Mechanism of eIF2α Kinase Inhibition
by Viral Pseudokinase PK2

by

John Junyi Li

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Department of Molecular Genetics, Faculty of Medicine
University of Toronto

© Copyright by John Junyi Li 2011
Mechanism of eIF2α Kinase Inhibition by
Viral Pseudokinase PK2

John Junyi Li

Master of Science
Department of Molecular Genetics, Faculty of Medicine
University of Toronto, 2011

Abstract

Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) is a conserved eukaryotic mechanism to limit protein synthesis under stress conditions. Baculovirus PK2, which resembles the C-terminal half of a protein kinase domain, inhibits eIF2α family kinases in vivo, thereby increasing viral fitness in the face of host immunological and stress responses. The precise mechanism by which PK2 modulates eIF2α stress response signalling remains unknown. To address this issue, I employed a combination of biochemical, biophysical and in vivo approaches, in collaboration with Dr. Thomas Dever’s lab, to probe the mechanism of PK2 inhibition on a prototypical human eIF2α kinase, the RNA dependent protein kinase (PKR). We have found that PK2 inhibits PKR catalytic activity by binding directly to the PKR kinase domain (PKRKD). This direct interaction requires both the kinase-like C-lobe fold of PK2 and a critical 22 residue N-terminal extension that precedes it. We show that the N-terminal extension is required but not sufficient for the ability of PK2 to: 1) directly bind PKRKD in vitro; 2) inhibit PKR kinase autophosphorylation activity and downstream eIF2α phosphorylation activity in vitro; and 3) inhibit PKR mediated growth arrest in yeast. Further detailed probing of the 22-residue N-terminal extension of PK2 shows that it has bipartite function: one region centered on Phe18 is required for high affinity binding to PKR while a second region centered on Lys2 and Pro3 is required for PKR inhibition but is dispensable for PKR binding. Taken together, these findings show that PK2 inhibits the eIF2α kinase PKR by direct interaction with its kinase domain in a manner that requires the PK2 N-terminal extension. We further show that this N-terminal extension mediated kinase inhibition is general to another eIF2α kinase PERK, and thus is likely general to PK2’s natural host eIF2α target kinase in insect cells.
Acknowledgement

First and foremost, I would like to thank my supervisor Dr. Frank Sicheri for his valued mentorship and for giving me the opportunity to work in his laboratory for the past three years. In kind, I would like to thank my collaborators, Dr. Thomas E. Dever and Chune Cao at NIH for their invaluable experimental support and advice on the PK2 project.

Next, I would like to thank all past and present members of the Sicheri lab for their camaraderie and insightful discussions. In no particular order, I wish to thank the following people who have made the Sicheri lab a great place to work over the last few years: Dave Chiovitti, Thanashan Rajakulendran, Dan Mao, Yang-Chieh Chou, Derek Ceccarelli, Steve Orlicky, Sebastian Guettler, Elton Zeqiraj, Mario Sanches, Leo Wan, Ken Lee, Elaine Chiu, Dante nesulai, Yu-chi Juang, Doug Briant, Cynthia Ho, Jamie Rawson, XiaoJing (Jean) Tang, and Khan Osman, Abiodun A. Ogunjimi, and Zhiqin Li.

Finally, I would like to thank my parents who supported me in pursuing a research career and for constantly being there for me when I needed it the most.
# Table of Contents

**Chapter 1 Introduction** ......................................................................................... 1  
1.1 The kinase domain and catalytic regulation .................................................. 1  
1.2 The eIF2α Kinase PKR and the Baculovirus Pseudokinase Inhibitor PK2 ........ 10  
1.3 Project Aims ............................................................................................... 15  

**Chapter 2 Methods and Materials** .................................................................. 16  
Protein Expression, Mutagenesis, and Purification ............................................ 16  
GST Pull-Down Assay ......................................................................................... 17  
MBP Pull-Down Assay ......................................................................................... 17  
PKR-PK2 Gel Filtration Co-elution Assay ............................................................. 17  
Limited Proteolysis of PK2 and Proteolytic Protection Assays ......................... 17  
eIF2α phosphorylation in vitro kinase assay ....................................................... 18  
Autophosphorylation *in vitro* Kinase Assay ..................................................... 18  
Yeast Toxicity *in vivo* Assay .............................................................................. 19  

**Chapter 3 Results** .......................................................................................... 20  
3.1 PK2 resembles the C-lobe of an eIF2α family kinase domain but contains a unique N-terminal extension sequence ......................................................... 20  
3.2 PK2 directly binds PKR kinase domain but not eIF2α in vitro ...................... 24  
3.3 The N-terminal extension of PK2 composes a protease sensitive region peripheral to the core C-lobe fold and is required but not sufficient for binding to PKR<sup>KD</sup> ........ 27  
3.4 PK2’s N-terminal extension, but not its helix αG, is important for inhibiting PKR function in a yeast model system ................................................................. 29
3.5 Detailed mutational analysis of the PK2 N-terminal extension reveals a binding element centered on Phe18 and an inhibitory element centered on Lys2 and Pro3 ……31

3.6.1 PK2 inhibits PKR\textsuperscript{KD} mediated autophosphorylation and eIF2\textalpha\ phosphorylation \textit{in vitro}…………………………………………………………………………………………..34

3.6.2 PK2 does not inhibit PKR dimerization…………………………………………………………..35

3.7 PK2 directly binds PERK\textsuperscript{KD} and inhibits PERK\textsuperscript{KD} autophosphorylation \textit{in vitro}…..38

Chapter 4 Discussion………………………………………………………………………………………41

4.1 Binding and Inhibitory Elements of the PK2 N-terminal Extension………………41

4.2 Mechanism of eIF2\textalpha\ kinase Inhibition by PK2…………………………………………………42

4.3 The eIF2\textalpha\ kinase fold of PK2……………………………………………………………………..44

4.4 Allosteric Pseudokinase Regulation of Protein Kinase Counterparts………………..44

4.5 PK2 and eIF2\textalpha\ kinase specificity………………………………………………………………46

Chapter 5 Concluding Remarks…………………………………………………………………………..47

References……………………………………………………………………………………………………49
List of Figures

Figure 1.1 Dendrogram of human protein kinases

Figure 1.2 A prototypical protein kinase domain

Figure 1.3 Protein kinase domain secondary and tertiary structures: Conservation of key residues and motifs.

Figure 1.4 Two key features of the activated configuration of the kinase domain

Figure 1.5 Catalytic switching mechanisms of protein kinases

Figure 1.6 elf2α family kinases have distinct regulatory but conserved kinase domains

Figure 1.7 elf2B mediated recycling of elf2 and regulation by elf2α kinases.

Figure 1.8 Atomic structure of the PKR kinase domain in complex with elf2α

Figure 3.1 PK2 resembles the C-terminal lobe of an elf2α kinase domain

Figure 3.2 PK2 directly binds PKR\textsuperscript{KD} but not elf2α \textit{in vitro}

Figure 3.3 The PK2 N-terminal extension is peripheral to its core C-terminal lobe fold and is required but not sufficient for PKR\textsuperscript{KD} binding \textit{in vitro}

Figure 3.4 The PK2 N-terminal extension, but not the PK2 putative elf2α recognition site helix αG, is required for inhibitory function in yeast

Figure 3.5 Detailed mutational analysis of the PK2 N-terminal extension reveals a binding element centered on Phe18 and an inhibitory element centered on Lys2 and Pro3

Figure 3.6 PK2 inhibits PKR\textsuperscript{KD} mediated Thr446 autophosphorylation and elf2α phosphorylation \textit{in vitro}, but does not outwardly inhibit PKR dimerization by crosslinking.

Figure 3.7 PK2 binds PERK\textsuperscript{KD} and inhibits PERK\textsuperscript{KD} Thr980 autophosphorylation \textit{in vitro}

Figure 4.1 Mode of PKR binding and inhibition by pseudokinase PK2

Figure 5.1 TR-FRET \textit{in vitro} kinase assay

Figure 5.2 PKR face-to-face αEF helix exchange mechanism: a model for PK2-PKR C-lobe to C-lobe interaction?

Figure 5.3 PK2 construct optimization, crystallization, and diffraction.

Figure 5.4 NMR backbone assignment of the kinase domain of PKR K296R (252-551)
# List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>λ</td>
<td>Lambda</td>
</tr>
<tr>
<td>μM</td>
<td>micro Molar</td>
</tr>
<tr>
<td>mM</td>
<td>milli Molar</td>
</tr>
<tr>
<td>A&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Absorbance at 600nm wavelength</td>
</tr>
<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> multiple nucleopolyhedrovirus</td>
</tr>
<tr>
<td>AGC</td>
<td>Protein kinase A, protein kinase G and protein kinase C family</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>β,γ-imidoadenosine-5′-triphosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Advanced photon source</td>
</tr>
<tr>
<td>aPK</td>
<td>Atypical protein kinase</td>
</tr>
<tr>
<td>A-seg, A-loop</td>
<td>Activation segment of the kinase domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BmNPV</td>
<td><em>Bombyx Mori</em> nucleopolyhedrovirus</td>
</tr>
<tr>
<td>C-lobe</td>
<td>Carboxyl terminal lobe of the kinase domain</td>
</tr>
<tr>
<td>CAMK</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/calmodulin-regulated kinases</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1 family</td>
</tr>
<tr>
<td>CMGC</td>
<td>Cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinase and CDK-like kinases</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic translation initiation factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor-alpha subunit</td>
</tr>
<tr>
<td>GCN2</td>
<td>General control non-derepressible 2</td>
</tr>
<tr>
<td>GEF</td>
<td>GTP exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxylethylpiperazine-N′-2′-ethane sulfonic acid</td>
</tr>
<tr>
<td>HRI</td>
<td>Heme-responsive initiator kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria bertani</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>N-lobe</td>
<td>Amino terminal lobe of the kinase domain</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>P+1 loop</td>
<td>Phosphor-acceptor peptide binding segment of the kinase domain</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PERK</td>
<td>Pancreatic, ER-resident protein kinase</td>
</tr>
<tr>
<td>PK</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>RNA-dependent protein kinase</td>
</tr>
<tr>
<td>PKR&lt;sup&gt;KD&lt;/sup&gt;</td>
<td>Kinase domain of RNA-dependent protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl Flouride</td>
</tr>
<tr>
<td>pSer</td>
<td>phosphorylated serine residue</td>
</tr>
<tr>
<td>pThr</td>
<td>phosphorylated threonine residue</td>
</tr>
<tr>
<td>RBD/RBM</td>
<td>dsRNA binding domain or motif</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STE</td>
<td>Related to yeast sterile kinases</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phosphine</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinases</td>
</tr>
<tr>
<td>TKL</td>
<td>Tyrosine kinase-like</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight to volume</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 The Kinase Domain and Catalytic Regulation

Protein phosphorylation is a covalent, reversible regulatory mechanism involved in almost all facets of eukaryotic cellular biology (Hunter, 1991). The core enzymatic reaction involves the transfer of the $\gamma$-phosphate of ATP onto the hydroxyl group of specific seryl, threonyl, or tyrosyl residues of target protein substrates, resulting in the modulation of conformation and/or enzymatic activity (Manning et al, 2002). Underscoring the importance of protein phosphorylation, ~2% of all eukaryotic genes encode proteins containing the conserved catalytic core necessary for phospho-transfer activity, referred to as the protein kinase domain. (Hunter, 1991; Hunter, 1995; Manning et al, 2002)

Approximately 518 known human protein kinases have been identified to date, first represented in the iconic dendrogram published by Manning et al. (Fig 1.1) and collectively known as the Human Kinome (Manning et al, 2002). Protein kinases are the third largest family of signaling proteins in the human genome (behind only immunoglobulin domain proteins and C2H2 Zinc finger domain proteins) and are further subdivided according to their sequence similarity into eight major groups: AGC, CAMK, CK1, CMGC, STE, TK, TKL and aPK (refer to list of abbreviations section for full names). Interestingly, approximately 10% of known protein kinases lack one or more key conserved motifs required for catalytic function. These kinases are termed pseudokinases and are thought to function not through their catalytic phosphate transfer activity but through their ability to modulate protein conformations of partner kinases (Manning et al, 2002; Zeqiraj and Van Aalten, 2010)
Figure 1.1

Dendrogram of human protein kinases. Genes harboring a protein kinase domain in the human genome were analyzed and grouped by sequence similarity (Manning et al., 2002). Seven distinct kinase families are depicted (refer to list of abbreviations for full names), while an eighth group of more divergent atypical kinases (aPK group) is not shown. Figure adapted from Manning et al., 2002. Shown in a red circle is the location of the eIF2α family kinases, which constitute the primary subject of investigation for this thesis (See Section 1.2)
Protein kinase domains possess a number of conserved, structural features, as exemplified by the kinase domain of the RNA dependent protein kinase (Fig 1.2; PDB ID 2A19; Dar et al., 2005; Hanks et al., 1988, Hubbard et al., 1999). The kinase fold consists of approximately 300 residues forming a bilobal tertiary structure in which a smaller N-terminal lobe is connected by a short hinge region to a larger C-terminal lobe. The hinge region is approximately 10 residues in length and confers flexibility between the N and C terminal lobes to modulate the plasticity of the interlobal space, which is also known as the catalytic cleft (Huse et al., 2002). The catalytic cleft is the site of ATP binding and phosphate transfer. ATP binding is tightly coupled to the binding of two divalent cations (usually Mg$^{2+}$), which in turn are coordinated by interactions with critical, conserved residues contributed by both the N-terminal and the C-terminal lobes of the kinase domain. The N-terminal lobe is minimally composed of five beta strands and a single helix known as the $\alpha$C helix. The regulated conformation of helix $\alpha$C can serve to switch the kinase domain between active and inactive states. The C-terminal lobe is predominantly alpha-helical in nature and contains many structural features required for catalysis, metal binding and substrate binding. A large flexible element referred to as the activation segment or A-loop is also found in the C-terminal lobe of the kinase domain. Similar to helix $\alpha$C, the regulated conformation of the A-loop is also tightly coupled to catalytic switching of the kinase domain (Nolen et al., 2004).

**Figure 1.2**

*Figure 1.2 A prototypical protein kinase domain.* Ribbons representation (left) and schematic representation (right) of the structural features of the PKR prototypical protein kinase domain (PDB ID 2A19). The N-terminal lobe, inter-lobal hinge, and the C-terminal lobe are colored cyan, green and blue, respectively. AMPPNP (a non-hydrolyzable ATP analogue) is colored orange while Mg$^{2+}$ ions are represented by grey spheres. Two key regulatory elements, namely the N-terminal lobe helix $\alpha$C and the C-terminal lobe activation loop, are colored yellow and red, respectively.
From extensive structural and sequence alignment analysis of many divergent protein kinase domains, several conserved motifs and residues that mediate kinase catalytic function have been identified. (Hanks et al. 1988; Taylor and Radzio-Andzelm, 1994). (Fig 1.3) In the N-terminal lobe, a loop known as the glycine-rich loop, located between strands $\beta_2$ and $\beta_3$ (Subdomain I), forms a cap over the bound ATP molecule while stabilizing its $\gamma$ phosphate moiety. Also within the N-terminal lobe, a conserved VAVK motif is involved in ATP coordination (Subdomain II). Specifically, the VAVK Lys residue within strand $\beta_3$ bridges a Glu residue in helix $\alpha_C$ (Subdomain III), while concomitantly interacting with the $\alpha$ and $\beta$ phosphate moieties of ATP.

In the C-terminal lobe of the kinase domain, the catalytic loop is comprised of the HRD motif (Subdomain VIb), for which the Asp residue is the catalytic base responsible for nucleophilic attack of the substrate OH acceptor group during catalysis. Also in the C-lobe, one of the most conserved motifs in the kinase domain consists of the DFG motif or Mg$^{2+}$ binding loop (Subdomain VII), whereby the Asp residue of DFG contacts all three phosphate moieties of ATP via coordination to divalent cations. The DFG metal binding loop defines the beginning of an important region of the kinase C-terminal lobe known as the activation segment or A-loop, typically 20 to 30 residues in length (Subdomains VII and VIII). The activation segment harbors a docking site for the phospho-acceptor substrate known as the P$^+$1 loop and ends with a conserved APE motif that defines helix $\alpha_{EF}$. In most kinases, the activation segment is also subject to phospho-regulation, in which the phosphorylation of specific residues by other kinases or by autophosphorylation is critical for the attainment of a productive conformation for catalysis. Typically, the A-loop will re-arrange into an open conformation that allows for 1) productive ATP binding 2) substrate docking and 3) kinase catalytic function. Re-arrangement is achieved by specific interactions of the A-loop phosphorylated residues with basic residues in the $\alpha_C$ helix, as well as the Arg residue in the HRD motif.
Figure 1.3 Protein kinase domain secondary and tertiary structures: Conservation of key residues and motifs. A. Schematic representation of the secondary structure elements of a protein kinase domain. The kinase N-terminal lobe, inter-lobal hinge, and C-terminal lobe are colored cyan, green and blue, respectively. β-sheets are represented by block arrows while α-helices are represented by rounded rectangles. Conserved kinase motifs and kinase subdomains are indicated. B Ribbon representation of the conserved structural motifs within a protein kinase domain (PKR: PDB ID 2A19). The N-terminal lobe, inter-lobal hinge, and C-terminal lobe are colored cyan, green and blue, respectively. AMPPNP (a non-hydrolyzable ATP analogue) is colored orange while two Mg$^{2+}$ ions are represented by grey spheres. The N-terminal lobe glycine-rich loop is colored dark brown. The N-terminal lobe helix αC is colored yellow, and the conserved Lys (β3)-Glu(αC) ion pair is represented by purple sticks. The C-terminal lobe activation loop is colored red. The metal binding DFG motif is represented by light pink sticks and the catalytic loop HRD motif is represented by light brown sticks. The A-loop phospho-regulatory site (Thr446 in PKR) is represented by magenta sticks and coordinated to αC via two basic yellow residues. Coordination of pThr446 with the catalytic loop Arg is not shown.
The ability to modulate catalytic switching of kinases between active and inactive states in order to control cellular behavior is of tremendous relevance for understanding human biology as well as for developing clinical intervention strategies for human diseases. As such, kinase regulation has been an area of intensive research for over four decades in both the academic community and pharmaceutical industry. From our current understanding of kinase domain catalytic switching mechanisms, kinases can adopt one of two main conformations (Johnson et al., 1996). Firstly, in the ATP-bound active state, only one conformation is favored across many different kinases. Indeed, most kinases are highly similar in the “ON” state conformation due to the conservation of specific residues required for ATP binding and catalysis that need to align in a particular orientation permissive for phospho-transfer. However, unlike the ubiquitous “ON” state conformation, the “OFF” state conformations of kinases harbor tremendous diversity. In the inactive apo state, kinases contain many different deformations that structurally disallow the correct alignment of conserved residues required for kinase activity. Indeed, the variety of structurally distinct “OFF” state conformations underlies the diversity of regulatory mechanisms by which kinase catalytic switching is achieved.

Figure 1.4

The active state of the kinase domain is often distinguished from its inactive state by the presence of the conserved Glu-Lys salt-bridge in the N-terminal lobe and by the
formation of a two-stranded β-sheet between the catalytic loop β6 and the activation segment β9 in the C-terminal lobe. (Fig 1.4) These two key structural features demark the active, productive conformations of both the αC helix and the activation segment of the kinase domain. In inactive states of kinases, one or both of these features are perturbed and subjected to regulation via diverse mechanisms. (Fig 1.5) These mechanisms can be broadly grouped into one of three categories: 1) **Direct modulation of kinase domain:** for example, the insulin receptor kinase contains an activation segment that collapses into the catalytic cleft in the unphosphorylated, deactivated state, which sterically blocks both ATP and substrate binding (Hubbard, 1997; Hubbard et al., 1994; Till et al., 2001). Upon phosphorylation, the activation segment of the insulin receptor kinase domain adopts an extended conformation that allows both nucleotide and substrate to bind to the active site. Furthermore, the phosphorylated tyrosine residue of the activation segment promotes the closure of N and C-terminal lobes through an interaction with helix-αC. (Fig 1.5a) 2) **Intramolecular modulation of the kinase domain:** for example, the Src family tyrosine kinases possess SH2 and SH3 domains that sequester the catalytic kinase domain in the auto-inhibited state (Sicheri and Kuriyan, 1997). Specifically, intramolecular interactions are established between the phosphorylated tail region of the Src kinase domain and the SH2 domain, and between the SH3 domain and the SH2-kinase domain linker. The auto-inhibited state promotes non-productive conformations of the A-loop (disordered state) as well as helix αC (no Glu-Lys ion pair) that are incompatible with catalysis, while release of the SH2 and SH3 domains interaction, mediated by Src tail region dephosphorylation, transitions the kinase domain to an active conformation that is further stabilized by A-loop phosphorylation. (Fig 1.5b) 3) **Intermolecular modulation of the kinase domain:** for example, the cyclin-dependent kinases (CDK) are allosterically regulated by an intermolecular interaction with proteins called cyclins (De bondt et al. 1993; Jeffrey et al. 1995). In the OFF state, the kinase domain αC is rotated into a conformation that disallows the formation of the regulatory Lys-Glu ion pair. Cyclins directly bind the kinase domain N-terminal lobe at a site centered on helix αC, resulting in the conformational reinstatement of the Lys-Glu ion pair required for catalysis. (Fig 1.5c)
Figure 1.5 Catalytic switching mechanisms of protein kinases. A schematic representation of the insulin receptor kinase (IRK), the sarcoma proto-oncogene kinase (Src), and the cyclin dependent kinase (CDK). A. In the inactive state of IRK, the activation loop sterically blocks ATP and substrate binding. Phosphorylation of the A-loop results in closure of the N- and C- terminal lobes. Moreover, the A-loop and helix αC conformationally re-orient to catalytically permissive states. B. In the inactive state of Src, intramolecular interactions are established between the phosphorylated tail region of the Src kinase domain and the SH2 domain, and between the SH3 domain and the SH2-kinase domain linker. These interactions contort helix αC into an inactive conformation while the A-loop remains disordered. Release of intramolecular interactions via dephosphorylation of the Src C-terminal tail results in activation of the kinase domain, aided by phosphorylation and conformational re-arrangement of the A-loop C. In the inactive state of CDK, the activation loop sterically blocks ATP and substrate binding. Intermolecular cyclin binding to the kinase N-terminal lobe helix αC results in the re-establishment of the Glu-Lys ion pair required for catalysis. Concomitantly, autophosphorylation of the A-loop stabilizes the active conformation of kinase
The tremendous diversity in catalytic switching mechanisms of protein kinases underlies the vast array of cellular processes controlled by them. Tight regulation of kinase catalytic switching is intuitively required, given the plethora of signal transduction pathways that kinases control within a cell. In disease states, kinases are often misregulated which can lead to aberrant cellular behavior. The kinase B-RAF, for example, is one of the most frequently mutated genes within the kinase superfamily in human cancers. These mutations are found with an astounding frequency in certain cancers (e.g. 66% of malignant melanomas, 30-53% of papillary thyroid cancer and 30% of serous ovarian cancer; Brose et al., 2002; Davies et al., 2002); and mostly result in the constitutive activation of B-RAF kinase activity that drives uncontrolled cellular growth and proliferation. As a result, these findings have identified intercepting aberrant RAF activation as an attractive therapeutic target. Since protein kinases are bona fide druggable targets with previous successes (i.e. Gleevec/Imatinib targeting BCR-Abl, Sprycel/Dasatinib targeting BCR-Abl & Src, Iressa/Gefitinib targeting EGFR, Sorafenib/Nexavar targeting several kinase families, etc), the development of RAF kinase inhibitors to treat cancer has garnered tremendous attention from the pharmaceutical industry. Many other kinases possess properties that strongly link them to the development of human disease, including eIF2α kinases who are the subject of investigation for this report. In particular, aberrant functions of members of the eIF2α kinase family have been implicated as potential targets for cancer, neurodegenerative disease, and diabetes (Takahisa et al. 2010; Scheper et al. 2007; Shi et al. 2003; Peel, 2004). From these studies and the culmination of decades of research on kinase structure/function & biology, it has become clear that efforts to understand how kinases turn ON and OFF in response to specific, regulated signals represent a key step towards bridging basic science research and targeted therapeutics development (Blume-Jensen and Hunter, 2001; Clemens, 2004; Druker, 2001; Hunter, 1998; Kurzrock et al., 2003).
Eukaryotic cells possess diverse mechanisms of molecular adaptation to stress conditions. eIF2α kinases are evolutionarily conserved signaling enzymes that convert stress signals into adaptive gene expression responses (Barber, 2001; Clemens, 2004; Zhan et al., 2004). Specifically, four members of the eIF2α kinase family have diverse regulatory domains that respond to different stress signals (Fig 1.6 green domains): 1) Heme-Regulated Inhibitor kinase (HRI) senses heme deficiency relative to globin levels (Han et al., 2001; Levin et al., 1980; Ehrenfeld et al., 1971); 2) RNA dependent Protein Kinase (PKR) senses viral invasion by engaging viral double stranded RNA (dsRNA) (Cole, 2006; Garcia et al., 2007; Barber et al., 1993); 3) General Control Nonrepressed-2 kinase (GCN2) detects amino acid starvation and regulates metabolite homeostasis (Dever et al., 1992); 4) and finally, PKR-like endoplasmic reticulum kinase (PERK) detects unfolded proteins in the ER and regulates the unfolded protein response (Brewer and Diehl, 2000).

Though eIF2α kinases may respond to different stress stimuli using a repertoire of divergent regulatory domains, their catalytic kinase domains are highly conserved (Fig 1.6 blue domains) and transmit an overlapping signal that potently inhibits cellular translation through specific phosphorylation of a common substrate eIF2α on Ser51 (Dhaliwal and Hoffman, 2003). eIF2 is a heterotrimeric (α, β, γ subunits) GTPase that delivers Met-tRNA to the 40S ribosome during translation initiation. Delivery is coupled to eIF2 GTPase activity, whereby hydrolysis of GTP to GDP renders eIF2 incompetent for subsequent rounds of initiation. The GTP-exchange factor (GEF) eIF2B recycles GDP-bound eIF2 back into the active GTP bound form. Upon stress-induced activation, eIF2α kinases inhibit translation initiation by specifically phosphorylating eIF2α on Ser51, which converts it from a substrate into a competitive inhibitor of its GEF eIF2B. Since eIF2B protein level is limiting compared to eIF2 in cells, phosphorylation of a small percentage of eIF2α results in the complete sequestration of eIF2B and hence a potent inhibition of protein synthesis (Fig 1.7). (Krishnamoorthy et al., 2001; Pavitt, 2005; Pavitt et al, 1998)
Figure 1.6 eIF2α family kinases have distinct regulatory but conserved kinase domains. A schematic representation of the eIF2α family kinases: RNA dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), Heme-Regulated Inhibitor kinase (HRI), and General Control Non-repressed 2 kinase (GCN2). eIF2α family kinases sense stress signals via their diverse repertoire of regulatory domains (green) and transmit an overlapping phosphorylation signal via their conserved kinase domain (blue). The result is the phosphorylation of eIF2α on Ser51 and attenuation of global translation for an adaptive stress response signaling. (Note: the schematic does not depict relative size nor position of the various regulatory and kinase domains.)

Figure 1.7 eIF2B mediated recycling of eIF2 and regulation by eIF2α kinases. eIF2 is a heterotrimeric (α: magenta, β: cyan, γ: yellow subunits) GTPase that delivers Met-tRNA to the 40S ribosome during translation initiation. Upon AUG codon recognition and GTP (green circle) hydrolysis, GDP (red square) bound eIF2 needs to be recycled back to the GTP bound form via eIF2B (purple). Phosphorylation of eIF2α on Ser51 turns eIF2α into a competitive inhibitor of eIF2B, which results in inhibition of translation. Figure adapted from Dever et al. 2002.
Inhibition of translation by eIF2α kinases allows cells to efficiently utilize resources when mounting an adaptive signaling response. The archetypal eIF2α kinase PKR has been extensively studied as a critical enzyme of innate immunity that regulates interferon signaling and translation attenuation in response to viral infection. PKR has a modular domain structure, with two conserved, dsRNA binding domains (regulatory) at the N-terminus, followed by a short interdomain linker region, and finally, a conserved 300-residue Ser/Thr kinase domain at the C-terminus (Dar et al., 2005; Dey et al., 2005).

When viral dsRNA engages the N-terminal dsRNA binding domains, PKR undergoes a specific, precise mode of kinase domain dimerization followed by autophosphorylation at a key regulatory residue Thr446 in the activation loop. (Dar et al., 2005; Mckenna et al., 2007; Nanduri et al., 1998; Nanduri et al., 2000; Romano, 1995; Zhang et al., 2001). Both dimerization and A-loop phosphorylation are required for maximal PKR kinase activity in vitro and in vivo. (Dar et al., 2005, Dey et al., 2005)

eIF2α kinases share sequence similarity only within their catalytic kinase domains and the minimal PKR_KD is sufficient to propagate a translational repression response in vivo when fused to an exogenous dimerization domain (Ung et al. 2001). PKR_KD has a prototypical kinase domain architecture consisting of a smaller N-terminal lobe and a larger C-terminal lobe. The crystal structure of phosphorylated PKR kinase domain in complex with eIF2α revealed two key aspects of kinase function. (Fig 1.8) First, a conserved interface on the N-terminal lobe mediates dimerization in a back-to-back configuration that is required for catalytic activity (Dar et al., 2005; Dey et al., 2005; Dey et al., 2006); and second, helix αG in the C-terminal lobe adopts a specific, non-canonical position that is critical for eIF2α recognition; notably, PKR helix αG is one turn longer and tilted by 40° compared to other non-eIF2α kinases (Dar et al., 2005; Dey et al., 2005). The conservation of both the dimerization interface and the unique αG helix conformation (both orientation and size) amongst all eIF2α kinases suggests a common mechanism of kinase regulation and substrate recognition (Fig 1.8; Dey et al., 2006).
Activated PKR attenuates viral propagation by specific phosphorylation of eIF2α on Ser51 and inhibition of cellular (and viral) translation. The importance of PKR’s ability to down-regulate translation during viral invasion is underscored by the vast array of DNA and RNA viruses that successfully target PKR for inhibition (Langland et al., 2006). In many cases, elucidating the mechanism of PKR inhibition by viral products has had a great impact on our understanding of how PKR naturally functions. In particular, viral mimicry appears to be a reoccurring theme (Elde et al., 2009; Langland et al., 2006). For example, analysis of the pseudosubstrate inhibitor K3L from vaccinia virus revealed key molecular determinants required for PKR recognition of its natural substrate eIF2α (Dar and Sicheri, 2002). Interestingly, K3L possesses dual inhibitory activity on PKR. As a structural mimic of eIF2α, K3L achieves 100% inhibition of PKR.
phosphorylation of eIF2α in a competitive manner. In contrast, K3L binding to PKR only partially inhibited kinase domain autophosphorylation in a non-competitive manner. Another example of viral mimicry is the PKR inhibitor E3L, also from vaccinia virus, which mimics the dsRNA binding domains of PKR. E3L functions by sequestering viral dsRNA and preventing PKR activation by dsRNA binding (Davies et al. 1993). Finally, VAI, a small structured RNA from adenovirus, can bind dsRNA domains of PKR in a manner that circumvents kinase domain activation (O’Malley et al., 1986; Sharp et al., 1993). Other viruses, such as influenza virus, reovirus, rotavirus, hepatitis C virus, and Human Immunodeficiency Virus HIV-1, have all developed mechanisms to inhibit PKR function in order to gain competitive advantage over their mammalian hosts, though the precise mechanisms by which these viral products function have not been fully elucidated (Langland et al., 2006).

Suppression of eIF2α phosphorylation is a common strategy employed by viruses to propagate in host cells. Insect viruses have also independently evolved strategies to shut down insect eIF2α kinase signaling (Aarti et al., 2010). Baculovirus of the nuclear polyhedrosis virus family (NPV), such as Bombyx mori and Autographica californica, encode a truncated pseudokinase called PK2 that inhibits eIF2α kinase function (Dever et al., 1998; Li et al., 1995). In a PKR overexpression model in yeast, PK2 can alleviate PKR mediated translational arrest and cell toxicity by inhibiting eIF2α phosphorylation in vivo (Dever et al., 1998). Based on previous sequence analysis, PK2 most closely resembles the minimal C-terminal lobe of the kinase domains of eIF2α family kinases (Dever et al., 1998). Indeed, my analysis reveals that PK2 also contains a non-canonical αG helix characteristic of eIF2α kinases, but lacks the entire kinase domain N-terminal lobe required for back-to-back dimerization. Based on these features, one would predict that PK2 might competitively sequester eIF2α. Surprisingly, however, PK2 co-immunoprecipitates with PKR, and PK2 co-expression with PKR inhibits PKR\textsuperscript{KD} autophosphorylation in vivo (Dever et al., 1998). Consistent with PKR rather than eIF2α being the direct target of PK2, a yeast slow growth phenotype caused by PK2 overexpression can be rescued by co-expression of a catalytically inactive PKR-K296R, and not by co-expression of eIF2α (Thomas Dever, personal communications; Dever et
Subsequently, PK2 was postulated to be a general eIF2α kinase inhibitor as it was shown to inhibit both yeast GCN2 and insect Bek, an HRI homologue from silkworm *Bombyx Mori.* (Dever *et al.*, 1998; Prasad *et al.*, 2003). In this thesis, I set out to investigate the mechanism of action underlying PK2 function by using the eIF2α kinase PKR as a model.

### 1.3 Project Aims

Though PK2 can inhibit eIF2α kinase signaling *in vivo,* little is known about the molecular basis for PK2 inhibitory action. Our current understanding of eIF2α kinase structure and function does not provide a framework for explaining how PK2, a kinase C-terminal lobe mimic, can inhibit eIF2α kinase activity. Specifically, whether PK2 binds to eIF2α and/or PKR directly and whether binding is specific and sufficient for PK2 inhibitory function remain open questions. In this study, I employed biochemical, genetic, and biophysical approaches in combination with *in vivo* yeast toxicity experiments to study how PK2 inhibits PKR, and by extension other eIF2α kinases. (described in Chapter 3).

The following are my specific project aims:

1) Express and purify PK2, eIF2α, and PKR recombinant proteins

2) Determine whether PK2 directly and specifically binds eIF2α and/or PKR *in vitro*

3) Map residues and/or regions on PK2 important for eIF2α kinase inhibitory function

4) Determine whether PK2 directly inhibits PKR kinase activity in a reconstituted system
Chapter 2
Materials and Methods

Protein Expression, Mutagenesis, and Purification

Expression and purification of human PKR^{KD} (a.a. 258-551, H412N, β4-β5 kinase insert delete) and yeast eIF2α (a.a. 3-175) was performed as described previously (Dar et al. 2005). Wildtype PK2 was expressed in *E.coli* BL21 cells as a N-terminal 6XHis-tagged fusion protein using the pProEx vector system. Cell cultures were grown to O.D. 0.8 in LB medium and induced with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18°C overnight. The bacterial pellet was isolated by centrifugation and resuspended in lysis buffer (1XPBS pH7.3, 300mM NaCl 3mM TCEP, 1mM PMSF) and purified by Ni-affinity chromatography Ni-NTA Superflow (Qiagen) according to the manufacturer’s instructions at 4°C. The eluted PK2 fusion protein was cleaved with TEV protease, dialyzed in lysis buffer and subjected to Ni-affinity subtraction purification to yield untagged PK2 protein in the flow through. Finally, PK2 was applied to a Superdex 75 gel filtration column at 4°C in running buffer 0.1M HEPES pH7, 100mM NaCl, 1mM TCEP for final purification and concentration.

The Quickchange kit (Stratagene, LaJolla, CA) was used to generate PK2 site-directed mutants. Expression and purification of these proteins were performed as described for wildtype PK2. The PK2 N-terminal deletion mutants were generated by PCR and sub-cloning the desired construct into the pProEx vector for expression and purification as described above.

The minimal PK2 N-terminal extension constructs with a non-cleavable C-terminal MBP tag was expressed and purified as a N-terminal GST-TEV tagged fusion protein. The expression and purification protocol was performed as described for human PKR with the following exception. C-terminally tagged PK2-MBP was released from its N-terminal GST tag by addition of TEV. The PK2-MBP protein was then subjected to MBP affinity purification to remove the TEV protease and contaminants.
GST-Pull Down Assay

In a 50 µL GST-pull down assay reaction using wash buffer (20mM HEPES pH 7.0, 100mM NaCl, 1 mM DTT, 1% v/v Glycerol), 10 µL bed volume of Glutathione-Sepharose 4B resin (GE Healthcare), 10µg of GST-fusion bait protein, and 50µg of untagged prey protein (PK2) were incubated at 25°C for 20 minutes with constant nutation. The reactions were then washed with 3X500mL wash buffer, mixed with 10µL 6X SDS loading buffer, boiled, and electrophoresed on 15% SDS polyacrylamide gels (Novex, Invitrogen). Proteins were visualized using coomassie blue staining.

MBP Pull Down Assay

In a 60µL MBP-pull down assay reaction using wash buffer (20mM HEPES pH 7.0, 100mM NaCl, 1 mM DTT, 1% v/v Glycerol), 10µL bed volume Amylose resin (NEB), 10µg of MBP-fusion bait protein, and 50µg of untagged prey protein were incubated at 25°C for 20 minutes with constant nutation. The reactions were then washed with 3X500mL wash buffer, mixed with 12µL 6X SDS loading buffer, boiled, and electrophoresed on 15% SDS polyacrylamide gels (Novex, Invitrogen). Proteins were visualized using coomassie blue staining.

PKR-PK2 Gel Filtration Co-elution Assay

500mL volumes of either 100µM PKR<sup>KD</sup> alone, 100µM PK2 alone, or a 1:1 stoichiometric 100µM PKR<sup>KD</sup>-PK2 complex were applied to a Superdex 75 gel filtration column at 4°C (buffer: 0.1M HEPES pH7, 100mM NaCl, 1mM TECP).

Limited Proteolysis of PK2 and Proteolytic Protection Assays

In a 30µL reaction, 2µg of wildtype PK2 protein was incubated at 25°C for 1hour in the absence or presence of 1ng thermolysin, chymotrypsin, or trypsin. The trypsin proteolyzed PK2 fragment was subjected to Edman sequencing. For the Proteolytic protection assay, proteolysis was performed as described with the exception of adding
either equimolar or four fold molar excess of either PKR$^{KD}$ or a 33kDa protein that does not to bind PK2. The reactions were then mixed with 6µL 6X SDS loading buffer, boiled, and electrophoresed on 15% SDS polyacrylamide gels (Novex, Invitrogen). Proteins were visualized using coomassie blue staining.

**eIF2α phosphorylation in vitro kinase assay**

In a 60µL reaction volume, 20nM PKR$^{KD}$ was incubated with 3µM wildtype or Ser51Ala mutant yeast eIF2α in the absence or presence of wildtype or mutant PK2s for 15 minutes at 25°C. Reaction buffer for the kinase reaction consists of 0.5mM PMSF, 10mM MgCl$_2$, 2mM ATP, 60mM HEPES pH7.5, 50mM NaCl, 0.2mM TCEP. The reactions were then mixed with 12µL 6X SDS loading buffer, boiled, and electrophoresed on 15% SDS polyacrylamide gels (Novex, Invitrogen). Phosphorylation of Ser51 on eIF2α was detected by immunoblot using primary anti-phospho-Ser51-eIF2α antibody from Cell Signaling (product #9721), followed by secondary HRP-conjugated goat anti-rabbit antibody from Pierce (product #1858415)

**Autophosphorylation in vitro Kinase Assay**

To produce proteins in their dephosphorylated states, human GST-PKR$^{KD}$ or mouse PERK$^{KD}$ were treated with recombinant 6xHis-tagged Lamda phosphatase in dephosphorylation buffer (50mM HEPES pH7.0, 100mM NaCl, 2mM MnCl$_2$, 1mM DTT), followed by Ni-affinity subtraction to remove the phosphatase. In a 60µL reaction, dephospho-GST-PKR$^{KD}$ was incubated in reaction buffer (0.5mM PMSF, 10mM MgCl$_2$, 2mM ATP, 60mM HEPES pH7.5, 50mM NaCl, 0.2mM TCEP, 1mM sodium orthovanadate) for 50 minutes at 30°C, in the absence or presence of wildtype or mutant PK2 proteins. In an analogous 60µL reaction, dephospho-PERK$^{KD}$ was incubated in reaction buffer (0.5mM PMSF, 10mM MgCl$_2$, 2mM ATP, 60mM HEPES pH7.5, 50mM NaCl, 0.2mM TCEP, 1mM sodium orthovanadate) for 20 minutes at 25°C, in the absence or presence of wildtype or mutant PK2 proteins. The reactions were then mixed with 12µL 6X SDS loading buffer, boiled, and electrophoresed on 15% SDS polyacrylamide gels (Novex, Invitrogen). Phosphorylation of Thr446 on PKR was detected by
immunoblot using primary anti-phospho-Thr446-PKR antibody from Cell Signaling (product #3076), followed by secondary HRP-conjugated goat anti-rabbit antibody from Pierce (product #1858415). Phosphorylation of Thr980 on PERK was detected using primary anti-phospho-Thr980-PERK antibody from Cell Signaling (product #9451), followed by secondary HRP-conjugated goat anti-rabbit antibody from Pierce (product #1858415)

**Yeast Toxicity in vivo Assay (Performed in collaboration with the Dever Lab)**

Vector (pEMBLyex4), and wildtype (pC201) or mutant PK2 expression plasmids were individually transformed into yeast strain H2544 (Mat*a ura3-52 trp-63 leu2-3, -112 <GAL-CYC1-PKR, LEU2>), and transformants were replica plated to glucose (SD) medium, where PKR and PK2 expression is repressed, or galactose medium (Sgal), where PKR and PK2 expression is induced. Plates were incubated for 2 days at 30°C for SD and 3 days at 30°C for Sgal. The Quickchange kit (Stratagene, LaJolla, CA) was used to generate PK2 site-directed mutants using the pC201 plasmid. PK2 N-terminal deletion mutants were generated by PCR and sub-cloning the desired construct into the pC201 plasmid for analysis of inhibition of PKR mediated yeast toxicity as described above.
Chapter 3
Results

3.1 PK2 resembles the C-lobe of an eIF2α family kinase domain but contains a unique N-terminal extension sequence

The DNA sequence of the baculovirus *Autographica californica* multiply embedded nuclear polyhedrosis virus (AcMNPV) contains an open reading frame (ORF123) that encodes a truncated protein kinase termed PK2. PK2 is a 25 kDa (215 a.a.) conserved protein present in other members of the MNPV baculovirus family such as *Bombyx mori*, *Rachiplusia ou*, and *Plutella xylostella*. PK2 resembles the C-terminal lobe of a prototypical protein kinase domain lacking the N-terminal lobe. Since conserved elements of both N-terminal and C-terminal lobes of the kinase domain are essential for ATP binding and phospho-transfer functions, PK2 is predicted to be kinase dead and hence relegated to the family of kinases termed pseudokinases that lack catalytic function. By BLAST analysis, PK2 shows greatest homology to the eIF2α family kinases (Dever et al., 1998). Figure 3.1a shows a multiple sequence alignment between three baculoviral PK2 orthologues, the kinase domains of each of the four human eIF2α kinase family members, and the kinase domain of an insect eIF2α kinase known as Bek. Sequence similarity between PK2 and each of the eIF2α kinase domains analyzed is comparable to that observed between pair-wise comparisons amongst the various eIF2α kinase domains themselves (approximately 24-27% identity). Interestingly, PK2 shows the highest sequence similarity to the insect eIF2α kinase Bek with 33% identity. Compared to non-eIF2α kinases, PK2 only shows approximately 19% sequence identity.

Figure 3.1b shows a homology model of PK2 based on the known crystal structure of the kinase domain of PKR. While many C-terminal lobe residues required for phospho-transfer function in genuine protein kinases are missing from PK2, such as the catalytic HRD motif (HHN in PK2) and the magnesium binding DFG motif (MFG in PK2), key secondary structural elements conserved across all prototypical kinase C-
terminal lobes are maintained. Importantly, the eIF2α kinase specific substrate recognition infrastructure centered on helix αG, which is one turn longer and tilted by 40 degrees in eIF2α kinases, appears to be conserved in PK2, suggesting that it may be able to recognize eIF2α (Fig 3.1, brown box). In addition to an eIF2α kinase-like C-terminal lobe fold, PK2 also possesses a unique 22 residue N-terminal extension that does not appear to correspond to any remnants of a kinase N-terminal lobe (Fig 3.1, orange box).
Figure 3.1 PK2 resembles the C-terminal lobe of an eIF2α kinase domain. A. Structure-based sequence alignment of three PK2 homologues from baculoviruses *B. mori*, *A. californica*, and *P. xylostella* with five eIF2α kinase domains from human PERK, HRI, PKR, GCN2, and insect *B. Mori*. Bek (HRI homologue). Secondary structural elements are represented by block arrows for β-sheets and rounded rectangles for α-helices. Residues conserved in all kinase domains are colored red while residues conserved specifically amongst eIF2α kinases are colored yellow. Conservation of the eIF2α recognition element (αF-αG linker and helix αG) is highlighted by a brown box. The PK2 unique N-terminal extension sequence is highlighted by an orange box. B. Homology model of PK2 structure based on a sequence alignment with the PKR kinase domain (PDB: 2A19). eIF2α is colored magenta while PKR N-terminal and C-terminal lobes are colored cyan and blue, respectively (left). PK2 is colored yellow (right). Importantly, PK2 also contains an eIF2α kinase specific, substrate recognition infrastructure centered on helix αG, as shown in brown. A unique extension peptide located at the N-terminus of PK2 is shown by a dashed light orange line, for which no homologous secondary structure could be modeled based on PKR sequence.
3.2 PK2 directly binds PKR kinase domain but not eIF2α in vitro

Previous in vivo studies showed that PK2 binds the isolated kinase domain of PKR (PKR\textsuperscript{KD}) by co-immunoprecipitation (Dever et al., 1998). My sequence analysis of PK2 predicts that it may bind eIF2α through a conserved infrastructure centered on helix αG. These observations raise the possibility that PK2 inhibits eIF2α signaling by directly binding the kinase domain of PKR, and/or by sequestering the substrate eIF2α. To determine whether PK2 directly binds to PKR and/or eIF2α, we employed in vitro binding assays using bacterially expressed and purified proteins. In a GST-pull down assay, both phosphorylated and dephosphorylated GST-PKR\textsuperscript{KD}, but not GST-eIF2α detectably bound to wildtype PK2 (Fig 3.2a lanes 6-8). Moreover, the PKR\textsuperscript{KD}-PK2 interaction was stable to co-purification by size exclusion chromatography with an apparent 1:1 stoichiometry. (Fig 3.2b) Surprisingly, in a reciprocal pull-down experiment, N-terminally tagged GST-PK2 did not bind to PKR\textsuperscript{KD}. (Fig 3.2c right panel lane 12) However, when an affinity MBP tag was placed at the C-terminus of PK2, I observed strong binding to PKR\textsuperscript{KD}. (Fig 3.2c left panel lane 5) The differential effect of tag placement on binding hinted at a potential role for PK2’s N-terminus in PKR recognition. Finally, although we could not detect binding between GST-PK2 and eIF2α, I could not definitively rule out a low-affinity transient interaction that is not detectable using my in vitro binding assays, since the positive control GST-PKR\textsuperscript{KD} also demonstrated no binding signal to eIF2α (Fig 3.2d lanes 5,7). Interestingly, a direct in vitro binding signal has never been shown between PKR and eIF2α, and since PKR is well appreciated to bind eIF2α through its substrate recognition infrastructure centered on helix αG, PK2 could still conceivably interact weakly with eIF2α through the same mechanism.
Figure 3.2

A

 GST-PKR
 GST-PKR K296R
 GST-eIF2α
 GST PK2

 Loading

 1 2 3 4 5 6 7 8 9

 kDa

 72 55 43 34 26

 B

 S75 Size Exclusion Chromatography

 Ve (mL) 7 8 9 10 11 12 13 14 15 16 17

 PKR-KD

 Ve (mL) 7 8 9 10 11 12 13 14 15 16 17

 PK2

 Ve (mL) 7 8 9 10 11 12 13 14 15 16 17

 PKR-KD

 PK2

 C

 PKC-Cterminal MBP
 MBP
 PKR-KD
 eIF2α

 Loading

 1 2 3 4 5 6 7 8

 kDa

 72 55 43 34 26

 D

 Loading

 GST-eIF2α
 PKR-KD
 GST PK2

 GST-eIF2α

 PKR-KD

 GST

 eIF2α
Figure 3.2 PK2 directly binds PKR<sup>KD</sup> but not eIF<sub>2α</sub> in vitro. A. GST-Pull-down Assay. Purified wildtype PK2 was incubated with GST-PKR wildtype, GST-PKR K296R (catalytically dead), GST-eIF2α, or GST, and bound to glutathione sepharose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. B. Size exclusion chromatography co-elution assay. 60uM amounts of PK2 alone, PKR alone, or PK2-PKR complex were applied to a Superdex S75 column. Fractions corresponding to elution volumes (Ve) are shown. PK2 co-eluted stoichiometrically with PKR<sup>KD</sup>. C. Left Panel: MBP-pull down assay. Either untagged PKR<sup>KD</sup> or untagged eIF2α was incubated with C-terminally tagged PK2-MBP or MBP alone, and bound to Amylose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. Right Panel: GST-pull down assay. Untagged PKR was incubated with N-terminally tagged GST-PK2 or GST alone, and bound to sepharose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. PK2 only bound to C-terminally MBP tagged PK2 but did not bind to N-terminally GST-tagged PK2. D. GST-Pull down Assay. Either untagged PKR or untagged PK2 was incubated with GST-eIF2α or GST alone, and bound to glutathione sepharose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. Both PK2 and PKR did not show detectable binding to eIF2α.
3.3 The N-terminal extension of PK2 composes a protease sensitive region peripheral to the PK2 C-terminal lobe tertiary fold and is required but not sufficient for binding to PKR\textsuperscript{KD}.

In the crystal structure of PKR\textsuperscript{KD} in complex with eIF2α, the N-terminal lobe of PKR mediates kinase domain dimerization while the C-terminal lobe mediates eIF2α recognition though helix αG. The observation that PK2 can directly interact with PKR\textsuperscript{KD} raises the question as to how this occurs given that PK2 lacks a recognizable N-terminal lobe. Since by sequence alignment analysis (Fig 3.1), PK2 has a 22-residue N-terminal extension in place of a kinase N-terminal lobe, we investigated the importance of this extension sequence in PKR recognition. In an \textit{in vitro} pull-down assay, wildtype but not N-terminally truncated (N\textsubscript{Δ}-L22) PK2 bound GST-PKR\textsuperscript{KD}, which demonstrates that the N-terminal extension of PK2 is directly required for PKR recognition (Fig 3.3a lanes 5-6). To ascertain whether the PK2 N-terminal extension is an independent external element, or an integral part of the PK2 kinase C-terminal lobe fold, we performed limited proteolysis. Using dilute concentrations of three different proteases, wildtype PK2 was cleaved from a 25kDa full-length form into a smaller 22kDa protease resistant form (Fig 3.3b). By Edman sequencing, the trypsin cleavage site was mapped to Arg15. Therefore, the N-terminal extension of PK2 is extraneous to the core fold of the kinase C-terminal lobe-like domain. Interestingly, the PK2 N-terminal extension becomes resistant to protease attack when PKR\textsuperscript{KD} is added to the cleavage reaction mixture, further supporting its involvement in PKR binding (Fig 3.3c lanes 5, 11). To investigate whether the N-terminal extension of PK2 alone, in the absence of the core C-terminal lobe domain of PK2, is sufficient to bind PKR\textsuperscript{KD}, I expressed and purified C-terminally MBP-tagged PK2 (a.a. 1-22, a.a.1-44, or full-length a.a. 1-215) and evaluated binding to free PKR\textsuperscript{KD}. While full length PK2-MBP (a.a. 1-215) bound PKR\textsuperscript{KD}, the N-terminal extension fragments of PK2 (a.a. 1-22 or a.a.1-44) did not bind PKR\textsuperscript{KD}. (Fig 3.3d lanes 6-8) Taken together, these results show that the N-terminal extension of PK2 is an external, peripheral element to the core PK2 C-terminal lobe fold, and that the PK2 N-terminal extension is required, but not sufficient for high affinity binding to PKR\textsuperscript{KD} \textit{in vitro}. Furthermore, since the minimal N-terminal extension alone cannot engage PKR\textsuperscript{KD}, the kinase C-lobe domain of PK2 is also inferred to participate in PKR binding.
Figure 3.3

A. GST-Pull-down Assay. Purified wildtype PK2 and N-terminal delete PK2 (ΔL22 PK2) were competitively incubated with GST-PKR wildtype, GST-PKR K296R (catalytically dead), GST-elf2α, or GST. Samples were bound to glutathione sepharose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. Only wildtype PK2 bound GST-PKR KD.

B. PK2 limited proteolysis. 2µg wildtype PK2 was incubated with 2ng of protease (trypsin, chymotrypsin, or thermolysin) for 30 min and samples were analyzed by SDS-PAGE analysis and coomassie staining. Edmon sequencing of the protease resistant band from the trypsin digest shows the protease cut site to be located at Arg15.

C. PK2 N-terminal extension proteolytic protection assay. 2µg of wildtype PK2 was subjected to chymotrypsin proteolysis in the presence or absence of equimolar or four-fold molar excess of PKR KD. As shown, PKR was able to protect PK2 from proteolysis of its N-terminus, while a non-PK2 binding protein was not.

D. MBP pull-down assay. Purified PKR and elf2α were competitively incubated with C-terminal MBP-tagged full length PK2, PK2 a.a. 1-22, PK2 a.a. 1-40, or simply MBP alone. Samples were bound to amylose resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. The minimal PK2 N-terminal extension constructs were insufficient for PKR binding.

Figure 3.3 The PK2 N-terminal extension is peripheral to its core C-terminal lobe fold and is required but not sufficient for PKR KD binding in vitro.  

3.4 The PK2 N-terminal extension, but not its putative eIF2α recognition site helix αG, is required for inhibitory function in yeast

PK2 may function by targeting the kinase PKR or the substrate eIF2α, or both. My initial in vitro studies showed that PK2 directly binds the kinase domain of PKR in a manner requiring its N-terminal extension. However, no direct interaction was detected between PK2 and the substrate eIF2α, although binding could not be definitively ruled out. Indeed, the predicted presence of an eIF2α kinase-specific helix αG required for eIF2α recognition suggests that a transient PK2-eIF2α interaction may still be possible. To explore the relative importance of the PK2 N-terminal extension (required for PKR binding) or the PK2 helix αG (required for putative eIF2α binding) for PK2 function, I designed a series of PK2 mutants that disable either PKR binding or putative eIF2α binding, and tested for their effects on function compared to wildtype PK2.

Firstly, to disable PKR binding, I designed PK2 N-terminal truncation mutants. The largest N-terminal truncation of 22-residues (N-ΔL22-PK2) was previously shown to abolish PK2 binding to GST-PKRKD in vitro (Fig 3.3c). Secondly, to disable putative eIF2α binding, I generated three PK2 mutants, namely T147D, T147A, and T147Q, which are predicted to disrupt helix αG function without disrupting the overall PK2 fold (Note: A fourth control mutant, PK2 E150A is situated in helix αG adjacent to the critical T147 residue, but is not expected to disrupt helix αG function). Indeed, the analogous αG Thr to Asp mutation is well appreciated to strongly disable eIF2α recognition by PKR without disrupting PKR fold (Dar et al., 2005; Dey et al., 2005). Finally, to test for PK2 loss of function, a yeast model system was adopted in collaboration with Dr. Thomas Dever (Dever et al. 1998). In yeast, exogenous expression of human PKR is toxic due to sustained phosphorylation of Ser51 on yeast eIF2α and subsequent inhibition of translation (Fig 3.4a row 1). Co-expression of wildtype PK2 in the same system suppresses PKR mediated toxicity and rescues growth (Fig 3.4a row 2). Using this experimental setup, wildtype PK2 was compared to either T147D/A/Q PK2 or N-terminally truncated PK2 for their relative abilities to inhibit PKR. Interestingly, all PK2 N-terminal truncation mutants, including deletions of as few as six amino terminal...
residues, showed a striking loss of ability to inhibit PKR mediated toxicity \textit{in vivo} (Fig 3.4a rows 3-8). In contrast, mutations of the putative eIF2\(\alpha\) binding infrastructure (helix \(\alpha G\) T147A, T147D, T147Q, E150A) did not disrupt PK2 inhibitory function \textit{in vivo}. (Fig 3.4a rows 9-12) Analogous results for PK2 \(\alpha G\) or N-terminal truncation mutants were observed using growth as a measure of yeast viability. (Fig 3.4b) Taken together, these results indicate that PK2 inhibits PKR signaling by directly binding PKR\textsuperscript{KD} and not by sequestering the substrate eIF2\(\alpha\) (Fig 3.4c).

**Figure 3.4**

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.4.png}
\caption{The PK2 N-terminal extension, but not the PK2 putative eIF2\(\alpha\) recognition element helix \(\alpha G\), is required for inhibitory function in yeast. A. PKR yeast toxicity assay. Galactose inducible expression of PKR in the yeast strain H2544 (\textit{Mata} \textit{ura3-52 trp-63 leu2-3, -112 <GAL-CYC1-PKR, LEU2>}) is toxic due to sustained eIF2\(\alpha\) phosphorylation. Transformation of an empty control vector (pEMBLyex4) has no effect on toxicity, while introducing a vector expressing PK2 counteracts PKR mediated yeast toxicity. PK2 mutants that lack the N-terminal extension, but not mutations in the \(\alpha G\) helix, are deficient for the ability to inhibit PKR mediated yeast toxicity. B. Growth curves of yeast transformants as described in 3.4a over 25 hours.}
\end{figure}
3.5 Detailed mutational analysis of the PK2 N-terminal extension reveals a binding element centered on Phe18 and an inhibitory element centered on Lys2 and Pro3

In our yeast toxicity studies, all PK2 N-terminal truncation mutants tested showed a striking loss of ability to inhibit PKR \textit{in vivo}. N-terminal deletion of as few as six residues was sufficient to abolish PK2 function. Using PK2 truncation mutants of various lengths, I investigated whether loss of function \textit{in vivo} correlated with compromised PKR binding \textit{in vitro}. Surprisingly, N-terminal deletion of the first 6 or 9 residues, which showed a strong loss of function phenotype in yeast, did not affect \textit{in vitro} binding to GST-PKR$^{\text{KD}}$ (5a lane 3-4); however, further deletion of 19 or 22 amino acids from the PK2 N-terminus abolished binding (Fig 3.5a lanes 5-6). These observations localize an important PKR binding element to a region encompassing PK2 residues 9 to 22, and interestingly, a PKR inhibitory element to a region encompassing PK2 residues 1 to 6 that is dispensable for PKR binding \textit{in vitro} but indispensable for inhibitory function in yeast.

To more finely probe residues important for PKR binding \textit{in vitro}, I performed an Alanine scan on the N-terminal extension of PK2 spanning residues 9 through 22. Consistent with my previous \textit{in vitro} binding analysis by PK2 truncation, I found a single amino acid substitution F18A in PK2 that abolished its ability to bind GST-PKR$^{\text{KD}}$ (Fig 3.5b lane 11). Moreover, further analysis revealed that PK2 F18A is not protected by PKR$^{\text{KD}}$ from N-terminal proteolysis (Fig 3.5c lane 8); and importantly, PK2 F18A also cannot inhibit PKR mediated toxicity in yeast (Fig 3.5d row 4). Interestingly, unlike PK2 F18A, mutation of phenylalanine 18 to isoleucine (F18I) had no effect on PK2 inhibitory function in yeast (Fig 3.5d row 3). The molecular basis for the requirement of a hydrophobic residue at position 18 of PK2 for its inhibitory function is not understood. Taken together, these results localize the PKR binding element to a region centered on Phe18 of PK2.

Since truncation of as few as six amino acids from the N-terminus of PK2 was sufficient to abolish its ability to inhibit PKR in yeast, I tested Alanine point mutants spanning PK2 residues 1 through 6 for ability to relieve PKR induced toxicity in yeast. Consistent with previous truncation analysis in yeast (Fig 3.4a), an inhibitory element
was located within the first six residues of PK2, centered on K2A and P3A, that is required for PK2 inhibitory function \textit{in vivo} (Fig 3.5d rows 5, 6). As expected, upon further analysis, none of the purified Alanine point mutants (residues 1-6) showed loss of binding to GST-PKR\textsuperscript{KD} \textit{in vitro}. These results localize the PKR inhibitory element to a region centered on Lys2 and Pro3 of PK2 that is dispensable for binding (Fig 3.5e).

Taken together, \textit{in vitro} binding experiments and \textit{in vivo} yeast experiments reveal that the N-terminal extension of PK2 has bipartite function, with Phe18 composing an important PKR binding element, and Lys2 and Pro3 composing an important PKR inhibitory element that is dispensable for binding.

\textbf{Figure 3.5}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.5.png}
\end{figure}
Figure 3.5 Detailed mutational analysis of the PK2 N-terminal extension reveals a binding element centered on Phe18 and an inhibitory element centered on Lys2 and Pro3

A. In vitro GST pull-down assay with various PK2 N-terminal truncation mutants using protocol as described in Fig 3.2a.  

B. In vitro GST pull-down assay with PK2 Alanine point mutants (residues 9-22) using protocol as described in Fig 3.2a.  

C. Proteolytic Protection Assay using wildtype and F18A PK2 with protocol as described in Fig 3.3b  

D. Yeast toxicity assay using protocol as described in 3.4a with PK2 point mutants F18A, F18I, K2A, P3A, E4A, Q5A, L6A.  

E. PK2 K2A, P3A, E4A and Q5A retain binding to GST-PKRKD.  GST pull-down assay was performed using protocol as described in Fig 3.2a.
3.6.1 PK2 inhibits PKR<sup>KD</sup> mediated autophosphorylation and eIF2α phosphorylation <em>in vitro</em>

Previous studies by Dever <em>et al.</em> showed that PK2 inhibits PKR autophosphorylation and eIF2α phosphorylation in yeast. Since I showed that PK2 directly binds PKR<sup>KD</sup> <em>in vitro</em>, I investigated whether PK2 binding, mediated by functional elements within the PK2 N-terminal extension, results in the <em>in vitro</em> inhibition of PKR kinase activity with respect to autophosphorylation and eIF2α phosphorylation.

In order to investigate inhibition of PKR<sup>KD</sup> autophosphorylation, GST-PKR<sup>KD</sup> was first dephosphorylated using λ phophatase, as assessed by both a downward mobility shift in SDS-PAGE analysis and a greatly reduced phosphoThr446 signal by immunoblot analysis (Fig 3.6a). Residual signal observed for dephospho-GST-PKR<sup>KD</sup> is comparable to the non-phosphorylated, catalytically inactive GST-PKR<sup>KD</sup> K296R control, and likely represents residual binding of the pThr446 primary antibody to the PKR dephospho-Thr446 epitope (Fig 3.6a).

Using dephospho-GST-PKR<sup>KD</sup> enzyme in an <em>in vitro</em> kinase assay, wildtype PK2 was found to inhibit PKR autophosphorylation at the key A-loop phospho-regulatory site Thr446 (Fig 3.6b lane 3). Moreover, consistent with previous <em>in vitro</em> binding and <em>in vivo</em> toxicity inhibition studies, PK2 mutants K2A, P3A, F18A and N-ΔL22 were deficient for the ability to inhibit GST-PKR autophosphorylation (Fig 3.6b lane 4, 5, 8). Finally, consistent with the inability of the PK2 N-terminal extension to bind PKR<sup>KD</sup>, the minimal N-terminal extension fused to MBP, did not inhibit GST-PKR<sup>KD</sup> autophosphorylation (Fig 3.6c lane 5-6).

I next investigated whether PK2 can inhibit eIF2α phosphorylation on Ser51 by activated, phosphorylated PKR. Activated PKR phosphorylated eIF2α on Ser51 in an ATP dependent manner (Fig 3.6d lanes 2-3), while mutation of Ser51 to Ala on eIF2α completely abolished Ser51 phosphorylation. (Fig 3.6c lanes 1) Wildtype PK2 inhibited eIF2α Ser51 phosphorylation by PKR to a level comparable to that of the no ATP negative control kinase reaction (Fig 3.6d lane 4). PK2 F18A and PK2 N-ΔL22, which
are compromised for PKR binding, were strongly deficient in their ability to inhibit eIF2α phosphorylation by PKR (Fig 3.6c lanes 11, 13). Interestingly, all PK2 mutants that still maintain ability to bind PKR (M1A, K2A, P3A, E4A, Q5A, T147D, N-ΔL9) show little to no loss of inhibitory function under the conditions tested (Fig 3.6d lanes 5-10, 12). Finally, consistent with the inability of the N-terminal extension to bind PKR\textsuperscript{KD}, the minimal PK2 N-terminal extension fused to MBP was not sufficient for inhibition of eIF2α phosphorylation. (Fig 3.6e lanes 6-7)

Taken together, these results show that PK2 directly inhibits PKR autophosphorylation and eIF2α phosphorylation \textit{in vitro} in a manner dependent on functional elements of the PK2 N-terminal extension. Specifically, the inhibition of PKR autophosphorylation requires both the binding element centered on Phe18 and the inhibitory element centered on Lys2/Pro3, whereas the inhibition of eIF2α phosphorylation requires only the PK2 binding element centered on Phe18.

3.6.2 PK2 does not outwardly inhibit PKR dimerization

Since back-to-back dimerization is an upstream requirement for eIF2α kinase activation by autophosphorylation on Thr446, we asked if PK2 inhibits PKR kinase activity by preventing dimerization. As coarsely assessed by non-specific cross-linking and immunoblot analysis, PKR forms a dimeric species when BS3 cross-linker is added in a concentration dependent manner (Fig 3.6f lanes 2-3). With the addition of PK2 at concentrations expected to fully inhibit PKR\textsuperscript{KD} autophosphorylation and eIF2α phosphorylation (15uM PK2 compared to 2.5uM PKR\textsuperscript{KD}), the formation of the dimeric PKR species by cross-linking is not significantly affected. (Fig 3.6f lanes 4-7). Interestingly, a 1:1 stoichiometric PK2-PKR cross-linked species also appeared, consistent with previous \textit{in vitro} binding experiments that show PK2 directly binds PKR\textsuperscript{KD}. Taken together, a crude cross-linking assay suggests that although PK2 directly binds PKR \textit{in vitro}, this binding activity does not appear to outwardly interfere with PKR’s intrinsic ability to form dimers.
Figure 3.6 PK2 inhibits PKR\textsuperscript{KD} mediated autophosphorylation and eIF2α phosphorylation \textit{in vitro}, but does not outwardly inhibit PKR dimerization by crosslinking. A. GST-PKR\textsuperscript{KD} Dephosphorylation Assay. Phospho-GST-PKR was incubated with λ phosphatase and 2mM Mn\textsuperscript{2+} as indicated. Samples were visualized using immunoblot analysis with a pThr446 specific PKR antibody. GST-PKR\textsuperscript{KD} K296R was used as a negative control. B and C. \textit{In vitro} PKR autophosphorylation assay. In a 20µL reaction at 30°C, 6µM dephosphorylated GST-PKR\textsuperscript{KD} was incubated +/- ATP in the presence of 30µM wildtype or mutant PK2 proteins. Samples were subjected to SDS-PAGE and immunoblot analysis using an anti-pThr446 PKR antibody. Phospho-signal intensities were determined relative to the no ATP reaction (Fig 3.6b lane 1) using ImageQuant software. D and E. \textit{In vitro} eIF2α phosphorylation. In a 60µL reaction at 25 °C, 10nM phosphorylated PKR\textsuperscript{KD} was incubated with 3µM eFl2α, +/- ATP in the presence of 6µM wildtype or mutant PK2 proteins. Samples were subjected to SDS-PAGE and immunoblot analysis using an anti-pSer51 eIF2α antibody. Phospho-signal intensities were determined relative to the no ATP reaction (Fig 3.6d. lane 2) using ImageQuant software. F. PKR dimerization assay by cross-linking. 15 molar excess of BS3 chemical cross-linker was added to 2.5µM PKR\textsuperscript{KD} at 25°C and incubated with and without 5µM and 15µM wildtype PK2. Samples were subjected to SDS-PAGE and immunoblot analysis using an anti-pThr446 PKR antibody.
3.7 PK2 directly binds PERK\textsuperscript{KD} and inhibits PERK\textsuperscript{KD} autophosphorylation \textit{in vitro}

Since PK2 is a baculovirus encoded protein, its target is likely an endogenous insect eIF2\(\alpha\) kinase (or multiple eIF2\(\alpha\) kinases) encoded by the insect host. Unlike vertebrates that harbor four eIF2\(\alpha\) kinases, insects lack PKR but contain GCN2, HRI (a.k.a. Bek), and PERK. My data and previous studies have shown that PK2 inhibits PKR in yeast and \textit{in vitro} (Dever \textit{et al.} 1998). Moreover, PK2 has also been found to inhibit GCN2 in yeast and Bek (insect HRI) in BmN insect cells (Dever \textit{et al.}, 1998; Prasad \textit{et al.}, 2003). Whether PK2 also inhibits PERK is unknown; however, the aforementioned studies already indicate that PK2 may be a broad-spectrum eIF2\(\alpha\) kinase inhibitor. To investigate whether the inhibitory mechanism I have elucidated underlying PK2 function is conserved to other eIF2\(\alpha\) kinases \textit{in vitro}, we investigated if PK2 can also directly bind PERK\textsuperscript{KD} and inhibit PERK autophosphorylation in a manner requiring the PK2 N-terminal extension.

Using a GST-pull down assay, I first showed that, similar to PK2 binding to GST-PKR\textsuperscript{KD}, PK2 also binds GST-PERK\textsuperscript{KD} in a manner that requires the PK2 N-terminal extension (Fig 3.7a lanes 2-3). Moreover, the minimal PK2 N-terminal extension alone was also not sufficient for PERK binding (Fig 3.7b lanes 6). In contrast to GST-PKR\textsuperscript{KD}, however, PK2 F18A show reduced but not complete loss of binding to GST-PERK\textsuperscript{KD}. (Fig 3.7a lane4). This residual binding may reflect the fact that wildtype PK2 appears to bind more tightly to GST-PERK\textsuperscript{KD} than GST-PKR\textsuperscript{KD} (Fig 3.7b lanes 2 and 6).

Since PK2 directly binds PERK\textsuperscript{KD}, we next tested whether PK2 can directly inhibit PERK autophosphorylation activity \textit{in vitro}. First, PERK\textsuperscript{KD} was fully dephosphorylated using \(\lambda\) phosphatase, as assessed by both a downward mobility shift in SDS-PAGE analysis and a near complete disappearance of phosphoThr980 signal (analogous to PKR Thr446) by immunoblot analysis (Fig 3.7c). Using dephospho-PERK\textsuperscript{KD} enzyme, we found that PK2 potently inhibits Thr980 autophosphorylation in a concentration dependent manner with an IC\textsubscript{50} of approximately 200 nM. (Fig 3.7d lanes 3-8) In addition, PK2 mutants K2A, P3A, and N-\(\Delta\)L22 proteins were all deficient for the ability to inhibit PERK\textsuperscript{KD} Thr980 autophosphorylation (Fig 3.7e lanes 5-8, 11-12). These
results show that functional elements of the PK2 N-terminal extension required for inhibition of PKR autophosphorylation are also required for inhibition of PERK autophosphorylation. Finally, consistent with the inability of the PK2 N-terminal extension to bind PERK$^{KD}$, the minimal PK2 N-terminal extension fused to MBP did not inhibit PERK$^{KD}$ Thr980 autophosphorylation (Fig 3.7f lanes 6-11).

Taken together, these results show that PK2 directly binds the kinase domain of PERK and inhibits autophosphorylation on its key A-loop regulatory site Thr980. As observed for PKR, functional elements within the PK2 N-terminal extension are also required but not sufficient for inhibition of PERK. These results demonstrate that PK2 employs a common mechanism for eIF2α kinase inhibition.
Figure 3.7 PK2 binds PERK\(^{KD}\) and inhibits PERK\(^{KD}\) Thr980 autophosphorylation \textit{in vitro}.  
\textbf{A.} GST-PERK\(^{KD}\) pull-down assay. GST-PERK\(^{KD}\) was immobilized and evaluated for ability to bind wildtype, N-AL22, or F18A PK2 proteins. \textbf{B.} MBP pull-down assay. Purified PERK kinase domain was incubated with C-terminal MBP-tagged full length PK2, PK2 a.a. 1-22, PK2 a.a. 1-40, or simply MBP alone. Samples were bound to amylase resin as indicated, washed, and then subjected to SDS-PAGE analysis and coomassie staining. \textbf{C.} PERK\(^{KD}\) Dephosphorylation Assay. Phospho-PERK\(^{KD}\) was incubated with \(\lambda\) phosphatase and 2mM Mn\(^{2+}\) as indicated. Samples were visualized using immunoblot analysis with pThr980 specific PERK antibody. \textbf{D.} \textit{In vitro} PERK autophosphorylation assay. In a 60\(\mu\)L reaction at 20\(^\circ\)C, 0.2\(\mu\)M dephosphorylated PERK\(^{KD}\) was incubated +/- ATP in the presence of increasing amounts of wildtype PK2. Samples were subjected to SDS-PAGE and immunoblot analysis using anti-pThr980 PERK antibody. \textbf{E} and \textbf{F.} \textit{In vitro} PERK autophosphorylation assay. In a 60\(\mu\)L reaction at 20\(^\circ\)C, 0.2\(\mu\)M dephosphorylated PERK\(^{KD}\) was incubated +/- ATP in the presence of various wildtype or mutant PK2. Samples were subjected to SDS-PAGE (lower panel) and immunoblot (upper panel) analysis as previously described.
Chapter 4
Discussion

4.1 Binding and Inhibitory Elements of the PK2 N-terminal Extension

PK2 is a truncated pseudokinase that inhibits eIF2α kinase signaling. In this study, we used both *in vitro* and *in vivo* approaches to probe PK2 mechanism of action, and found a labile region at the N-terminus of PK2 critical for its function, which we termed the PK2 N-terminal extension.

Using *in vitro* binding assays, we first found that PK2 directly binds the kinase domain of the eIF2α kinase PKR in a manner requiring the N-terminal extension of PK2. However, the N-terminal extension alone was not sufficient for binding. Detailed mutagenesis analysis of the N-terminal extension revealed a binding element centered on Phe18 that is essential for high affinity binding to PKR$^{KD}$.

In an *in vivo* yeast toxicity assay, we validated our *in vitro* binding data and confirmed the importance of the PK2 N-terminal extension for PKR inhibition. In this *in vivo* yeast assay, toxicity mediated by over-expression of PKR can be reversed by co-expression of PK2. We found that the N-terminal extension of PK2, specifically the binding element centered on Phe18, was required for this activity. Unexpectedly, we also uncovered an additional functional element in the N-terminal extension of PK2 centered on Lys2 and Pro3 that was also required for PKR inhibitory function in yeast but upon subsequent analysis, was dispensable for PKR binding *in vitro*.

Taken together, our *in vitro* and *in vivo* studies show that the N-terminal extension of PK2 is important and has bipartite functions: one region centered on Phe18 is required for high affinity binding to PKR, while a second region centered on Lys2 and Pro 3 is important for PKR inhibition but dispensable for PKR binding.
4.2 Mechanism of eIF2α kinase inhibition by PK2

Since PK2 binds PKR in vitro in a manner requiring the binding element Phe18 and inhibits PKR in vivo in a manner requiring both the Phe18 binding element and an additional inhibitory element centered on Lys2/Pro3, I investigated whether these functional elements of the PK2 N-terminal extension mediate inhibition of PKR kinase activity in vitro. I found that PK2 directly inhibited autophosphorylation of PKR on Thr446 within the activation loop of the kinase domain. This inhibitory activity was dependent on both the Phe18 binding element and the Lys2/Pro3 inhibitory element. By inhibiting PKR A-loop autophosphorylation, PK2 can prevent PKR activation and restrict the kinase domain in a catalytically repressed state. (Fig 4.1) In addition to its ability to inhibit PKR autophosphorylation, I also found that PK2 inhibited activated PKR from phosphorylating its substrate eIF2α on Ser51. However, this inhibitory function is solely dependent on the Phe18 binding element, and not the Lys2/Pro3 inhibitory element. By also inhibiting activated PKR from phosphorylating eIF2α, PK2 may provide a two-pronged mechanism to prevent eIF2α phosphorylation and downstream translation inhibition (Fig 4.1).

Taken together, my results reveal a dual mechanism of PK2 inhibitory function that ensures shutdown of eIF2α signaling from both inactive and activated PKR. In addition, the different regions of PK2 required for inhibition suggests that PK2 inhibits PKR autophosphorylation in a manner that is distinct from how it inhibits eIF2α phosphorylation.
Figure 4.1. Mode of PKR binding and inhibition by pseudokinase PK2. PK2 is shown in yellow and contains an important N-terminal extension sequence colored orange. The binding element Phe18 (orange box) is required for high affinity binding to PKR, while the inhibitory element Lys2/Pro3 (yellow boxes) are required for inhibition of PKR in yeast. The PKR N-terminal and C-terminal lobes are shown in cyan and blue, respectively, while eIF2α is shown in magenta. In this inhibition model, PK2 can bind to dephosphorylated PKR and prevent PKR activation by autophosphorylation at the key A-loop regulatory site Thr446. In addition, PK2 can also bind phosphorylated PKR and inhibit its ability to catalyze phosphorylation of its substrate eIF2α at the translational regulatory site Ser51.
4.3 The eIF2α kinase fold of PK2

We initially postulated that the tertiary structure of PK2 (kinase domain C-terminal lobe) may act to sequester eIF2α via its eIF2α recognition infrastructure centered on helix αG. However, using *in vitro* pull-down assays, we could not detect binding between PK2 and eIF2α. In addition, targeted mutation of the proposed eIF2α recognition infrastructure T147D/A/Q on helix αG did not perturb PK2 inhibitory function in yeast (analogous mutation on PKR T487D strongly perturbs eIF2α recognition by PKR without affecting PKR autophosphorylation activity: Dar *et al.*, 2005; Dey *et al.*, 2005). These observations disfavor a model whereby PK2 acts in part by a mechanism of substrate sequestration. Instead, my studies reveal a direct role for the PK2 N-terminal extension in binding and inhibition of PKR$^{KD}$ *in vitro* and *in vivo*. These results raise the question as to why the kinase C-terminal lobe fold is conserved at all in PK2 orthologues. Partially addressing this question, I demonstrated that although the PK2 N-terminal extension is required, it is not sufficient for binding or inhibition of PKR$^{KD}$. These results indicate that the kinase-like fold of PK2 is likely functional and mechanistically involved in PKR inhibition. How precisely the N-terminal extension and the kinase C-terminal lobe of PK2 function to inhibit PKR awaits the high-resolution structure of the PKR-PK2 complex.

4.4 Pseudokinases can allosterically regulate bona fide kinases

10% of all human protein kinases are designated pseudokinases because they lack one or more conserved residues required for ATP binding and/or catalytic function (Manning *et al.*, 2002). Presumed to be catalytically dead, pseudokinases were initially thought to function as passive scaffolds or adaptors (Pawson and Scott, 1997); however, recent findings suggest that some pseudokinases actually possess ATP binding and kinase regulatory functions (Zeqiraj *et al.*, 2009; Zeqiraj and Van Aalten, 2010). An emerging framework for re-evaluating pseudokinase function is highlighted by the finding that some pseudokinases allosterically activate or inhibit their kinase-competent counterparts. For example, the human epidermal (HER) family of tyrosine receptor kinases contains
the Her3/Erb3 pseudokinase that forms heterodimers with their genuine kinase counterparts Her1/Erb1, Her2/Erb2, and Her4/Erb4 (Shi-Fumin et al., 2009). The C-terminal lobe of the Her3 pseudokinase domain directly binds the N-terminal lobe of other Her1, Her2, or Her4 kinase domains, which results in the allosteric re-orientation of helix αC that is permissive for catalytic activity. As such, Her3 is a direct allosteric activator of Her1, 2 and 4 kinase activities.

Another well-studied example of a kinase domain regulation by a pseudokinase domain consists of the Janus family of Kinases (JAKs). JAK kinases contain an N-terminal pseudokinase domain JH2 that binds and inhibits the catalytic activity of the C-terminal kinase domain JH1 (Pipsa et al., 2003). Interestingly, this auto-inhibitory mechanism requires the C-terminal lobe of the JH2 pseudokinase domain in vivo, while the JH2 N-terminal lobe is dispensable for function. Therefore, JAK JH1 is an allosteric inhibitor of JAK JH2 kinase activity. The structural basis by which this inhibitory activity is achieved remains to be determined.

Her3 and JAK-JH2 are examples of pseudokinase domains that modulate bona fide kinase domain activities. PK2 is also a pseudokinase, but it differs from all previously examined pseudokinases for which mechanism of action has been studied. Specifically, whereas the entire kinase domain fold is maintained for most pseudokinases, PK2 contains only the C-terminal lobe of the kinase domain. Consistent with PK2 being deficient for phospho-transfer function (as it lacks the kinase N-terminal lobe), PK2 also lacks both the DFG metal binding loop and the HRD catalytic loop, which are typical features of the C-terminal lobes of active protein kinases. Whether PK2 functions by allosteric regulation of eIF2α kinases remain an open question; but consistent with this model, PK2 binds the kinase domain of eIF2α kinases such as PKR and PERK and is able to repress their catalytic activities. Understanding exactly how the pseudokinase PK2 engages eIF2α kinases to perturb catalytic function will require the atomic structure of PK2 in complex with an eIF2α kinase domain. Moreover, elucidating the underlying mechanism of action of PK2 may provide insight into the normal functions of eIF2α kinases in general, since PK2 likely mimics/short-circuits normal eIF2α kinase function.
4.5 PK2 and eIF2α kinase specificity

PKR plays a pivotal role in innate immunity by regulating translation during viral invasion. In order to gain a selective advantage over their hosts, many mammalian viruses have targeted PKR for inhibition. PK2 is an eIF2α kinase inhibitor that independently evolved in insect viruses. Since insects lack an obvious orthologue of PKR (i.e. an eIF2α kinase that possesses dsRNA binding domains), PK2’s natural target must be another member of the eIF2α kinase family that is present in the insect host, such as GCN2, PERK, or Bek (an insect HRI homologue). My studies and previous studies have shown that PK2 binds and inhibits both PKR\textsuperscript{KD} and PERK\textsuperscript{KD} (Dever \textit{et al.} 1998). Moreover, PK2 has also been found to inhibit GCN2 in yeast and Bek (insect HRI) in BmN cells (Dever \textit{et al.}, 1998; Prasad \textit{et al.}, 2003). Taken together, these results indicate that PK2 may be a broad-spectrum eIF2α kinase inhibitor.

When baculovirus infects insect hosts, PK2 may function to inhibit endogenous eIF2α kinase activity to facilitate virus propagation. Consistent with a productive role for PK2 in baculovirus infection, a previous study showed that \textit{B. mori} nuclear polyhedral virus (BmNPV) deleted for PK2 has reduced budded virus production in silkworm \textit{B. mori} BmN cells (Katsuma \textit{et al.}, 2008). Our current model suggests that PK2 may inhibit eIF2α kinases by direct binding to their kinase domains via the N-terminal extension of PK2. An ultimate test of the structure/function relationships I discovered in my studies would be to introduce PK2 mutants (F18A, K2A, P2A, NΔL22) into the BmN cell infection system and evaluate their respective function as compared to wildtype PK2.
eIF2α kinases are evolutionarily conserved enzymes that initiate stress response signaling via inhibition of cellular translation. The representation of eIF2α kinase family members varies from unicellular eukaryotes to metazoans (Zhan et al., 2004). For example, GCN2 is represented in all eukaryotes, while PKR is only present in higher vertebrates such as mammals and fish. HRI and PERK, the other two well-characterized eIF2α kinase family members, are periodically distributed throughout the eukaryotic lineage. The conservation at least one eIF2α family kinase member in all eukaryotic species studied to date highlights the importance of translation inhibition as an adaptive stress-response strategy.

Misregulation of eIF2α kinases has been implicated in many human diseases such as cancer, neurodegeneration, and diabetes (Takahisa et al., 2010; Scheper et al., 2007; Shi et al., 2003; Peel, 2004). As a result, tremendous effort has been devoted to understanding the underlying regulatory and catalytic switching mechanisms of these protein kinases. In particular, PKR has been used extensively as a model to study eIF2α kinase regulation in the context of translation inhibition in response to viral invasion.

In order to gain a selective advantage over hosts, viruses are known to encode specific factors that inhibit PKR signaling by short-circuiting or mimicking PKR natural functions (i.e. Vaccinia virus K3L mimics eIF2α and reveals molecular determinants required for PKR\textsuperscript{KD} substrate recognition; Dar et al., 2005). Thus, we can learn much about PKR normal functions by studying how viruses overcome them. In collaboration with the Thomas Dever lab, my studies have shown that PK2 is a viral factor that
directly inhibits PKR autophosphorylation and substrate phosphorylation. Structurally
distinct from other viral factors characterized to date, PK2 has a core fold that mimics the
C-terminal lobe of an eIF2α kinase domain. Intriguingly, PK2 also contains a labile N-
terminus for which I have shown to be required for its inhibitory function in
vitro and in vivo. Specifically, the N-terminal extension has bipartite functions, with one
region centered on Phe18 required for binding of PKR$^{KD}$, while another region centered
on Lys2 and Pro3 required for inhibition of PKR$^{KD}$ but dispensable for binding. Exactly
how PKR is affected by PK2 binding and what role does the PK2 C-terminal lobe play in
this inhibitory model are future areas of investigation. Our long term goal and hope is
that by studying how PK2 functions as an eIF2α kinase inhibitor, we can gain a better
understanding of the mechanisms of regulation underlying eIF2α kinase function.

*Arthur Kornberg*, Nobel Laureate 1959:

“Depend on viruses to open windows”
References


initiation factor 2B (eIF2B) recognize overlapping surfaces on eIF2alpha. Mol Cell Biol 25, 3063-3075.


(eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol Cell Biol 21, 5018-5030.


