or from COS-7 cells cotransfected with PDK1 and wild-type CIB1 (CIB1+PDK1), CIB1-4A (CIB1-4A+PDK1), or CIB1-G2A (CIB1-G2A+PDK1). The precipitated proteins were detected with anti-PDK1 and anti-CIB1 antibodies as indicated.

We next performed mutational analysis to further confirm whether the FRIFDF hydrophobic motif of CIB1 was required for binding to PDK1. Several residues within the CIB1 hydrophobic motif were mutated to alanines (F112A/RIF115A/D116A/F117A; termed CIB1-4A) and either wild-type CIB1 or CIB1-4A was then cotransfected with myc-PDK1 into HeLa cells. Mutation of several key residues within the CIB1 hydrophobic motif resulted in a significantly decreased co-immunoprecipitation of PDK1 with CIB1. However, additional amino acids may also contribute to the binding of CIB1 and PDK1 since we did not observe a complete loss of the CIB1-PDK1 interaction (Fig. 3-4 B).

Previous studies showed that CIB1 is myristoylated and membrane bound (Stabler et al., 1999); we therefore generated another CIB1 construct that will become relevant to our later subcellular localization study described in Chapter IV, a non-myristoylated mutant of CIB1 (CIB1-G2A), which lacks the N-terminal myristoylated glycine, and tested its ability to bind PDK1. Fig. 3-4 B shows that this mutant still binds PDK1 with the same capacity as wild-type CIB1.

3) CIB1 Stimulates PDK1 Kinase Activity

PDK1 function appears to be tightly controlled by its interacting proteins and subcellular distribution (Review in (Toker and Newton, 2000). Instead of being constitutively active as previously thought, PDK1 kinase activity was demonstrated to be regulated by associating proteins or peptides, such as PKC-related kinase-1/2 (PRK1/PRK2) (Wick et al., 2000b), PDK1 interacting fragment of PRK2 (Balendran et al., 1999a), RSK2 (Frodin

et al., 2000), Hsp90 (Fujita et al., 2002), and 14-3-3 (Sato et al., 2002a). Therefore, we asked whether CIB1 affects PDK1 kinase activity. Purified recombinant PDK1 alone exhibited basal autophosphorylation, and kinase activity. However, increasing concentrations of purified recombinant CIB1 significantly increased PDK1 autophosphorylation (Fig. 3-5 A) and kinase activity (Fig. 3-5 B) above basal levels, indicating that CIB1 directly stimulates PDK1 autophosphorylation and kinase activity. SGK was used as the substrate for the PDK1 kinase assay with SGK phospho-specific antibodies recognizing the T-loop PDK1 phosphorylation site (Ser255/Thr256) (Prasad et al., 2000).

We next asked whether CIB1 can stimulate PDK1 kinase activity in cells. HeLa cells were transfected with control cDNA or CIB1 cDNA. Endogenous PDK1 activity was determined by immune complex kinase assays using SGK as the substrate. Consistent with previous reports (Casamayor et al., 1999), vector-transfected cells exhibited basal levels of PDK1 activity. However, CIB1-transfected cells showed increased PDK1 kinase activity that was further stimulated with increased CIB1 expression levels (Fig. 3-5 C), suggesting that CIB1 can stimulate PDK1 activity in cells.

4. Conclusion and discussion:

In this section, we have identified a PDK1 binding protein, CIB1. We demonstrated their binding both *in vitro* and in cells. We characterized the binding motif of PDK1 within CIB1 and also showed that this binding stimulates PDK1 activity towards its substrates.

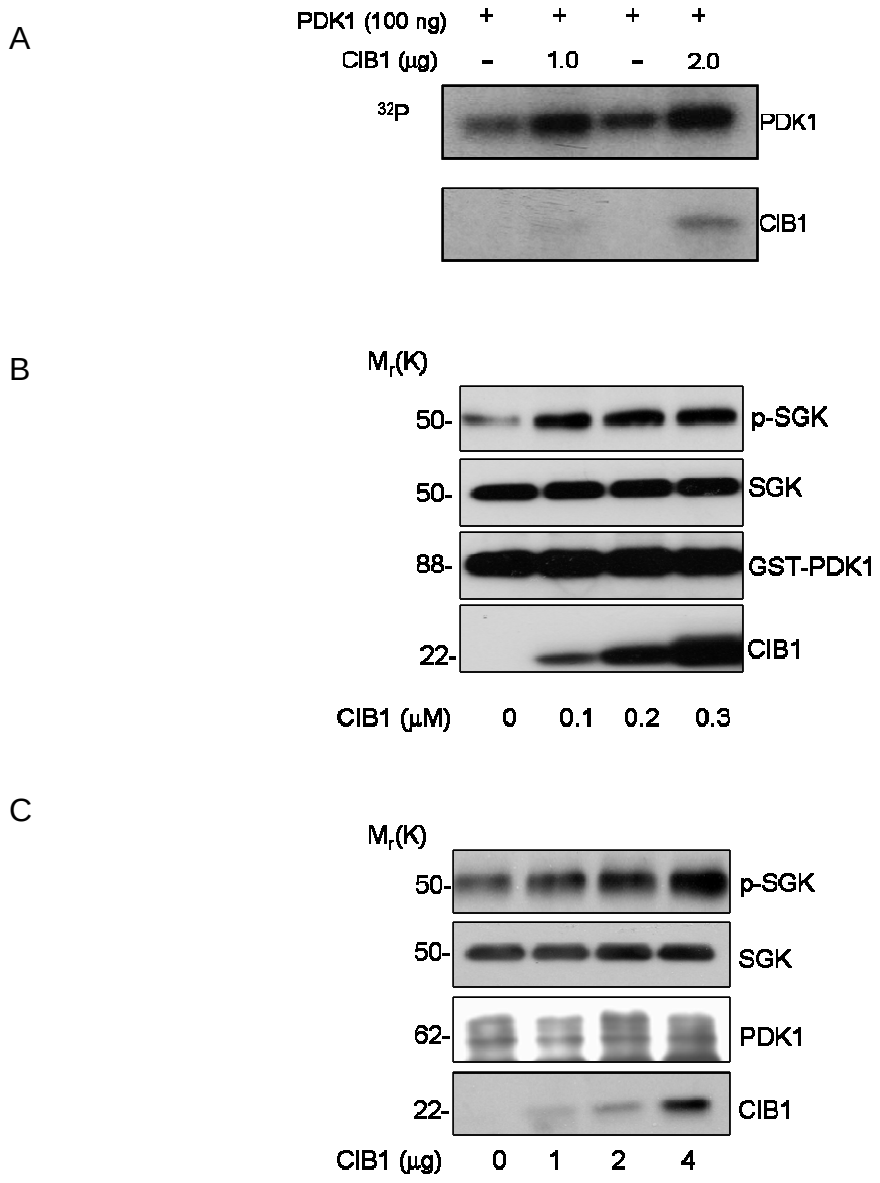
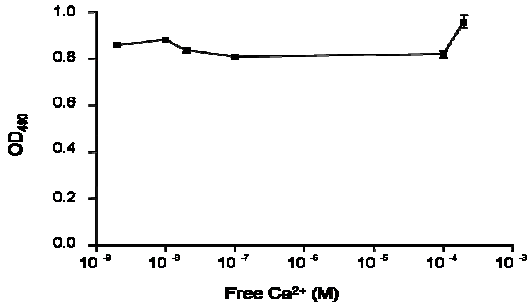


Figure 3-5 CIB1 activates PDK1 kinase activity *in vitro* and in cells. (A) Purified CIB1 increases PDK1 autophosphorylation. Purified PDK1 was incubated with ^{32}P-ATP in the absence or presence of purified CIB1 at 30°C for 20 min. Reactions were stopped and products were separated on SDS-PAGE and subjected to autoradiography. **(B)** Purified CIB1 increases PDK1 kinase activity. GST-PDK1 and inactive SGK substrate were incubated with increasing concentrations of recombinant CIB1. Reactions were stopped and products were separated on SDS-PAGE and probed for phospho-Ser255/Thr256-SGK (P-SGK), SGK, PDK1, and CIB1. **(C)** CIB1 increases PDK1 kinase activity in cells. Endogenous PDK1 was immunoprecipitated from lysates prepared from vector- and CIB1-transfected HeLa cells. Inactive SGK was added as a specific PDK1 substrate. P-SGK, SGK, immunoprecipitated PDK1, and CIB1 were detected by Western blotting with respective antibodies.

CIB1, a widely expressed calcium-binding protein homologous to calmodulin, interacts with a variety of serine/threonine and tyrosine kinases, suggesting that CIB1 may play an important role in regulating kinase function. In support of this, high stringency *in silico* analysis of the CIB1 protein sequence predicted a consensus PDK1 hydrophobic binding motif (FxxF[D/E/S][Y/F]) between residues 112 and 117 of CIB1 (FRIFDF). This binding motif contains a negatively charged glutamic acid residue, which is preferred by PDK1. This hydrophobic motif is present within many PDK1 substrates such as Akt, PKC isozymes, PRK and RSK (Biondi et al., 2000) and non-substrate binding proteins such as Grb14 (King and Newton, 2004) and mAKAPα (Michel et al., 2005). Mutation of the CIB1 glutamic acid residue 116 together with the flanking phenylalanine residues to alanines dramatically decreased the binding between CIB1 and PDK1. With protein-protein interaction emerging as a key regulatory mechanism of PDK1, we provide another example of PDK1 binding to a non-substrate regulatory protein, CIB1, via a PDK1 consensus hydrophobic binding motif that may significantly modulate PDK1 function.

CIB1 contains two Ca^{2+}-binding EF hands that appear to be important for the Ca^{2+}-dependent binding of CIB1 to PAK1 (Leisner et al., 2005). In contrast, the binding of CIB1 to PDK1 was found to be Ca^{2+}-independent, since chelation of free Ca^{2+} by incubation with increasing concentrations of EDTA did not significantly reduce the interaction of CIB1 and PDK1 (Fig. 3-6 A). Fluorescence binding assays also show a slight decrease in the interaction between CIB1 and PDK1 in the presence of Ca^{2+} (Fig. 3-6 B and C), which may be due to the conformational change of the dye itself in the

A



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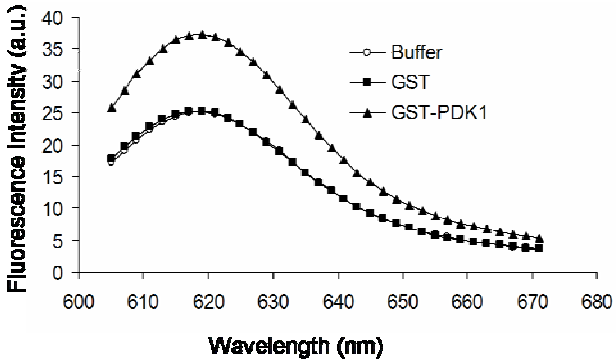

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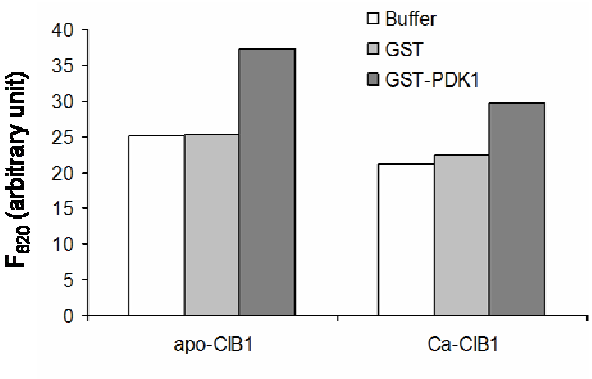


Figure 3-6. CIB1 and PDK1 interaction is Ca^{2+}-independent. (A) Purified GST-PDK1 was incubated in buffer containing 0-50 mM EGTA for 30 minutes before addition to immobilized CIB1. Binding of soluble PDK1 to immobilized proteins was detected using anti-PDK1 antibodie. Approximate free Ca^{2+} concentrations were calculated using the MaxChelator program (Bers et al., 1994). **(B)** Fluorescence binding assay. GST-PDK1 (500 nM), GST (500 nM), or control buffer was incubated with 1 mM EDTA to chelate residual Ca^{2+}. Environment-sensitive fluorescent dye covalently bound to CIB1-cystine134 (100 nM) was then added and incubated at 37°C for 5 minutes. Fluorescent intensity (tens of thousands of counts per second) was obtained as the binding capacity between GST-PDK1 and CIB1. Samples were excited at 595 nm and fluorescence emission was collected from 605 nm to 680 nm. **(C)** GST-PDK1 (500 nM), GST (500 nM), or control buffer was incubated with 1 mM EDTA to chelate residual Ca^{2+} (apo-CIB1), or excess Ca^{2+} (5 mM) was added in the presence of 1 mM EDTA (Ca-CIB1). Dye was added and fluorescent intensity was obtained as described in B. Fluorescence emission maxima (tens of thousands of counts per second) at 620 nm (F_{620}) is plotted vs. different groups.

presence of Ca^{2+} (personal communication with Hahn). This unexpected finding may be due to the fact that the PDK1 binding site within CIB1 (amino acid 112-117) overlaps the Ca^{2+}-binding loop of the third EF hand of CIB1 (amino acid 116-124) (Gentry et al., 2005), which may block PDK1 binding.

The mechanism by which CIB1 activates PDK1 is unclear. Several reports indicate that the PH domain of PDK1 might sterically block access to the kinase domain and act as a negative regulator for PDK1 autophosphorylation and catalytic activity (Filippa et al., 2000;Wick et al., 2000a;Gao and Harris, 2006). CIB1 binding to the PH domain of PDK1 may result in a conformational change in PDK1 that releases the autoinhibitory function of the PH domain. However, our coimmunoprecipitation data do not support this hypothesis since CIB1 still binds the ΔPH-PDK1 mutant even more efficiently than wild-type PDK1 (Fig. 3-7).

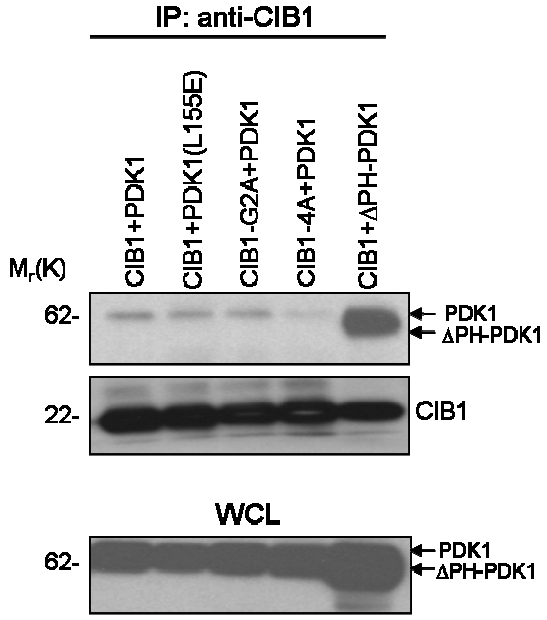


Figure 3-7 Identification of CIB1-binding sites within PDK1. CIB1 was immunoprecipitated from COS-7 cells co-transfected with respective CIB1 and myc tagged PDK1 constructs: CIB1+PDK1, CIB1+PDK1 (L155E), CIB1-G2A+PDK1, CIB1-4A+PDK1, and CIB1+DPH-PDK1. The precipitated proteins were detected with anti-myc (upper panel) and anti-CIB1 (middle panel) antibodies as indicated. Bottom panel shows the various PDK1 constructs expression in the whole cell lysate.

An alternative mechanism by which CIB1 may activate PDK1 is by binding to the hydrophobic pocket of PDK1 (PIF-binding pocket) within the PDK1 catalytic domain. The PIF-binding pocket is an important binding site for some PDK1 substrates such as S6K and occupation of the PIF-binding pocket increases PDK1 autophosphorylation and kinase activity (Engel et al., 2006;Biondi et al., 2000;Frodin et al., 2000). Mutation of a critical leucine residue (PDK1-L155E) within the PIF-binding pocket markedly decreases the binding of S6K and SGK to PDK1 (Biondi et al., 2001). We tested whether CIB1 also binds the PIF-binding pocket and thereby activates PDK1, but we found that PDK1-

L155E co-immunoprecipitated with CIB1 at similar levels to wild-type PDK1 (Fig. 3-7), suggesting that CIB1 binds to different sites than S6K and SGK to PDK1. In addition to Leucine155, several other residues including Lys115, Ile119, Gln150, and Ala277 were also predicted to make up part of the hydrophobic pocket of PDK1 (Biondi et al., 2001). Besides, a weak interaction can still be detected between SGK1 and PDK1-L155E, indicating that SGK may significantly interact with additional sites within PDK1 other than the PIF-binding pocket (Biondi et al., 2001). This means that CIB1 may still interact with the PIF pocket of PDK1 by binding to residues distinct from those required by S6K or SGK. If CIB1 does occupy the PIF pocket, this binding energy may initiate an allosteric transition that stabilizes the functionally active conformation of PDK1.

In addition, since PDK1 is also negatively regulated by some of its binding proteins such as the adaptor protein 14-3-3, another possibility by which CIB1 may activate PDK1 is by competition with 14-3-3 for binding to PDK1, thus removing 14-3-3 from the PDK1/14-3-3 complex. This possibility remains to be further tested.

5. Methods and materials

Plasmid constructs

Plasmid cDNAs pcDNA3.1-CIB1 and pGEX-2T-CIB1 were generated in a previous study (Leisner et al., 2005). Constructs were kindly provided as follows: pCMV5-myc-hyman PDK1 (Dr. Gary Bokoch), pGEX-2T-PDK1 (Dr. John Blenis), pEBG2T-GST-PDK1 (Dr. Dario Alessi). The pcDNA3.1-CIB1 112A/RIF115A/D116A/F117A (CIB1-4A) was generated by site-directed mutagenesis per manufacturer's instructions

(Stratagene, Site-directed mutagenesis kit) and confirmed by DNA sequencing. To make lentivirus particles expressing PDK1 and CIB1, myc-PDK1 and CIB1 cDNAs were amplified by PCR and subcloned into FG12 lentiviral vector between the *Xba*I and *Xho*I sites (Qin et al., 2003). The double-cassette FG12 expression vectors express green florescent protein (GFP) via a separate promoter. All the constructed plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

Cell culture, antibodies, reagents, and transfection

Cells were cultured in DMEM-F12 (PC-3 cells) or DMEM medium (HEK-293 T, Cos-7, HeLa and NIH 3T3 cells) supplemented with 10% fetal bovine serum (Sigma). Cells were transfected by Fugene 6 (Roche) according to the manufacturer's protocol. Antibodies and reagents were purchased as follows: rabbit anti-PDK1 antibody (Ab) (Cell Signaling); goat anti-PDK1 Ab, rabbit anti-SGK Ab, and rabbit anti-phospho-SGK Ab (Upstate Biotechnology); monoclonal anti-myc (9E10) Ab, polyclonal anti-myc (A-14) Ab, and goat anti-actin Ab (Santa Cruz). Both monoclonal and polyclonal antibodies to CIB1 were generated in a previous study (Leisner et al., 2005). HRP-conjugated (Amersham) and Alexa-conjugated (Molecular Probes) secondary antibodies were used. Recombinant human SGK1 (Δ1-59, S422D, unactive) was purchased from Upstate. All other reagents were from Sigma.

Expression and purification of proteins

Glutathione *S*-transferase (GST) fusion proteins were expressed in bacteria and purified on a gtathione Spharose (Amersham) or Ni^{2+} column (Qiagen) as recommended by the

manufacturers. Briefly, GST-CIB1 and GST-PDK1 fusion proteins were generated as follows: pGEX2T1-CIB1 and pGEX2T1-PDK1 plasmid cDNAs were transformed into *Escherichia coli* BL21 cells and expression of proteins was induced in cultured cells using 1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG) at 30 °C for 4 hours for GST-CIB1 and at 23°C for 16 hours for GST-PDK1, respectively. Cells were pelleted and lysed using Novogen's Bugbuster containing 1 mg/ml lysozyme and 1 μl/ml benzonase, and fusion proteins were isolated from the supernatant using gutathione-Sepharose beads. GST beads were washed in buffer (20 mM Tris/HCl, pH7.4, 150 mM NaCl, 2 mM DTT, 0.5% TritonX-100 with protease inhibitor) twice, once in buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% TritonX-100 with protease inhibitor), once in buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) and then resuspended in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 25% glycerol. GST-PDK1 was eluted from the resin at RT in buffer (10 mM reduced glutathione, 20 mM Tris/HCl, pH 8.0, 150 mM NaCl). Part of the GST fusion proteins were cleaved from the beads by incubating with 10 units of thrombin at 23°C overnight. Protein concentration was estimated by BCA and purified protein was resolved using SDS-PAGE and immunoblotting (See co-immunoprecipitation and immunoblotting section).

GST-PDK1 fusion protein was also generated from a mammalian cell system [described previously (Alessi et al., 1997)]. Briefly, twenty 10 cm diameter dishes of HEK-293 cells were transiently transfected with 10 μg of the pEBG-2T-PDK1 plasmid DNA and the cells were lysed 48h post-transfection in 1ml of ice-cold lysis buffer A (50 mM Tris/HCl pH7.5, 1 mM EDTA, 1 mM EGTA, 1% (by volume) Triton X-100, 1 mM sodium

orthovanadate, 10 mM sodium beta-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM PMSF, 10 ug/ml leupeptin and 0.1% (by volume) 2-mercaptoethanol). The lysates were centrifuged at 13000g for 10 min at 4°C, and the supernatants were pooled and incubated for 1 hour on a rotating platform with 800 μl of glutathione-Sepharose equilibrated previously in lysis buffer. The beads were washed three times with lysis buffer A containing 0.5M NaCl, three times with buffer B (50 mM Tris/HCl pH7.5, 0.1 mM EGTA, 0.03% (volume) Brij-35, 0.27 M sucrose and 0.1% (volume) 2-mercaptoethanol), and then GST-PDK1 was eluted from the resin at RT with three 1 ml portions of Buffer B containing 20 mM glutathione pH 8.0. About 0.5 mg GST-PDK1 protein can be obtained, more than 90% homogenous, as judged by SDS-PAGE and commassie staining. Eluted GST-PDK1 was then dialyzed against buffer C (50 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol) at 4°C overnight and then aliquoted and snap frozen at -80°C.

Recombinant His-CIB1 proteins were expressed in bacteria and purified on a Ni^{2+} column (Qiagen) as recommended by the manufacturers. Briefly, pQE-His-CIB1 plasmid cDNA was transformed into *Escherichia coli* BL21 cells and expression of proteins was induced in cultured cells using 1 mM IPTG at 30 °C for 4 hours. Cells were pelleted and lysed in buffer 1 (20 ml Bugbuster (Novagen), 20 mM imidazole pH 8.0, 1 mg/ml lysozyme, and1 μl/ml benzonase). The lysates were centrifuged at 13000g for 10 min at 4°C, and the supernatants were pooled and incubated for 1 hour on a rotating platform with Ni-NTA columns equilibrated previously in lysis buffer. The Ni beads were washed once with buffer 2 (50 mM NaH_2PO_4, 200 mM NaCl, 30 mM imidazole,

0.5% TX-100), once with Buffer 3 (50 mM NaH_2PO_4, 200 mM NaCl, 30 mM imidazole), and once with Buffer 4 (50 mM NaH_2PO_4, 200 mM NaCl, 40 mM imidazole). His-CIB1 proteins were eluted by Elution Buffer (50 mM NaH_2PO_4, 200 mM NaCl, 250 mM imidizole, pH 8.0, 10 % glycerol) and dialyzed overnight in 50 mM Hepes/150mM NaCl/5% glycerol. His tag was then cleaved off from CIB1 by His-TEV.

In vitro binding assay

Microtiter wells were coated with and without 5 μg/ml CIB1 or PDK1 overnight at 4°C, and were blocked with 3% BSA. Increasing amounts of PDK1 or CIB1 protein were added and incubated for 1 hour at RT. For peptide competition binding studies, soluble GST-PDK1 was pre-incubated with wild-type CIB1 peptide or scrambled CIB1 peptides I and II for 30 minutes at RT before adding to CIB1-coated wells. For Ca^{2+}-dependent binding studies, GST-PDK1 was diluted in buffer containing 0.2 mM Ca^{2+} with increasing concentrations of EGTA (0–50 mM) before addition to CIB1-coated wells. In all cases, wells were washed with TBST (0.1% Tween 20), and binding was detected with either an anti-PDK1 antibody or anti-CIB1 antibody followed by an HRP-conjugated goat anti-rabbit or anti-chicken IgG. The reactions were developed by using o-phenylenediamine (Sigma) as a substrate, and absorbance was measured at 490 nm (OD_{490}).

Co-immunoprecipitation and immunoblotting

Cells were lysed for 30 min at 4 °C in ice-cold lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM CHAPS or 1% Brij35, 50 mM NaF, 0.5 mM Na_3VO_4, and Protease

Inhibitors Cocktail Set III [Calbiochem-Novabiochem]). Lysates were clarified at 13,000g for 10 min. Immunoprecipitations were carried out using the antibodies indicated. After 1 hour or overnight incubation with antibodies at 4 °C, 20 µl of protein G beads (Amersham) or protein Y beads (Aves lab) were added to cell lysates for 1 hour to capture the antibody-antigen complex. The beads were washed once with lysis buffer and three times with immunoprecipitation washing (IPW) buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CHAPS or 0.2% Brij35, 0.5 mM Na_3VO_4) and boiled in Laemmli sample buffer. For immunoblotting, samples were separated on 10% gels, and transferred to an Immobilon-P PVDF membrane (Millipore). Membranes were blocked, incubated with respective primary and secondary antibodies, and visualized by enhanced chemiluminescent detection (Amersham Life Science).

***In vitro* kinase assays and immunoprecipition kinase assays**

For the autophosphorylation assay, about 100 ng of purified PDK1 was incubated with 1 µg or 2 µg of purified CIB1 in reaction buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$). Ten µM ATP and 10 µCi [γ-^{32}P] ATP (4,500 Ci mmol^{-1}, New England Nuclear) were added in 30 µl reaction buffer. After a 20-min incubation at 30 °C, reactions were stopped by adding Laemmli sample buffer and separated by SDS-PAGE. Coomassie blue-stained gels were dried, and samples were visualized by autoradiography. *In vitro* PDK1 kinase activity toward an exogenous substrate, inactive SGK, was assayed similarly except that kinase activity was detected by anti-phospho-SGK antibody. To detect PDK1 kinase activity in cells, equal amounts of endogenous PDK1 was immunoprecipitated from HeLa cells transfected with increasing amount of plasmid

encoding CIB1, washed twice with immunoprecipitation washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 mM Na_3VO_4) and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.25 μM PKI, 1 μM microcystin-LR, 10 mM magnesium acetate and 0.1 mM ATP). The reactions were started by the addition of 30 μl of kinase buffer and inactive SGK as exogenous substrate. The reactions were carried out as above. The samples were immunoblotted with anti-phospho-SGK and anti-SGK antibodies. The activity of PDK1 was measured by SGK phosphorylation.

Chapter IV

CIB1 Protects Cells from Apoptosis via Signaling to PDK1

1. Abstract

Our previous findings showed that CIB1$^{-/-}$ MEFs proliferate at a significantly slower rate than wild-type MEFs, indicating a role for CIB1 in regulating cell growth and survival. Here we identified a new CIB1 binding partner, PDK1, a master kinase for regulating the AGC protein kinase family. The PDK1/AGC kinase signaling pathway is involved in fundamental roles such as survival, proliferation, and growth. We found that CIB1 depletion significantly enhances cell apoptosis in response to a variety of apoptotic stimuli, and this response was partially reduced by PDK1 overexpression. In addition, CIB1 overexpression protects cells from apoptotic stimuli, which is largely dependent on PDK1. Upon cell adhesion to fibronectin, CIB1 and PDK1 translocate and colocalize at the plasma and perinuclear membranes. However, expression of a non-membrane-bound CIB1 mutant markedly redistributes PDK1 from the membrane to the cytosol. In addition, the CIB1 mutant with diminished binding to PDK1 does not co-localize with PDK1, and redistributes PDK1 from the membrane to the cytosol. These two mutants of CIB1 can no longer protect cells from apoptosis. Moreover, depletion of CIB1 disrupts PDK1 downstream signaling to both Akt and RSK3, suggesting a CIB1/PDK1/Akt or RSK3 dependent pathway. In conclusion, our results demonstrate that CIB1 is a critical regulator of cell survival, which appears to require both membrane localization and binding to PDK1.

2. Introduction

We previously found that CIB1$^{-/-}$ mouse embryo fibroblasts (MEFs) proliferate more slowly than CIB1$^{+/+}$ MEFs and CIB1$^{-/-}$ mice demonstrated increased apoptosis in male germ cells (Yuan et al., 2006), suggesting that CIB1 is required for maximal rates of MEF proliferation and may promote cell survival and protect cells from apoptosis by an unknown mechanism. Studies show that PDK1 also promotes cell proliferation (Zeng et al., 2002), whereas inhibition or depletion of PDK1 results in apoptosis (Crowder and Ellis, 2005;Kucab et al., 2005;Zhu et al., 2004a;Flynn et al., 2000). In addition, we have found that both CIB1 and PDK1 are highly expressed in MCF-7 breast cancer and PC3 prostate cancer cell lines (Fig. 4-1)). Taken together, these findings suggest potential roles for CIB1 and PDK1 in cell proliferation and survival. We therefore examined the role of CIB1 in protection from apoptosis, and determined if this effect is mediated through the PDK1 pathway.

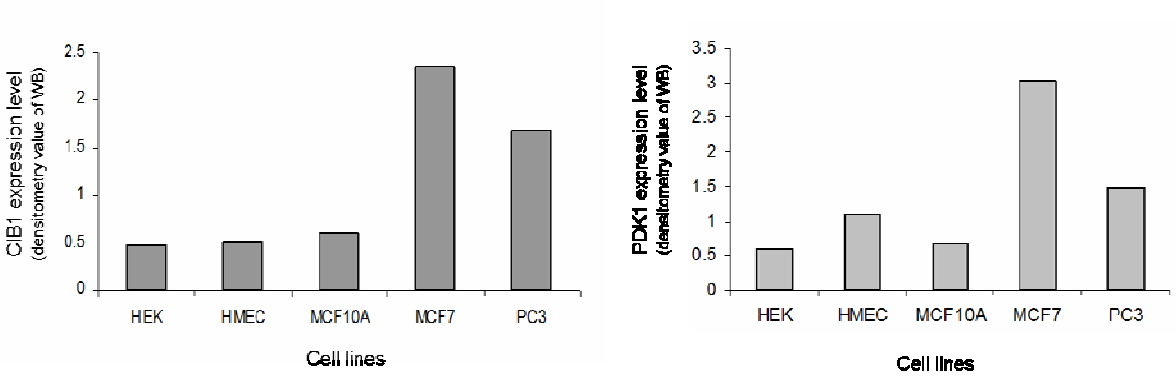


Figure 4-1 CIB1 and PDK1 are highly expressed in MCF-7 and PC3 cancer cell lines. Cell lysates prepared from different cell lines are subjected to Western blot and the densitometry value (CIB1/PDK1 relative to actin) was obtained for each cell line. HEK, human embryonic kidney cell line; HMEC, human mammary epithelial cell line; MCF-10A, human mammary epithelial cell line; MCF-7, human breast adenocarcinoma cell line, and PC3, human prostate cancer cell line.

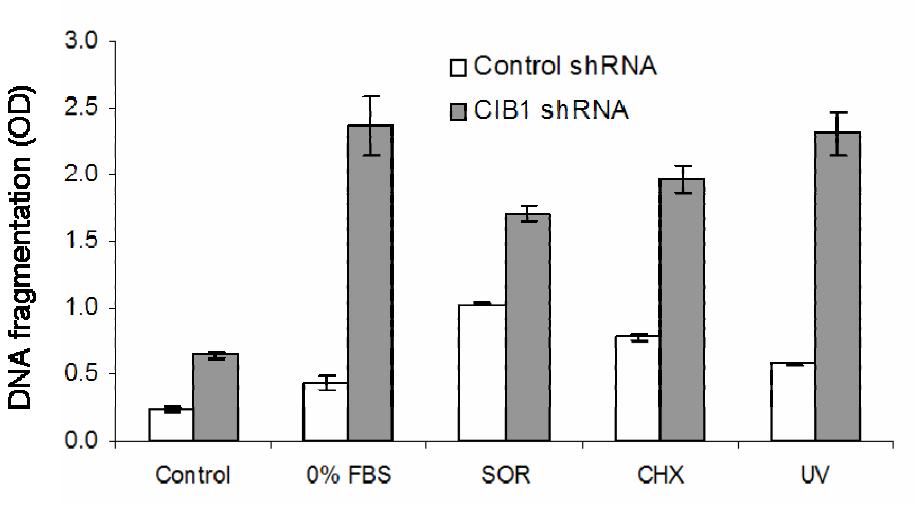
3. Results

1) CIB1 Decreases Cell Sensitivity to Apoptosis

To determine if CIB1 protects cells from apoptosis, we first examined the effect of CIB1 knockdown on apoptosis in human cancer cell lines. Depletion of CIB1 from HeLa cells using lentivirus increased apoptosis as revealed by DNA fragmentation (Fig. 4-2A). Exposure of cells to a variety of apoptotic stimuli further increased cell apoptosis, and CIB1-depleted HeLa cells are significantly more susceptible to apoptosis induced by serum withdrawal, the high osmotic pressure solute sorbitol (SOR), the protein synthesis inhibitor cycloheximide (CHX), and ultraviolet (UV) irradiation (Fig. 4-2A). Knocking down CIB1 in PC3 cells using lentivirus gave the similar results (data not shown).

Analysis of the cleavage of PARP, a key mediator of apoptosis, showed that there is a marked increase of PARP cleavage in CIB1-depleted cells compared to control cells either uninduced or induced by serum withdrawal, sorbitol (SOR), and cycloheximide (CHX) (Fig. 4-2B). Taken together, these results suggested a potential role for CIB1 in anti-apoptotic signaling events, and loss of CIB1 increases cell sensitivity to apoptosis.

A



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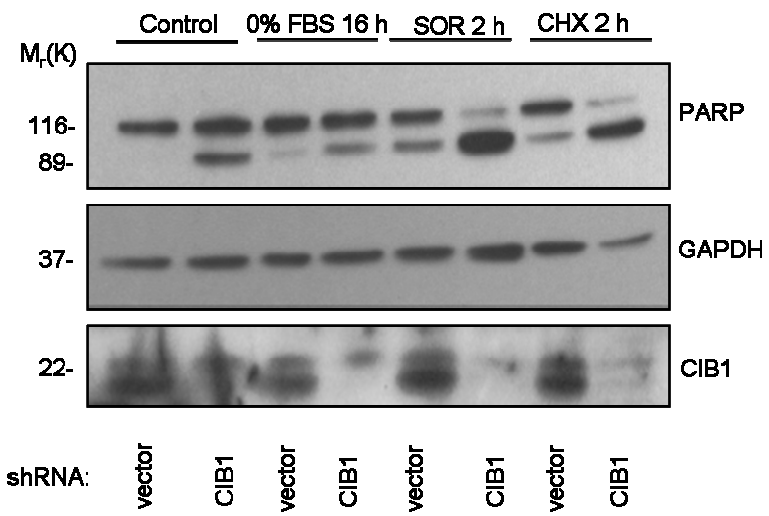


Figure 4-2 Knockdown of CIB1 increases cell sensitivity to apoptotic stimuli. (A) Depletion of CIB1 via shRNA sensitizes cells to apoptotic stimuli. HeLa cells transduced with lentivirus expressing either CIB1 shRNA or control shRNA were left untreated, treated with 0.2 M sorbitol (SOR), 5 μg/ml cyclohexamide (CHX), or UV irradiation (120 J/m^2), and apoptosis was quantitated 24 h following treatment. Apoptosis induced by serum starve (0% FBS) was quantitated 48 h after serum deprivation. Apoptosis was measured by quantitation of DNA fragmentation using the cell death detection ELISA method (see Materials and Methods). Results are represented as the mean ± SEM of 3 independent experiments. **(B)** HeLa cells transduced with lentivirus expressing either CIB1 shRNA or control shRNA were left untreated, treated with serum deprivation (0% FBS) for 16 h, 0.2 M sorbitol (SOR) or 5 μg/ml cyclohexamide (CHX) for 2 h. Whole cell extracts were prepared and subjected to Western blot analysis using anti-PARP, anti-GAPDH, and anti-CIB1 antibodies. Data represent three independent experiments.

The above results show that depletion of CIB1 increases cell sensitivity to apoptotic stimuli. To test whether overexpression of CIB1 reduces cell sensitivity to apoptotic stimuli, we transduced NIH3T3 fibroblasts, which express very low levels of endogenous CIB1 (data not shown), with lentivirus bearing either CIB1 or empty vector. We found that cells transduced with CIB1 showed decreased sensitivity to both sorbitol and cycloheximide as compared to control cells transduced with empty vector (Fig. 4-3). Thus, these results indicate that CIB1 overexpression decreases cell sensitivity to apoptosis and that CIB1 may function to protect cells from a variety of apoptotic stimuli. Transduction efficiency for both knockdown and overepression was greater than 95% (Fig. 4-4).

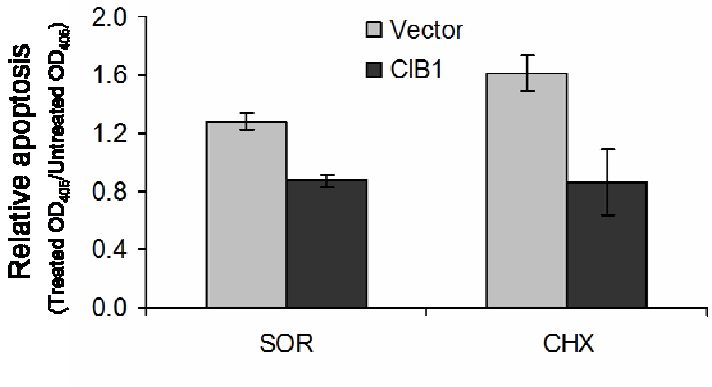
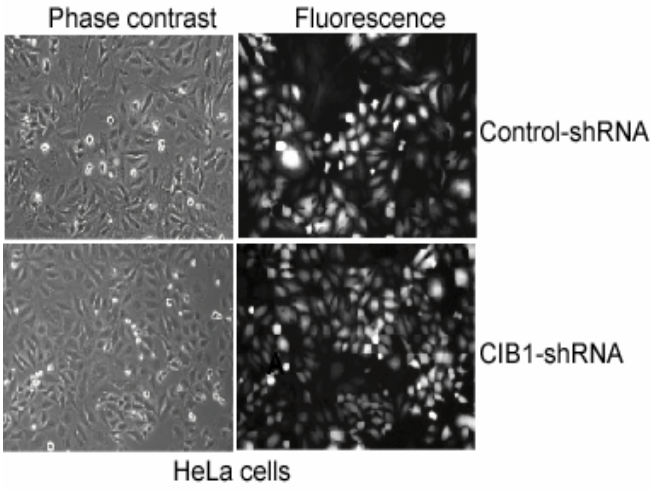


Figure 4-3 CIB1 protects cells from apoptotic stimuli. Overexpression of CIB1 inhibits apoptosis triggered by various stimuli. NIH 3T3 cells were transduced with lentivirus expressing either CIB1 cDNA or control cDNA. Forty hours post-transduction, cells were treated with 0.2 M sorbitol (SOR) or 5 μg/ml cyclohexamide (CHX). Results are expressed as the ratio of treated (OD405) to untreated (OD405) cells and represented as the mean ± SEM of 3 independent experiments.

A



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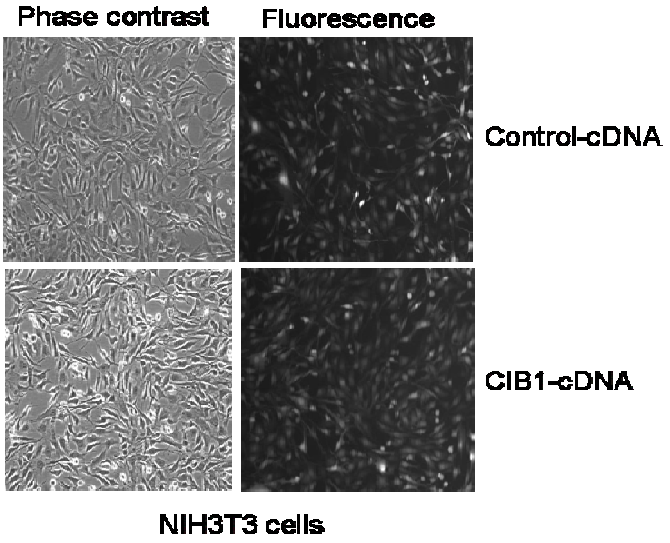


Figure 4-4 Transduction efficiency of overexpression or knockdown of CIB1 via lentivirus. (A) HeLa cells at 50% confluency were transduced with lentivirus expressing either control shRNA or CIB1 shRNA with GFP exrpession via a separate promoter. Transduction efficiency assessed by GFP expression is greater than 95% after 48-72 hours. The left panel shows the phase contrast images of cells and the right panel shows the fluorescence images of cells. **(B)** NIH3T3 cells at 30% confluency were transduced with lentivirus overexpressing either control cDNA or CIB1 cDNA with GFP expressed via a separate promoter. After 48 to 72 hours, GFP expression was detected in greater than 95% of the cells. The left panel shows the phase contrast images of cells and the right panel shows the fluorescence images of cells.

2) Depletion of CIB1 Decreases Cell Proliferation

Consistent with our previous observation that *Cib1*$^{-/-}$ mouse embryo fibroblasts (MEFs) proliferate at a decreased rate, we also found that HeLa cells depleted of CIB1 via lentivirus expressing shRNA have decreased cell proliferation during a 4-day period compared to HeLa cells induced with lentivirus expressing shRNA vector alone (Fig. 4-5).

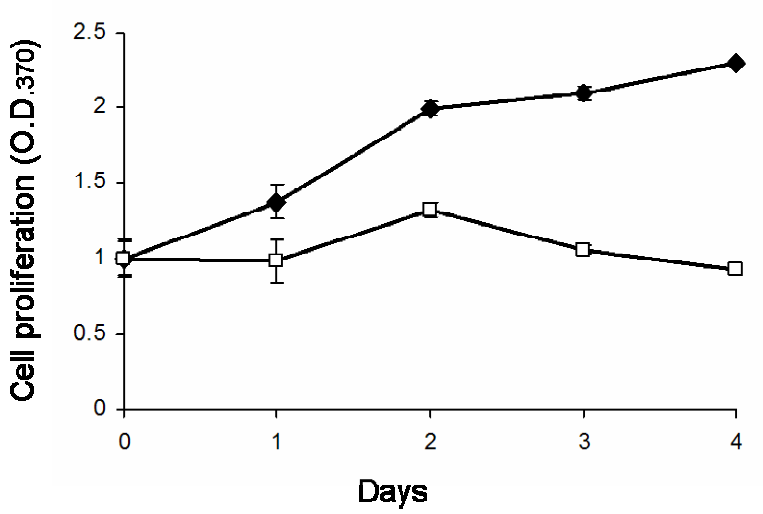


Figure 4-5 Depletion of CIB1 decreases cell proliferation rate. HeLa cells were depleted of CIB1 via lentivirus expressing shRNA, and cell proliferation rate was analyzed using BrdU proliferation assay during a 4-day period compared to HeLa cells induced with lentivirus expressing vector alone. Solid diamond represents HeLa cells infected with vector control, and open square represents HeLa cells infected with shRNA-CIB1.

3) CIB1 Does Not Cause Cell Morphological Transformation

Since depletion of CIB1 increases cell sensitivity to apopototic stimuli and decreases cell proliferation, we next tested if overexpression of CIB1 has transforming activity towards epithelial cells and fibroblasts. Using H-Ras as a positive control, which has strong transforming activity (Fig. 4-6 right panel), we did not see dramatic cell morphological transformation by retrovirus overexpression of CIB1 towards either COMMA-1D mouse mammary epithelial cells or NIH3T3 fibroblasts (Fig. 4-6 middle panel).

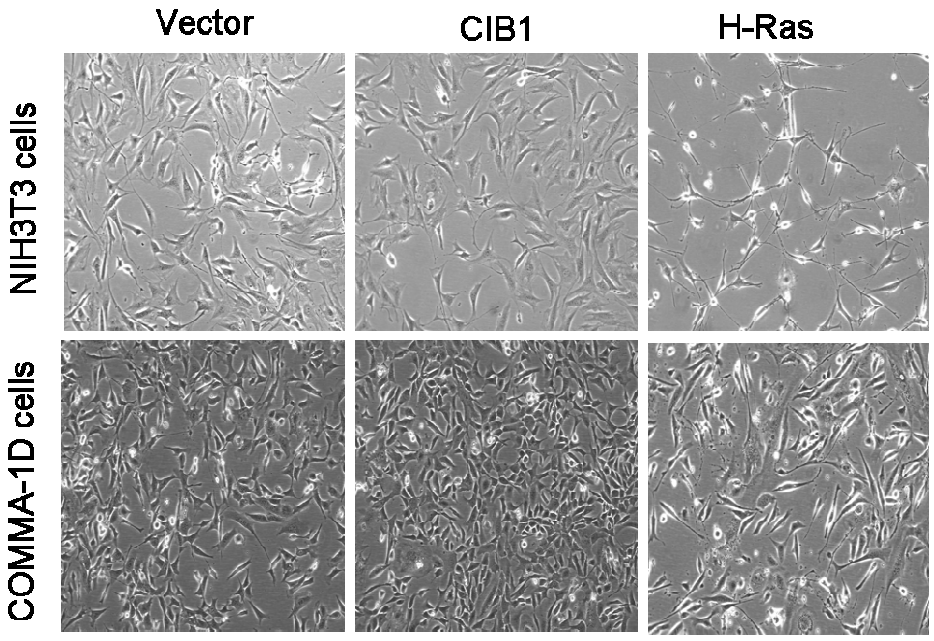


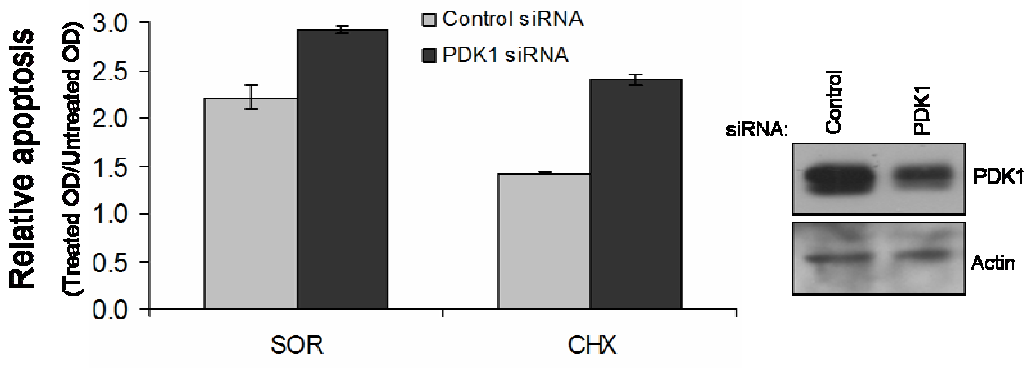
Figure 4-6 CIB1 does not induce cell morphological transformation in either fibroblasts or epithelial cells. NIH3T3 cells or COMMA-1D mouse mammary epithelial cells were transduced by retrovirus expressing vector, CIB1, or H-Ras, and phase contrast pictures were taken after 3 days of infection. H-Ras was used as positive cell morphological transformation control. Expression of CIB1 proteins was verified by immunoblot.

4) CIB1 Protects Cells from Apoptosis via Signaling to PDK1

As a first step in determining whether CIB1 protects cells from apoptosis via PDK1, we examined whether PDK1 depletion affected apoptosis under our conditions. PC3 cells were transfected with either control or PDK1 siRNA and treated with sorbitol or staurosporine to induce apoptosis. Consistent with previous reports that PDK1 depletion (Liang et al., 2006;Zhang et al., 2006) or inhibition (Crowder and Ellis, 2005;Kucab et al., 2005;Zhu et al., 2004a) increased either cell death or sensitivity to anti-cancer agents, we also found that PDK1 depletion increased cell sensitivity to both sorbitol and cycloheximide (Fig. 4-7 A).

Since the loss of either CIB1 or PDK1 increased apoptosis in response to apoptotic stimuli, we next asked whether PDK1 acts as a downstream effector in CIB1-mediated cell survival. PDK1 was overexpressed in CIB1-depleted HeLa cells, and cells were subjected to a wide range of apoptotic stimuli. As shown in Figure 4-7B, PDK1 overexpression in CIB1-depleted cells significantly decreased the number of apoptotic cells induced by serum withdrawal (0% FBS), sorbitol (SOR), cycloheximide (CHX), or ultraviolet (UV) irradiation, compared to CIB1-depleted cells expressing a control vector, suggesting that PDK1 overexpression can rescue CIB1 depletion-induced cell sensitivity to apoptosis. Overexpression and depletion of proteins was confirmed by Western blot (Fig. 4-7).

A



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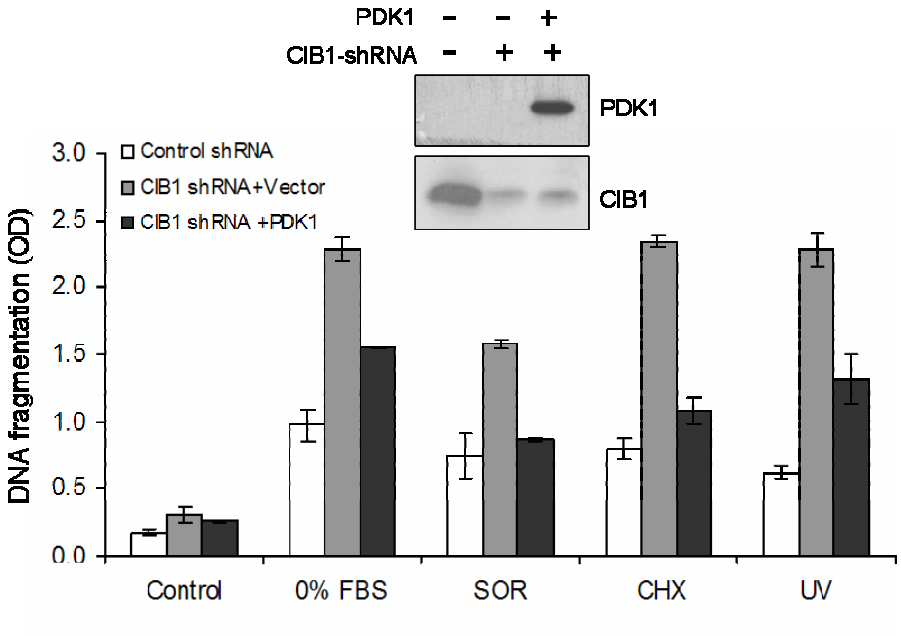

Figure 4-7 PDK1 rescues apoptosis from CIB1-depleted cells exposed to apoptotic stimuli. (A) Depletion of PDK1 increases cell sensitivity to apoptotic stimuli. PC3 cells transfected with PDK1 or control siRNA were treated with sorbitol (SOR, 0.2M) or 5μg/ml cyclohexamide (CHX) for 15 hours. Apoptotic cells were identified by ELISA and analyzed as described in Fig. 4-3. **(B)** HeLa cells were transduced with lentivirus expressing either CIB1 shRNA or control shRNA, and sequentially infected with lentivirus expressing PDK1-cDNA or vector-cDNA after 24 h. Cells were left untreated, treated with 0.2 M sorbitol (SOR), 5 μg/ml cycloheximide (CHX), UV irradiation (120 J/m2) and apoptosis was quantitated 24 h following treatment. Apoptosis induced by serum starve (0% FBS) was quantitated 48 h after serum deprivation. Apoptosis was measured by quantitation of DNA fragmentation using the cell death detection ELISA method. Western blot confirms 90% of CIB1 knockdown and PDK1 overexpression. Data represent means mean ± SEM of 2 independent experiments.

To confirm whether downstream signaling to PDK1 is required for CIB1-mediated cell survival, CIB1 was overexpressed in either PDK1-depleted or control PC3 cells. As shown in Fig. 4-8, in the absence of PDK1, overexpression of CIB1 did not significantly rescue cell apoptosis. These results indicate that PDK1 may not only act as a downstream effector of CIB1 but also appears to be required for CIB1 promoting cell survival. Overexpression and depletion of proteins was confirmed by Western blot (Fig. 4-8).

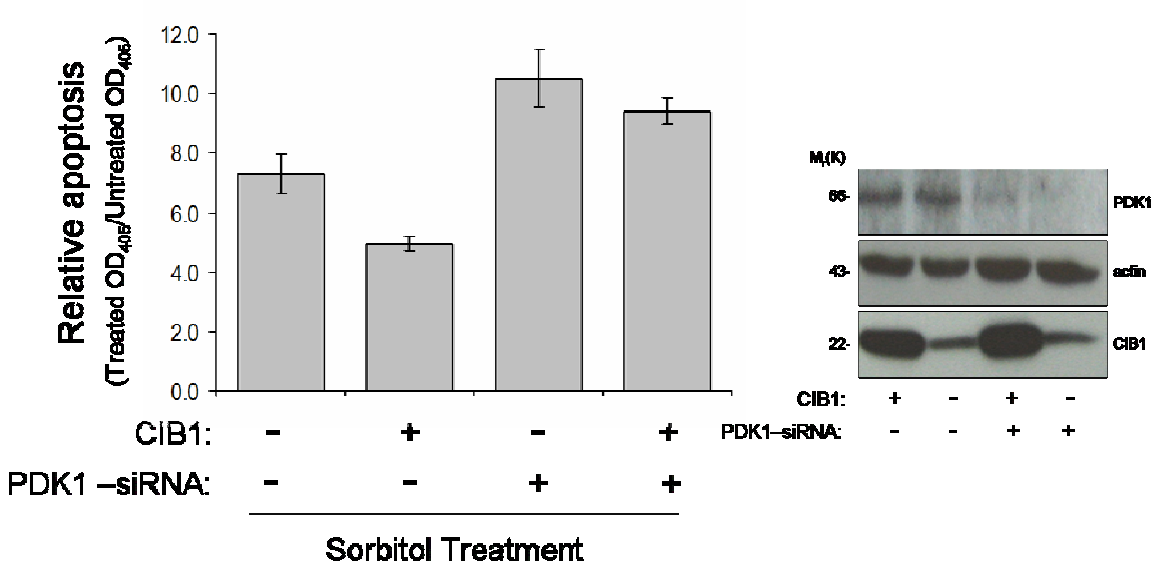

Figure 4-8 CIB1 protects cells from apoptotic stimuli in a PDK1-dependent manner. Overexpression of CIB1 in PDK1-depleted cells fails to protect cells from apoptosis induced by sorbitol. PC3 cells were transduced with lentivirus overexpressing either CIB1 cDNA (+) or control cDNA (-), and sequentially transfected with PDK1-siRNA (+) or control-siRNA (-) after 24 hours. Cells were treated and analyzed as in Fig. 4-3.

To further evaluate the role of PDK1 in CIB1-mediated cell survival, we tested the ability of the CIB1-4A mutant that does not bind PDK1 to protect cells from apoptosis. Overexpression of wild-type CIB1, but not the CIB1-4A mutant protected cells from apoptosis induced by sorbitol and cycloheximide (Fig. 4-9 A). Interestingly, like the CIB1-4A mutant, overexpression of a non-myristoylated CIB1-G2A mutant only partially protected cells from these apoptotic stimuli (Fig. 4-9 A). These results indicate that CIB1 requires not only PDK1, but also membrane association in order to protect cells from apoptosis. To ensure that these results are not due to differences in protein expression, we confirmed equal expression levels of wild-type and mutant CIB1 proteins by Western blotting (Fig. 4-9 B). Taken together, these results indicate that CIB1-mediated PDK1 signaling plays an important role in protecting cells from apoptosis.

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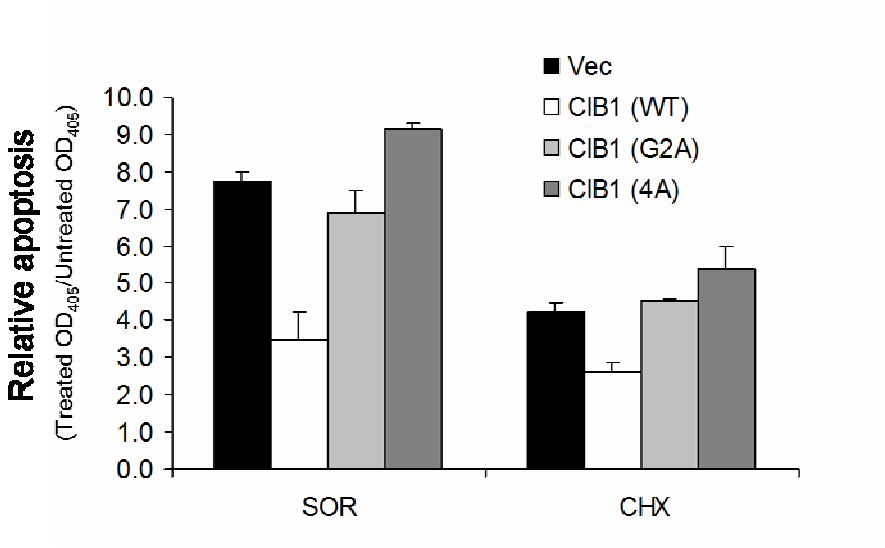

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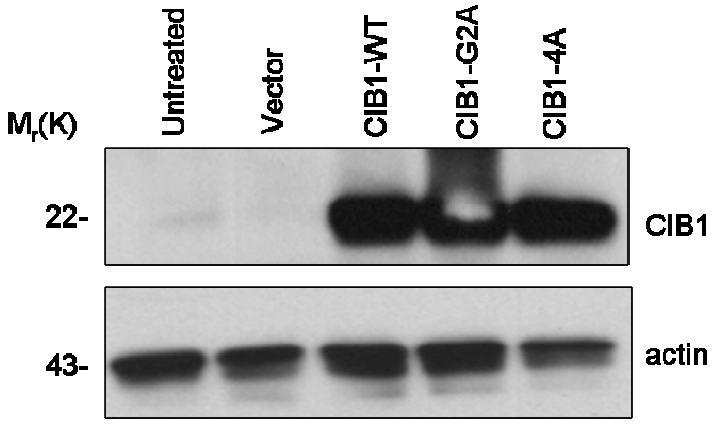

Figure 4-9 CIB1 must be myristoylated and capable of binding PDK1 to protect cells from apoptosis. (A) NIH 3T3 cells were transduced with lentivirus expressing either empty vector (Vector), wild-type CIB1 (CIB1-WT), CIB1-4A, or CIB1-G2A. After 40 hour of transduction, cells were treated and apoptosis detected as in Fig. 4-3. **(B)** Western blot shows equal expression of CIB1 constructs in cells.

5) CIB1 Regulates PDK1 Cellular Localization upon Adhesion to Fibronectin

Direct protein-protein interactions not only contribute to the regulation of PDK1 kinase activity, but also participate in the subcellular localization of PDK1 in order to restrict its activity towards specific downstream targets. For example, upon growth factor stimulation, PDK1 undergoes membrane redistribution, moving from the cytosol to intracellular membranes where it can activate downstream effector proteins such as Akt (Anderson et al., 1998a;Klippel et al., 1997;Vanhaesebroeck and Alessi, 2000). Previous studies have shown that upon adhesion to fibronectin (FN), overexpressed CIB1 localizes to membrane domains (Leisner et al., 2005). Since CIB1 interacts with PDK1 both *in vitro* and in cells, we asked whether cell adhesion to FN regulates PDK1 cellular localization, and whether CIB1 is required for proper PDK1 localization. Myc-PDK1 and CIB1 proteins were expressed individually or together in HeLa cells and the subcellular localization of these proteins was examined. When expressed individually and maintained in serum-starved culture conditions, both CIB1 (upper panels) and PDK1 (lower panels) were diffusely distributed in the cytoplasm (Fig. 4-10 A). However, when cells were detached and replated onto FN for 30 min, each protein translocated to the plasma membranes (Fig. 4-10 B). When expressed together, both CIB1 and PDK1 translocated to the plasma membrane within 30 min of readhesion to FN (Fig. 4-10 C upper panels). Furthermore, adhesion to FN at 30 min (Fig. 4-10 C upper panels) and 2 h (Fig. 4-10 C, lower panels) resulted in significant colocalization of CIB1 and PDK1 at these membrane sites. These results suggest that PDK1 subcellular translocation can be regulated not only by growth factors, as previously studied, but also by cellular adhesion

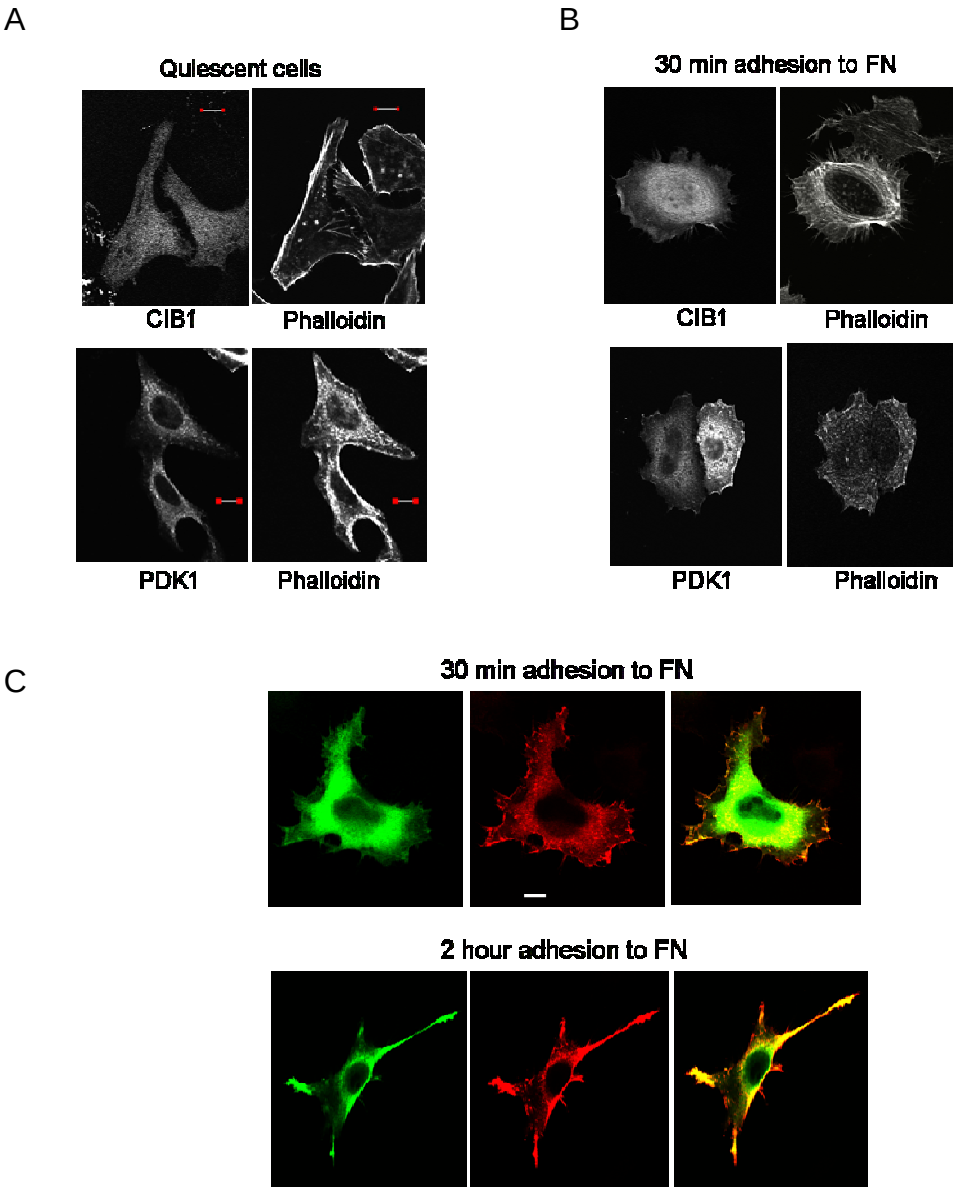

Figure 4-10 CIB1 and PDK1 translocate to and co-localize at membranes upon FN adhesion. **(A)** Confocal images showing CIB1 and PDK1 localization to the cytosol in quiescent cells. HeLa cells transfected with either CIB1 or myc-PDK1 were maintained in serum-starved culture conditions overnight. Cells were stained for CIB1 or PDK1 and phalloidin to visualize F-actin. Bars, 10mm. **(B)** CIB1 or PDK1 translocates to intracellular membranes upon cells adhesion to FN. HeLa cells transfected with CIB1 or myc-PDK1 were serum-starved overnight and replated on FN for 30 min. Cells were stained as described above. **(C)** Colocalization of CIB1 and PDK1 at the plasma membrane upon cell adhesion to fibronectin (FN). HeLa cells transfected with CIB1 and myc-PDK1 were serum-starved overnight and replated on FN for 30 min (upper panels) or 2 h (lower panels). Cells were costained for CIB1 (green) and myc-PDK1 (red). The merged images show colocalization of CIB1 and PDK1 (yellow).

to extracellular matrix proteins, such as FN; and that CIB1 and PDK1 colocalize at plasma membranes upon cell adhesion to FN.

To determine if CIB1 might function to target PDK1 to membrane structures, we first asked if we could alter CIB1 subcellular localization by mutating the N-terminal myristoylation site (CIB1-G2A). The CIB1-G2A mutant showed predominantly nuclear staining and diffuse cytoplasmic staining, with a dramatic reduction in membrane association, both at the quiescent conditions and upon cell replated to adhere to FN (Fig. 4-11 A), consistent with previous reports (Zhu et al., 2004b). When PDK1 was coexpressed with CIB1-G2A in cells adhered to FN, PDK1 was distributed diffusely within the cytosol (Fig. 4-12 A, middle panels), in sharp contrast to its predominantly plasma membrane distribution when expressed alone (Fig. 4-10 B) or with wild-type CIB1 (Fig. 4-10 C). Unlike CIB1-G2A, however, PDK1 was not localized to the nucleus, indicating that CIB1-G2A cannot direct nuclear localization of PDK1 (Fig. 4-12 A, middle panels). We also tested the subcellular localization of CIB1-4A mutant, which has minimal binding to PDK1. Like the wild-type CIB1, CIB1-4A was diffusely distributed in the cytoplasm in quiescent cells (Fig. 4-11 B, left panels) but translocated to membranes upon adhesion to FN (Fig. 4-11 B, right panels). However, when PDK1 was coexpressed with CIB1-4A in cells, PDK1 lost membrane localization (Fig. 4-12 A, lower panels).

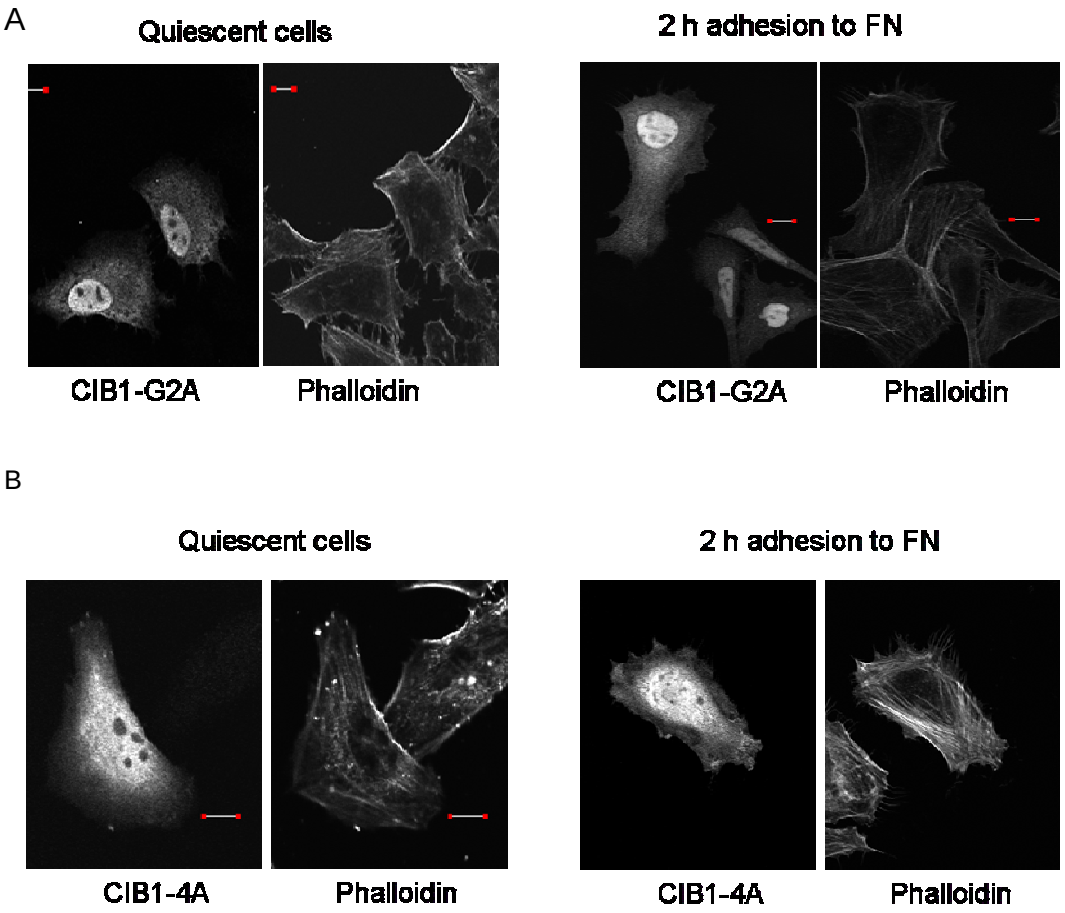

Figure 4-11 Subcellular localization of CIB1 mutants. (A) Non-myristolylated CIB1-G2A localizes mainly in nucleus. HeLa cells transfected with CIB1-G2A were either maintained in serum-starved culture conditions overnight (left panels) or replated onto FN-coated coverslips for 2 h. Cells were stained for CIB1-G2A and phalloidin. **(B)** CIB1-4A translocates to plasma membranes upon cells adhesion to FN. HeLa cells transfected with CIB1-4A were either maintained in serum-starved culture conditions (left panels) or replated onto FN-coated coverslips for 2 h (right panels). Cells were stained for CIB1-4A and phalloidin.

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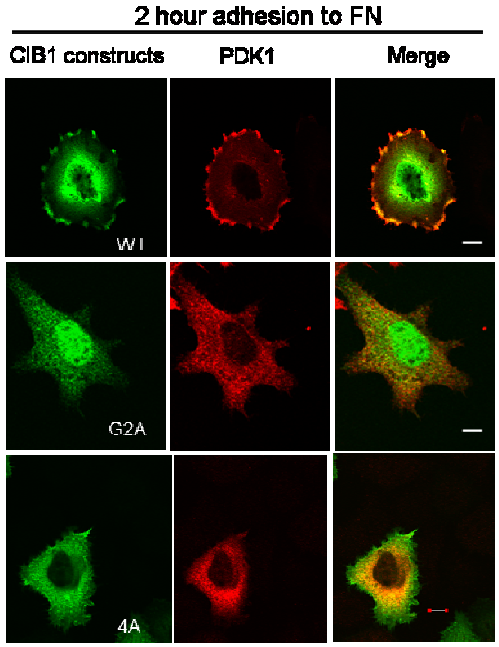

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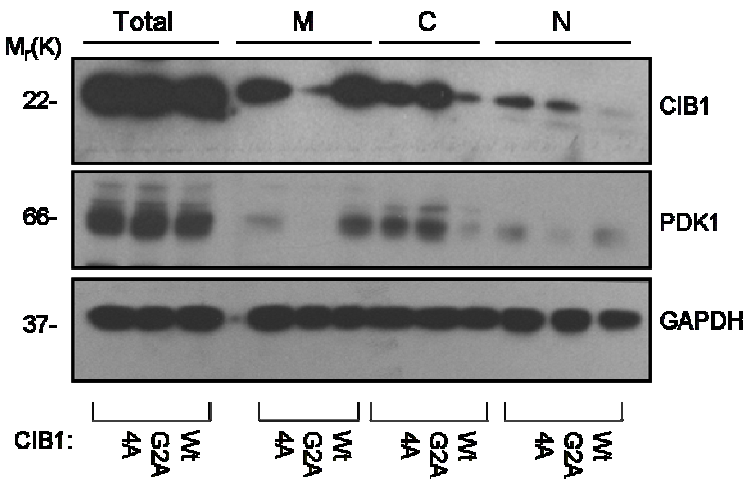

Figure 4-12 Redistribution of PDK1 from membrane to cytosol by CIB1 mutants. (A) Redistribution of PDK1 to the cytosol by CIB1-G2A and CIB1-4A. HeLa cells co-transfected with either WT-CIB1, CIB1-G2A, or CIB1-4A together with myc-PDK1 were serum-starved overnight and replated on FN for 2 h, and then stained for CIB1 constructs (green) and myc-PDK1 (red). The merged images are shown in yellow. **(B)** Redistribution of endogenous PDK1 by CIB1-G2A or CIB1-4A. HeLa cells expressing WT-CIB1, CIB1-G2A, or CIB-4A were serum-starved overnight, followed by adhesion to FN for 1 h, and then subjected to subcellular fractionation using the CNMCS Compartmental Protein Extraction Kit (Biochain Institute). Total protein lysate (total), membrane (M), cytosolic (C), and nuclear (N) fractions were analyzed by immunoblot with respective antibodies with GAPDH as a loading control.

Subcellular fractionation analysis in HeLa cells was performed to confirm the effect of CIB1 constructs on the localization of endogenous PDK1. Consistent with our immunofluorescence data, upon cell adhesion to FN for 1 hour, significant amount of endogenous PDK1 was detected in the membrane fraction in cells overexpressing WT-CIB1 (Fig. 4-12 B). However, translocation of endogenous PDK1 to the membrane fraction was significantly reduced when cells overexpressed the non-myristoylated CIB1-G2A mutant (Fig. 4-12 B). In addition, overexpressing the CIB1-4A mutant that shows minimal binding to PDK1 also decreased the amount of membrane-bound PDK1, although the CIB1-4A mutant maintained the ability to localize to membranes upon adhesion to FN (Fig. 4-12 B). There was no significant difference in the total abundance of endogenous PDK1 protein and exogenous CIB1 proteins among these cells. These data suggest that CIB1 may recruit PDK1 to the plasma and perinuclear membranes during cell adhesion, allowing PDK1 to phosphorylate its membrane-associated substrates and promote cell survival.

PDK1 binds to membranes through its PH domain (Anderson et al., 1998b). To determine whether the PH domain of PDK1 is necessary for its translocation to membrane structures upon adhesion to FN, we overexpressed a PDK1 mutant lacking the C-terminal PH domain (myc-ΔPH-PDK1) either alone or together with CIB1. Upon cell adhesion to FN, the majority of ΔPH-PDK1 was found in the cytosol and a small fraction localized to the plasma membrane (Fig. 4-13 A). Surprisingly, in cells co-expressing CIB1, most of the ΔPH-PDK1 translocated to the plasma membrane (Fig. 4-13 B), suggesting that the PH domain of PDK1 is not required for its recruitment to membranes

upon adhesion to FN and that interaction with CIB1 is sufficient for and can even enhance its membrane localization.

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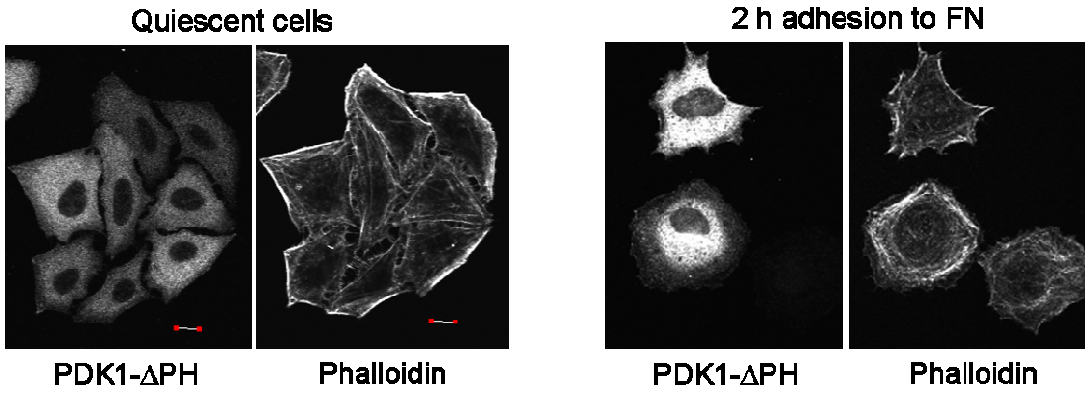

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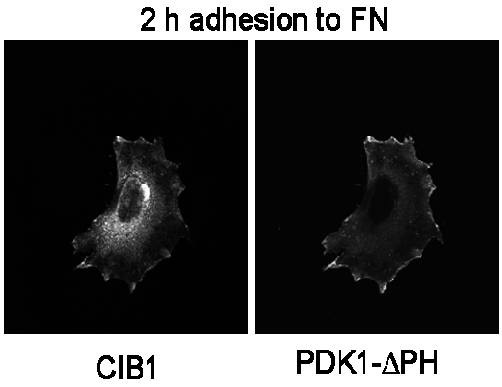

Figure 4-13 PDK1 does not require its PH domain to translocate to the membranes upon FN adhesion. (A) HeLa cells transfected with PDK1-ΔPH were either maintained in serum-starved culture conditions (left panels) or replated onto FN-coated coverslips for 2 h (right panels). **(B)** PDK1-ΔPH and CIB1 were co-transfected into HeLa cells (lower panels), serum starved and then replated on FN for 2 h. Bars, 10mm.

6) CIB1 Affects PDK1 Downstream Signaling to Akt and RSK3

PDK1 activates a variety of AGC family of protein kinases including Akt, p70S6K, p90RSK, PKC isoforms, and SGK. Many of these kinases are proposed to have anti-apoptotic function to promote cell survival (Brunet et al., 1999;Tan et al., 1999;Brunet et al., 2001;Harada et al., 2001;Zhang et al., 2005). To characterize the CIB1-dependent downstream signaling pathways mediated through PDK1, we depleted endogenous cellular CIB1 and tested the phosphorylation status of a number of AGC and non-AGC kinases using phospho-specific antibodies recognizing the T-loop PDK1 phosphorylation site. We first tested the phosphorylation state of Akt, the best characterized PDK1 substrate that plays a critical role in promoting cell survival. Stimulation of HeLa cells with IGF-1 induced Akt phosphorylation at Thr308, which was markedly decreased in the absence of CIB1 (Fig. 4-14A). Since both CIB1 and PDK1 translocate to and colocalize at cell membranes upon cell adhesion to FN, we asked whether CIB1 is required for FN adhesion-induced phosphorylation of Akt by PDK1. As shown in Figure 4-14B, adhesion of cells to FN increased Akt phosphorylation by PDK1 in a time dependent manner, however, loss of CIB1 dramatically decreased the phosphorylation of Akt at all time points. Akt was not the only PDK1 downstream target that was affected by CIB1, since depletion of CIB1 also decreased RSK3 phosphorylation by PDK1 (Fig. 4-14C). ERK1/2 is another upstream regulator of RSK and phosphorylates RSK within the C-terminal kinase domain. Depletion of CIB1 also modestly decreased ERK1/2 phosphorylation (Fig. 4-14C). We also examined the phosphorylation state of S6K and PKCα, two other PDK1 downstream kinases, but no dramatic difference was detected between CIB1-depleted cells and control cells.

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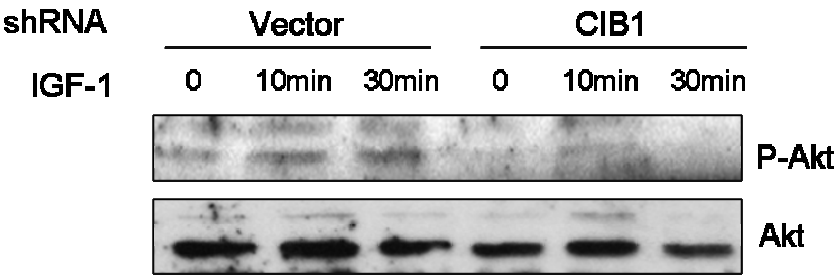

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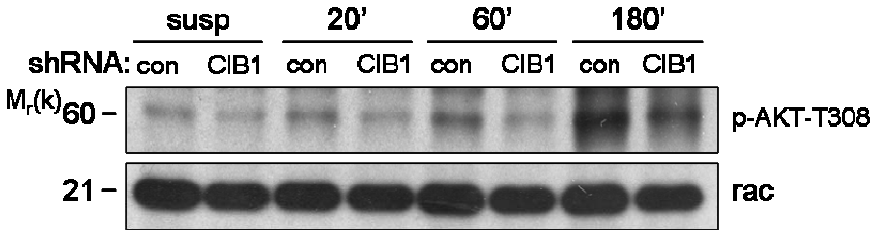

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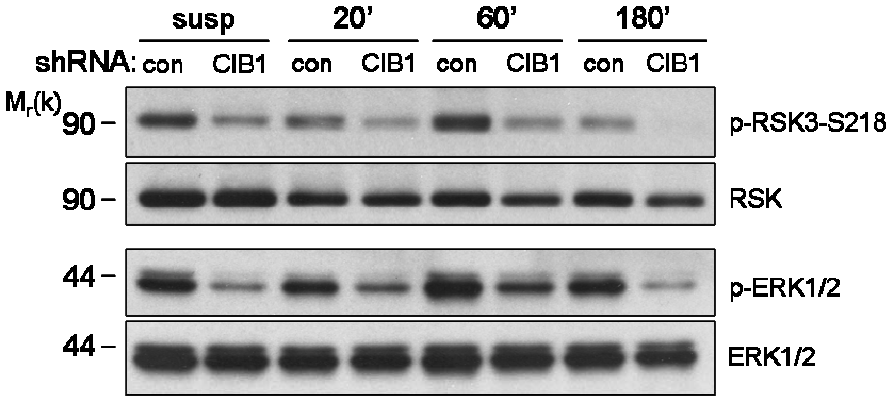

Figure 4-14 CIB1 regulates PDK1 downstream signaling to Akt and RSK3. (A) Control shRNA or CIB1 shRNA-transduced HeLa cells were serum starved overnight and treated with 100 ng/ml IGF1 for the indicated time. Cell lysates were analyzed by Western blotting with antibodies recognizing phospho-Akt-T308 and Akt. **(B)** Control shRNA or CIB1 shRNA-transduced HeLa cells were kept in suspension for 2 h and then either remained in suspension (susp) or adhered to FN-coated plates for the indicated time. The cell lysates were analyzed by Western blotting with antibodies recognizing phospho-Akt-T308. Rac was used as loading control. **(C)** Control shRNA or CIB1 shRNA-transduced HeLa cells were kept in suspension for 2 h and then either remained in suspension (susp) or adhered to FN-coated plates for the indicated time. The cell lysates were analyzed by Western blotting with antibodies recognizing phospho-RSK3-S218, pan-RSK, phosphor-ERK1/2, and ERK1/2 respectively.

Together, these results suggest that CIB1 is required for both soluble growth factor and integrin-mediated adhesion-induced phosphorylation of Akt and RSK3, two critical PDK1 downstream signals that play an important role in promoting cell survival.

4. Conclusion and Discussion

In this section, we have determined that CIB1 promotes cell survival and protects cells from programmed cell death. What might be the mechanism by which CIB1 protects cells from apoptosis? The ability of CIB1 to bind and target PDK1 to membranes might underlie the anti-apoptotic effects of CIB1. Our immunofluorescence and subcellular fractionation studies demonstrate that the non-myristoylated CIB1-G2A mutant that is expressed predominantly in the cytosol induces the redistribution of PDK1 from the plasma membrane to the cytosol. Cells expressing the CIB1-G2A mutant are more sensitive to apoptotic stimuli. The CIB1-4A mutant that retains the ability to localize to the plasma and perinuclear membranes, but does not bind PDK1, also decreases the level of membrane-bound endogenous PDK1. Cells expressing the CIB1-4A mutant are also much more sensitive to apoptotic stimuli, suggesting that both binding to PDK1 and CIB1-dependent recruitment of PDK1 to the membrane may be required for the CIB-mediated protection of cells from apoptosis.

What might be the downstream effectors of PDK1 that contribute to the cell survival function of CIB1? CIB1-depletion decreased both Akt and RSK3 phosphorylation at the PDK1-dependent phosphorylation site in cells stimulated by growth factor or adhered to FN, and this is consistent with the capacity of both Akt and RSK mediating cell survival

function. Akt is well known to translocate from cytosol to the plasma membrane upon extracellular growth factors stimulation or matrix adhesion. On the contrary, RSK is mainly located to cytosol. However, recent experimental evidence have shown that serum and mitogen stimulation can promote translocation of RSK to cellular membranes (Chen et al., 1992;Richards et al., 2001). In addition, RSK membrane translocation and PDK1-dependent phosphorylation of RSK was observed in rat hippocampal neurons that may play a role in cell survival during ischemia (Qi et al., 2007). Therefore, it is possible that upon integrin engagement, CIB1 may recruit PDK1 to the plasma membrane, where it phosphorylates Akt and RSK3 and transduces survival signals into the cell. In addition to Akt and RSK3, we also found that depletion of CIB1 modestly inhibited FN induced phosphorylation of ERK1/2. However, since expression of constitutively active PDK1 significantly rescued cells from apoptosis induced by CIB1 depletion, and CIB1 mutant deficient in either PDK1 binding or membrane localization lost the ability to protect cells from apoptosis, we concluded that PDK1 is the primary target of CIB1 in protection of cells from apoptosis, by sending signal to both Akt and RSK3.

A handful of studies have shown that extracellular growth factors can regulate PDK1 subcellular localization (Anderson et al., 1998b;King and Newton, 2004;Kim et al., 2001). In contrast, another study showed that PDK1 is constitutively localized at the membrane through its PH domain (Currie et al., 1999). To our knowledge, our study is the first to provide evidence that PDK1 subcellular localization can be regulated by cell adhesion to the extracellular matrix. However, our data indicate that instead of the PH domain, the

binding of PDK1 to membrane-bound CIB1 is required for FN-induced membrane translocation.

5. Materials and Methods

Lentiviral vector production and transduction

HEK-293 T cells were cotransfected with lentiviral packaging vectors pRSVREV, pMDLg/pRRE, and the VSV-G expression plasmid pHCMVG (kindly provided by Dr. XiaoFeng Qin), and the corresponding FG12 expression vectors that contain either shRNA or cDNA construct of interest. Culture supernatants were collected 24-hour post-transfection, and the virus stocks were titered on HEK-293T cells based on GFP expression. NIH3T3 cells, HeLa cells, and PC3 cells were infected with filtered supernatants in the presence of 3 μg/ml (for NIH3T3 and HeLa cells) or 6μg/ml (for PC3 cells) polybrene (Sigma). Virus supernatants were replaced with normal growth media after 3 hour incubation, and the cells were incubated for the indicated times before harvesting.

Knockdown of CIB1 and PDK1

Depletion of CIB1 was generated from a lentivirus expression system as described in the 'Lentiviral vector production and transduction section'. Specifically, human CIB1 shRNA (5'accggagcgaatctgcatggtcttcaagagagaccatgcagattcgctccttttttc3') was cloned into a mammalian FG12 expression vector, and mouse R-Ras shRNA (5' accgctcttcacacagatcctcttcaagagagaggatctgtgtgaagagctttttc) cloned into FG12 vector was used as a vector control, which does not have homology to any sequence in human

genome. For knocking down PDK1, siGENOME SMARTpool targeting human PDK1 (Dharmacon) was used, and siCONTROL Non-Tarteting siRNA (Dharmacon) was used as a negative control. These siRNAs were transfected at a final concentration of 50 nM (HeLa) and 75 nM (PC3) by using siPORT NeoFX siRNA transfection reagent from Ambion. Assays were performed 40 h posttransfection.

Immunofluorescence

HeLa cells were transiently transfected with plasmids encoding CIB1 and myc-PDK1. Twenty-four hours post-transfection, cells were serum starved in DMEM containing 0.1% BSA overnight. The next morning, cells were seeded onto coverslips that were either with or without 10 μg/ml FN and allowed to adhere for various times at 37°C. Cells were then fixed with 3.7% paraformaldehyde for 20 min at RT and permeablized with 0.2% Triton X-100 in PBS for 5 min, blocked with normal serum, and incubated with mouse anti-CIB1 monoclonal (1:1000) and rabbit anti-myc polyclonal (1:1500) antibodies. After extensive washing, cells were incubated with goat anti-mouse AlexaFluor594, goat anti-rabbit AlexaFluor488 (Molecular Probes) to detect bound primary antibodies. Filamentous actin was visualized by AlexaFluor594 phalloidin staining (Molecular Probes), and images were collected with an Olympus IX70 laser scanning confocal fluorescence microscope using Fluoview 300 software using a 60x objective with 1.5x optical zoom (PlanApo; Olympus). Confocal images were processed using Adobe Photoshop.

Apoptosis Assay

Cells were plated at $5X10^3$ cells per well in multiple 96-well plates and treated with different apoptotic stimuli for indicated time. Apoptosis was measured by detection of DNA fragmentation using a cell death detection enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals), which quantitively detects the enrichment of nucleosomal DNA fragmentation in the cytoplasm (OD_{405}). The enrichment of mono- and oligonucleosomes released into cytosol was calculated using the formula suggested by the manufacturer: (absorbance of sample cells)/(absorbance of negative control cells). Mean and standard error values from three independent experiments were calculated as percentage of wild-type control within each experiment.

Apoptosis was also detected by anti-PARP and anti-cleaved PARP antibodies. PARP is one of the main cleavage targets of caspase-3 *in vivo*, and cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis.

Chapter V

Concluding Remarks and Future Directions

Two critical findings of this study with broad implications are:

1. From a CIB1 focus: Here we have identified a novel CIB1 binding partner, PDK1. During the process of characterizing this interaction, we discovered a new biological function for CIB1 in promoting cell survival and inhibiting cell apoptosis. We found that CIB1 mediates cell survival through a PDK1-dependent signaling pathway, indicating that CIB1 might be a potential target for anticancer therapy. In addition, we also propose that CIB1, a calcium and integrin binding protein, may act as a coordinator for matrix-based signals and growth factor signals to promote cell survival.

CIB1 was first identified as a protein that binds to the platelet integrin αIIb cytoplasmic domain and was later found to inhibit αIIbβ3 activation, suggesting that CIB1 may negatively regulate integrin inside-out signaling (Naik et al., 1997;Yuan et al., 2006b). In recent studies it was found that CIB1 also binds with high affinity to additional α integrin cytoplasmic tails including α5, a receptor for fibronectin (Gentry, unpublished data). In the current study, we have demonstrated that upon cell adhesion to fibronectin and integrin engagement, CIB1 may recruit PDK1 to the plasma membrane, where it phosphorylates Akt and RSK3 to promote cell survival. This emphasizes a role of CIB1 in integrin outside-in signaling, and also indicates that CIB1 might be a potential target for anticancer therapy.

2. From a PDK1 focus: Here we have demonstrated that protein-protein interactions and subcellular localization play a role in regulating PDK1 activity and restricting its specificity, thereby demonstrating that PDK1 function can be regulated on multiple levels.

Since the first discovery of PDK1 a decade ago, considerable progress has been made towards understanding its biological functions and how the diverse range of PDK1 downstream effectors contribute to these functions [Review in (Alessi, 2001)]. However, many questions remain regarding the mechanism by which PDK1 is regulated to orchestrate its various functions and achieve specificity. Although several studies have shown that protein-protein interaction can regulate PDK1 kinase activity, the biological significance of this interaction remains unclear (Frodin et al., 2000;Fujita et al., 2002;Sato et al., 2002). PDK1 has also been shown to be targeted to different subcellular compartments. For example, in response to PI3K stimulation, PDK1 is targeted to the plasma membrane via its PH domain, where it can phosphorylate and activate Akt (Anderson et al., 1998). PDK1 has also been found to shuttle between the cytoplasm and nucleus upon insulin stimulation (Lim et al., 2003). Nuclear sequestration of PDK1 may act to negatively regulate PDK1 function, but its substrates in the nucleus have not yet been identified. In this study, we demonstrated that PDK1 can be targeted to plasma and perinuclear membranes upon cell adhesion to the extracellular matrix protein fibronectin, and this targeting seems to be CIB1-dependent and appears to achieve its specificity via transmitting signals to the PDK1 downstream effectors Akt and RSK3, resulting in cell survival.

However, a number of questions remain to be answered.

1. Although we demonstrated that CIB1 promotes cell survival via signaling to PDK1, and knocking down CIB1 reduces Akt and RSK3 phosphorylation by PDK1, we haven't provided direct evidence that Akt or RSK3 mediates cell survival in a CIB1-dependent manner. To answer this question, genetic approaches could be utilized. First, constitutively active Akt or RSK3 could be overxpressed in cancer cell lines that have been depleted of CIB1 by shRNA and then exposed to a variety of apoptotic stimuli as used in the current study. This will allow us to determine whether Akt or RSK3 can rescue CIB1-depleted cells from sensitivity to apoptosis. To determine if CIB1 promotes cell survival in an Akt or RSK3-dependent manner, we could deplete Akt or RSK3 expression by siRNA or shRNA in cancer cells where it is highly expressed, and test if CIB1 fails to protect cells from apoptosis. To further confirm whether Akt or RSK3 is required, we could overexpress the silent mutation of Akt or RSK3 back into those cells and test if it can fully recover CIB1 survival function. We would also examine if the CIB1/PDK1/Akt or CIB1/PDK1/RSK3 complex exists in cells by immunoprecipitation and immunofluorescence analysis.

2. Is PDK1 the only downstream effector contributing to CIB1-mediated cell survival? A modest inhibitory effect on FN-induced ERK1/2 phosphorylation was also observed in CIB1-depleted cells (Fig. 4-15 D). Overexpression of PDK1 significantly but not completely rescued cells from apoptosis induced by the loss of CIB1 (Fig. 4-7 B), suggesting that PDK1 is the primary but not the only target of CIB1 in protecting cells from programmed cell death induced by apoptotic stimuli. Effectors other than PDK1,

such as ERK1/2 may also play a modest role. Further experiments on whether overexpression of ERK1/2 can rescue CIB1-depletion-induced cell death would help to address this question. Loss of CIB1 dramatically inhibited FN-induced phosphorylation of Akt and RSK3, indicating that CIB1 might regulate cell survival through a PDK1-Akt or RSK3 signaling pathway. Another upstream kinase activating RSK is ERK1/2, further supporting the idea that there may be crosstalk between the ERK1/2 and PDK1 pathways regulated by CIB1 upon FN stimulation.

Akt and RSK3 might not be the only downstream effectors of PDK1 that contribute to the survival function of CIB1. PDK1 propagates the input of extracellular signals by phosphorylating and activating a variety and growing list of downstream effectors, many of which have anti-apoptotic function. Among these, we tested the phosphorylation status of several well-characterized effectors including Akt, S6K, RSK and PKCα by PDK1 and found that Akt and RSK3 phosphorylation by PDK1 was inhibited in CIB1-depleted cells, but not that of S6K and PKCα. It is possible that some critical substrates that contribute to the CIB/PDK1-mediated cancer cell survival were missed. Moreover, it has also been suggested that PDK1 can activate downstream effectors via a kinase-independent mechanism. For example, the non-catalytic N-terminus of PDK1 binds the N-terminal region of the Ral-GEF, RalGDS, and relieves the auto-inhibitory effect of RalGDS on the catalytic domain to increase its GEF activity (Tian et al., 2002). Thus, it is possible that the phosphorylation status of a potential target remains the same in CIB1-depleted cells as in CIB1 wild-type cells, but the subcellular localization of the target protein might be different. This possibility cannot be detected by a simple depletion of

CIB1 and test of the phosphorylation status of the possible targets. Instead, a beneficial approach would be to use proteomics to identify proteins that could be 'pulled-down' in complex with CIB1 when CIB1 is expressed in different cancer cells under distinct conditions (such as different apoptotic stimuli), and analyzed by mass spectrometry. This approach has several advantages. First, potential CIB1 interacting proteins that contribute to its survival functions might require post-translational modifications such as phosphorylation or myristoylation, and these post-translational modifications can be preserved in mammalian cell systems. If the interaction of CIB1 with its various effectors requires these modifications, they can be detected by mass spectrometry and detailed mechanisms of interaction can also be obtained. Second, whether CIB1 is dynamically involved in different complexes in different cell types under distinct stimuli, and whether cell context and stimuli-specific targets exist for CIB1-mediated survival function can also be characterized. Another useful approach to identify potential targets for CIB1-mediated cell survival would be to use a human breast or prostate cancer cDNA library, since CIB1 was found highly expressed in the MCF-7 human breast cancer and the PC3 prostate cancer cell lines (Fig. 4-1).

3. Another question of interest would be to identify possible survival factors downstream of CIB1/PDK1/Akt or RSK3. The AGC family of kinases including Akt, RSK, S6K and SGK regulate cell survival by converging on one or more of the anti-apoptotic and pro-apoptotic factors (Fig. 2-5). RSKs can phosphorylate and inactivate pro-apoptotic factors such as Bad, procaspase-9, and Forkhead (FKHR) transcription factors, as well as phosphorylate and activate anti-apoptotic factors such as cyclic-AMP response element-

binding protein (CREB) and IkB kinase (IKK) (Ghoda et al., 1997;Bonni et al., 1999;Shimamura et al., 2000) (Fig. 2-5). As a result, these factors have been found to play important roles in apoptosis, cell cycle and DNA repair. Therefore, further detailed experiments are needed to dissect which one(s) of these factors may contribute to the CIB1-induced survival function.

4. Our data demonstrate that: 1) CIB1 and PDK1 translocate to the membrane upon cell adhesion to fibronectin, 2) the ability of CIB1 to promote cell survival requires both PDK1 binding and membrane localization, and 3) CIB1 binds to the α5 cytoplasmic tail of the integrin fibronectin receptor *in vitro* (Gentry, unpublished data). Therefore, we hypothesize that CIB1 may act to coordinate matrix-based signals (fibronectin-integrin) and growth factor signals (PDK1-Akt/RSK or ERK/RSK) to promote cell survival. To test this hypothesis, it will be necessary to determine whether CIB1 binds to the integrin α5 cytoplasmic tail *in vivo*. In addition, an anti-α5 functional blocking antibody can be used to inhibit fibronectin binding to determine whether signaling through the α5β1 receptor is required for CIB1 and PDK1 translocation to the membrane to promote cell survival.

5. Since our studies indicate that CIB1 activates PDK1 kinase activity both *in vitro* and in cell lysates, does PDK1 kinase activity contribute to CIB1-mediated cellular survival function? To answer this question, we can treat cells with PDK1 kinase inhibitors or overexpress a kinase-dead version of PDK1. However, no specific PDK1 inhibitors are commercially available at this time. Furthermore, kinase-dead PDK1 does not affect

endogenous PDK1 activity, demonstrating that the kinase-dead PDK1 mutant does not exert a dominant-negative effect on the Ser/Thr kinase activity of endogenous PDK1 (Yamada et al., 2001). Therefore, experiments such as the depletion of endogenous PDK1 with siRNA/shRNA followed by the overexpression of a silent kinase-dead PDK1 mutant would provide an alternative method to answer this question. In addition, PDK1 kinase domain knock-in mice can be generated, which would express endogenous kinase-dead PDK1 and address whether specific biological functions of PDK1 are mediated through its kinase activity.

PDK1 has a high basal activity in non-stimulated cells. CIB1 overexpression can further increase its kinase activity. However, we did not see a significant reduction in PDK1 kinase activity in CIB1-depleted cells. In contrast, knocking down CIB1 dramatically increased cell sensitivity to apoptotic stimuli and decreased Akt and RSK3 phosphorylation by PDK1. These data indicate that CIB1 regulation of cell survival might not be directly due to its regulation of PDK1 kinase activity *per se*, but may be due to the ability of CIB1 to localize PDK1 in close proximity to Akt and RSK3 thereby facilitating activation of Akt and RSK3 by PDK1. An example for this hypothesis is provided from a study showing that constitutive localization of PDK1 in the nucleus does not affect its kinase activity; however, its tumorigenic ability to promote anchorage-independent growth and protect against UV-induced cell apoptosis is dramatically reduced (Lim et al., 2003). These data indicate that PDK1 translocation from the cytosol to the nucleus may sequester it from activating cytosolic downstream effectors, and therefore may act as a "brake" or negative regulator of PDK1-mediated cell signaling and

biological functions. In this regard, we found that the non-myristoylated CIB1 mutant that retains binding to PDK1, but localizes mainly to the cytosol and nucleus shows decreased ability to protect cells from apoptosis. While we did not observe a CIB1-dependent translocation of PDK1 to the nucleus in these cells, these results suggest that PDK1 kinase activity in the cytosol may not be sufficient to protect cells from apoptosis. These results also indicate that PDK1 kinase activity at the plasma membrane may be required to promote CIB1-induced cell survival function. Collectively, these finding also reinforce the concept that PDK1 is regulated on multiple levels, in addition to the regulation of its kinase activity.

6. Our experiments indicate that CIB1 might act as a survival signal, but is it sufficient for tumorigenesis in living systems? What is the therapeutic significance of CIB1 in cancer? CIB1 is upregulated in MCF-7 breast cancer and PC3 prostate cancer cell lines (Fig. 4-1). However, we found that retrovirus infection of CIB1 in COMMA-1D mouse mammary epithelial cells was not transforming (Fig. 4-6). We have found that CIB1 protects cells from apoptotic stimuli, indicating that instead of inducing transformation, it plays a supportive role in tumorigenesis by reducing the apoptotic potential of cells and promoting cell survival. It will be helpful to use mouse cancer model systems to confirm this hypothesis. The well-characterized MMTV-PyMT mouse model (transgenic expression of polyoma middle T antigen under the control of the mouse mammary tumor virus long terminal repeat promoter in mammary epithelial cells) can be used, since it develops tumors with 100% penetrance and causes aggressive tumor formation within 50-60 days (Guy et al., 1992;Tynan et al., 2005). We have already crossed PyMT mice

with our CIB1 knockout mice to determine if the absence of CIB1 reduces aggressive tumor growth and promote apoptosis in these PyMT mice. Preliminary experiments showed promising results in that the PyMT mice with depleted CIB1 develop significantly fewer breast tumors compared to the PyMT mice with normal CIB1 expression (Zayed,, unpublished data). Similarly, heterozygous PTEN (+/-) mice develop a wide range of tumors. Reducing the expression of PDK1 in PTEN (+/-) mice by crossing the hypomorphic PDK1 mice with $PTEN^{+/-}$ mice dramatically protects these animals from developing a variety of tumors (Bayascas et al., 2005). It will be interesting to see whether depletion of CIB1 by crossing CIB1 knockout mice with PTEN (+/-) mice will produce similar results. In contrast, we could also express CIB1 in mice having the mutant PyMT that is not coupled with PI3K stimulation, by either retrovirus infection or transgene expression, to detect if CIB1 can restore aggressive tumorigenesis. If positive effects are observed, it will further confirm our cell culture data, and support the hypothesis that CIB1 plays an accessory function in tumorigenesis by raising the threshold of the pro-apoptotic signal.

CIB1 may thus be targeted for anti-cancer therapy. However, in our previous studies, CIB1 has been shown to negatively regulate cell migration through the PAK1/LIM kinase pathway in cancer cells. This raises the problem that inhibiting CIB1 might increase the metastatic potential of an otherwise localized tumor. In contrast, a recent finding in our lab showed that endothelial cells derived from CIB1-/- mice have impaired migration and ischemia-induced angiogenesis (Zayed, manuscript in preparation). These seemingly contradictory data suggest that CIB1 function might be agonist and cell-type specific.

7. An interesting question that has not been addressed in this study is whether PAK1 contributes to CIB1/PDK1-induced cell survival. PAK1 is a well-known regulator of cytoskeletal remodeling and cell motility (Kiosses et al., 1999;Sells et al., 1999). However, increasing evidence has demonstrated that PAK1 plays important roles in cell proliferation, apoptosis and cell cycle control, which results in tumorigenesis and invasiveness (Balasenthil et al., 2004). PAK1 can be activated by a variety of proteins, including the small GTPases Cdc42/Rac (Manser et al., 1994), phospholipid sphingosine (Bokoch et al., 1998), PI3K (Papakonstanti and Stournaras, 2002), actin-binding protein filamin A (FLNa) (Vadlamudi et al., 2002), the SH2/SH3-containing adaptor protein Nck (Lu and Mayer, 1999), PAK-interacting exchange factors Cool/PIX (Bagrodia et al., 1998), as well as PDK1 (King et al., 2000) and CIB1 (Leisner et al., 2005). In addition, PAK1 transduces signals to a variety of downstream effectors such as ERK, p38, and JNK (Tang et al., 1997), and promotes cancer-cell survival via multiple mechanisms such as NF-kB, Bad, and FKHR (Schurmann et al., 2000;Mazumdar and Kumar, 2003). Although purified PDK1 phosphorylates and activates PAK1 *in vitro* and overexpressed PDK1 activates PAK1 in vascular smooth muscle cells, whether endogenous PDK1 is a physiological activator for PAK1 is not clear (King *et al.*, 2000). However, like PDK1, PAK1 is also highly expressed in several breast cancer cells (Mira et al., 2000;Balasenthil *et al.*, 2004). Inducible expression of PAK1 or PDK1 in mammary epithelial cells results in similar phenotypes, which include anchorage-independent growth in soft agar, breast cancer cell proliferation and aggressive cell phenotype, as well as invasiveness of breast cancer cells stimulated by growth factors (Mira *et al.*, 2000;Vadlamudi et al., 2000;Adam et al., 2000;Zeng et al., 2002;Xie et al., 2006). In addition, PDK1 and PAK1 share some

common effectors for anti-apoptotic function. Therefore, it is likely that PAK1 plays a role in CIB1/PDK1-induced cell survival. To test this possibility, we could examine the survival function of CIB1/PDK1 using PAK1-/- mice that are available in our lab, or in cancer cell lines depleted of PAK1 by siRNA or shRNA. Further elucidation of the role of CIB1, PAK1, and PDK1 in cancer cells would contribute to our understanding of CIB1 biological functions and identify new therapeutic targets.

8. Another intriguing question that has not been addressed in my current study is, in addition to acting as a cell survival regulator, does CIB1 play a role in regulating cell cycle control in cancer cells? CIB1-/- mice have a defect in spermatogenesis that is associated with increased Cdc2 expression both at the mRNA and protein level in testis tissues (Yuan et al., 2006a). Preliminary data also showed that histone phosphorylation profiles are altered upon UV exposure in CIB1-/- testis tissue and MEFs (Yuan, unpublished data). Therefore, these findings indicate that CIB1 might play an important role in cell cycle control and DNA damage. To test this possibility, we can use flow cytometry analysis to compare cell cycle progression in CIB1-/- and CIB1+/+ MEFs, as well as in cancer cell lines depleted of CIB1. If CIB1 is found to play an important role in cell cycle control, we will further determine if CIB1 achieves its function via existing binding proteins or via novel proteins.

An intriguing finding from our study shows that a non-myristoylated mutant of CIB1 that is unable to target to membranes and therefore is predominantly localized in the cytosol and nucleus has decreased ability to protect cells from apoptosis (Fig. 4-10 and 4-13).

Whether this is due to sequestering CIB1 from anti-apoptotic factors localized in the cell membrane, or due to placing CIB1 close to a pro-apoptotic factor in the cytosol and nucleus is not known. Further identification of new CIB1 nuclear interacting proteins and substrates will help elucidate CIB1 functions in apoptosis, cell cycle control, and DNA damage, and provide the basis for the rational targeting of the cancer-related aberrant functions of nuclear CIB1.

9. Finally, we found that PDK1 kinase activity was not dramatically impaired in CIB1 knockout mice; and CIB1 knockout mice did not have the same lethal phenotype as PDK1 knockout mice. How do we explain this discrepancy? One possible explanation may be through the compensation by other CIB1 isoforms. Sequence analysis revealed that CIB2, CIB3, and CIB4 also have a PDK1 consensus binding motif, indicating that these CIB1 isoforms may also bind PDK1 in cells. If one or more of the CIB isoforms does bind PDK1 and has a redundant role as CIB1, it might explain the lack of a marked phenotype in CIB1 knockout mice. It will also be important to test whether depletion of CIB1 upregulates the expression levels of the CIB1 isoforms in order to provide insight into possible compensatory mechanisms in the CIB1 knockout mouse. To do this, a specific antibody needs to be generated to detect endogenous levels of CIB1 isoforms. Furthermore, a double-knockout (CIB1 together with CIB2/3/4) mouse model may also help to answer the question. In addition, since the CIB1 gene is knocked out early in development this may allow sufficient time for the mice to adapt. These potential compensatory mechanisms may explain the differences in results obtained from the CIB1 knockout mice versus the results obtained from acute removal of the CIB1 gene.

Strategies using an inducible gene knock-in could be developed to target important binding sites within CIB1 such as the potential PDK1 binding site, the possible integrin binding site, as well as the EF-hand motif of CIB1, to investigate CIB1 biological functions and how these different motifs/residues in CIB1 contribute to its functions. This inducible gene knock-in strategy will not only get around the problem of compensation and adaptation, but also mimic the small molecule inhibitor effect more accurately, since CIB1 binds many other proteins including a variety of serine/threonine and tyrosine kinases. CIB1 may also act as an adaptor for regulating these proteins in a temporal and spatial-dependent manner, which might be overlooked by a simple genetic disruption of the CIB1 gene.

In summary, in this study I have identified a novel PDK1 interacting protein CIB1, and have attributed CIB1 with a novel anti-apoptotic function. A model is proposed to explain my results as follows (Fig. 5-1): upon cell adhesion to extracellular matrix protein fibronectin and integrin engagement, CIB1 may recruit PDK1 to the plasma membrane, where it phosphorylates Akt and RSK3. ERK1/2 also contributes to RSK3 phosphorylation. Activated Akt and RSK3 then dissociate from the membrane, translocate to the cytosol and nucleus and promote cell survival. As discussed in this chapter, this picture is far from complete, and a number of questions remain to be answered (Fig. 5-2). Short-term questions include: (1) Is FN adhesion required for recruiting CIB1/PDK1/Akt or RSK3 to the membrane and thereby promoting cell survival? (2) Does the CIB1/PDK1/Akt or RSK3 multiprotein complex exist within the membrane upon integrin engagement and growth factor stimulation and does Akt or

RSK3 rescue CIB1-depletion-induced cell sensitivity to apoptotic stimuli? (3) Does PAK1 contribute to CIB1/PDK1-induced cell survival? In the long-term, we will determine if CIB1 plays roles in addition to promoting cell survival, such as cell cycle control in cancer cells. In addition, *in vivo* mouse cancer models will be developed from different tumor types. Such models are likely to help elucidate the roles of CIB1 in the process of tumorigenesis and validate CIB1 as a potential anti-cancer drug target.

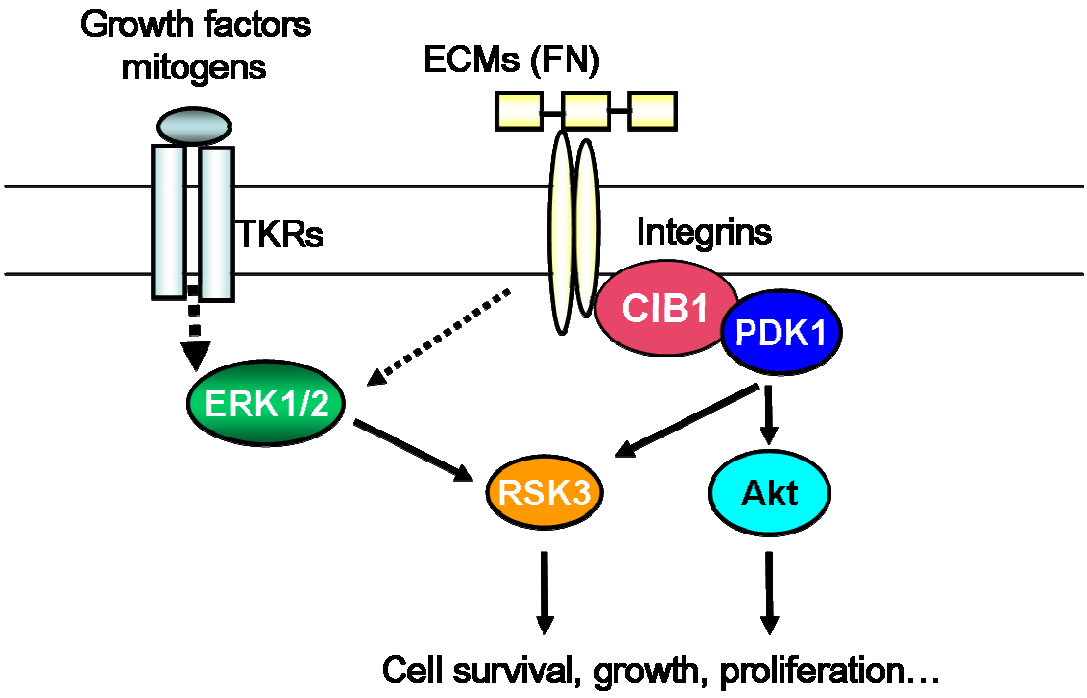

Figure 5-1 Proposed model for CIB1 promoting cell survival. Upon cell adhesion to extracellular matrix protein fibronectin and integrin engagement, CIB1 may recruit PDK1 to the membranes, where PDK1 phosphorylates Akt and RSK3. ERK1/2 also contributes to RSK3 phosphorylation. Activated Akt and RSK3 dissociate from membrane, translocate to the cytosol and nucleus and promote cell survival.

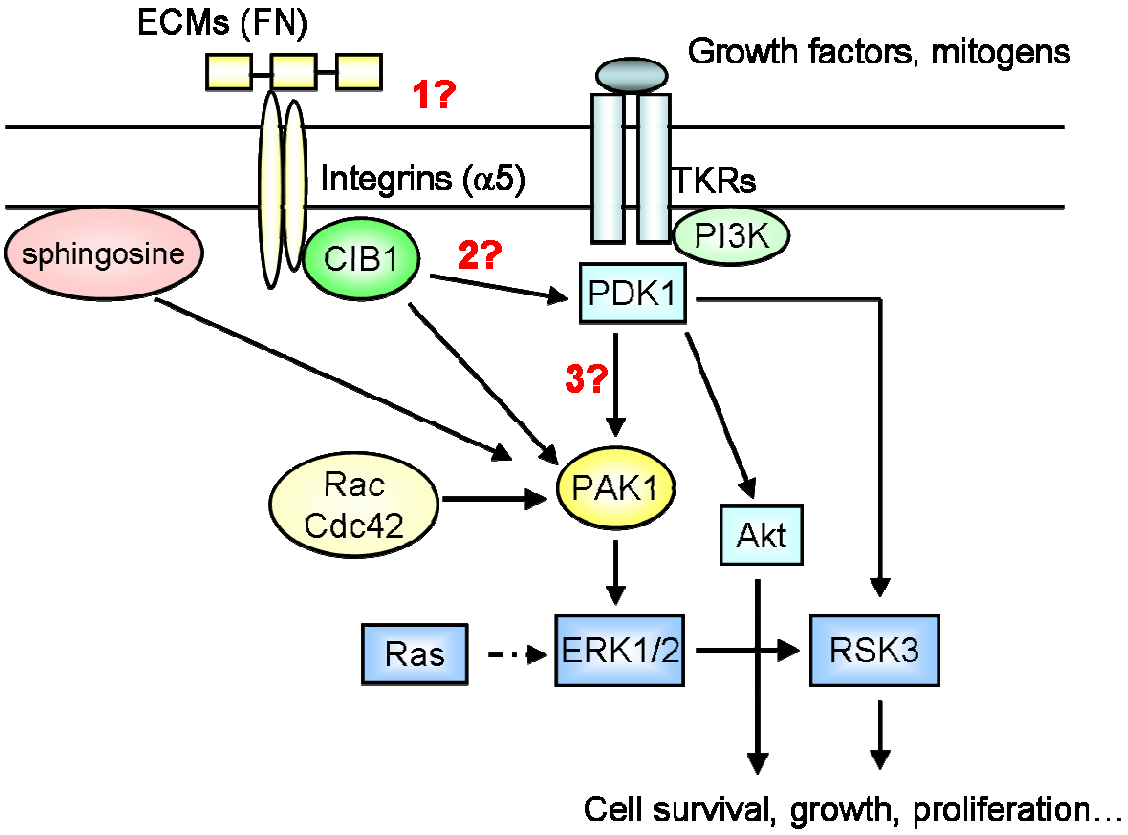

Figure 5-2 Future questions to complete the current model. 1) Is cellular adhesion to FN required for recruiting CIB1/PDK1/Akt or RSK3 to the membrane and thereby promote cell survival? 2) Does the CIB1/PDK1/Akt or CIB1/PDK1/RSK3 multiprotein complex exist within the membrane upon integrin engagement and growth factor stimulation and does Akt or RSK3 rescue CIB1-depletion-induced cell sensitivity to apoptotic stimuli? 3) Does PAK1 contribute to CIB1/PDK1-induced cell survival?

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