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REGULATION OF PROTEIN SYNTHESIS
AND INDUCTION OF ONCOGENESIS
BY A CELLULAR PROTEIN KINASE
INHIBITOR

by

Norina Mei Ngon Tang

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by



Chairperson of Supervisory Committee

Program Authorized
to Offer Degree

Microbiology

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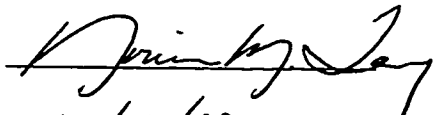
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Doctoral Dissertation

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Abstract

REGULATION OF PROTEIN SYNTHESIS
AND INDUCTION OF ONCOGENESIS BY A
CELLULAR PROTEIN KINASE INHIBITOR

by Norina Mei Ngon Tang

Chairperson of the Supervisory Committee: Professor Michael G. Katze
Department of Microbiology

P58^{IPK} is a cellular inhibitor of the interferon-induced, dsRNA-activated, serine/threonine kinase, PKR. Earlier, P58^{IPK} was shown to block both PKR autophosphorylation and the phosphorylation of eIF-2 α substrate *in vitro*, in a dose-dependent manner. Furthermore, murine cell lines overexpressing P58^{IPK} were tumorigenic in nude mice, suggesting that P58^{IPK} is an oncoprotein. The mechanism by which P58^{IPK} inhibits PKR is unknown. In order to elucidate this mechanism as well as define the physiological role of the P58^{IPK} protein, a structure/function study of the P58^{IPK} molecule was undertaken. Using site directed mutagenesis, eleven different P58^{IPK} mutants were generated. Using both *in vitro* and *in vivo* assays, each of the mutants was characterized for the ability to block PKR phosphorylation activity *in vitro*, stimulate cellular protein synthesis *in vivo*, and induce cellular transformation and tumorigenesis in nude mice. The findings presented in this dissertation demonstrate that the sixth TPR motif (TPR6) was required for P58^{IPK} activity *in vitro*. Although the DnaJ homology

region at the carboxyl terminus of P58^{IPK} was dispensable for function *in vitro*, the *in vivo* experiments indicated that this region, along with TPR6, was required for the stimulation of cellular protein synthesis. Thus, the carboxyl terminus of P58^{IPK} may serve a regulatory function to modulate P58^{IPK} activity in the cell.

To define the physiological role of the P58^{IPK} protein, stable NIH 3T3 cell lines expressing either wild type, bovine P58^{IPK} or Δ TPR6, a P58^{IPK} variant lacking TPR6, were generated and characterized. Both of the cell lines displayed *in vitro* hallmarks of cellular transformation and induced tumors in nude mice. By three different *in vivo* assays, PKR was found to be active in the Δ TPR6-expressing cells, suggesting that P58^{IPK}-induced oncogenesis may occur via a PKR-independent mechanism.

This study has identified two cellular functions for P58^{IPK}: (i) the regulation of protein synthesis through an inhibition of PKR-mediated eIF-2 α phosphorylation, and (ii) the suppression of apoptosis. One or both of these activities may regulate cellular growth and contribute to P58^{IPK}-induced neoplastic transformation.

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ACKNOWLEDGMENTS

The author wishes to thank Dr. Michael Katze, the thesis advisor, for his guidance, training and support. As any mentor-student relationship goes, we've also had our share of good times and bad times. But through it all, we've kept our sense of humor and respect for one another. I've learned a great deal from Dr. Katze, both scientifically, professionally and personally, which I will carry with me from here on.

I am greatly indebted to all of the members of my committee: Dr. David Morris, Dr. Carol Sibley, Dr. Adam Geballe and Dr. Stephen Lory. All were intellectually insightful, available when needed, and offered diverse and useful advice. I particularly want to acknowledge Drs. Morris and Sibley for their unfledling support and valuble insights, both inside and outside of the lab.

My sincerest thanks to the various members of the Katze lab who have contributed their time, patience and scientific discourse; in particular, Ms. Marlene Wambach. I would also like extend my appreciation to the many people of the Microbiology deparatment, past and present, who have befriended me, lent support and provided sobering perspectives. Special thank you's to Drs. Steve Moseley, James Champoux, Elizabeth Beattie, Leah Turner, and Sam Whiting for the myriad of terrific advice. A word of acknowledgement also to the Molecular and Cellular Biology Training

Grant at the University of Washington for their financial support and training the past two years.

Finally, I would like to thank my family. To my parents and four brothers, thank you for your patience and encouragement. To my husband, James Sugai who had to put up with alot, thank you also for the encouragement, support and never ending love. Last but not least, to my daughter Amanda. Thank you for bringing such wonderful joy into my life.

DEDICATION

The author wishes to dedicate this dissertation to the memory of Dr. Keith Y.K. Tang, my brother, who died tragically at the prime of his life. Keith, you have always been an inspiration to me. It was through your enthusiasm, boldness and zest for life that I am at this juncture. Thank you. I love and miss you very much.

INTRODUCTION

During influenza virus infection, cellular proteins are not made but viral proteins are efficiently synthesized. The strategies employed by influenza virus to selectively and efficiently translate its own mRNAs are numerous and complex. My studies have focused on one of these strategies: the maintenance of host cell translational competence during influenza virus infection via activation of P58^{IPK}, a cellular inhibitor of the double stranded RNA-activated protein kinase, PKR.

P58^{IPK} was identified from influenza virus-infected Madin Darby bovine kidney cells and cloned in 1992. When expressed as a histidine-tagged fusion protein in *Escherichia coli*, P58^{IPK} was shown to inhibit in a dose dependent manner both PKR autophosphorylation and PKR phosphorylation of eIF-2 α substrate *in vitro*. In addition, murine cell lines overexpressing P58^{IPK} were tumorigenic in nude mice, suggesting P58^{IPK} to be an oncoprotein.

The mechanism by which P58^{IPK} inhibits PKR is unknown. In order to elucidate this mechanism as well as define the physiological role of the P58^{IPK} protein, a structure/function study of the P58^{IPK} molecule was undertaken. Specifically, a series of P58^{IPK} mutants were constructed and analyzed for PKR inhibitory activity. Using both *in vitro* and *in vivo* assays, the ability to inhibit PKR kinase phosphorylation activity was

quantitated for each of the P58^{IPK} mutants. In addition, P58^{IPK} mutant-expressing cell lines were generated and their oncogenicity correlated with their ability to inhibit PKR kinase activity.

The goals of the P58^{IPK} structure/function study were to define the functional domains for P58^{IPK} and to examine the biological role of the P58^{IPK} protein. In achieving these goals, this study provided additional insights into the complex pathway of PKR regulation in both virus-infected and uninfected cells. Moreover, this study identified novel functions for P58^{IPK} in two areas: transcriptional regulation and apoptotic cell death. These novel functions may very well be independent of the ability of P58^{IPK} to modulate PKR activity.

Chapter 1: Background

Introduction

Protein synthesis, also known as translation, is a vital component of the cellular gene expression machinery. Translational control, which is the selective translation of a specific subset of available mRNAs, is one method by which the eukaryotic cell can regulate a variety of its cellular processes. Among these processes is host antiviral defense. When a virus infects a cell, interferon is made and secreted by the infected cell. The secreted interferon binds to receptors on neighboring uninfected cells and sets into motion a series of events intended to inhibit the proliferation of both viruses and cells (Sen and Lengyel 1992; Samuel 1991). As one of greater than 30 proteins induced by interferon (Meurs et al. 1990; Katze et al. 1987), the protein kinase PKR (Protein Kinase, RNA-dependent) plays a critical role in the establishment of the antiviral state.

PKR is a serine/threonine kinase that is activated by dsRNA (or polyanions, in some cases). When activated, PKR autophosphorylates and phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α). These events lead to limitations in functional eIF-2, which then results in dramatic decreases in protein synthetic rates inside the cell (Hershey 1991; Merrick 1992; Merrick and Hershey 1996). Through this mechanism, PKR restricts viral protein synthesis and replication. As a counter strategy, many eukaryotic viruses have devised mechanisms to downregulate PKR activity and

many eukaryotic viruses have devised mechanisms to downregulate PKR activity and avoid decreases in protein synthetic rates during infection (Samuel 1991; Katze 1992; Katze 1993a; Katze 1993b; Katze 1995; Gale, Jr. and Katze 1998). One of these viruses is influenza virus, which activates a cellular protein, called P58^{IPK}, in order to inhibit PKR activity.

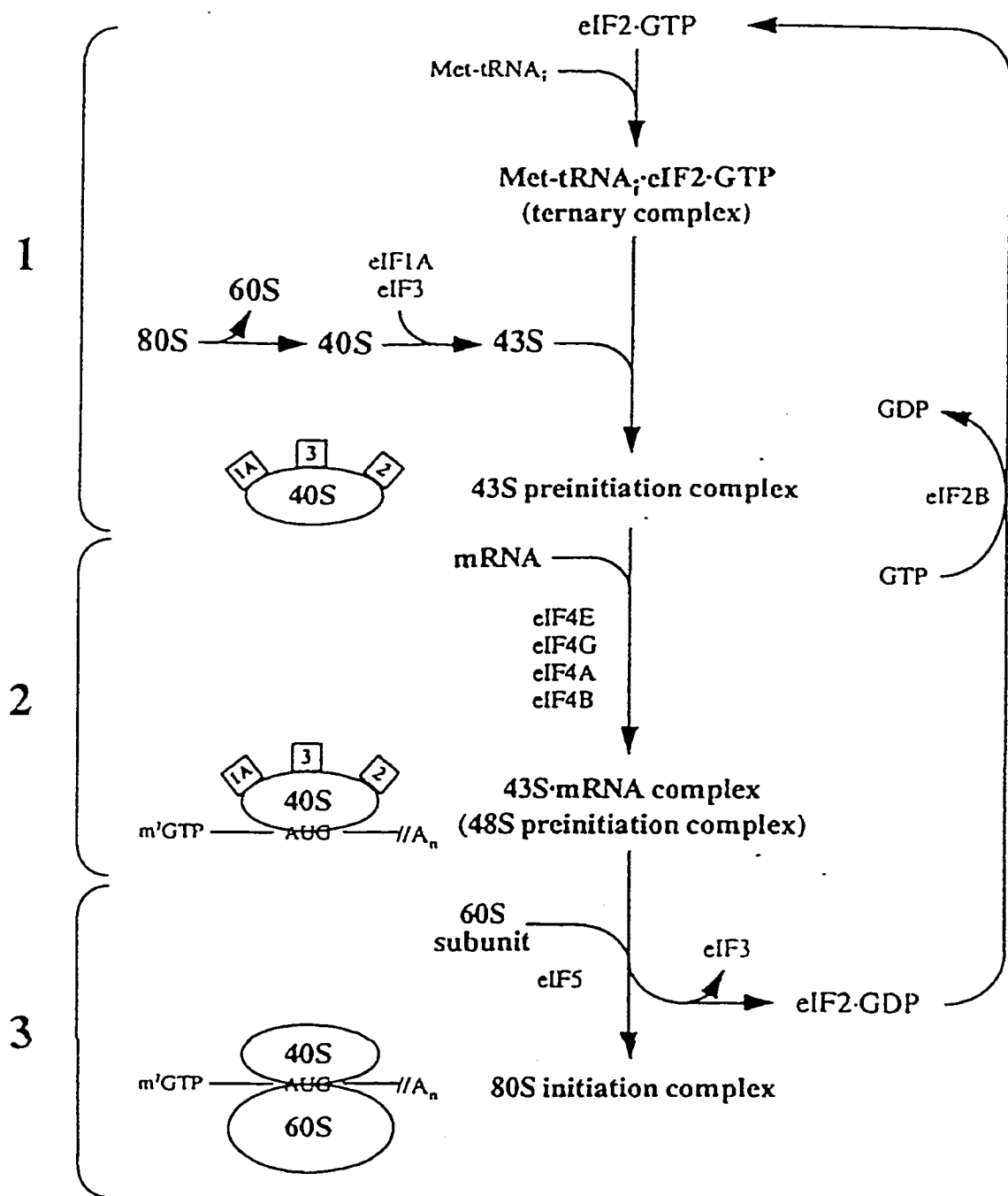
What follows is a brief review of eukaryotic translational initiation, and what is known about the structure and functional activities of PKR in both virus-infected and uninfected cells. A summary of the regulation of PKR by viral and cellular-encoded proteins is also provided with special emphasis on P58^{IPK}, the influenza virus-activated cellular inhibitor of PKR. Finally, other translational control strategies employed by influenza virus will also be discussed.

Translational Initiation In Eukaryotic Cells

Mechanisms of translational initiation

Eukaryotic protein synthesis can be divided into three stages: initiation, elongation and termination. Initiation entails the binding of the ribosome to the mRNA to form an 80S initiation complex that contains initiator methionyl-tRNA (Met-tRNA_i). This is accomplished through a series of reactions diagrammed in figure 1.1. As shown in figure 1.1, eukaryotic translational initiation itself can be divided into three stages: (1) formation of the 43S pre-initiation complex through the association of Met-tRNA_f and several initiation factors with the 40S ribosomal subunit; (2) formation of the 48S

Figure 1.1. Mechanism of eukaryotic translational initiation. Stage 1, formation of 43S pre-initiation complex. Stage 2, formation of 48S pre-initiation complex. Stage 3, formation of 80S ribosome at initiation codon poised to commence the elongation stage of translation. The continuity of initiation events requires the recycling of initiation factor molecules. In stage 3, eIF-2 is released as a binary complex with GDP and requires a guanine nucleotide exchange factor, eIF-2B, to catalyse the regeneration of the eIF-2•GTP complex required to recruit the next Met-tRNA_i molecule (modified from Pain 1996).



preinitiation complex through the binding of the 43S preinitiation complex to mRNA, followed by its migration to the correct AUG initiation codon; and (3) formation of the 80S initiation complex at the initiator AUG through the joining of the 60S ribosomal subunit to the 48S preinitiation complex. When these three stages of eukaryotic initiation are completed, the 80S ribosome is ready to commence translation of the coding sequence.

Eukaryotic translational initiation begins with the formation of the 43S preinitiation complex, whereby eIF2 forms a ternary complex with GTP and Met-tRNA_i. The ternary complex then binds a 40S ribosomal subunit containing initiation factors eIF-3 and eIF-1A (formerly known as eIF-4C) to form the 43S preinitiation complex (Jagus et al. 1981; Hershey 1991). At some unknown point during the assembly of the 43S preinitiation complex, the second stage of protein synthesis initiation begins, whereby the 40S ribosomal subunit binds the 7-methyl-guanylic acid (m⁷G) "cap" present at the 5' end of eukaryotic mRNAs with the help of eIF-4E, eIF-4G, eIF-4A (collectively known as eIF-4F), eIF-4B and ATP hydrolysis. The hydrolysis of ATP provides the energy requirements for mRNA unwinding and utilization. Following the binding of the ribosome to the mRNA cap, the ribosome (along with the associated protein factors) scans linearly down the mRNA until it reaches the initiator codon. At this point, the third stage of initiation begins. When an appropriate match is made between the anticodon of the initiator tRNA and the AUG start codon, eIF-2 is poised to allow hydrolysis of its bound GTP to GDP. This hydrolysis event is triggered by eIF-5. GTP hydrolysis also triggers

the release of initiation factors from the surface of the 40S subunit, allowing for 60S subunit joining. The joining of the large 60S ribosomal subunit to the mRNA-bound, small 40S ribosomal subunit is facilitated by eIF-4D. Once the two ribosomal subunits are joined on the mRNA, commencement of translation of the coding sequence (elongation) begins. (For an in-depth review of eukaryotic translational initiation, see (Merrick and Hershey 1996)).

Regulation of translational initiation

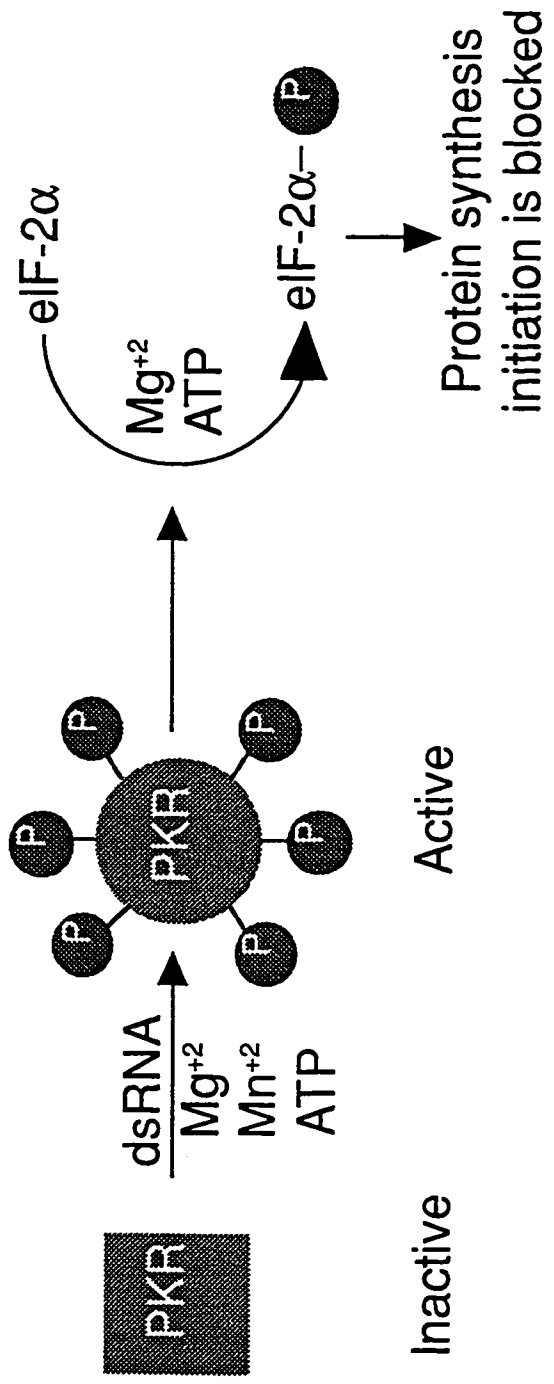
Because initiation is usually the rate-limiting step of translation, much of translational control centers around the regulation of the protein factors associated with initiation. One well documented method of translational control at the level of initiation is the regulation of protein factors eIF-2 and eIF-4F through phosphorylation. eIF-2 consists of three subunits: alpha (α), beta (β) and gamma (γ). Phosphorylation of the specific residue Ser⁵¹ on the alpha subunit of eIF-2 (eIF-2 α) correlates with decreased factor activity *in vitro* and with repression of protein synthesis *in vivo* (Hershey 1989; Hershey 1991). This occurs because phosphorylated eIF-2 α binds tightly to and sequesters eIF-2B, a guanine nucleotide exchange factor. As illustrated in figure 1.1, GTP is required for the catalytic utilization of eIF-2. Thus, the sequestration and loss of eIF-2B activity as a result of eIF-2 α phosphorylation leads to functional limitations of eIF-2, an essential component of protein synthesis. Because cellular levels of eIF-2B are lower than those of eIF-2, only a portion of eIF-2 needs to be phosphorylated in order to sequester and block eIF-2B activity (Merrick 1992). eIF-2 α is the natural substrate of the protein

kinase PKR. Thus, one method of translational regulation is via the activation of PKR, which phosphorylates eIF-2 α , leading to inhibition of protein synthesis initiation (Figure 1.2). Another method of translational regulation is through the phosphorylation of eIF-4E, a component of eIF-4F. In contrast to the phosphorylation of eIF-2 α , the phosphorylation of eIF-4E results in increased translation rates (Thach 1992).

Alternative Methods of Translational Initiation

It should be noted that, in addition to the scanning model, there are two other methods of getting the 40S ribosome to the initiating AUG codon. Both of these methods occur infrequently. The first method is reinitiation. Reinitiation occurs on mRNAs that are polycistronic; i.e. having at least two open reading frames (ORFs). It is postulated that the first initiation event occurs as diagrammed in figure 1.1. After completion of the polypeptide chain (which is always short), the 40S subunit continues to scan, or move down, the mRNA. At some point, a new ternary complex is acquired, both to serve as the initiator tRNA and to locate the next initiating AUG. Once this occurs, presumably all of the components necessary for AUG selection and subsequent subunit joining are in place. Reinitiation plays a key role in regulating the translational efficiency of the yeast GCN4 mRNA (Hinnebusch 1988) and the cytomegalovirus gp48 transcript (Cao and Geballe 1995). (For review, see (Geballe 1996)). The second rare method is a cap-independent event called internal initiation. Simply put, this kind of initiation event entails the binding of the 43S pre-initiation complex to a portion of the mRNA distal from the 5' cap and the subsequent scanning (if necessary) of the mRNA to locate the initiating

Figure 1.2. PKR activation and phosphorylation of substrate. In the presence of Mg^{+2} , Mn^{+2} and dsRNA, ATP binds to PKR, leading to its autophosphorylation. The autophosphorylated PKR then catalyzes phosphorylation of exogenous substrates, such as eIF-2 α . The square and circle forms of PKR represent inactive and active PKR, respectively. The P in a circle represents the PO_4^{2-} group.



AUG codon. This type of translation is seen with picornaviruses, which lack 5' m⁷G cap structures.

Overview of PKR

Introduction to PKR

PKR (Protein Kinase, RNA-dependent) was first identified over 20 years ago when it was shown that a protein kinase mediated the inhibition of protein synthesis when double-stranded (ds) RNA is added to the reticulocyte lysate cell-free translation system (Hunter et al. 1975; Clemens et al. 1975; Farrell et al. 1977; Levin and London 1978). Interest in PKR rose dramatically when it was demonstrated that PKR, not only acted in immature red cells, but also is induced by interferons in a wide range of cell types (Farrell et al. 1978; Roberts et al. 1976; Zilberstein et al. 1976; Samuel 1979). Later studies showed PKR to be responsible for at least part of the antiviral and antiproliferative effects of interferons (Samuel et al. 1984; Rice et al. 1985; Krust et al. 1984; Hovanessian 1989; Der and Lau 1995; Baca et al. 1994).

The name for PKR was standardized in 1993 (Clemens et al. 1993). Previously, PKR was also referred to as P68, dsI, DAI, dsRNA-PK, eIF-2PK_{ds} and p1/eIF-2 kinase. PKR belongs to a small family of enzymes that have a common substrate, eIF-2 α (Samuel 1993; Clemens et al. 1994; Clemens 1996). There are homologs of PKR in human, mouse, rat, rabbit and plant cells (Langland et al. 1995; Langland et al. 1996). More distant relatives of PKR, which also phosphorylate eIF-2 α ,

are yeast GCN2 and reticulocyte heme-regulated kinase HCR (also referred to as HRI). GCN2 is involved in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*, and HCR is involved in the regulation of reticulocyte protein synthesis. As previously mentioned, by phosphorylating eIF-2 α , PKR participates in translational downregulation. In addition to its translational regulatory activity, PKR is implicated in transcriptional regulation and signal transduction pathways. These activities can mediate a wide range of biologic effects, including growth and tumor suppression.

Structure and localization of the *pkc* gene and protein

The human *pkc* gene consists of 17 exons and 16 introns (Kuhlen et al. 1996a; Kuhlen et al. 1996b) and is found on chromosome 2p21-p22 (Barber et al. 1993; Squire et al. 1993). The promoter region of *pkc* contains a number of potential regulatory elements. Sequences for interferon stimulated response element (ISRE) and gamma interferon activated site (GAS) confer interferon inducibility for the *pkc* gene (Kuhlen and Samuel 1997). Other enhancer elements present in the promoter region suggest that PKR may be regulated at the transcriptional level by the cytokine IL-6 or by key regulators of the cell cycle (E2F) or cell growth (NF- κ B, Ets, MyB) (Kuhlen and Samuel 1997).

The human PKR protein is a 551 amino acid molecule, which can be divided roughly in half into two functional domains: an amino-terminal regulatory domain, and a carboxyl-terminal catalytic domain (Figure 1.3). The amino-terminal regulatory half contains two subdomains (DSRM1 and DSRM2) which bind both activator and inhibitor RNAs (Katz et al. 1991). The carboxyl-terminal catalytic half is the enzymatic domain

and contains the eleven catalytic subdomains characteristic of all protein serine/threonine kinases (Hanks et al. 1988; Meurs et al. 1990). PKR shares sequence homology to two other protein kinases which also phosphorylate eIF-2 α . These are GCN2, the PKR homologue in yeast which regulates amino acid biosynthesis (Dever et al. 1992; Hinnebusch 1988), and HCR, the heme regulated inhibitor which shuts down protein synthesis in rabbit reticulocyte lysates during heme deficiency (Chen et al. 1991a). PKR sequence identity to each of these two proteins is 38% and 42%, respectively (Chen et al. 1991b; Chong et al. 1992).

Most mammalian cells and tissues express low, constitutive levels of PKR (Meurs et al. 1992). The amount of PKR protein can be enhanced by treatment of cells with type I interferon (Samuel et al. 1982; Samuel and Knutson 1983), with the level of PKR phosphorylation activity increasing 5-10 fold in the presence of interferon (Samuel 1979). PKR protein is mainly found in the cytoplasm, with the majority associated with ribosomes (Levin et al. 1980; Galabru and Hovanessian 1987; Jeffrey et al. 1995). PKR protein has also been detected in the nucleolus (Jeffrey et al. 1995; Jiménez-García et al. 1993), constituting one fifth of the total enzyme in Daudi cells before interferon treatment (Jeffrey et al. 1995). Interferon treatment results in a several-fold increase in the ribosomal-associated, cytoplasmic pool of PKR protein, but no change in the nuclear pool. The cytoplasmic pool of PKR proteins also show multiple phosphorylated forms. On the other hand, the nuclear pool is relatively homogeneous and underphosphorylated (Jeffrey et al. 1995). These findings suggest that nucleolar PKR may not be active and, therefore,

Figure 1.3. Domain structure of PKR. PKR is a 551 amino acid protein. The amino-terminal regulatory domain contains two double-stranded RNA binding motifs termed DSRM1 (amino acids 55-75) and DSRM2 (amino acids 145-166). The carboxyl-terminal catalytic domain contains the eleven catalytic subdomains characteristic of all protein serine/threonine kinases.

PKR



may not participate in cellular functions mediated by interferons.

Activation of PKR by double-stranded RNA

PKR is activated via interaction of the kinase with double-stranded RNA (dsRNA). The exact nature of this interaction is not understood. However, it has been shown *in vitro* that low levels of dsRNA (in the nanogram per ml range) activate PKR, while high concentrations of dsRNA (1-10 μ g/ml) inhibit the kinase. These characteristics give rise to a bell-shaped curve for the activation of PKR as a function of dsRNA concentration (Clemens et al. 1975; Levin et al. 1980). It has been suggested that PKR activation entails a conformational change in the kinase molecule upon binding to dsRNA (Manche et al. 1992; Langland and Jacobs 1992; Romano et al. 1995). Such a change may relieve an inhibition of PKR activity imposed by an internal segment of the kinase molecule, a situation found in many members of the serine/threonine kinase family (Tiley et al. 1994).

Although some forms of dsRNA can act as activators of PKR, others inhibit kinase activity. It is unclear what the physical characteristics are that distinguish activator RNAs from inhibitor RNAs. Because PKR exhibits differing affinities for dsRNAs of different lengths, it has been postulated that only dsRNAs that are long enough to allow binding to both DSRM regulatory subdomains will induce a conformational change in PKR that results in activation of the kinase (Green and Mathews 1992). An RNA length of about 85 base pairs is optimal for binding to and activation of PKR, while dsRNAs containing less than 30 base pairs do not bind to the kinase in a stable manner

(Manche et al. 1992). This hypothesis is consistent with observations that both DSRM domains are required for efficient binding of the dsRNA activator to PKR (Green and Mathews 1992). Although length may be an important determinant of whether the dsRNA species acts as an activator or inhibitor of PKR, it may not be the only criterion. It appears that RNA secondary structure or, perhaps, tertiary structure, may also be of importance. For instance, some viral RNAs with extensive secondary structure (such as reovirus s1 mRNA (Bischoff and Samuel 1989; Henry et al. 1994), hepatitis delta RNA (Robertson et al. 1996; Circle et al. 1997) and transcripts from the Bam HI W repeat region of the Epstein-Barr virus genome (Elia et al. 1996)) do not contain 30 or more uninterrupted base pairs, although they are potent activators of PKR. Moreover, hepatitis delta genomic RNA contains a pseudoknot tertiary structure which may be important for PKR recognition and activation (Circle et al. 1997).

In addition to dsRNA, PKR can be activated by polyanions (heparin, dextran sulphate, chondroitin sulphate and poly(*L*-glutamine) (Hovanessian and Galabru 1987; Hovanessian 1989; Patel et al. 1994; George et al. 1996). Binding of PKR to polyanions likely occurs through different sites than those identified for dsRNA interaction since PKR mutants that do not bind dsRNA can be activated by heparin (Patel et al. 1994; George et al. 1996). There is *in vitro* evidence that some cellular RNAs, presumably those with extensive secondary structure, can activate PKR (Katze et al. 1991; Davis and Watson 1996; Pratt et al. 1988). The exact identities of these cellular RNAs are presently unknown with the exception of the 3' untranslated region of α -trophomyosin, which has

been recently shown to activate PKR in purified, *in vitro* kinase assays (Davis and Watson 1996).

Dimerization of PKR

There is evidence to suggest that active PKR is a dimer of two PKR monomers. As early as 1989, kinetic studies showed that PKR autophosphorylation is a second order reaction dependent on kinase concentration (Kostura and Mathews 1989). Since then, biochemical analyses have shown that partially phosphorylated PKR purifies as a dimer, whereas unphosphorylated PKR exists as a monomer (Langland and Jacobs 1992). Moreover, PKR exists in solution predominantly as a dimer (Carpick et al. 1997). Other studies have also shown PKR to self-associate *in vitro* and *in vivo* (Cosentino et al. 1995; Patel et al. 1995; Ortega et al. 1996). Most recently, our lab has shown that P58^{IPK} prevents PKR dimerization, which correlated with an inhibition of PKR phosphorylation activity (Tan et al. 1998). These studies strongly suggest that dimerization may be a prerequisite for PKR activation and/or function.

In light of the fact that PKR activation follows a bell-shaped curve as a function of dsRNA concentration, it is thought that dimerization mediates dsRNA-dependent activation of PKR through cross-phosphorylation between two PKR monomers brought into close proximity by binding to a single RNA molecule. This model is consistent with observations that short dsRNAs and very low or high concentrations of long dsRNAs both fail to activate PKR. The PKR dimerization model is also consistent with findings that mutants of the dsRNA binding motifs of PKR can functionally

complement each other in yeast (Romano et al. 1995). Finally, the PKR dimerization model has been invoked to explain the dominant negative effects of some inactive PKR mutants (Barber et al. 1995). It has been suggested that expression of non-functional mutants of PKR inhibit kinase function through the formation of inactive heterodimers with endogenous wild type PKR. Although more direct evidence needs to be provided showing that the active form of PKR is a dimer, the evidence presented thus far is, nevertheless, extremely strong.

Substrates of PKR

Thus far, only one *in vivo* substrate of PKR has been identified: the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α). As discussed earlier in this chapter, phosphorylation of the Ser⁵¹ residue of eIF-2 α by PKR results in the sequestration of the guanine nucleotide exchange factor eIF-2B. This event blocks the regeneration of the ternary complex (eIF-2•GTP•Met-tRNA_i), resulting in the global inhibition of protein synthesis initiation.

Because PKR has multiple biologic effects (discussed later in this chapter), it is likely that eIF-2 α may not be the only substrate for PKR *in vivo*. A recent study has shown that PKR can phosphorylate the inhibitor of nuclear factor kappa B (I κ B α) *in vitro* (Kumar et al. 1994). Moreover, several studies, using selective ablation of PKR mRNA or cells from PKR knockout mice, have shown that PKR is required for the poly(rI):poly(rC)-induced activation of nuclear factor kappa B (NF- κ B) *in vivo* (Yang et al. 1995; Maran et al. 1994; Der et al. 1997). Phosphorylation of I κ B α releases NF- κ B

from an inactive complex in the cytoplasm such that NF- κ B can translocate to the nucleus to activate transcription of target genes. These studies, therefore, suggest that PKR may function in the cell to activate NF- κ B activity through the phosphorylation of I κ B α . Further studies are needed to clarify the role of I κ B α as a cellular substrate of PKR.

Biologic Effects Of PKR

Antiviral defense

PKR is an interferon-inducible enzyme which mediates, in part, the antiviral properties of interferon. Together with another known interferon-inducible enzyme, 2'5'-oligoadenylate synthetase, PKR, likely, participates in the host shutoff of protein synthesis following virus infection (Hovanessian 1991). The mechanism of antiviral action by PKR is as follows. During virus infection, viral intermediates, in the form of dsRNA, can activate PKR. Once activated, PKR autophosphorylates and phosphorylates eIF-2 α , resulting in dramatic decreases in protein synthetic rates. The shutdown of protein synthesis by PKR blocks viral replication by preventing viral protein synthesis. (For reviews on PKR and antiviral defense, see (Samuel 1991; Katze 1992; Katze 1996; Katze 1995; Mathews 1996; Schneider 1996).) It should be noted that different viruses show different susceptibility to PKR activation. For instance, replication of encephalomyocarditis virus is inhibited by kinase activity (Meurs et al. 1992), while vaccinia virus replication is unaffected by the presence of PKR (Rice and Kerr 1984; Paez

and Esteban 1984). These variations may stem from differences in the virus' ability to activate PKR or suppress PKR activity. The need to downregulate PKR activity is of such importance that many viruses have evolved one or more strategies to counter PKR activation during virus infection. These strategies are discussed below and summarized in Table I.

Viral Strategies to Counter PKR Activation

Among the eukaryotic viruses that have evolved strategies to downregulate PKR activity are adenovirus, Epstein-Barr virus, human immunodeficiency virus (HIV-1), reovirus, vaccinia virus, poliovirus and influenza virus (Katze 1992; Katze 1993a; Katze 1993b; Katze 1995; Gale, Jr. and Katze 1998).

Adenovirus and Epstein-Barr virus: Adenovirus and Epstein-Barr virus (EBV) both use viral RNAs to bind PKR and block its activity. During wildtype adenovirus infection, a polymerase III transcribed viral gene product, VAI RNA is made which directly binds to PKR to inactivate the kinase (Katze et al. 1987). However, when the cells were infected with an adenovirus mutant lacking VAI RNA (dl331), both viral and cellular protein synthesis were blocked (Thimmappaya et al. 1982). This inhibition was explained by showing that the high levels of PKR autophosphorylation led to abnormally high levels of eIF-2 α phosphorylation, which then led to a depletion of functional eIF-2 (Mathews and Shenk 1991). Although it is generally agreed that the central domain of VAI RNA is vital for PKR inactivation, it is still somewhat controversial since this region is thought to have less secondary structure than most other areas of the VAI RNA. Because all studies done,

Table I. PKR Inhibitors And Their Mechanisms of Action

<i>Inhibitor</i>	<i>Mode of Action</i>	<i>Physiological Role</i>
Viral RNAs		
Adenovirus VAI	Binds directly to PKR	Inhibition of PKR in adenovirus-infected cells
Epstein-Barr EBER-1	Binds to PKR	Inhibition of PKR in Epstein-Barr virus-infected cells
Viral Proteins		
HSV-1 γ_1 34.5	Enhances activity of protein phosphatase 1 α	Reduces phosphorylation of eIF-2 α in HSV-1-infected cells
Vaccinia E3L	Binds dsRNA activators; dimerizes with PKR	Inhibition of PKR in vaccinia virus-infected cells
Vaccinia K3L	Acts as a pseudosubstrate <i>in vitro</i>	Enables virus to replicate in the presence of interferon
Reovirus σ 3	Binds dsRNA activators	Inhibition of PKR in reovirus-infected cells
Influenza NS1	Binds dsRNA	Inhibition of PKR in influenza virus-infected cells
HIV-1 Tat	Binds to and is phosphorylated by PKR <i>in vitro</i> ; downregulates PKR <i>in vivo</i>	Reduces phosphorylation of eIF-2 α in HIV-1-infected cells
Cellular Proteins		
P58 ^{IPK}	Binds directly to PKR	Regulation of PKR and induction of oncogenesis
TAR-BP	Binds dsRNA; dimerizes with PKR	Possible role in oncogenesis
La antigen	Binds and unwinds dsRNA (?)	Control of translation and transcription
p67	Binds to eIF-2	Blocks eIF-2 α phosphorylation by PKR
dRF	Inhibits dsRNA and ATP binding	Possible role in cellular differentiation

thus far, show that VAI RNA binds to the same sites on PKR as activator RNAs, VAI RNA, likely, acts as a powerful competitive inhibitor. Interestingly, EBV encoded EBER-1 RNA is also a polymerase III transcript which can, at least in part, functionally substitute for VAI RNA in adenovirus mutants (Bhat and Thimmappaya 1983; Bhat and Thimmappaya 1985). Like VAI RNA, EBER-1 RNA can form a stable complex with PKR *in vitro* (Clarke et al. 1991). In addition, at high concentrations, EBER-1 RNA can prevent the dsRNA-mediated inhibition of protein synthesis initiation in rabbit reticulocytes (Clarke et al. 1990). These studies suggest that EBER-1 RNA may function in a manner similar to VAI RNA. In contrast to these *in vitro* studies, more recent *in vivo* work have shown that EBV mutants with deletions in EBER-encoding genes did not adversely affect EBV replication or immortalization of cultured B cells (Swaminathan et al. 1991). In addition, these EBV mutants had wildtype sensitivity to the antiviral effects of interferon (Swaminathan et al. 1992). Because the *in vivo* work was done in cell culture, it is still possible that EBER-1 RNA may modulate the effects of interferon by acting as a competitive inhibitor to block PKR activation.

Human immunodeficiency virus (HIV): Regulation of PKR in HIV-infected cells is shrouded in controversy with molecular details uncertain. Very early work in the late 1980s by Parkin et al. and Edery et al. showed that a fusion of the *trans*-activation response element (TAR) in the 5'-untranslated region of HIV mRNAs to a heterologous mRNA (CAT) exhibited *cis*- and *trans*-inhibitory effects on mRNA translation *in vitro* (Parkin et al. 1988; Edery et al. 1989). These inhibitory effects were dependent on RNA

secondary structure and were reversed by the addition of eIF-2B recycling factor, suggesting that TAR-containing mRNAs can activate PKR *in vitro*. In contrast to these studies, later work by Gunnery and colleagues in a cell free system showed that high concentrations of synthetic TAR RNA blocked PKR activation and prevented dsRNA-mediated inhibition of protein synthesis (Gunnery et al. 1990; Gunnery et al. 1992), suggesting that TAR RNA may act similarly to VAI RNA and EBER-1 RNA to inhibit PKR. Recently, work by Maitra et al. with chemically synthesized TAR RNAs unambiguously demonstrated that TAR RNAs can activate PKR efficiently (Maitra et al. 1994). Thus, the regulatory properties of TAR are probably due to its extensive secondary structure, which permits TAR to bind to PKR and form a stable complex with the kinase (Roy et al. 1991). In addition to regulation by TAR, PKR levels and/or activity may also be modulated by the HIV-encoded regulatory gene product, Tat (Roy et al. 1990). The mechanism behind Tat inhibitory activity appears to be the direct binding of Tat to PKR (McMillan et al. 1995) and the utilization of Tat as an alternative substrate for the kinase (Brand et al. 1997). Since PKR has been implicated as a tumor suppressor protein (details discussed in a later section), it is interesting that a cellular protein called TAR-BP, which was cloned based on its ability to bind to TAR RNAs (Gatignol et al. 1991), was shown to be an inhibitor of PKR that also malignantly transformed cells (Park et al. 1994; Benkirane et al. 1997). Because TAR-BP contains an RNA binding motif conserved in PKR, it may block PKR activation by competing for common RNA substrates. In addition to this mechanism, TAR-BP may also form a hetero-complex with

PKR, thus blocking PKR autophosphorylation and functions (Benkirane et al. 1997).

Reovirus and vaccinia virus: Both reovirus and vaccinia virus downregulate PKR by encoding virus-specific dsRNA-binding proteins. The reovirus $\sigma 3$ protein acts by sequestering viral dsRNA activators (Imani and Jacobs 1988; Lloyd and Shatkin 1992). The vaccinia virus *E3L* gene product is a protein of 190 amino acids that has sequence similarity to PKR. The *E3L* gene product has been shown to bind to dsRNA, and may well function in a similar manner to reovirus $\sigma 3$ protein (Chang and Jacobs 1993; Davies et al. 1993). In addition to the *E3L* gene product, vaccinia virus also contains the *K3L* gene product, an 88 amino acid protein with sequence similarity to eIF-2 α . It has been postulated that the *K3L* gene product acts as a pseudosubstrate for PKR to block kinase function (Beattie et al. 1991; Davies et al. 1992; Davies et al. 1993; Carroll et al. 1993)

Poliovirus and influenza virus: In contrast to the previously mentioned virus-encoded regulators, both poliovirus and influenza virus appear to recruit cellular proteins to downregulate PKR activity. In the case of poliovirus, a cellular protease may be activated which acts in concert with poliovirus-specific dsRNAs to degrade the RNA-bound PKR (Black et al. 1993). In the case of influenza virus, a cellular inhibitor of PKR, termed P58^{IPK}, may be recruited/activated through the release from its own inhibitor (I-P58). It is not known at this time whether a specific viral gene is involved in the recruitment/activation of P58^{IPK} or whether P58^{IPK} recruitment/activation is a response to the "stress" of influenza virus infection. Additional details on P58^{IPK} is provided later in this chapter.

Control of cell growth and differentiation

Most of the work on PKR has focused on the role of the kinase in mediating the inhibitory interferon response to viral infection. However, PKR is constitutively expressed in eukaryotic cells in the absence of interferon (Meurs et al. 1992) and there is accumulating evidence implicating PKR in normal cellular processes. These include cellular differentiation, growth, and proliferation. In mouse 3T3-F442A preadipocyte fibroblasts, PKR activity is correlated with terminal differentiation of the preadipocytes to adipocytes (Petryshyn et al. 1984). Conversely, inhibition of PKR autophosphorylation activity, possibly due to the presence of the 15 kDa dRF inhibitor in 3T3-F442A cells, is correlated with undifferentiation of the preadipocytes (Judware and Petryshyn 1992). Overexpression of PKR is growth suppressive in yeast (Chong et al. 1992), and toxic in insect (Barber et al. 1992) and mammalian cells (Koromilas et al. 1992). The yeast growth suppressive phenotype is correlated with increases in eIF-2 α phosphorylation (Chong et al. 1992). Overexpression of GCN2, the yeast homolog for PKR, also results in growth suppression (Tsamarias and Thireos 1988). These studies suggest that PKR promotes cellular differentiation and suppresses growth and proliferation.

Signal transduction and transcriptional regulation

PKR is also implicated in the mechanisms by which certain growth factors and cytokines mediate their effects on target cells (Williams 1995). Of all the growth factors and cytokines studied thus far, the evidence supporting the involvement of PKR in

platelet-derived growth factor (PDGF) signaling is the most compelling. It has been shown that dsRNA or serum induction of *c-fos*, *c-myc* and JE genes is blocked by 2-aminopurine, an inhibitor of PKR (Mundschau and Faller 1995; Zinn et al. 1988; Zullo et al. 1985; Hall et al. 1989; Mahadevan and Edwards 1991). Similarly, in *ras*-transformed cells (in which a cytoplasmic inhibitor of PKR autophosphorylation is induced), transcriptional induction of these growth-related genes by PDGF is also blocked (Mundschau and Faller 1992). In a more direct approach, it was shown that the introduction of antisense oligonucleotides to murine PKR, before treatment with PDGF, results in the inhibition of *c-myc* and JE induction (Mundschau and Faller 1995). Interestingly, PDGF can induce PKR phosphorylation in digitonin permeabilized cells (Mundschau and Faller 1995). These data are consistent with a role for PKR in the induction of immediate early genes by PDGF.

In addition to PDGF signaling, PKR is also involved in interferon and dsRNA signaling. PKR has been shown to mediate the expression of interferon- γ -induced genes encoding for such proteins as interferon regulatory factor 1 (IRF-1), guanylate-binding protein and immunoglobulin κ light chain (Kumar et al. 1997; Koromilas et al. 1995). In addition, PKR is required for both interferon-induced IRF-1 (interferon regulatory factor 1) activation (Kumar et al. 1997; Der et al. 1997) and dsRNA-induced NF- κ B activation (Yang et al. 1995; Der et al. 1997). Thus, PKR participates in a variety of signaling pathways through the modulation of gene expression at both the transcriptional and translational levels.

Tumor suppression and induction of apoptosis

Not surprisingly, for a protein that inhibits cell growth and proliferation, recent studies have implicated PKR in tumor suppression (for a review, see (Lengyel 1993)). NIH 3T3 cells expressing various inactive mutant forms of PKR display transformed phenotypes *in vitro* and form tumors *in vivo* when injected into nude mice (Koromilas et al. 1992; Meurs et al. 1993; Barber et al. 1995). Human glioblastoma cells lost tumorigenicity when transfected with the gene for interferon- α , which correlated with the upregulation of PKR expression (He et al. 1996). These data suggest that PKR suppresses tumor growth and that transdominant PKR mutants exert their oncogenic effects by blocking PKR-mediated tumor suppression.

In contrast to these findings, other studies have questioned whether PKR is a tumor suppressor protein. The *pkr* gene has been localized to chromosome 2p21-p22. Although there are known malignancies associated with chromosomal abnormalities of 2p21, it remains to be determined whether these abnormalities specifically involve the *pkr* gene. Examination of mice with a targeted disruption in the *pkr* gene revealed no evidence of increased spontaneous tumor formation (Yang et al. 1995). Moreover, fibroblast cell lines derived from the PKR knockout mice do not form tumors when injected into nude mice. These findings suggest that the absence of PKR is insufficient for cellular transformation. If *pkr* is a tumor suppressor gene, it may turn out that a yet to be identified functional homolog of PKR exists in the cell which maintains tumor suppressor function in the absence of PKR.

The mechanism by which PKR exerts its tumor suppressive properties is unknown. One possible mechanism is through the induction of apoptotic cell death. It has been shown that PKR induces apoptosis in certain cell types, under certain conditions. For instance, in HeLa cells infected with a recombinant vaccinia virus expressing wild type PKR under an inducible promoter, apoptosis occurred immediately following induction. In contrast, HeLa cells infected with virus expressing a dominant negative PKR mutant (PKR-DII) did not undergo programmed cell death (Lee and Esteban 1994). Furthermore, the PKR-DII mutant protected cells from apoptosis following influenza virus infection (Takizawa 1996). Lastly, several studies have implicated PKR in the induction of apoptosis by dsRNA as well as TNF- α (Yeung et al. 1996; Kibler et al. 1997). These studies suggest that one mechanism by which PKR suppresses tumor growth is through the induction of programmed cell death.

Regulators of PKR

Because PKR has been implicated in a variety of biologic effects, the regulation of PKR is currently an intense area of research. Both RNA activators and RNA inhibitors have already been discussed previously in this chapter (see *Activation of PKR by double-stranded RNA* and *Viral defense strategies to counter PKR activation*). To date, only one example of a protein that activates PKR has been published: the Semliki Forest virus capsid protein (Favre et al. 1996). In contrast, several protein inhibitors of PKR have been described.

Cellular protein inhibitors

All of the protein inhibitors identified thus far have been cellular in origin. Their mechanisms of action fall broadly into two classes: (I) those that sequester PKR activators through binding to dsRNA (Hatada and Fukuda 1992; Mabrouk et al. 1995; House and Kemp 1990), and (II) those that block kinase activity via protein-protein interaction with the kinase or its substrate eIF-2 (Katze 1996; Park et al. 1994; Cosentino et al. 1995; Xiao et al. 1994; Wu et al. 1996; Judware and Petryshyn 1992). As shown in Table I, those cellular inhibitors of PKR which act by sequestering dsRNA activators include the HIV-1 TAR RNA binding protein (TAR-BP) and La autoantigen. Interestingly, expression of TAR-BP in NIH 3T3 cells has been shown to induce tumor formation in nude mice (Benkirane et al. 1997), supporting the notion that PKR is tumor suppressive. The other class of proteins which act via protein-protein interactions include p67, dRF and P58^{IPK}. p67 has been shown to bind eIF-2, thus protecting eIF-2 α substrate from being phosphorylated by PKR (Wu et al. 1996). dRF is a 15 kDa protein found in mouse 3T3-F442A preadipocyte fibroblasts, which blocks PKR autophosphorylation and prevents PKR-mediated terminal differentiation of the 3T3-F442A cells (Judware and Petryshyn 1992). P58^{IPK} binds PKR to block both its autophosphorylation and the phosphorylation of eIF-2 α substrate.

Overview Of P58^{IPK}

Identification of P58^{IPK}

P58^{IPK} (Inhibitor of Protein Kinase) is a cellular inhibitor of PKR that was first identified in influenza virus-infected cells. The notion that influenza virus may encode a mechanism to downregulate PKR activity first surfaced when cells were coinfecting with influenza virus and a mutant form of adenovirus which lacked VAI RNA. (VAI RNA is the adenovirus-encoded RNA which blocks PKR activity by binding to and sequestering the kinase.) It was observed in these coinfecting cells that influenza virus was able to reduce the high levels of PKR autophosphorylation and activity normally seen in mutant adenovirus-infected cells (Wu and Kaufman 1997). Subsequently, it was shown that a similar suppression occurred in cells infected with influenza virus alone (Katze et al. 1988). Following up on these observations, the inhibitor of PKR activity was purified from influenza virus-infected Madin Darby bovine kidney (MDBK) cells (Lee et al. 1990). Unexpectedly, the purified inhibitor was shown to be cellular, not viral, in origin. This cellular inhibitor, referred to as P58^{IPK}, based on its M_r of 58,000, is present in equal amounts in infected and uninfected cells. However, during influenza virus infection, P58^{IPK} is hypothesized to be activated through a dissociation from its own inhibitor called I-P58 (Lee et al. 1992).

Using reverse genetics, P58^{IPK} was cloned from a MDBK cDNA expression library (Lee et al. 1994). When expressed in *Escherichia coli* (*E. coli*) as a histidine-tagged recombinant protein, purified P58^{IPK} was shown to inhibit *in vitro* both

PKR autophosphorylation and the phosphorylation of eIF-2 α substrate by an activated kinase in a dose-dependent manner (Lee et al. 1994). These observations suggested that the cloned P58^{IPK} cDNA encoded a protein inhibitor of PKR phosphorylation activity.

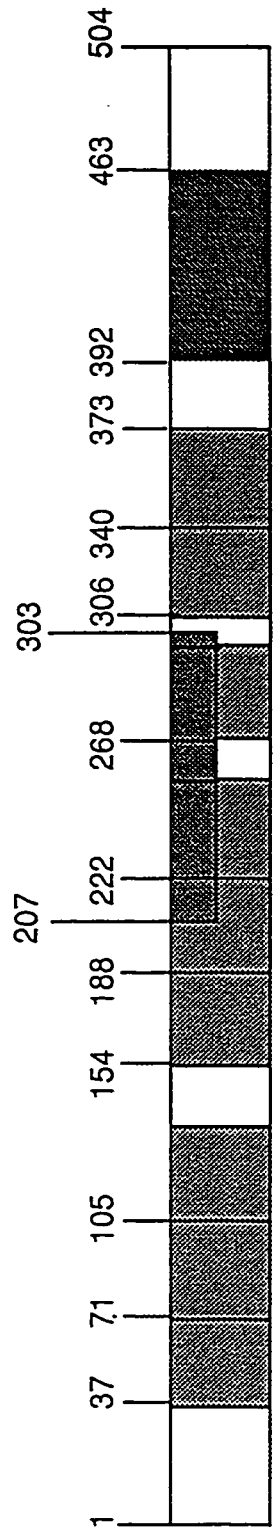
Structure of P58^{IPK}

The mechanism by which P58^{IPK} inhibits PKR activity is unknown. It was shown that P58^{IPK} is not a protease, nor does P58^{IPK} function as an ATPase, ribonuclease, or phosphatase (Lee 1993). In addition, P58^{IPK} does not appear to act by sequestering the dsRNA activators. It is possible that P58^{IPK} may be working through a direct interaction with PKR.

Based on sequence similarities to known proteins, several possible mechanisms come to mind as to how P58^{IPK} can inhibit kinase function. The open reading frame of the bovine P58^{IPK} cDNA encodes a protein of 504 amino acids with a predicted molecular weight of 57,668 daltons (Figure 1.4). Though P58^{IPK} is a novel protein, it does share some interesting homologies with other known proteins. Spanning the P58^{IPK} molecule are nine tandemly arranged motifs referred to as tetratricopeptide repeats, or TPRs for short. TPRs are 34 amino acid motifs which have been postulated to form amphipathic alpha helices (Sikorski et al. 1990). These have been shown to participate in protein-protein interactions (Lamb et al. 1995; Abe et al. 1993; Ratajczak and Carrello 1996). The current TPR family includes a diverse group of eukaryotic proteins (from yeast to human) which participate in a wide variety of cellular functions, including mitosis, protein transport, RNA synthesis, steroid receptor signaling, and neurogenesis (Goebel and

Figure 1.4. Domain structure of P58^{IPK}. The bovine P58^{IPK} cDNA encodes a protein of 504 amino acids containing similarity to TPR (nine repeats, amino acids 37-372), eIF-2 α (amino acids 207-303) and DnaJ (amino acids 392-463) proteins. The homology to eIF-2 α is 31% over a length of 97 amino acids, with five gaps. The homology to DnaJ is 41% over a length of 84 amino acids, with no gaps.

eIF-2 α homology



TPR motifs

DnaJ homology

Yanagida 1991; Chen et al. 1996). In addition, other TPR family members have been implicated in cellular transformation (Honore et al. 1992; Chen et al. 1995; Hirano et al. 1990). Although no functional role has been assigned to TPR motifs, it is tempting to speculate that these may mediate P58^{IPK}-PKR interactions, leading to kinase inactivation.

In addition to the nine TPR motifs, the middle region of P58^{IPK} (from amino acids 207-303) shows limited homology to the amino terminal region of eIF-2 α , the natural substrate of PKR. The homology between these two proteins is 31%, with 5 gaps, over a length of 97 amino acids. Interestingly, the serine residue at position 241 of P58^{IPK} is part of an ELS tripeptide, just like serine 51 of eIF-2 α . Since serine 51 is phosphorylated by PKR and its phosphorylation is vital for mediating PKR's blockage of protein synthesis initiation (Choi et al. 1992), it is possible that P58^{IPK}'s inhibition of a putative P58^{IPK} substrate site with the PKR catalytic region.

Finally, the carboxyl terminus of P58^{IPK} shows significant homology to the conserved domain of the *E. coli* DnaJ heat shock family of proteins (Ohki et al. 1986; Silver and Way 1993). The sequence identity to the DnaJ amino terminal region is extensive: 41% over a length of 84 amino acids, with no gaps. The DnaJ heat shock protein plays an essential role in the chaperone function of the Hsp70-like DnaK protein of *E. coli* (Benveniste et al. 1986; Cheetham et al. 1992). DnaJ homologs have been found in yeast (Luke et al. 1994; Rothblatt et al. 1989; Sadler et al. 1989) and humans (Cheetham et al. 1992; Raabe and Manley 1991). It has been suggested that eukaryotic DnaJ homologs may participate in protein-protein interactions and play a role in directing the activity of Hsp70 to different

substrates (Silver and Way 1993). Moreover, as part of the chaperone system, DnaJ has been shown to function in a molecular complex, along with DnaK and GrpE (Langer et al. 1992). Thus, it is possible that P58^{IPK} is participating in a molecular complex, similar to DnaJ-DnaK-GrpE, with the conserved motifs participating in protein-protein interactions. Furthermore, it is possible that P58^{IPK}'s mechanism of PKR inhibition is through the DnaJ region which serves to direct PKR away from its substrates, such as eIF-2 α . The significance of the DnaJ homology domain of P58^{IPK} remains to be determined.

OTHER TRANSLATIONAL CONTROL STRATEGIES EXERTED BY INFLUENZA VIRUS

Although this thesis study focuses on P58^{IPK}, influenza virus has employed other additional strategies to exert translational control. Some of these strategies are summarized in Table II and can be broken up into two major objectives: (1) to stop the translation of cellular mRNAs and to selectively translate viral mRNAs, and (2) to maintain translational competence in the infected cell. The second objective is achieved through the inhibition of PKR by the activation of P58^{IPK} (discussed previously).

To achieve the first objective and stop translation of cellular mRNAs, influenza virus has employed three different strategies. It has been reported that cellular mRNA degradation occurs in influenza virus-infected cells, especially late after infection (Inglis 1982; Beloso et al. 1992). In addition, newly synthesized cellular mRNAs which are in the nucleus are degraded early after infection such that these never reach the cytoplasm of virus-infected

Table II. Influenza virus translational regulatory mechanisms
(taken from Katze 1996)

- Host shutoff of cellular protein synthesis
- Inhibition of cellular mRNA transport/degradation of mRNAs in nucleus
- Inhibition of PKR
- Recruitment of P58^{IPK}
- Cap-dependent, selective translation of influenza virus mRNAs
- Inhibition of cellular mRNA translation at initiation and elongation stages
- Degradation of cellular mRNAs in the cytoplasm
- Structure of influenza virus mRNAs
- Dephosphorylation of eukaryotic initiation factor 4E
- Temporal control of influenza virus protein synthesis

cells (Katze and Krug 1984). Cellular nuclear RNA degradation is probably the result of cellular nuclease activity on decapped RNAs, which are generated by the cleavage of the 5' ends of cellular RNA polymerase II transcripts by the viral cap-dependent endonuclease. The third strategy utilized by influenza virus to stop translation of cellular mRNAs is through the inhibition of protein synthesis at both the initiation and elongation steps (Katze et al. 1986). To selectively translate viral mRNAs, it was shown that influenza virus mRNAs are intrinsically better initiators of translation than cellular mRNAs. This is likely due to the presence of a 20 nucleotide sequence conserved in all 5'-untranslated regions of influenza virus messages (Garfinkel and Katze 1993). The ability of the host translational apparatus to distinguish between viral and cellular mRNAs, based on the sequence and/or structure of the 5'-UTR, may depend on the *trans*-acting factors that interact with these sequences. One of these *trans*-acting factors may be the initiation factor eIF-4E. It has been observed that eIF-4E is slightly dephosphorylated in influenza virus-infected cells (Feigenblum and Schneider 1993). Because dephosphorylation of eIF-4E leads to a functional limitation of eIF-4F (one activity of which is to unwind secondary structure in the 5'UTRs of mRNAs), it is possible that the selective translation of influenza virus mRNAs may be due to their reduced requirement for functional eIF-4E, as a result of reduced higher order structure.

Although numerous viruses utilize translational control strategies to subvert the host, few have been shown to directly recruit cellular factors for this deed. Influenza virus is unique in that it is the only eukaryotic virus identified thus far whose

cellular cohort in the subversion of host translational regulation is known. For this reason, the study of P58^{IPK} is particularly interesting. Moreover, because few cellular regulators of PKR have been identified and cloned, an in-depth study of P58^{IPK} may illuminate important biologic processes regulated by PKR.

Objectives

P58^{IPK} is a cellular protein identified from influenza virus-infected cells which plays a role in the regulation of PKR activity. Expression of P58^{IPK} from a cloned bovine cDNA sequence demonstrated that P58^{IPK} has two inhibitory properties *in vitro*: (i) the inhibition of PKR autophosphorylation, and (ii) the inhibition of eIF-2 α substrate by an already activated kinase. P58^{IPK} has also been shown to transform NIH 3T3 cells and induce tumor formation in nude mice. The mechanism(s) of P58^{IPK} action is unknown. This study describes initial steps to elucidate the mechanism by which P58^{IPK} inhibits PKR activity and to define the physiologic role of P58^{IPK} by elucidating the pathway by which P58^{IPK} induces malignant cellular transformation. To achieve these goals, the following specific aims were undertaken:

Specific aim 1. Identify functional domains of P58^{IPK} through *in vitro* analyses of P58^{IPK} mutants. Utilizing the bovine P58^{IPK} cDNA as template, a series of P58^{IPK} mutant constructs were generated using site-directed mutagenesis. These mutant constructs were expressed as histidine-tagged fusion proteins in *E. coli*; affinity purified

using metal-chelation chromatography; and assayed for the ability to inhibit kinase phosphorylation activity *in vitro*.

Specific aim 2. Perform *in vivo* analysis on wild type and mutant P58^{IPK} constructs to correlate *in vitro* and *in vivo* results. Wild type or mutant P58^{IPK} DNA were cotransfected with reporter DNA into COS-1 cells, and examined for the ability to stimulate protein synthesis, one indicator of inhibition of PKR activity.

Specific aim 3. Define the physiologic role of P58^{IPK} through an examination of the oncogenic properties of the various P58^{IPK} mutant constructs. The mechanism by which P58^{IPK} induces oncogenesis is unknown. Because PKR may be anti-tumorigenic via its ability to downregulate protein synthesis and suppress cellular growth, one possible mechanism by which P58^{IPK} induces oncogenesis is through the inhibition of PKR. Thus, P58^{IPK} activity may cause increased synthesis of proteins which function to promote cell growth and malignancy. To test this hypothesis, P58^{IPK} mutant-expressing cell lines were generated and analyzed for oncogenic potential. The oncogenic potentials of the various P58^{IPK} mutant cell lines were correlated with their ability to inhibit murine PKR phosphorylation of endogenous eIF-2 α , the natural substrate of PKR. The studies outlined in this aim are not intended to precisely define the molecular pathway by which P58^{IPK} induces oncogenesis, but rather to investigate whether eIF-2 α phosphorylation/protein synthesis control plays a significant role in regulating cell growth and tumorigenesis. It is quite possible that P58^{IPK} induces oncogenesis via some other pathway, such as the NF- κ B signal transduction pathway (see sections on *Control*

of cell growth and Proliferation and Tumor suppression and induction of apoptosis for details).

The proposed studies serve to identify the functional domains of P58^{IPK} as well as elucidate the mechanism of P58^{IPK} oncogenicity. In accomplishing these goals, this work adds significantly to the general understanding of protein synthesis control and further defines the molecular mechanisms of cell growth control and tumorigenesis. Moreover, this work may provide a basis for designing therapeutic strategies and drugs to combat human cancer by identifying two molecular targets (P58^{IPK} and PKR) for medical intervention.

Chapter 2: Mutational Analysis Of P58^{PK} Inhibitory Activity In

Vitro

Introduction

The *pk*r gene is one of more than 30 interferon-induced genes. The PKR protein is also one of two enzymes induced by interferon that is regulated by dsRNA (Hovanessian and Galabru 1987; Sen and Lengyel 1992). As a result of dsRNA activation, PKR undergoes autophosphorylation and catalyzes the phosphorylation of its eIF-2 α substrate (Galabru and Hovanessian 1987; Samuel 1993). Phosphorylation of the serine 51 residue of eIF-2 α blocks the eIF-2B-mediated exchange of GDP in the inactive eIF-2-GDP complex (Hershey 1991; Merrick 1992; Merrick and Hershey 1996). This leads to functional limitations of eIF-2, a critical component of the protein synthesis machinery. Thus, activation of PKR leads to reductions of protein synthetic rates inside the cell.

Because viruses must make their proteins in order to replicate, many eukaryotic viruses have evolved strategies to inhibit PKR activity during virus infection. Some of these strategies include viral-encoded RNAs or proteins which bind directly to PKR to sequester and inactivate the kinase (Katze et al. 1987; Ghadge et al. 1991; Katze 1995; Schneider 1996; Sharp et al. 1993; Clemens et al. 1994; Clarke et al. 1991; Clarke

et al. 1990; Davies et al. 1992; Davies et al. 1993; Carroll et al. 1993). Other strategies utilize viral-encoded proteins which bind to and sequester dsRNA activators (Chang and Jacobs 1993; Davies et al. 1993; Mabrouk et al. 1995; Hatada and Fukuda 1992; Lu et al. 1995). There are still other viral strategies which take advantage of host proteins to downregulate PKR activity. One such strategy may be utilized by influenza virus.

The first indication that influenza virus encoded a mechanism to downregulate PKR activity came from studies of cells that were coinfecting with the adenovirus *d1331* VAI RNA-negative mutant and influenza virus (Wu and Kaufman 1997). It was observed in these coinfecting cells that influenza virus was able to reduce the high levels of PKR autophosphorylation and activity normally seen in adenovirus *d1331* mutant-infected cells (Thimmappaya et al. 1982). Subsequently, it was shown that a similar suppression occurred in cells infected with influenza virus alone (Katze et al. 1988). Attempts to purify the PKR inhibitor from influenza virus-infected cells led to the unexpected finding that the PKR inhibitor was cellular in origin. Western blot analysis using virus-specific antibodies did not detect a reactive protein in the purified cellular fractions containing kinase inhibitory activity (Lee et al. 1990). Furthermore, Western blot analysis using a polyclonal peptide antibody against the biochemically purified product showed that the detected 58 kDa protein was present in both influenza virus-infected and uninfected MDBK cells in equal amounts. The PKR inhibitor is referred to as P58^{IPK} on the basis of the M_r of 58,000.

P58^{IPK} was cloned and the cDNA sequence was reconstructed based on the information from two overlapping open reading frames (Lee et al. 1994). The reconstructed P58^{IPK} bovine cDNA contains 1,680 nucleotides, with one open reading frame. A putative mRNA instability sequence, ATTTA, is present near the 3' end of the P58^{IPK} sequence at nucleotides 1494-1495, suggesting possible post-transcriptional regulation of the P58^{IPK} mRNA. There are 115 nucleotides upstream of the start AUG codon which constitute the 5' untranslated region (UTR) of the cDNA. The single open reading frame of the bovine P58^{IPK} cDNA sequence predicts a protein coding sequence of 504 amino acids. This predicted protein is composed of extremely hydrophilic regions, with one major hydrophobic region located at the amino terminus (amino acids 11-24). There is one potential N-glycosylation site (Asn-Pro-Ser) at amino acid 135 and two potential protein kinase C phosphorylation sites at amino acids 214 and 390. There is also one potential cAMP-dependent protein kinase phosphorylation site at amino acid 79 and three casein kinase II phosphorylation sites at amino acids 241, 274 and 365. Preliminary *in vitro* and *in vivo* data suggest that P58^{IPK} may be a phosphoprotein (see Chapter 5 for details).

To confirm that the reconstructed cDNA encoded an inhibitor of PKR activity, the cDNA was expressed in *E. coli* as a histidine-tagged P58^{IPK} fusion protein. The P58^{IPK} recombinant protein was purified using nickel metal affinity chromatography. The material that eluted off the nickel column was subjected to dialysis. This purified material, when analyzed by Coomassie Blue staining showed one predominant protein

band at 58 kDa, with several shorter polypeptides around 20-30 kDa size. Western blot analysis revealed these shorter polypeptides to be P58^{IPK} degradation products which were reactive with a P58^{IPK}-specific antibody (Lee 1993). When the purified, dialyzed P58^{IPK} material was analyzed in an *in vitro* kinase assay for PKR inhibitory activity, it was found that recombinant P58^{IPK} inhibited PKR phosphorylation activity in a dose-dependent manner.

The mechanism by which P58^{IPK} inhibits PKR is unknown. P58^{IPK} does not appear to act as a protease, ATPase, ribonuclease or phosphatase (Lee 1993). P58^{IPK} also does not appear to act by sequestering dsRNA activators. In order to elucidate the mechanism of P58^{IPK} action, a structure/function study of the cloned bovine P58^{IPK} molecule was undertaken. Using site-directed mutagenesis, eight different P58^{IPK} mutants were generated. These were expressed in *E. coli* as histidine-tagged fusion proteins and metal affinity purified. A more reliable *in vitro* assay was developed to measure the ability of purified, wild type and mutant P58^{IPK} recombinant proteins to specifically inhibit PKR phosphorylation activity. Using such an assay, the middle region of P58^{IPK}, from amino acids 166 to 302, was shown to be required for P58^{IPK} inhibitory function *in vitro*. In addition, the P58^{IPK} functional data was correlated with the binding of P58^{IPK} to PKR *in vitro*. Taken together, these data demonstrate that P58^{IPK} inhibits PKR through a direct interaction. Moreover, the ability of P58^{IPK} to modulate PKR activity *in vitro* requires the central domain.

Materials And Methods

Expression and purification of the His-P58^{IPK} fusion protein in *Escherichia coli*

Histidine-tagged P58^{IPK} fusion protein was expressed in *E. coli* BL21(λ DE3)pLysS after induction with 0.2mM isopropylthiogalactopyranoside (IPTG) for 2 hours at 30°C. Because the major pool of P58^{IPK} fusion protein was found in the insoluble fraction, this insoluble fraction was subjected to denaturation in 6M guanidine-HCl. The fusion protein was purified by Ni(II) column (Novagen) according to manufacturer's instructions. The purified protein was renatured on ice after fifty-fold dilution in renaturation buffer (20 mM Tris-HCl, pH 7.8, 20% glycerol, 0.1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin). The renatured protein was aliquoted and stored at -80°C until assayed.

Purification of PKR

Approximately 10⁹ Daudi cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, supplemented with non-essential amino acids and sodium pyruvate. Cells were treated for 18 hours with 500 units/ml human interferon, after which they were spun down, washed with 1X PBS, and lysed in 10ml of Buffer A (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 μ g/ml aprotinin, 20% glycerol, 1% Triton X-100). The cells were frozen at -80°C and thawed. The thawed lysate was

spun in a cooled (4°C) ultracentrifuge for 1 hour at 32,000 rpm using a SW41 rotor. The clarified lysate was filtered through a 0.45µm filter and the material antibody affinity purified using a PKR monoclonal antibody conjugated to sepharose beads (Galabru and Hovanessian 1987). The purified material was then dialyzed in Dialysis Buffer (20 mM HEPES, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 100µg/ml aprotinin. Aliquots were made and frozen in liquid nitrogen until use.

Protein concentration determination

The protein concentration of purified PKR and purified recombinant P58^{IPK} proteins were determined using the Pierce BCA Protein Assay Reagent according to manufacturer's protocol (Pierce, Rockford, IL).

Construction of P58^{IPK} mutants

The P58ΔN1 and P58-SER241 mutants were prepared using the MUTA-GENE M13 *in vitro* mutagenesis kit (Bio Rad) and the following synthesized oligonucleotides: ΔN1: 5'-GTTTGTGTTTGGGATCATGAATATAACAATT-3', and SER241: 5'-GACCATGAACTGGXTCTCAGTGAAG-3' (where X = G, A or C). After mutagenesis, a 1.6 kb *XbaI-BamHI* fragment containing the mutated P58^{IPK} gene was isolated and cloned into *XbaI-BamHI*-digested pET15b bacterial expression vector. The N-terminal and C-terminal P58^{IPK} deletion mutants were constructed previously by Dr. Tae Gyu Lee (Lee 1993). The precise identity of all mutant constructs was verified by a

combination of restriction enzyme digest mapping and sequence analysis using the dideoxy chain termination technique.

***In vitro* assay for wild type P58^{IPK} and mutant P58^{IPK} activity**

Purified wild type or mutant P58^{IPK} recombinant proteins were preincubated with purified PKR for 10 minutes at 30°C in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 2 mM MnCl₂, 2 μM ATP, 5 μg/ml aprotinin, 1 mM dithiothreitol, 500 μg/ml bovine serum albumin. Subsequently, activator (0.1 mg/ml poly(rI):poly(rC)) was added in the presence of 0.05 μM [γ -³²P]ATP and incubated for an additional 10 minutes. Finally, exogenous substrate (10 μg of histones (Sigma, type IIA from calf thymus) or purified eIF-2 (gift from Dr. Rosemary Jagus, University of Maryland)) was added with an additional 0.05 μM [γ -³²P]ATP, and the mixture was allowed to incubate for another 10 minutes. The reaction was stopped by the addition of 2X disruption buffer (180 mM Tris-HCl, pH 6.8, 4.5% SDS, 23% (v/v) glycerol, 17 mM EDTA, 20 μg of RNase A, 3 M 2-mercaptoethanol) and boiling. The samples were then fractionated by SDS-PAGE (14% acrylamide) and visualized by autoradiography.

Immunoblot analysis

Purified recombinant P58^{IPK} proteins were resuspended and diluted into five volumes of water and one volume of 6X Sample Buffer (0.25 M Tris-HCl, pH 6.8, 7.1% SDS, 25% glycerol, 0.43M dithiothreitol, 0.01% bromophenol blue). Diluted

samples were loaded onto a 17% SDS polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis, polypeptides were transferred to nitrocellulose (Towbin et al. 1979) and detected with a rabbit polyclonal antibody generated against the fusion protein GST-P58^{IPK} (Lee et al. 1994).

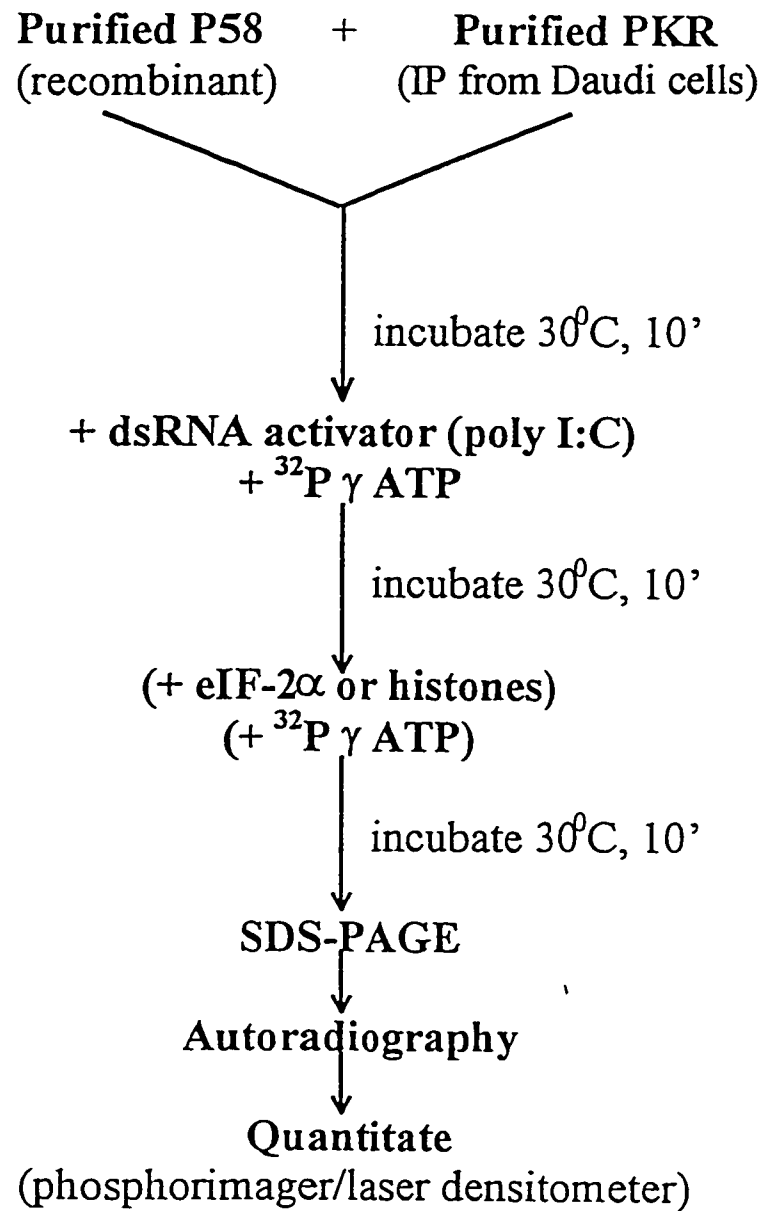
Results

Development of a more reliable *in vitro* assay to measure inhibition of PKR phosphorylation activity

Using purified components, an *in vitro* kinase assay was developed to more reliably measure the ability of recombinant (wild type or mutant) P58^{IPK} to inhibit PKR phosphorylation activity. This assay is outlined in figure 2.1. Briefly, the *in vitro* assay entailed the preincubation of purified histidine-tagged P58^{IPK} proteins with approximately equimolar amounts of purified native PKR protein. After 10 minutes at 30°C, dsRNA activator in the form of poly(rI):poly(rC) was added to the reaction, along with ³²P-γ-ATP as tracer for the kinase autophosphorylation reaction. Subsequently, exogenous substrate (if used) in the form of purified eIF-2 or histones was added, along with additional ³²P-γ-ATP to track PKR phosphorylation of substrate. The material from the final reaction was fractionated by SDS-PAGE, visualized by autoradiography, and the

Figure 2.1. *In vitro* assay used to measure P58^{IPK} activity. P58^{IPK} was purified as His-P58^{IPK} recombinant proteins using a nickel column. PKR was purified from Daudi cells using a monoclonal antibody conjugated to sepharose beads.

In Vitro Assay for Inhibition of PKR Activity



radiolabeled proteins quantified by laser densitometry scanning or phosphorimager analysis.

As shown in figure 2.2, recombinant P58^{IPK} protein inhibited both PKR autophosphorylation and PKR-mediated phosphorylation of histone substrate in a dose dependent manner. In contrast, control protein (material purified in the same manner as wild type P58^{IPK} using extracts from *E. coli* transformed with the vector lacking the P58^{IPK} cDNA gene) did not show any inhibition of kinase activity. Whether purified eIF-2 α (data not shown) or histones proteins were used for the kinase substrate, this assay consistently showed that recombinant P58^{IPK} was able to inhibit PKR phosphorylation activity *in vitro*.

The central domain of P58^{IPK} is required for inhibition of PKR activity *in vitro*

P58^{IPK} contains nine tandemly arranged 34 amino acid repeats, referred to as TPR motifs (Figure 2.3). Members of the TPR family of proteins participate in a wide variety of cellular functions, including mitosis, transcription, protein import, RNA splicing and stress (for reviews, see (Goebel and Yanagida 1991; Sikorski et al. 1991). The functional role of the TPR motif has not been determined. P58^{IPK} also shares similarity to the J-domain of bacterial DnaJ protein at the carboxyl terminus, and there is partial similarity between the central region of P58^{IPK} (amino acids 207 to 303) and the natural substrate of PKR, eIF-2 α (amino acids 26 to 120). Interestingly, the serine 51 residue of eIF-2 α is in a similar context as serine 241 of P58^{IPK}. Since serine 51 of eIF-2 α

Figure 2.2. Inhibition of PKR autophosphorylation and PKR-mediated histone phosphorylation by P58^{IPK}. Increasing amounts of His-P58^{IPK} proteins (0, 3.4 and 6.8 pmol, corresponding to 0, 2 and 4 μ l, respectively) were tested for the ability to inhibit PKR autophosphorylation and PKR-mediated histone phosphorylation as described under "Materials and Methods". As a control, extracts from *E. coli*, transformed with the vector lacking the P58^{IPK} cDNA, were passed over a nickel column and treated identically to the extracts containing the P58^{IPK} inhibitor. In this experiment, 0, 2 and 4 μ l (corresponding to approximately 0, 3 and 6 pmol) of control material were tested and compared to His-P58^{IPK}.

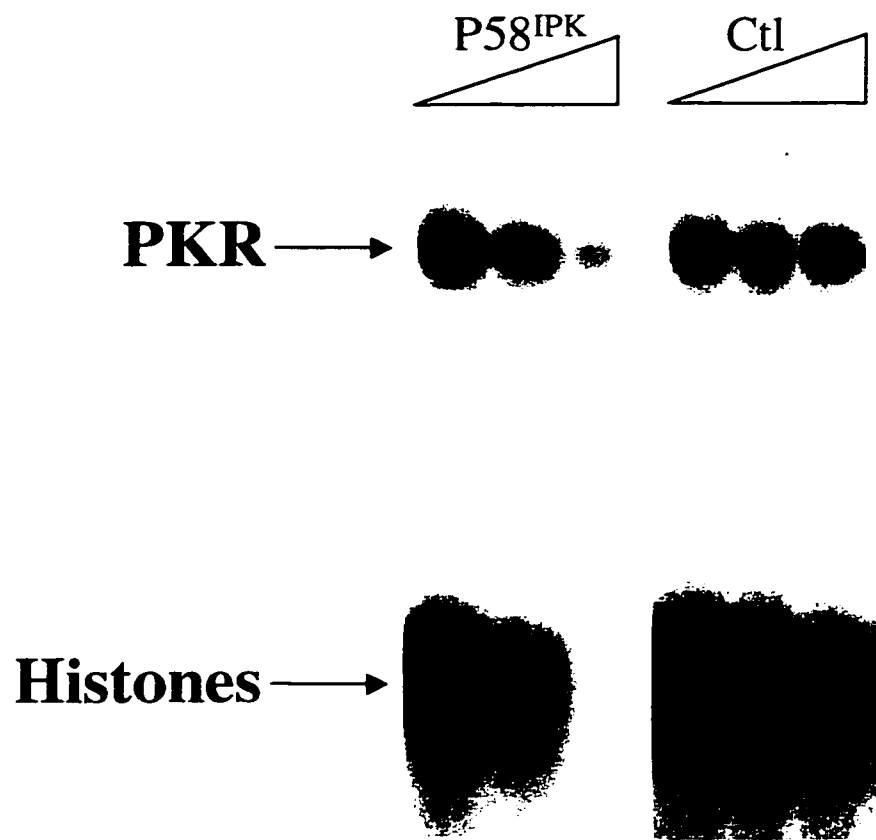
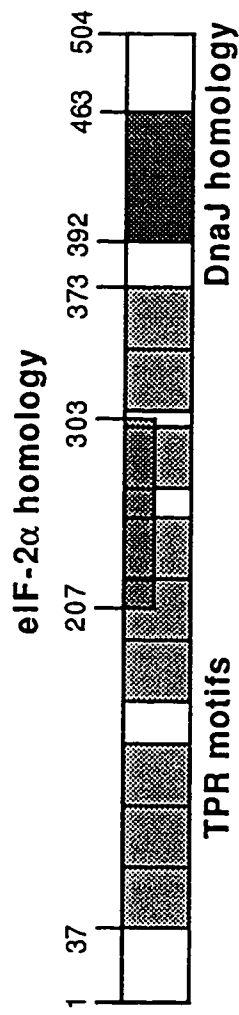


Figure 2.3. Schematic representation of full-length wild type P58^{IPK} (P58-WT) and mutants. Each P58^{IPK} construct was fused to the histidine tag (MGSSHHHHSSGLVPRGSH) at its amino terminus. The three amino-terminal deletion mutants (8-1, 8-2 and 8-3) were constructed by engineering stop codons at the indicated positions. The three carboxyl-terminal deletion mutants (9-1, 9-2 and 9-3) and the one internal deletion mutant (Δ N1) were constructed using site directed mutagenesis as described under "Materials and Methods". The SER241 mutant contains a single amino acid mutation from a serine to alanine at position 241. The total number of TPR motifs present in each of the construct is indicated at the *right*.



Construct	# of TPRs
P58-WT	9
P58-8-1	3
P58-8-2	6
P58-8-3	9
P58-9-1	5
P58-9-2	2
P58-9-3	0
P58-SER241	9
P58-ΔN1	6

■ : histidine-tag
 * : stop codon

P58-WT

P58-8-1

P58-8-2

P58-8-3

P58-9-1

P58-9-2

P58-9-3

P58-SER241

P58-ΔN1

166 (*)

277 (*)

390 (*)

168

278

390

SER → X
 ALA

187

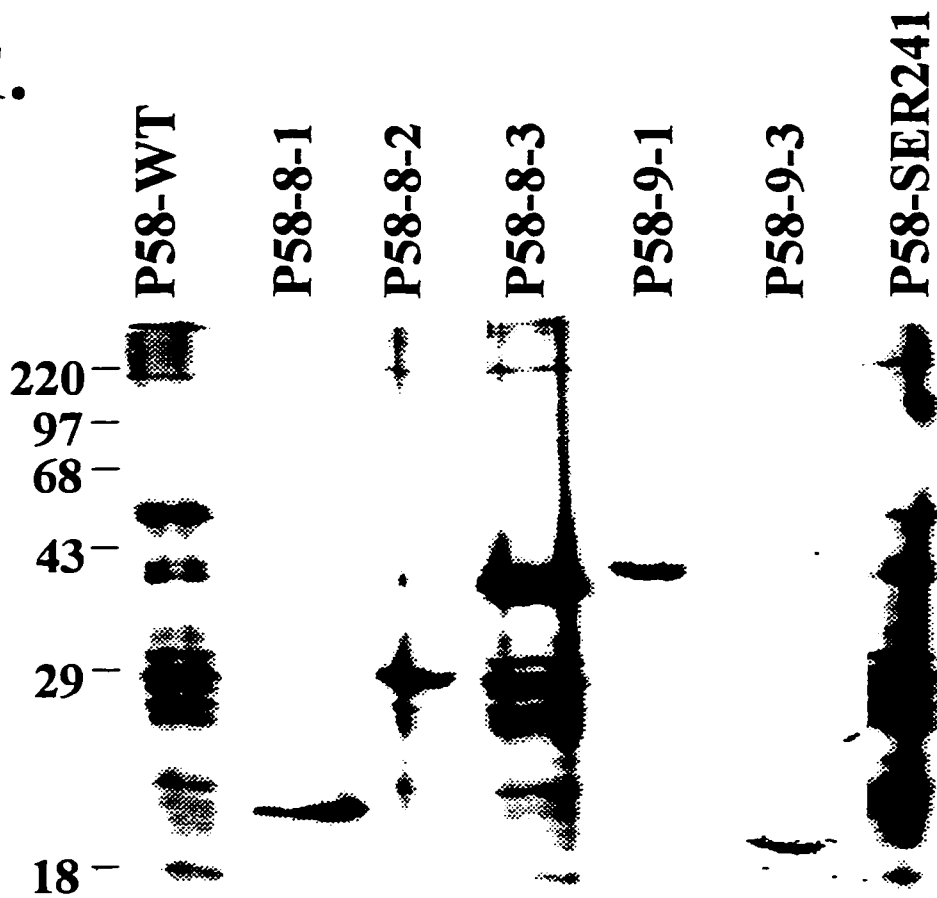
302

is phosphorylated by PKR *in vivo*, the serine 241 residue of P58^{IPK} may act in a similar manner, serving as a substrate for PKR.

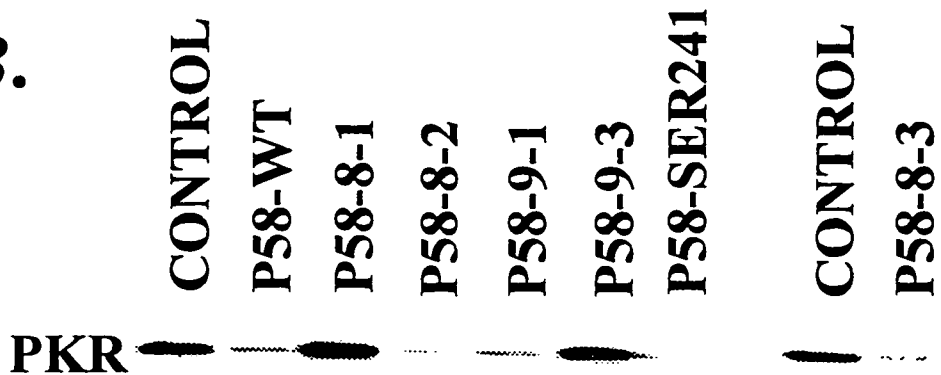
To determine the functional significance of the various similarities, seven P58^{IPK} mutants were initially constructed and tested for the ability to inhibit PKR phosphorylation activity using the *in vitro* assay described above. These mutants are depicted in figure 2.3. Wild type and mutant P58^{IPK} proteins were expressed in *E. coli* as histidine-tagged fusion proteins and purified on nickel columns (Figure 2.4A). Approximately equimolar amounts of mutant P58^{IPK} proteins were used in the *in vitro* assay to test their effects on PKR autophosphorylation in this series of experiments (Figure 2.4B). Consistently, there was a correlation between levels of PKR autophosphorylation and eIF-2 α phosphorylation in these assays (data not shown). As shown in figure 2.4B, constructs 8-2, 8-3 and 9-1 were all functional. In addition, the serine-to-alanine mutant (SER241) also retained activity. In contrast, constructs 8-1 and 9-3 lost their PKR inhibitory activity. Together, these data demonstrate that not all TPR motifs are required for function *in vitro*, nor is the DnaJ similarity region essential. On the other hand, all functional constructs contained amino acids 168 to 277, suggesting a critical role for this part of the P58^{IPK} protein. All of the functional constructs also contained 5 or more TPR motifs, indicating that perhaps a critical/minimal number of TPR motifs are essential for P58^{IPK} activity *in vitro*. The fact that a point mutational change of serine 241 to an alanine still conferred inhibitory activity does not rule out the possibility that P58^{IPK} may be acting as a substrate to sequester PKR. Perhaps, putative

Figure 2.4. P58^{IPK} structure-function analysis. (A) Western blot analysis was performed on the P58^{IPK} constructs after purification on the nickel columns by using a polyclonal P58^{IPK} antiserum. It was consistently observed that electrophoresis of the individual proteins did not always correlate with the migration of molecular weight standards, possibly because of the differential folding of the constructs and/or residual guanidine in the protein preparations. (B) Approximately equimolar amounts (10 pmol) of the various P58^{IPK} protein constructs, plus control extracts, were tested for the ability to inhibit PKR (approximately 5 pmol) autophosphorylation activity as described in Materials and Methods. Each construct was assayed a minimum of three times, and at least three independent preparations of mutants were assayed over a range of concentrations. Results of a representative assay are shown.

A.



B.

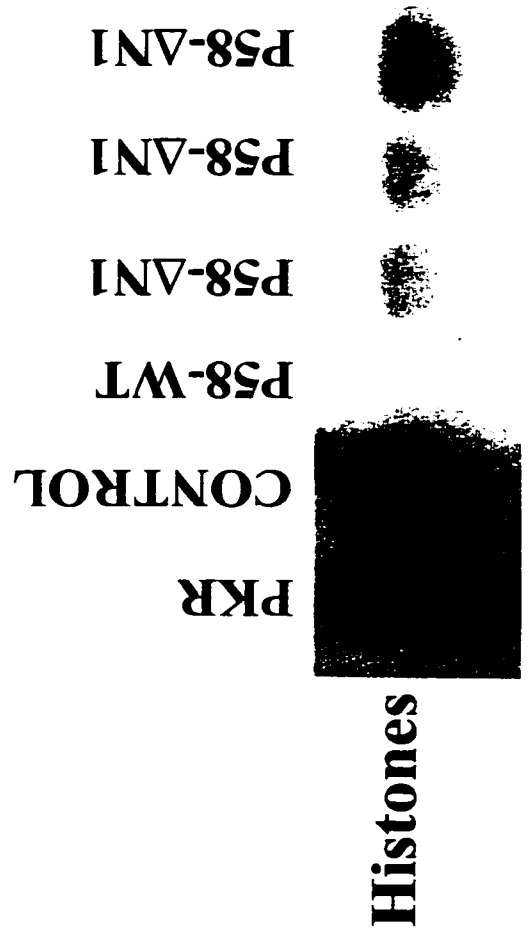


phosphorylation of other amino acids may be more important or a more radical change of serine 241 is required to abolish P58^{IPK} function. Until finer and more detailed mutagenic analysis is performed, these results should be interpreted with some caution.

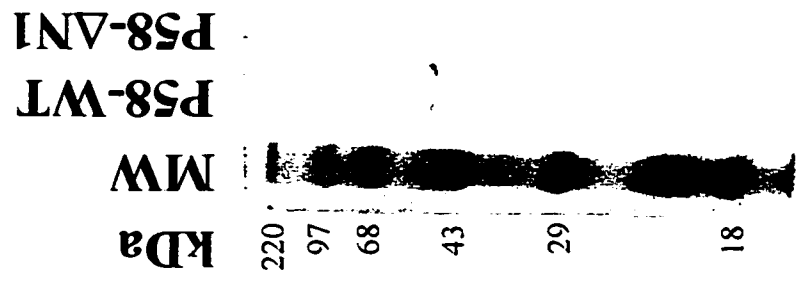
The above functional results led to the construction and testing of an eighth P58^{IPK} mutant. This eighth mutant is referred to as Δ N1 and is schematically illustrated in figure 2.3. Δ N1 lacks amino acids 188-301, which encompasses most of the middle region (amino acids 168-277) previously shown to be important for P58^{IPK} inhibition of PKR activity *in vitro*. Moreover, Δ N1 represents an in-frame deletion of TPRs 5, 6 and 7. This is significant since single amino acid substitutions in these TPRs have previously been shown to be important for biological function of the yeast CDC23 gene (Sikorski et al. 1991; Sikorski et al. 1993). Thus, the Δ N1 mutant was constructed with the intention of addressing the following two questions: (i) is the middle region of P58^{IPK}, from amino acids 188-277 important for P58^{IPK} function *in vitro*?, and (ii) are TPRs 5, 6 and 7 necessary for P58^{IPK} function *in vitro*? As shown in figure 2.5A, using three different preparations of purified His- Δ N1 proteins, the Δ N1 construct was deficient in blocking PKR phosphorylation of histone substrate. By titrating Δ N1 over a range of molar concentrations, it was determined that Δ N1 was approximately 30 times less active than wild type P58^{IPK} protein. By Western analysis with a P58^{IPK}-specific polyclonal antibody, one predominant His- Δ N1 protein product was detected at approximately

Figure 2.5. Δ N1, a P58^{IPK} variant lacking TPRs 5, 6 and 7, is deficient in blocking PKR-mediated histone phosphorylation. (A) 4 μ l of His- Δ N1 protein from three different preparations (corresponding to approximately 20, 20 and 10 pmol) were tested in the *in vitro* assay for the ability to inhibit PKR phosphorylation of histone substrate. As positive and negative controls, 4 μ l of His-P58^{IPK} (6 pmol) and 4 μ l of control extracts (6 pmol), respectively, were also tested. (B) Western blot analysis using a polyclonal P58^{IPK} antiserum was performed on material purified from the nickel columns. The recombinant His- Δ N1 protein shown here migrates at ~40-kDa, slightly faster than the predicted size of 45-kDa.

A.



B.



40-kDa (Figure 2.5B), very close to the predicted size of 45-kDa. When the $\Delta N1$ construct was translated in rabbit reticulocytes, the protein product migrated at 45-kDa when analyzed by SDS- PAGE (data not shown). It is likely that the migration pattern observed for $\Delta N1$ in figure 2.5B reflects residual guanidine in the His- $\Delta N1$ protein preparation and/or differential folding of the construct. The trace amount of shorter polypeptides detected in the $\Delta N1$ purification were degradation products of similar size to those detected in the wild type P58^{IPK} purification. Thus, the deficient inhibitory activity observed for the $\Delta N1$ construct was not due to a lack of intact, recombinant $\Delta N1$ protein or presence of other contaminating proteins. Interestingly, the 9-2 variant (deleted for amino acids 1 to 277) also showed deficient/intermediate functional activity (data not shown). Because $\Delta N1$ and 9-2 both lack TPRs 5, 6 and part of 7, the results suggest that these three TPR motifs play important roles in the inhibition of PKR activity *in vitro*. Taken together, the functional data suggest that the central region of P58^{IPK} is important for PKR inhibitory activity.

Discussion

This study confirms earlier work showing that the cloned bovine P58^{IPK} cDNA encodes an inhibitor of PKR activity *in vitro*. The ability of P58^{IPK} to block PKR phosphorylation activity *in vitro* requires the central domain from amino acids 168 to

302. This region encompasses TPRs 5, 6 and 7, as well as the eIF-2 α similarity region.

Evidence that P58^{IPK} inhibits PKR via a direct interaction

Thus far, the precise mechanism of PKR inhibition by P58^{IPK} has not been determined. P58^{IPK} does not function as a protease, phosphatase or ATPase (Lee 1993). P58^{IPK} also does not function by degrading or sequestering dsRNA, nor does P58^{IPK} compete for dsRNA binding sites. Thus, P58^{IPK} may inhibit PKR through a direct interaction. This hypothesis is supported by the observation that P58^{IPK} does not inhibit the other eIF-2 α kinase, HCR, making it unlikely that P58^{IPK} interacts with eIF-2 (Lee et al. 1994). By demonstrating that His-P58^{IPK} blocks both PKR autophosphorylation and the phosphorylation of histone substrate *in vitro*, this study provides additional evidence that P58^{IPK} may inhibit PKR through a direct interaction.

In order to test the hypothesis that P58^{IPK} functions through a direct interaction with PKR, P58^{IPK}-PKR complex formation was examined *in vitro* using coprecipitation of recombinant wild type or mutant P58^{IPK} proteins. It was shown that recombinant P58^{IPK} (histidine tagged or GST-tagged) can efficiently complex with PKR. Moreover, there was a correlation between the ability of P58^{IPK} to inhibit PKR *in vitro* and the ability of P58^{IPK} to bind the PKR protein kinase (Polyak et al. 1996). These data suggest that P58^{IPK} inhibits PKR activity through a direct binding of P58^{IPK} to the kinase molecule.

The observations made in this study, along with previous studies, have led to the model depicted in figure 2.6 for the regulation of PKR in influenza virus-infected cells. Previous work showed that the P58^{IPK} level does not change after influenza virus infection (Lee et al. 1990). In addition, P58^{IPK} is subjected to regulation, probably as a result of interaction with another cellular factor(s) which is referred to as I-P58 (Inhibitor of P58^{IPK}) (Lee et al. 1992). In the present study, evidence is provided that P58^{IPK} regulates PKR activity via a direct interaction. Putting these observations together, we speculate that during influenza virus infection, P58^{IPK} is activated via dissociation from I-P58. Activated P58^{IPK} then binds PKR, leading to the inhibition of PKR autophosphorylation and the inhibition of PKR-mediated phosphorylation of eIF-2 α substrate by an already autophosphorylated kinase.

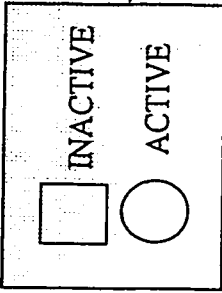
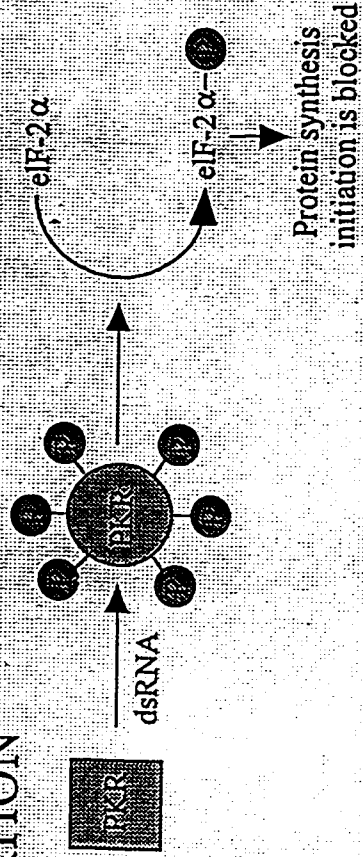
Besides influenza virus, the only other virus thus far identified which utilizes cellular gene products to downregulate PKR activity is poliovirus. In the case of poliovirus, PKR is selectively degraded by a yet to be identified cellular protease whose maximal activity, likely, requires a viral dsRNA component (Black et al. 1993). As previously mentioned in chapter 1, most other viruses encode their own gene products to inactivate PKR (see chapter 1, subsection titled *Viral inhibitors*).

Role of P58^{IPK} central domain in the regulation of PKR

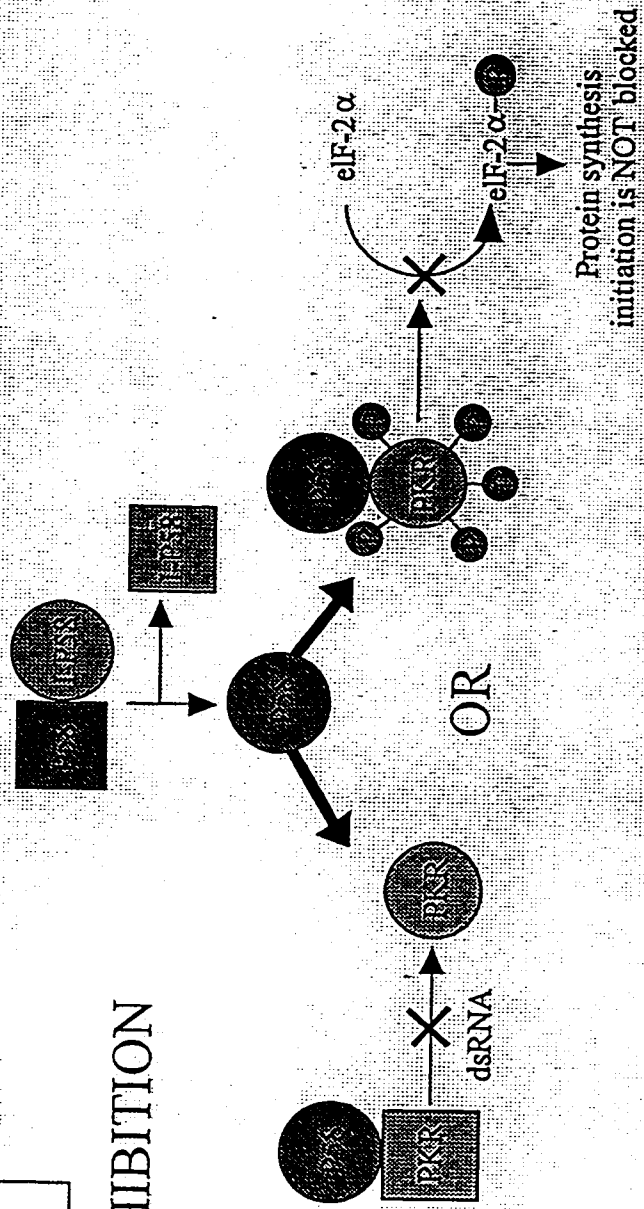
As a member of the TPR family of proteins, P58^{IPK} contains internal 34-amino acid repeats which are predicted to form an amphipathic alpha helix

Figure 2.6. Model for the regulation of PKR activity by P58^{IPK} in influenza virus-infected cells. (A) In the uninfected cell, the activation of PKR by dsRNA leads to the phosphorylation of eIF-2 α and the subsequent inhibition of protein synthesis initiation. **(B)** During influenza virus infection, P58^{IPK} (denoted as P58 in the diagram) is activated via a dissociation from its own inhibitor I-P58. Once activated, P58^{IPK} can block both PKR autophosphorylation and the phosphorylation of eIF-2 α by an already activated kinase, thus preventing PKR-mediated inhibition of protein synthesis initiation.

A. PKR ACTIVATION



B. PKR INHIBITION



(Goebel and Yanagida 1991). Each TPR motif can be further divided into two sub-alpha helical regions: domains A and B, which forms a hole and knob, respectively. The knob of one TPR subdomain can fit into the hole of another TPR subdomain, thus facilitating protein-protein interactions between TPR family members (Lamb et al. 1995). The alpha helical nature of the TPR motif can also serve as a general helix-associating domain to promote interactions between TPR proteins and non-TPR-containing proteins. Thus far, mutagenesis studies of two other TPR proteins, CDC23 (Sikorski et al. 1993) and SSN6 (Schultz et al. 1990), showed that only particular TPR motifs are required for biological function. Interestingly, our mutagenesis studies of P58^{IPK} also suggest that not all of its TPR motifs are required for function: both 9-1 and 8-2 mutants contain only five and six TPR motifs, respectively, yet both inhibit PKR phosphorylation activity *in vitro*. Given the alpha helical nature of the TPR motifs (which are predicted to direct protein-protein interactions), it is possible that the central TPR motifs of P58^{IPK} (TPRs 5, 6 and 7) act to direct interactions between PKR and P58^{IPK}, leading to the subsequent inhibition of kinase activity. Particular TPR motifs may also act to direct P58^{IPK} interactions with other proteins such as I-P58, its own regulator.

Role of DnaJ similarity region in P58^{IPK} function and regulation

Although our mutant experiments revealed that the DnaJ similarity region at the carboxy-terminus of P58^{IPK} is dispensable for kinase inhibitory function *in vitro*, it remains possible that this region is still essential for PKR inhibition *in vivo*. It is also possible that this region is required for other P58^{IPK} functions or P58^{IPK} regulation. For

instance, the DnaJ region may mediate interactions between P58^{IPK} and I-P58 in our model (Figure 2.6), in the same manner that DnaJ participates in a molecular complex with the DnaK and GroEL proteins (Langer et al. 1992).

Because P58^{IPK} shares homology with the DnaJ family of heat shock proteins as well as to the stress protein STI1, it is tempting to speculate that P58^{IPK} may itself be a stress protein which is regulated during influenza virus infection to modulate protein synthesis initiation activity. Indeed, there are precedents in the literature for the participation of stress proteins in translational initiation as well as the regulation of protein synthesis initiation under stress conditions. For instance, the *S. cerevisiae* DnaJ homolog, SIS1, has been shown to be required for yeast translational initiation (Zhong and Arndt 1993). Studies involving PKR and HCR (the PKR homolog in reticulocytes) show that both are regulated under conditions of heat shock or stress (Dubois et al. 1991; Samuel 1993; Hinnebusch 1994) with HCR being associated with hsp90, a member of another family of stress proteins (Matts and Hurst 1989). Finally, the yeast PKR homolog GCN2 is also regulated under the stress conditions of amino acid starvation (Dever et al. 1992). Thus, the DnaJ sequence at the carboxyl region of P58^{IPK}, although dispensable for the inhibition of PKR activity *in vitro*, may participate in the regulation of P58^{IPK}, thereby modulating translational initiation activities.

It is noteworthy to add that very recent observations from the Katze lab have surfaced which implicate P58^{IPK} as a molecular chaperone (Melville et al.

unpublished). For instance, P58^{IPK} has been shown to form a hetero-complex *in vitro* with other molecular chaperones, such as Hsp40 and Hsp70. Moreover, P58^{IPK} can modulate *in vitro* the ATPase and protein refolding activities of Hsp70. Although it remains to be determined whether P58^{IPK} (like other molecular chaperones) is regulated under conditions of heat shock or stress, evidence is nevertheless mounting which implicates P58^{IPK} as a stress protein with a role in translational regulation.

Chapter 3: The 58 Kilodalton Cellular Inhibitor Of The Double Stranded RNA-Dependent Protein Kinase Requires The Tetratricopeptide Repeat 6 And Dna-J Motifs To Stimulate Protein Synthesis In Vivo

Introduction

PKR is a cAMP-independent serine/threonine kinase that is induced by interferon treatment (Hovanessian 1991; Meurs et al. 1990). On activation by double stranded RNA, PKR undergoes autophosphorylation and catalyzes the phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor 2 (eIF-2 α), resulting in inactivation of the latter (Clemens 1996; Galabru and Hovanessian 1987). These events lead to dramatic decreases in protein synthetic rates inside the cell. This situation is not favorable to viruses, which must make their proteins to replicate. Much of the focus on PKR has related to the role of the kinase in mediating the inhibitory interferon response to viral infection (Katze 1996; Katze 1995; Hinnebusch 1996; Schneider 1996). However, PKR is constitutively expressed in eukaryotic cells in the absence of interferon (Meurs et al. 1992), and there is accumulating evidence implicating PKR in the regulation

of normal cellular processes. These include signal transduction (Williams 1995; Kumar et al. 1994), cellular differentiation (Petryshyn et al. 1984), growth and proliferation (Chong et al. 1992; Tsamarias and Thireos 1988; Jaramillo et al. 1995), and gene expression at the transcriptional level (Zinn et al. 1988). Finally, the importance of PKR in regulating cell growth and gene expression is underscored by evidence suggesting PKR to be a tumor suppressor gene (Koromilas et al. 1992; Meurs et al. 1993; Lengyel 1993; Barber et al. 1995).

PKR, which is efficiently activated by both cellular (Li and Petryshyn 1991; Davis and Watson 1996) and viral (Katze 1995; Black et al. 1989; Ederly et al. 1989; Rice et al. 1985) RNAs, is, in turn, subjected to stringent regulation by both viral and cellular gene products. For example, adenovirus encodes a polymerase III gene product, VAI RNA, which binds to and inactivates PKR (Katze et al. 1987; Ghadge et al. 1991). In addition, vaccinia virus encodes the K3L protein, which also binds to PKR and inhibits its activity (Carroll et al. 1993; Davies et al. 1993). Influenza virus has evolved an unusual mechanism to down-regulate PKR and thus ensure that viral protein synthesis is not compromised during infection. Influenza virus recruits or activates a cellular protein termed $P58^{IPK}$, based on its M_r of 58,000 (Lee et al. 1992; Lee et al. 1990). $P58^{IPK}$ is in a complex with its own inhibitor, $I-P58^{IPK}$, and is thus normally inactive in uninfected cells. On influenza virus infection, $P58^{IPK}$ is dissociated from $I-P58^{IPK}$, and available to complex with PKR, thereby preventing phosphorylation of eIF-2 α (Lee et al.

1992; Lee et al. 1990; Polyak et al. 1996). Sequence analysis of P58^{IPK} cloned from bovine, human, and mouse cells, showed P58^{IPK} to be a novel, highly conserved 504-amino acid protein (Korth et al. 1996; Lee et al. 1994). Spanning the P58^{IPK} molecule are nine tandemly arranged motifs referred to as tetratricopeptide repeats (TPRs). TPRs are 34-amino acid motifs that have been demonstrated to form amphipathic alpha helices that can direct protein-protein interactions (Lamb et al. 1995). The middle region of P58^{IPK} contains limited homology to the amino-terminal region of eIF-2 α , the natural substrate of PKR. Finally, the carboxyl terminus of P58^{IPK} shows extensive homology to the conserved J domain of the bacterial DnaJ protein (Silver and Way 1993). Like PKR, P58^{IPK} likely regulates cellular gene expression in the absence of virus infection. It was demonstrated that overexpression of P58^{IPK} in NIH 3T3 cells led to the malignant transformation of these cells, suggesting P58^{IPK} to be involved in cellular growth control (Barber et al. 1994).

Using *in vitro* assays with purified components, we previously showed that native or recombinant P58^{IPK} inhibited both the autophosphorylation of PKR and the phosphorylation of eIF-2 α by an already activated kinase (Lee et al. 1990; Lee et al. 1994). We have not, however, directly demonstrated that P58^{IPK} functions *in vivo*, inside the cell, to block PKR activity and stimulate protein synthetic rates. We therefore developed an *in vivo* cotransfection assay using cDNAs encoding P58^{IPK} and an alkaline phosphatase reporter gene. This allowed us both to analyze P58^{IPK} inhibitory activity and

to precisely map P58^{IPK} functional domains. We can now report that PKR inhibition and the resultant translational stimulatory activity appear to require two distinct regions of the P58^{IPK} molecule: the TPR6 domain located in the central region (amino acids 222-255) and the DnaJ similarity region at the COOH terminus (amino acids 391-504). Based on these data, a model of PKR regulation by P58^{IPK} will be presented.

Materials And Methods

Construction of plasmids

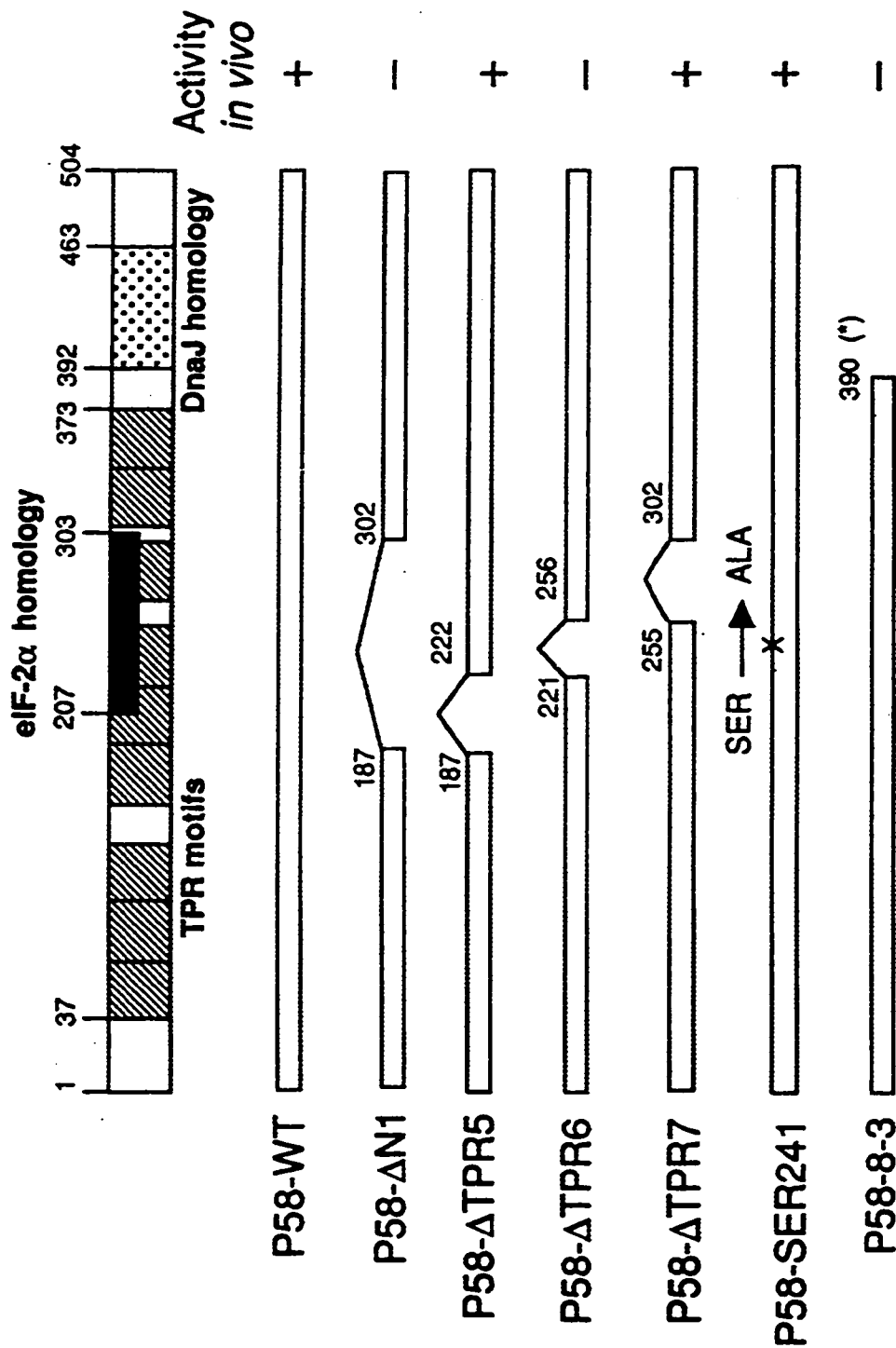
A 1.6-kb *EcoRV-XbaI* fragment containing 115 nucleotides of the bovine P58^{IPK} 5'-untranslated region plus 1.5 kb of coding sequence was cloned into pcDNA1/Neo, as described earlier (Lee et al. 1994). To construct the Ser241-mutant, a 1.7-kb *Bgl II-BamHI* fragment was subcloned from P58-Ser-241 (Lee et al. 1994) into pcDNA1/Neo (Invitrogen). To construct P58^{IPK}-8-3, a 1.3-kb *BstEII-BamHI* fragment containing the P58^{IPK} gene with a premature stop codon engineered at amino acid 390 was excised from P58^{IPK}-8-3 (Lee et al. 1994) and cloned into *BstEII-BamHI* digested WT plasmid. Plasmids Δ N1, Δ TPR5, Δ TPR6, and Δ TPR7 were created using the MUTA-GENE M13 *in vitro* mutagenesis kit (Bio-Rad) and the following synthesized oligonucleotides: Δ N1-GTTTGTGTTTGGGATCATGAATATAACAATT, Δ TPR5-

GTTTGTGTTTGGGATAC TGAGGCATTTTAT, Δ TPR6-TTGAAAAACGATAATA-AAAGGTGTTTTGCA, and Δ TPR7-CTTGACCAGGATCATCATGAATATAACAATT.

The Δ N1 oligonucleotide was designed to delete the central region of P58^{IPK} from amino acids 187-301. This region encompasses both TPRs 5-7 and the eIF-2 α homology region. The Δ TPR5 variant lacked amino acids 188-221; The Δ TPR6 variant lacked amino acids 222-255, and the Δ TPR7 variant lacked TPR7 plus the 12 amino acids preceding it. After mutagenesis, a 1.4-kb or 1.6-kb *Xba*I-*Bam*HI fragment containing the mutated P58^{IPK} gene was isolated and cloned into pET15b bacterial expression vector (Novagen). Subsequently, a smaller, 1.0- or 1.2-kb, internal *Bst*EII-*Bam*HI fragment containing the respective deletion was excised from the pET15b constructs and cloned into pcDNA1/Neo. The precise identity of all mutant constructs was verified by a combination of restriction enzyme digest mapping and sequence analysis using the dideoxy chain termination technique. See Figure 3.1 for a schematic of the wild type and mutant P58^{IPK} constructs used.

The glutathione *S*-transferase (GST) fusion constructs for the *in vitro* studies were made as follows. Full-length, wild type P58^{IPK} was cloned into pGEX2T plasmid (Pharmacia Biotech Inc.) as described previously (Lee et al. 1994). The GST- Δ TPR6 construct was made by digesting the Δ TPR6 plasmid (described above) with *Hind*III and *Eco*RI restriction enzymes and subcloning this 0.9kb internal fragment into the GST-P58^{IPK} backbone.

Figure 3.1. Schematic of the wild type and mutant P58^{IPK} constructs. Wild type P58^{IPK} contains nine tandemly arranged 34-amino acid repeats termed TPR domain. P58^{IPK} amino acids 207-303 are homologous to the amino-terminal region of eIF-2 α (amino acids 26-120). The DnaJ homology region resides at the COOH terminus (amino acids 392-463). The Ser-241 mutant contains a single amino acid mutation from a serine to alanine at position 241. A summary of the *in vivo* translational stimulatory activity of the various P58^{IPK} variants is indicated at the *right*.



* Stop Codon

Transfection procedures and SEAP assays

COS-1 cells were transfected using the DEAE-dextran/chloroquine method (Cullen 1987). Monolayers of COS-1 cells were washed once with serum-free Dulbecco's modified Eagle's medium. DNA was added to the cells in serum-free Dulbecco's modified Eagle's medium in the presence of 250 $\mu\text{g/ml}$ DEAE-dextran. After incubation for 2 h, chloroquine was added to a final concentration of 80 μM . After another 2-h incubation, the transfection mixture was removed and the cells were "shocked" with a solution of 20% glycerol in HEPES-buffered saline for 2 min. The cells were then washed twice with Hank's balanced salt solution (HBSS) and then incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. To measure SEAP activity, culture medium was removed from the cells at 40 h after transfection. The cells were then washed and incubated in prewarmed fresh media for 30 min. The media were then collected from the transfected cells, heated to 65°C for 5 min, and then assayed for SEAP activity as previously published (Berger et al. 1988).

Analysis of protein synthesis by ³⁵S-pulse labeling and immunoprecipitation

Transfected cells were labeled for 30 min with [³⁵S]-methionine (605 $\mu\text{Ci/ml}$) in methionine-free Dulbecco's modified Eagle's medium. The labeled cells were washed twice with ice-cold Hank's balanced salt solution and lysed in Triton lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM MgCl₂, 100 $\mu\text{g/ml}$ aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 1% Triton X-100). The clarified extract was then subjected to immunoprecipitation with protein A-agarose that had been pre reacted

with a polyclonal antibody against placental alkaline phosphatase (Dako). The immunoprecipitates were washed 4X with high salt buffer I (20 mM Tris, pH 7.5, 50 mM KCl, 0.4 M NaCl, 1% Triton X-100, 1 mM EDTA; 10 µg/ml aprotinin, 1 mM DTT, 0.2 mM PMSF, and 20% glycerol) and three times with high salt buffer II (10 mM Tris, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10 µg/ml aprotinin, and 20% glycerol). Bound, radiolabeled proteins were separated on a SDS-9% polyacrylamide gel and visualized using autoradiography. The secreted embryonic alkaline phosphatase (SEAP) protein signal was quantified using PhosphorImager analysis (Molecular Dynamics).

Western blot (immunoblot) analysis

Transfected cells were washed twice with ice-cold Hank's balanced salt solution and lysed in Triton lysis buffer. After SDS-polyacrylamide gel electrophoresis, polypeptides were transferred to nitrocellulose (Towbin et al. 1979) and detected with P58^{IPK} monoclonal antibodies (Barber et al. 1994) using the ECL chemiluminescence system (Amersham Corp.).

Northern blot RNA analysis

Poly(A)⁺ RNA was isolated from the transfected cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). The poly(A)⁺ RNA was denatured, electrophoresed in a 1% agarose gel containing 0.2 M formaldehyde, and transferred to a nylon membrane (Hybond N, Amersham). The blot was hybridized to a ³²P-labeled 1.6-kb *HindIII-XhoI* fragment

containing the SEAP gene. Afterwards, the same blot was stripped and reprobed with a ^{32}P -labeled 1.7-kb *Bam*HI fragment containing the human actin gene. Both the radiolabeled SEAP and actin RNA signals were quantified using PhosphorImager analysis (Molecular Dynamics).

***In vitro* assay for P58^{PK} activity**

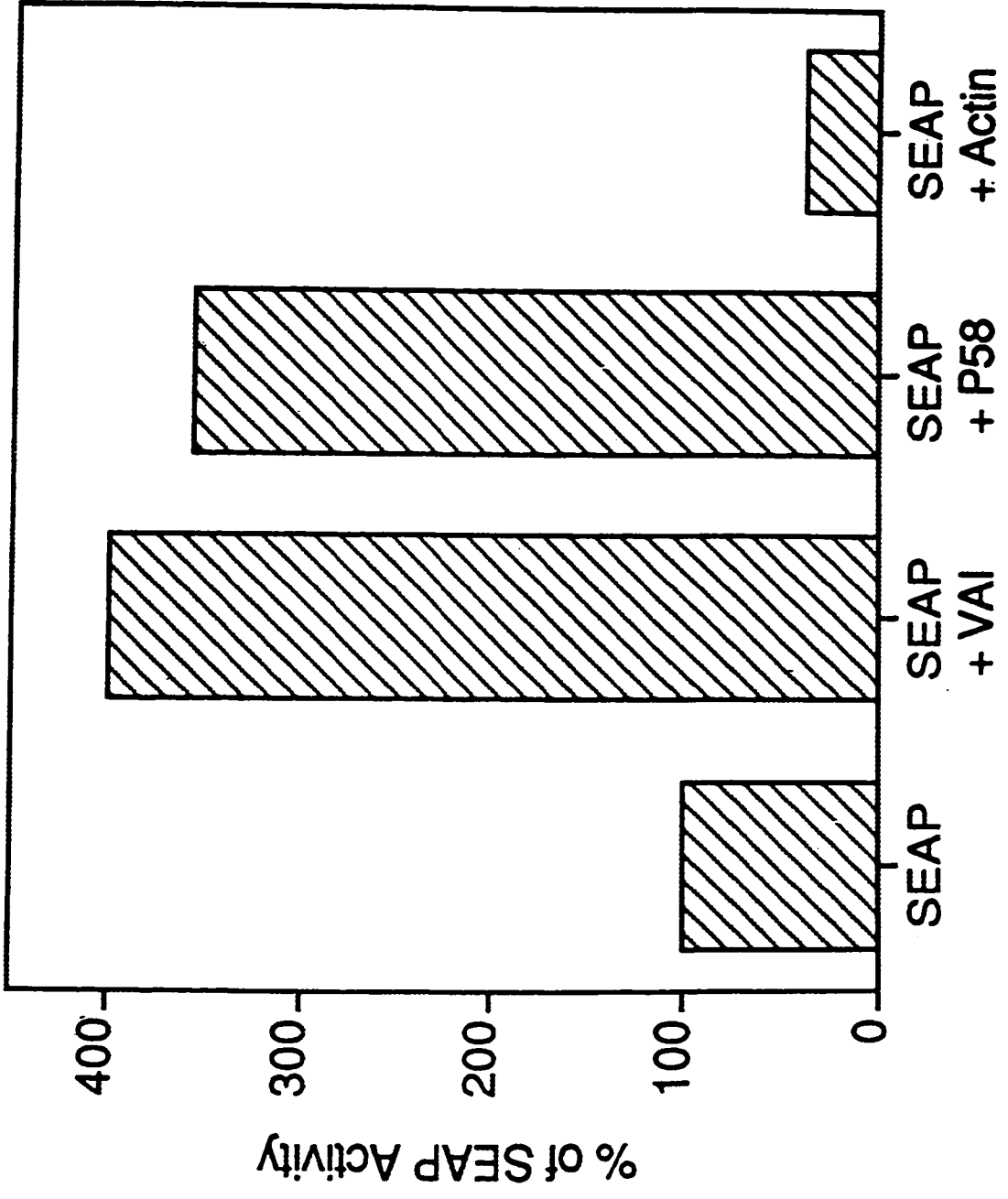
Both WT P58^{PK} and the ΔTPR6 variant were expressed in *Escherichia coli* and purified as GST fusion proteins as described previously (Lee et al. 1994). To test for kinase inhibitory activity, purified GST fusion proteins were preincubated with purified PKR (Galabru and Hovanessian 1987) for 10 min at 30°C in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 2 mM MnCl₂, 2 μM ATP, 5 $\mu\text{g/ml}$ aprotinin, 1 mM dithiothreitol, and 500 $\mu\text{g/ml}$ bovine serum albumin. Subsequently, an activator (0.1 $\mu\text{g/ml}$ poly(rI):poly(rC)) was added in the presence of 5 μCi [γ - ^{32}P]ATP and incubated for an additional 10 min. Finally, exogenous substrate (10 μg of calf thymus histone IIA) was added with an additional 10 μCi [γ - ^{32}P]ATP, and the mixture was allowed to incubate for another 20 min. The reaction was stopped by the addition of 2X disruption buffer (180 mM Tris-HCl, pH 6.8, 4.5% SDS, 23% (v/v) glycerol, 17 mM EDTA, 20 μg RNase A, and 3 M 2-mercaptoethanol) and boiling. The samples were then separated by 14% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Results

Development of an *in vivo* P58^{IPK} functional assay

A cotransfection assay was developed to measure the ability of P58^{IPK} to inhibit endogenous PKR function, thereby stimulating mRNA translation inside the cell. A similar assay has been frequently and successfully used with other PKR inhibitors, including adenovirus VAI RNA and the reovirus $\sigma 3$ protein (Svensson and Akusjarvi 1985; Kaufman 1985; Giantini and Shatkin 1989; Lloyd and Shatkin 1992; Seliger et al. 1992). This *in vivo* assay involved cotransfecting into COS-1 cells, a constant amount of SEAP reporter gene cDNA together with increasing amounts of P58^{IPK} cDNA. SEAP enzymatic activity was then measured as described under “Materials and Methods”. Although this assay does not directly measure PKR enzymatic activity, this protocol provides a quantitative measure of mRNA translational stimulation which occurs as a result of PKR inhibition. To test the efficacy of our assay, we first measured the SEAP activity of cells cotransfected with plasmids encoding the adenovirus VAI RNA and SEAP, and compared it to the activity obtained when cells were transfected with SEAP cDNA alone. VAI RNA stimulated SEAP activity approximately 4-fold (Fig 3.2). Similarly, after cotransfection with a cDNA encoding P58^{IPK}, SEAP enzymatic levels increased approximately 3.5-fold (Fig 3.2). This level of reporter gene stimulation was comparable to that previously observed for reovirus $\sigma 3$, another protein inhibitor of PKR activity (Seliger et al. 1992). As a negative control, we cotransfected human actin cDNA

Figure 3.2. Comparison of SEAP translational stimulatory activities of the adenovirus VAI RNA and the cellular P58^{IPK} protein. SEAP enzymatic activity, secreted during a 30-min pulse into the media of cells transfected with cDNAs, was analyzed as described under “Materials and Methods”. Activity is expressed as the percentage of SEAP activity in cells cotransfected with SEAP cDNA alone. 1 µg of SEAP DNA was cotransfected with either 6 µg of vector (*SEAP*), VAI RNA (*SEAP* + *VAI*), P58^{IPK}-WT (*SEAP* + *P58*) or actin cDNA (*SEAP* + *Actin*). The results shown represent the mean activity obtained from two separate dishes of transfected cells.

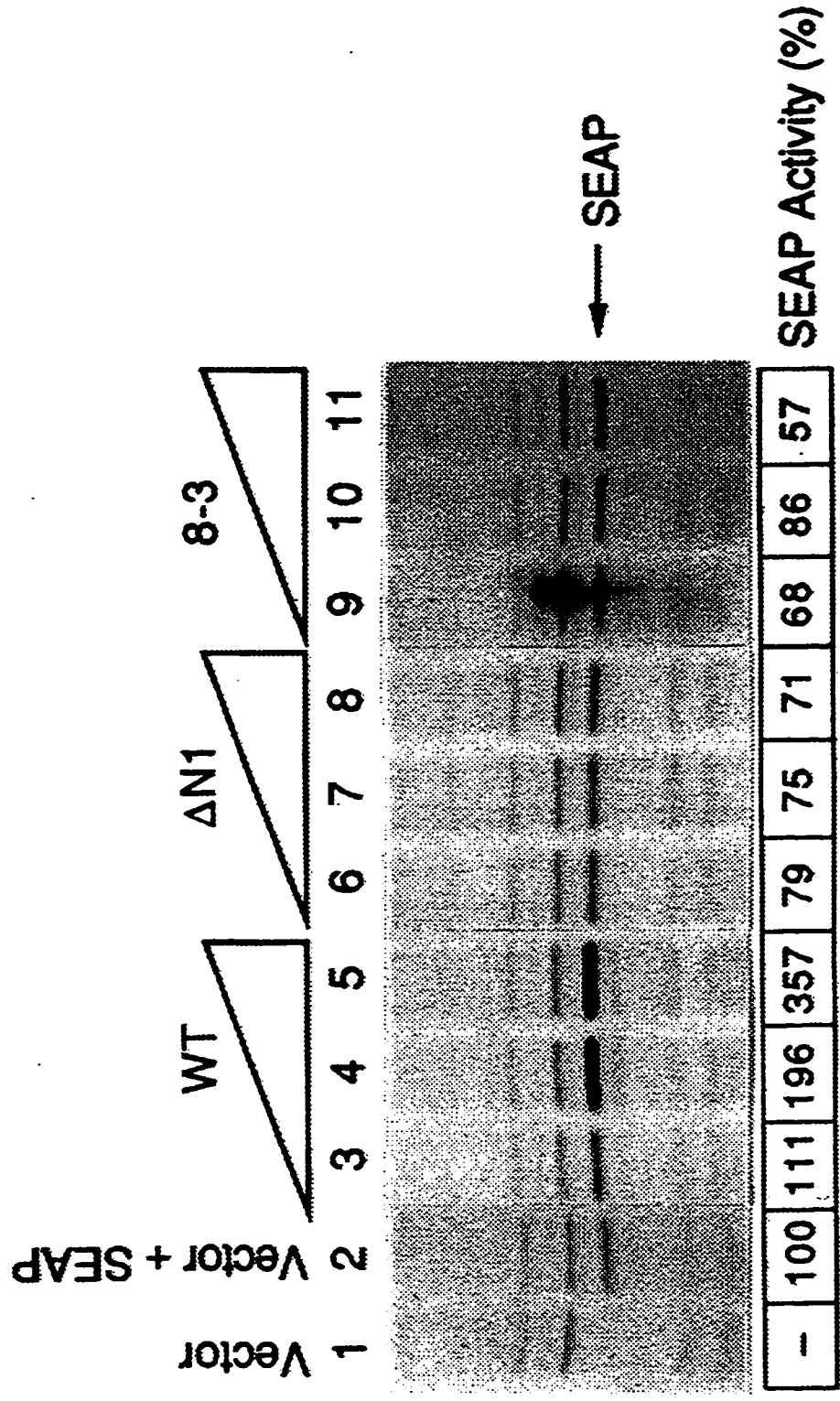


along with SEAP cDNA into COS-1 cells. In contrast to P58^{IPK} or VAI RNA, the cotransfection of actin DNA did not result in stimulation of SEAP reporter activity. Instead, SEAP activity actually declined relative to the SEAP alone control, probably as a result of enhanced PKR activation which occurs in transfected cells (Kaufman and Murtha 1987). To further demonstrate that the observed stimulatory activity was specific for P58^{IPK}, a titration of P58^{IPK} cDNA transfected into the cells was attempted. Both SEAP activity measurements and Western analyses showed there was an increase in SEAP reporter activity which correlated with increased P58^{IPK} expression (data not shown). This was not the case for the actin control, in which increasing amounts of actin expression did not result in an enhancement of SEAP enzymatic activity (data not shown).

P58^{IPK} stimulates SEAP protein synthesis in cotransfected COS cells

The previous analysis measured only the enzymatic activity of SEAP secreted into the culture media during a 30-min pulse. To determine whether the increase in SEAP enzymatic activity was due directly to enhanced SEAP mRNA translation, cotransfected cells were pulse-labeled with [³⁵S]-methionine. Cytoplasmic extracts were prepared and subjected to immunoprecipitation utilizing a SEAP-specific polyclonal antibody as described under "Materials and Methods". When increasing amounts of P58^{IPK} cDNA were cotransfected into the cells with a constant amount of SEAP cDNA, there was a corresponding increase in SEAP protein synthesis (Fig 3.3, *lanes 3-5*). This increase in the amount of SEAP protein synthesized (3-6-fold as determined by PhosphorImager analysis) paralleled the up to 3-4-fold increase in SEAP enzymatic

Figure 3.3. Analysis of P58^{IPK}-mediated increase in the amount of SEAP protein in COS-1 cells cotransfected with P58^{IPK} and SEAP cDNAs. COS-1 cells were pulse-labeled for 30 min with [³⁵S]methionine at 40 h after transfection, followed by immunoprecipitation of the lysates using a polyclonal antibody against placental alkaline phosphatase. The COS-1 cells were cotransfected with 1 µg of SEAP reporter cDNA plus 2 µg (*lanes 3, 6, and 9*), 4 µg (*lanes 4, 7, and 10*), or 6 µg (*lanes 5, 8, and 11*) of the P58^{IPK} cDNA construct indicated at the *top*. *Lane 1*, immunoprecipitation from cells transfected with empty vector alone. The total cDNA content of all the transfected samples was supplemented with the pcDNA1/Neo vector DNA to bring the total to 7 µg of cDNA for all transfections. The migration of the SEAP protein is indicated at the *right*. SEAP enzymatic activity (relative to the 100% activity of cells transfected with SEAP alone) was concurrently measured and is indicated at the *bottom*.

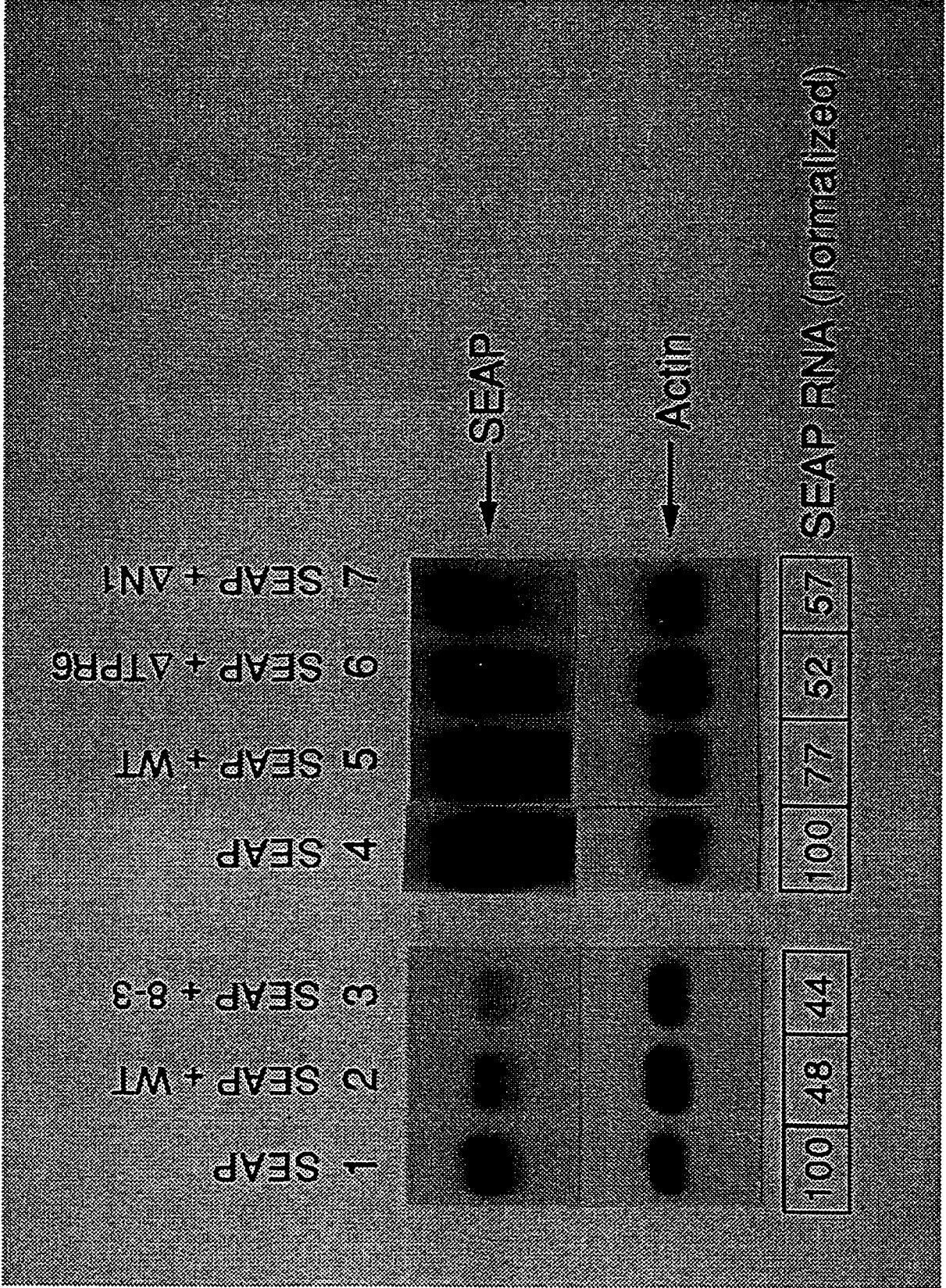


activity (Fig 3.3, *bottom*). To determine whether the increased synthesis of SEAP protein synthesis was a result of an increase in SEAP mRNA levels, Northern blot analysis of poly(A)⁺ RNA from the P58^{IPK}- and SEAP-cotransfected cells was performed (Fig 3.4). SEAP RNA levels were quantitated using PhosphorImager analysis and normalized according to control actin mRNA levels. Cotransfection of WT P58^{IPK} cDNA with SEAP cDNA did not result in increased but, rather in decreased levels of SEAP mRNA (Fig 3.4, compare *lanes 1* and *2* or *4* and *5*). These data demonstrate that cotransfection with P58^{IPK} cDNA enhanced the translation of SEAP mRNA and caused minor decreases in SEAP mRNA levels.

The P58^{IPK} central domain and TPR6 motif are critical for PKR inhibitory activity and enhanced mRNA translation *in vivo*

Previous data from our laboratory, using *in vitro* assays to map the functional domains of P58^{IPK}, implicated P58^{IPK} amino acids 168-277 as critical for function (Lee et al. 1994). To directly test the importance of the central region (which contains the eIF-2 α homology region) for PKR inhibitory activity and mRNA translational stimulation *in vivo*, we tested the Δ N1 mutant (deleted for amino acids 188-301; see Fig. 3.1) in the cotransfection assay. The Δ N1 variant lacks not only the central domain of P58^{IPK}, but also TPR motifs 5-7. The Δ N1 mutant was first tested in our *in vitro* assay and, as expected, was demonstrated to be approximately 30-fold less active than the WT P58^{IPK} protein (data not shown). Similarly, the Δ N1 variant, over a range of

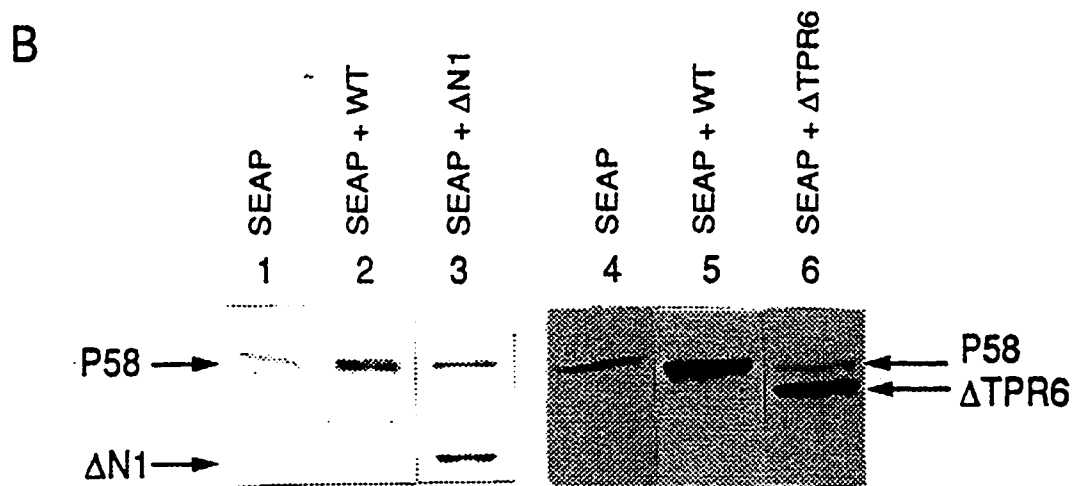
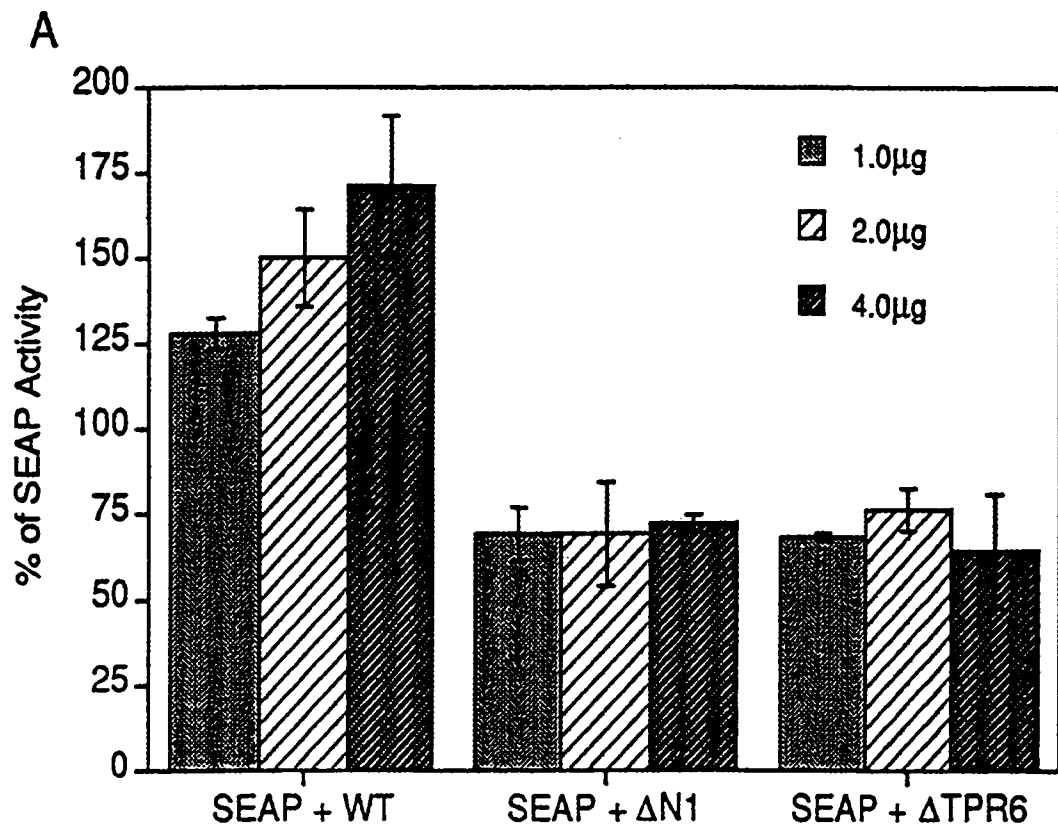
Figure 3.4. Northern blot analysis of RNA extracted from cells cotransfected with SEAP and WT or mutant P58^{PK} cDNAs. Approximately 2 µg of Poly(A)⁺ RNA was electrophoresed on a 1% formaldehyde agarose gel, which was subsequently blotted onto nylon filters. The blots were first probed with a ³²P-labeled 1.6-kb *HindIII-XhoI* fragment containing the SEAP gene, stripped, and then reprobed with radiolabeled actin DNA. The amounts of SEAP and actin mRNAs present in each of the samples were quantified using PhosphorImager analysis. To correct for loading variation, the SEAP mRNA levels were normalized relative to actin mRNA levels. A value of 100 was arbitrarily assigned to levels of SEAP mRNA present in cells transfected with SEAP cDNA alone.



concentrations, was unable to stimulate SEAP protein synthetic rates (Fig. 3.3, compare *lanes 6-8* with *lanes 3-5*) or enzymatic activity (Figs. 3.3, *bottom of lanes 6-8*, and 3.5A). Western blot analysis confirmed that the $\Delta N1$ protein was expressed at approximately the same level as WT P58^{IPK} (Fig. 3.5B; compare *lanes 2* and *3*). Consistent with the pulse-labeling experiments, Northern blot studies revealed only minor decreases in SEAP mRNA levels between cells transfected with the $\Delta N1$ mutant and wildtype P58^{IPK} (Fig. 3.4, compare *lanes 5* and *7*). We conclude that the P58^{IPK} central domain, spanning amino acids 188-301, is important for inhibitory function both *in vitro* and *in vivo*.

To more precisely map P58^{IPK} functional domains, we generated three smaller TPR deletion mutants: $\Delta TPR5$, $\Delta TPR6$, and $\Delta TPR7$ (see Fig. 3.1). Similar to $\Delta N1$, the $\Delta TPR6$ mutant protein was unable to stimulate SEAP enzymatic activity compared to WT P58^{IPK} in cotransfected cells. Indeed SEAP levels were reduced in $\Delta TPR6$ - and SEAP-cotransfected cells compared to cells transfected by SEAP cDNA alone (Fig. 3.4). Western blot analysis confirmed efficient synthesis of the $\Delta TPR6$ variant (Fig. 3.5B; compare *lanes 5* and *6*). In addition, Northern blot analysis confirmed that the inactivity of this mutant was at the level of translation, since only minor decreases in SEAP mRNA levels were observed when compared to the WT (52 versus 77%, respectively, of mRNA compared to levels in cells transfected by SEAP cDNA alone; Fig. 3.4, compare *lanes 5* and *6*). In contrast to $\Delta TPR6$, the $\Delta TPR5$ and $\Delta TPR7$ mutants induced enhanced translational stimulatory activity compared to WT P58^{IPK} (Fig.

Figure 3.5. The P58^{IPK} central domain and TPR6 motif are required for P58^{IPK} function. (A) SEAP enzymatic activity secreted from cells cotransfected with SEAP (0.2 μ g) and WT or mutant P58^{IPK} cDNAs in the amounts indicated. SEAP activity was measured as the percentage of activity secreted from cells transfected with SEAP cDNA alone. The results shown here were derived from five independent transfection experiments, with each transfection performed and sampled in duplicate. (B) Western blot analysis of WT and mutant P58^{IPK} protein expression. Proteins from the indicated cotransfected cells were separated by SDS-polyacrylamide gel electrophoresis on a 10-20% gradient gel. The separated proteins were transferred to nitrocellulose membranes and probed with a combination of two P58^{IPK}-specific monoclonal antibodies, 2F8 and 9F10 (Barber et al. 1994). *Lanes 1-3*, 2 μ g of empty vector (*lane 1*), P58^{IPK}-WT (*lane 2*), or Δ N1 (*lane 3*) DNA was cotransfected with SEAP cDNA. *Lanes 4-6*, 4 μ g of empty vector (*lane 4*), P58^{IPK}-WT (*lane 5*), or Δ TPR6 (*lane 6*) was cotransfected with SEAP cDNA. Because the endogenous P58^{IPK}, present in COS-1 cells, migrates at a similar molecular weight as the transfected bovine P58^{IPK}, expression of the transfected WT construct is indicated by the increase in exogenous P58^{IPK} expression (*lanes 2 and 5*) above the endogenous P58^{IPK} background levels (*lanes 1 and 4*). Migration of P58^{IPK}-related proteins is indicated on the *sides*.

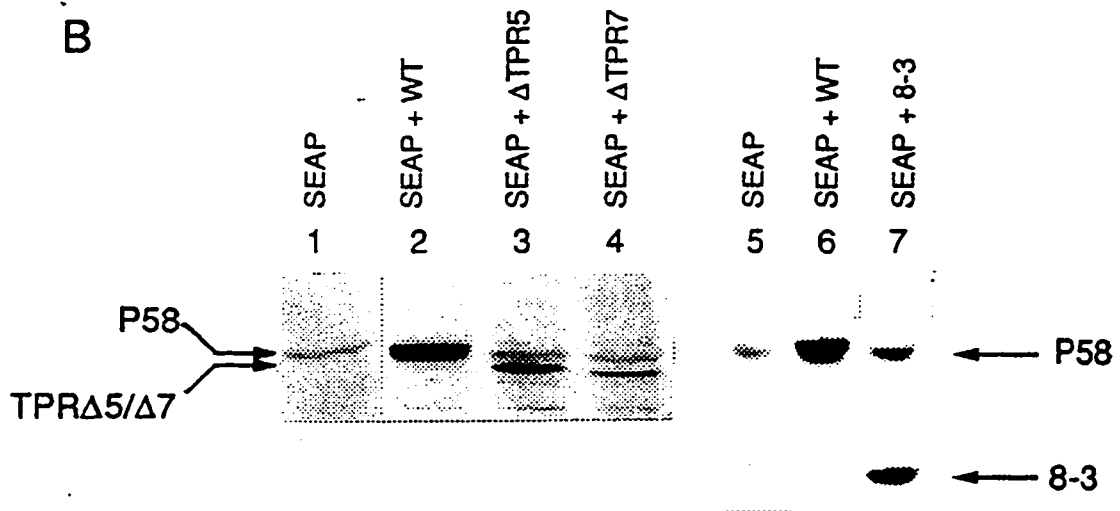
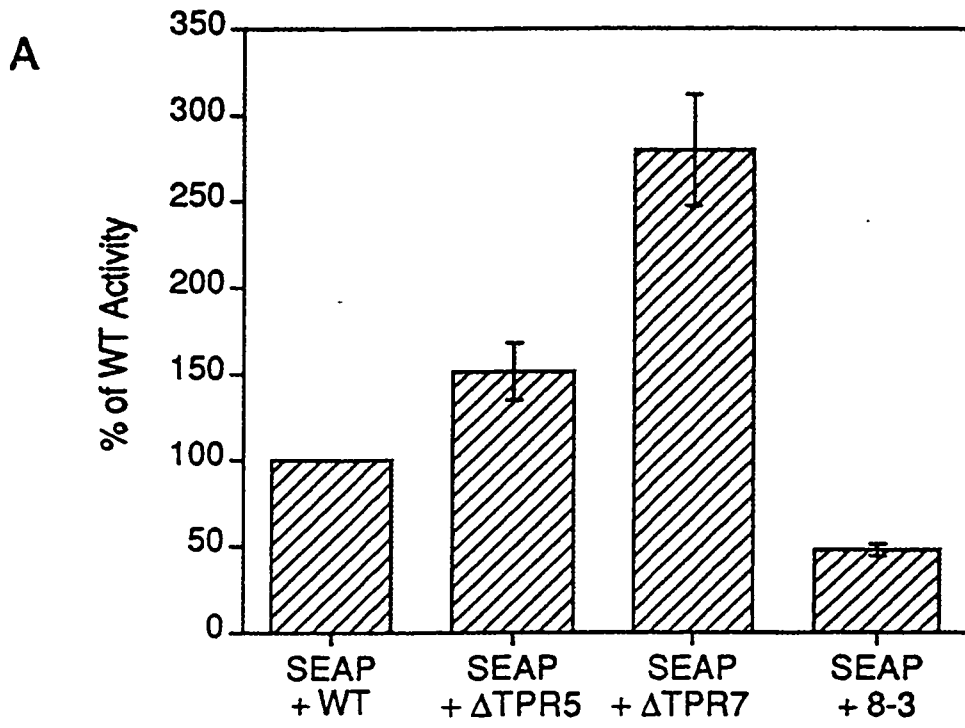


3.6A). This enhanced activity was not due to enhanced variant expression as Western blot analysis revealed that Δ TPR5 and Δ TPR7 were expressed to lower levels than the WT P58^{IPK} (Fig. 3.6B). It is tempting to speculate that this stimulation by the two mutants is due to lack of binding of negative regulatory factors which normally interact with these TPR domains. In support of this notion, we recently identified a novel gene product that was found to interact with the TPR7 domain using a yeast two-hybrid screen (Gale, Jr. et al. 1998). This novel protein, called P52^{IPK}, can block P58^{IPK}'s inhibition of PKR activity in the *in vitro* kinase assay.

The DnaJ homology region is required for P58^{IPK} activity *in vivo* but not *in vitro*

The carboxyl terminus of P58^{IPK} shows considerable homology to the conserved J domain of the *E. coli* DnaJ heat shock family of proteins (Silver and Way 1993; Ohki et al. 1986). Previous *in vitro* assays determined that the DnaJ homology region at the COOH terminus was dispensable for P58^{IPK} inhibitory activity *in vitro* (Lee et al. 1994).. However, because it has been suggested that eukaryotic DnaJ homologs participate in protein-protein interactions (Silver and Way 1993), we next determined whether the DnaJ similarity region contributed to the PKR inhibitory function inside the cell. The P58^{IPK}-8-3 mutant (which lacks the DnaJ region and amino acids 391-504; Fig. 3.1) failed to stimulate SEAP protein synthetic rates in a pulse-labeling analysis (Fig. 3.3, lanes 9-11) or in a SEAP enzymatic activity assay (Figs. 3.3, bottom of lanes 9-11, and 3.6A). Western blots revealed efficient synthesis of the 8-3 variant protein (Fig. 3.6B,

Figure 3.6. Analysis of the function of P58^{IPK} variants that lack TPR motifs 5 and 7 and the DnaJ similarity region. (A) SEAP enzymatic activity in cotransfected cells. In this experiment, activity is described as the percentage of activity found in cells cotransfected with WT P58^{IPK} and SEAP cDNAs. The results shown here were derived from three separate transfection experiments, with each performed and sampled in duplicate. SEAP cDNA was cotransfected with 2 μ g of WT or mutant P58^{IPK} cDNAs. (B) Western blot analysis of WT and mutant P58^{IPK} protein expression. Analysis was performed as described in the legend to Fig. 3.5A. Migration of P58^{IPK} related proteins is indicated on the *sides*.



lane 7) and Northern blot analysis failed to show significant differences in SEAP mRNA levels between cells cotransfected with SEAP cDNA and wild type P58^{IPK} cDNA or 8-3 mutant DNA (Fig. 3.4, compare *lanes 2* and *3*). These data demonstrate that, like the TPR6 domain, the DnaJ similarity region is required for PKR inhibitory activity and the resultant translational stimulation *in vivo*.

***In vitro* analysis of PKR inhibitory function**

To provide a direct link between our *in vivo* cotransfection assay and the ability of P58^{IPK} to regulate PKR, we examined the PKR inhibitory activity of the key Δ TPR6 mutant using an *in vitro* kinase inhibition assay. Purified GST-P58^{IPK} or GST- Δ TPR6 fusion proteins were preincubated with purified PKR for 10 min at 30°C, after which poly(rI):poly(rC) activator and histone substrate were added as described under “Materials and Methods”. As shown in Fig. 3.7, neither the vector control (GST, *lanes 2* and *3*) nor the GST- Δ TPR6 fusion construct (*lanes 6* and *7*) was able to reduce the amount of PKR-mediated histone phosphorylation. In contrast, GST-P58^{IPK} (Fig. 3.7, *lanes 4* and *5*) efficiently blocked PKR phosphorylation activity. At the higher concentration of GST-P58^{IPK}, the percent inhibition of PKR activity was 71% as determined by PhosphorImager analysis. These data confirmed our cotransfection analyses demonstrating the inability of the Δ TPR6 P58^{IPK} mutant to inhibit PKR.

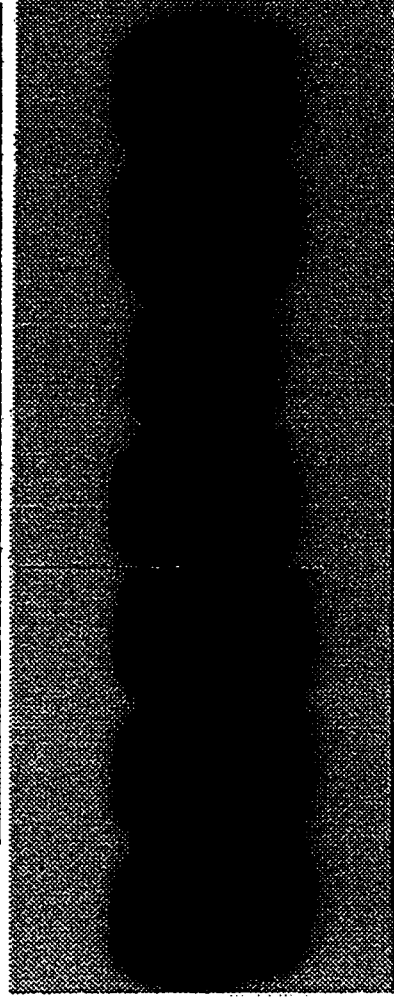
Figure 3.7. *In vitro* analysis of P58^{IPK} function. Equimolar amounts (0.6 and 1.2 pmol) of the P58^{IPK} wild type and Δ TPR6 variant were tested for their ability to inhibit PKR-mediated histone phosphorylation as described under “Materials and Methods”. As a negative control, we tested the PKR inhibitory activity of material that bound to and eluted from glutathione-agarose beads exposed to *E. coli* extracts that expressed the GST fusion vector alone. The histone bands were subjected to PhosphorImager analysis for quantitation. The degree of histone phosphorylation in lane PKR when no GST fusion protein had been added was arbitrarily set at 100%. Relative to this PKR control, the GST vector and the Δ TPR6 mutant showed little or no inhibition, whereas WT P58^{IPK} inhibited PKR-mediated histone phosphorylation by more than 70% at the 1.2 pmol concentration.

P
K
R

GST

P58

Δ TPR6



Histones —→

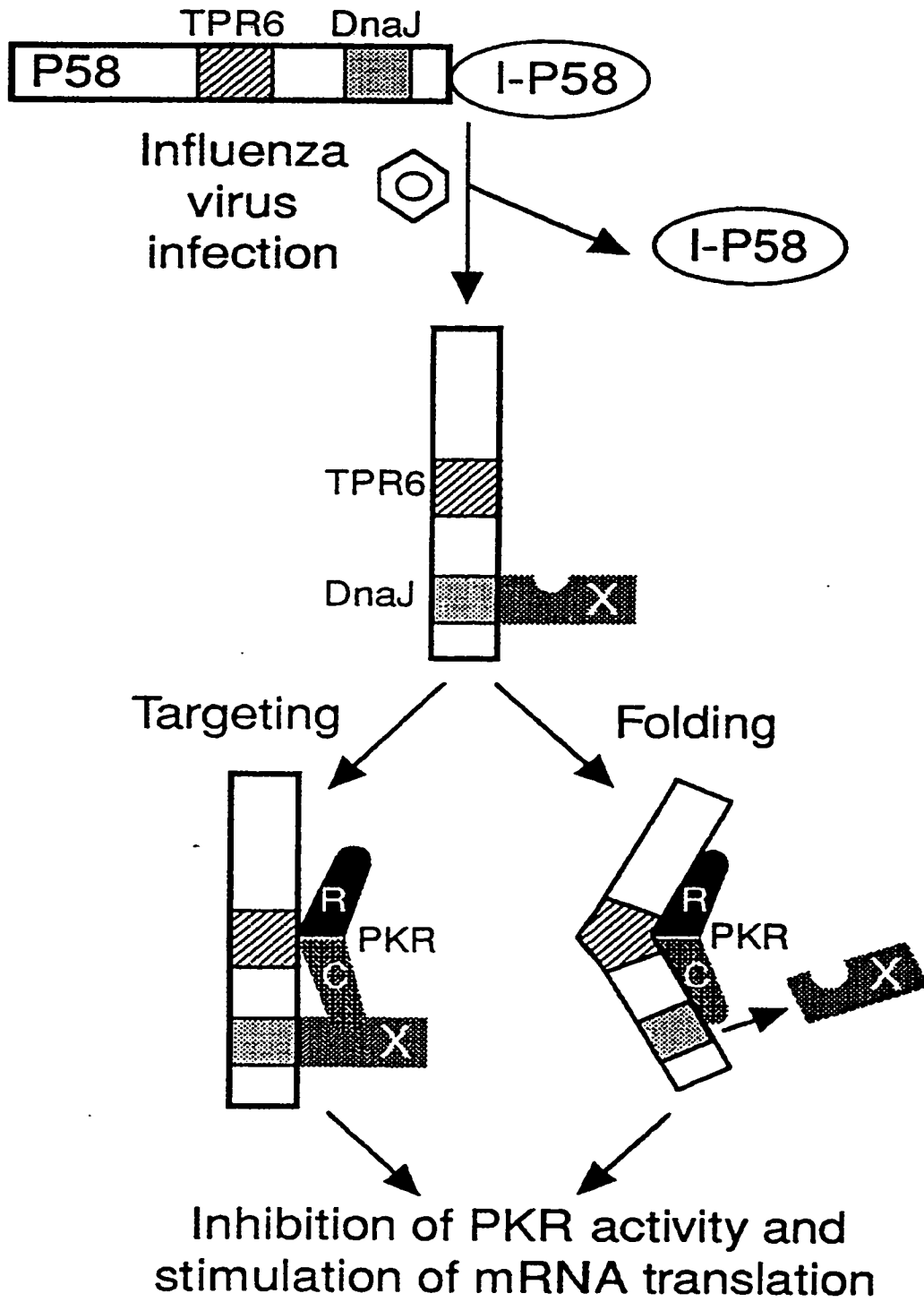
Discussion

In the present study, we developed a cotransfection assay to analyze P58^{IPK} function inside the cell. As was the case in similar assays with viral-encoded PKR inhibitors, such as the reovirus $\sigma 3$ protein (Giantini and Shatkin 1989; Lloyd and Shatkin 1992; Seliger et al. 1992) and the adenovirus VAI RNA (Svensson and Akusjarvi 1985; Kaufman 1985; Svensson and Akusjarvi 1984), P58^{IPK} down-regulated endogenous PKR activity leading to the stimulation of reporter gene mRNA translation. Although cotransfection with wild type P58^{IPK} and variants caused minor decreases in SEAP mRNA levels (possibly due to competition for transcription factors; Ref. (Giantini and Shatkin 1989)), the predominant effect of P58^{IPK} action was at the level of mRNA translation. This was perhaps most dramatically demonstrated by the cotransfections with WT P58^{IPK} and SEAP, in which the levels of SEAP mRNA were reduced approximately 2-fold, whereas the levels of SEAP mRNA translation were concurrently stimulated up to 3-6-fold (Fig. 3.3). Unexpectedly, we found that both the central region, containing the TPR6 motif, and the COOH terminus, encompassing the DnaJ similarity region were required for P58^{IPK} activity *in vivo* (summarized in Fig. 3.1). In accordance with this functional data, we found that P58^{IPK} lacking the TPR6 motif failed to interact with PKR utilizing the yeast two-hybrid system to analyze *in vivo* binding (Gale, Jr. et al. 1996). In addition,

the Δ TPR6 variant failed to inhibit PKR phosphorylation activity *in vitro* (Fig. 3.7). In contrast, the COOH region of P58^{IPK} is not required for P58^{IPK}-PKR interactions (Polyak et al. 1996; Gale, Jr. et al. 1996) nor is the DnaJ region required for P58^{IPK} function *in vitro* (Lee et al. 1994). This region shows homology as high as 55% identity and 80% similarity to select DnaJ proteins that play an essential role in the chaperone function of the hsp70-like DnaK protein of *E. coli* (Silver and Way 1993; Ohki et al. 1986; Cyr et al. 1994).

Based on our *in vitro* and *in vivo* functional and binding data, we present what has become an increasingly complex model of PKR regulation by P58^{IPK} (Fig. 3.8). As we have previously shown, P58^{IPK} likely exists in an inactive complex with its own inhibitor, I-P58^{IPK}, in uninfected cells. After influenza virus infection, P58^{IPK} dissociates from I-P58^{IPK}, allowing P58^{IPK} to interact with PKR. Although we found that P58^{IPK} can interact with PKR in the absence of other protein or RNA factors *in vitro* (Polyak et al. 1996; Lee et al. 1994), the present study suggests that additional factors must interact with P58^{IPK} and/or PKR to allow for P58^{IPK}-PKR interactions inside the cell. We hypothesize that such a factor (Fig. 3.8, X) binds to the P58^{IPK} DnaJ region, a region already known to promote protein-protein interactions (Silver and Way 1993). Two alternative pathways can further explain how factor X facilitates interactions between P58^{IPK} and PKR. In one scenario, we propose that X binds to P58^{IPK} and targets P58^{IPK} to the correct cellular compartment and then to the protein kinase. This model would likely

Figure 3.8. Model for the regulation of PKR activity and mRNA translation by the cellular P58^{IPK} inhibitor. P58^{IPK} is normally in an inactive complex with its own regulator, referred to as I-P58^{IPK}. Subsequent to influenza virus infection, I-P58^{IPK} dissociates from P58^{IPK}, allowing P58^{IPK} to interact with and inhibit PKR. Both the TPR6 and DnaJ regions are required for P58^{IPK} function *in vivo*. Based on the data presented in the current report, we hypothesize that for P58^{IPK}-PKR interactions *in vivo*, protein X must first interact with the DnaJ region of P58^{IPK}. Protein X then facilitates P58^{IPK}-PKR interaction either: (i) by targeting P58^{IPK} to PKR inside the cell, or (ii) by inducing the proper folding of P58^{IPK} such that the inhibitor can then interact with PKR. Earlier work has found that the TPR6 motif is critical for P58^{IPK} binding to PKR, and that P58^{IPK} binds to PKR in a region spanning the protein kinase regulatory (*R*) and catalytic (*C*) domains. See text for additional details.



require that X interacted with both P58^{PK} and PKR. Such a model could explain why P58^{PK} variants, lacking the DnaJ region, can still interact with and inhibit PKR *in vitro*. Alternatively, factor X, which might itself be a molecular chaperone (Craig et al. 1993; Morimoto et al. 1994), may bind to the DnaJ region of P58^{PK} and alter the conformation or folding of P58^{PK}, such that it can now interact with PKR and inhibit the protein kinase. Recent data from our lab showing heterocomplex formation between P58^{PK} and other molecular chaperones favors the latter model (see last paragraph of chapter 2). Earlier studies by our lab has also mapped the P58^{PK}-interactive site of PKR to amino acids 244-296, which spans the regulatory (R) and catalytic (C) borders of PKR, and includes the ATP binding region of the protein kinase catalytic domain (Gale, Jr. et al. 1996). Which ever scenario should the case may be, the end result of either of these two model pathways would be the P58^{PK} induced inhibition of PKR activity and resultant stimulation of mRNA translation. The interplay between P58^{PK}, PKR, and protein X is reminiscent of other systems in which a TPR protein either interacts with other TPR proteins or else another regulatory protein, such as $\alpha 2$, to control its activity (Lamb et al. 1994; Smith et al. 1995). Moreover, there is even precedence in the literature for the interaction of another TPR protein, Hip, with the molecular chaperone Hsc70 (Höhfeld et al. 1995).

Before closing it is worth commenting on the requirement of the TPR6 motif for both P58^{PK} binding and function. Curiously, the central TPR motifs, often

including TPR6, were found critical for the function of other TPR proteins including Nuc2, CDC-23, CDC-27, and BimA (Hirano et al. 1990; Sikorski et al. 1991; Sikorski et al. 1993; Goebel and Yanagida 1991). Furthermore, single amino acid substitutions in TPR domains 5-7 have been shown to abolish the biological function of the yeast CDC23 gene (Sikorski et al. 1991; Sikorski et al. 1993). These data suggest a critical function for this region of these TPR proteins despite their diverse regulatory roles. The P58^{IPK} TPR6 domain contains sequences with limited similarity to a region of eIF-2 α , the natural PKR substrate. This homologous region, however, is not extensive, with a maximum 31% identity with five gaps. Nonetheless, this region of P58^{IPK} does contain the PKR consensus eIF-2 α phosphorylation motif ELS (P58^{IPK} amino acids 239-241 and eIF-2 α amino acids 49-51). Since the eIF-2 α serine 51 is phosphorylated by PKR (Colthurst et al. 1987), these data suggested P58^{IPK} may function as a PKR substrate (Kemp et al. 1991). This would require the presence of serine 241 to functionally mimic the eIF-2 α phosphorylation site. However, we found that a serine to alanine mutation at amino acid 241 (see Fig 1.1) did not inactivate P58^{IPK} function either when P58^{IPK} was assayed *in vitro* (Lee et al. 1994) or *in vivo* (data not shown), suggesting that P58^{IPK} probably does not function as a PKR pseudosubstrate. In accordance with these results, we found that P58^{IPK} likely binds at or near the ATP binding domain on PKR, a region clearly distinct from the substrate binding site. In contrast, the vaccinia virus-encoded PKR inhibitor K3L likely does function as PKR substrate (Carroll et al. 1993; Davies et al. 1993; Beattie et al. 1991). This argument is supported by our recent evidence that,

unlike P58^{IPK}, K3L binds to a region of PKR spanning amino acids 367-551, which localizes to the large lobe of the PKR protein kinase, thought to be largely responsible for substrate recognition and binding (Gale, Jr. et al. 1996; Taylor et al. 1993).

Chapter 4: Inhibition Of PKR Activity Is Not Required For P58^{IPK}-Induced Oncogenesis

Introduction

P58^{IPK} was first identified as a cellular inhibitor of PKR that is activated during influenza virus infection (Lee et al. 1990; Lee et al. 1992). PKR is an interferon-induced, dsRNA-activated protein kinase, which when activated, autophosphorylates and then phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α), leading to dramatic decreases in protein synthesis initiation (Hovanessian 1991; Meurs et al. 1990; Clemens 1996). These events are not favorable to viruses, which must make their proteins in order to replicate. For this reason, many viruses, including influenza virus, have evolved strategies to downregulate PKR activity (Mathews 1996; Schneider and Shenk 1987; Katze 1996; Katze 1995). We have shown that activated P58^{IPK} can block both PKR autophosphorylation and phosphorylation of eIF-2 α substrate *in vitro* (Lee et al. 1994). In addition, P58^{IPK} stimulates reporter gene protein synthesis initiation *in vivo* (Tang et al. 1996). Thus, P58^{IPK} acts in both the virus-infected and uninfected cell to regulate protein synthesis.

In addition to these roles, P58^{IPK} has also been shown to possess oncogenic properties. Overexpression of P58^{IPK} transforms NIH 3T3 cells; and injection of these cells into nude mice produces tumors (Barber et al. 1994). The mechanism by which P58^{IPK} malignantly transforms cells is unknown. One hypothesis is that P58^{IPK} induces oncogenesis via the inhibition of PKR phosphorylation of eIF-2 α . The basis for this hypothesis are the various studies implicating PKR as a tumor suppressor protein via its ability to phosphorylate eIF-2 α and suppress cellular growth. First, expression of PKR in yeast is growth suppressive (Dever et al. 1993; Chong et al. 1992), with growth suppression correlated with increases in eIF-2 α phosphorylation (Chong et al. 1992). Second, NIH 3T3 cells which have been malignantly transformed by the expression of one of several transdominant PKR mutants show reduced eIF-2 α phosphorylation (Donzé et al. 1995; Barber et al. 1995). Third, expression of a non-phosphorylatable mutant of eIF-2 α (eIF-2 α^{ala}) in NIH 3T3 cells also leads to eIF-2 α hypophosphorylation and neoplastic transformation (Donzé et al. 1995). These observations suggest that the inhibition of PKR phosphorylation of eIF-2 α can lead to oncogenesis.

Although PKR can suppress cellular growth and tumorigenesis by regulating gene expression at the translational level, there is evidence that PKR also participates in transcriptional regulation. For example, the protein kinase inhibitor 2-aminopurine blocks both PKR activity (DeBenedetti and Baglioni 1983) and virus-induced transcription of *c-fos* and *c-myc* proto-oncogenes (Zinn et al. 1988). PKR can

also phosphorylate the inhibitor of nuclear factor-kappa B ($I\kappa B\alpha$) *in vitro* (Kumar et al. 1994); and cells deleted for PKR (through mRNA ablation or gene knockout (Maran et al. 1994; Yang et al. 1995)) cannot activate NF- κ B *in vivo* in response to poly(rI):poly(rC) inducer (Maran et al. 1994; Der et al. 1997). Finally, PKR has been shown to associate with the signal transducer and activator of transcription (STAT1); and increases in STAT1 DNA binding are associated with decreases in PKR-STAT1 interaction (Wong et al. 1997). Thus, it is also possible that P58^{IPK} can induce oncogenesis through the inhibition of PKR-mediated transcriptional activity. Although no link has been made between PKR's ability to regulate transcription and oncogenesis, it is conceivable that the growth and tumor suppressive properties observed for PKR may be a direct result of PKR's ability to regulate cellular gene expression at both the transcriptional and translational levels.

In addition to the above activities, PKR has been shown to mediate programmed cell death in certain cell types, under certain conditions. For instance, in HeLa cells infected with a recombinant vaccinia virus expressing wild type PKR under an inducible promoter, apoptosis occurred immediately following induction. In contrast, HeLa cells infected with virus expressing a dominant negative PKR mutant (PKR-DII, also known as K296R) did not undergo programmed cell death (Lee and Esteban 1994). Furthermore, the PKR-DII mutant protected cells from apoptosis following influenza virus infection (Takizawa 1996). Lastly, several studies have implicated PKR in the

induction of apoptosis by dsRNA as well as TNF- α (Yeung et al. 1996; Kibler et al. 1997; Der et al. 1997). Despite the fact that no work has been done to investigate whether PKR's ability to mediate apoptotic cell death participates in tumor suppression, it has nevertheless been suggested that one mechanism by which PKR suppresses tumor growth is through the induction of programmed cell death. Thus, it is also possible that P58^{IPK} induces tumorigenesis via the inhibition of PKR-mediated programmed cell death.

To determine whether P58^{IPK} induces oncogenesis via the inhibition of PKR phosphorylation of eIF-2 α and/or through the inhibition of some other PKR-mediated event, NIH 3T3 cell lines expressing a P58^{IPK} mutant that does not bind to or inhibit PKR (Δ TPR6) were generated. The Δ TPR6 cell lines were analyzed for oncogenic potential and these results were correlated with three different PKR activities in the cells. We report that expression of Δ TPR6 malignantly transformed NIH 3T3 cells. However, no decrease in eIF-2 α phosphorylation was observed in the Δ TPR6-expressing cells. In addition, the Δ TPR6-expressing cells did not show an inhibition of poly(rI):poly(rC)-induced NF- κ B activation, and they did not show an inhibition poly(rI):poly(rC)-induced programmed cell death. Thus, these three PKR activities are not required for P58^{IPK}-induced oncogenesis. Interestingly, when the mouse cell lines were treated with TNF- α , Δ TPR6-expressing cells were partially refractory to the induced apoptotic cell death, whereas P58^{IPK}-expressing cells were significantly more resistant. Taken together, our data suggest that P58^{IPK}-induced oncogenicity occurs via a PKR-

independent pathway, which may be enhanced through the inhibition of PKR.

Materials And Methods

Plasmid construction

The cloning and sequencing of the 1.6-kb wild type bovine P58^{IPK} cDNA into pcDNA1/Neo (Invitrogen, Carlsbad, CA) has been described previously (Lee et al. 1994). In addition, the cloning and sequencing of the Δ TPR6 mutant into pcDNA1/Neo has also been previously described (Tang et al. 1996).

Construction of wild type and mutant P58^{IPK}-expressing cell lines

Murine NIH 3T3 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD). 1.3×10^6 cells each were plated into 100-mm dishes and transfected by calcium phosphate preparation (Ausubel et al. 1988) using 20 μ g of wild type P58^{IPK} or Δ TPR6 mutant DNA. After 20 hrs of incubation, the DNA mix was removed and complete medium (DMEM containing 10% FBS) was added to the cells. This medium was removed 24 hrs later and replaced by complete medium supplemented with 600 μ g/ml of G418 (Geneticin; Gibco, Gaithersburg, MD). After 9 days of drug selection, the surviving G418-resistant cells were trypsinized and individual clones harvested from 24-well plates. Clonal cell lines

were maintained in complete medium containing 400µg/ml of G418.

Western blot (immunoblot) analysis of P58^{IPK} protein levels

Monolayer cell lines or dissociated tumor cells were washed twice with ice-cold Hank's balanced salt solution and lysed in Triton lysis buffer (10mM Tris-HCl, pH 7.5, 50mM KCl, 1mM dithiothreitol, 2mM MgCl₂, 100µg/ml aprotinin, 1mM phenylmethylsulfonyl fluoride, and 1% Triton X-100). After SDS-polyacrylamide gel electrophoresis, polypeptides were transferred to nitrocellulose (Towbin et al. 1979) and detected with the 2F8 P58^{IPK} monoclonal antibody (Barber et al. 1994) or the P58^{IPK} polyclonal antibody described below.

Preparation of polyclonal antibody to P58^{IPK}

100µg of purified GST-P58^{IPK} fusion protein and 100µg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine in 2.5ml were mixed with an equal volume of incomplete Freund's adjuvant. The emulsified antigen mixture was injected subcutaneously in multiple sites into New Zealand White rabbits. Two subsequent boosts of 100mg of GST-P58^{IPK} in incomplete Freund's adjuvant were administered monthly monthly.

Analysis of eIF-2α phosphorylation levels

The state of eIF-2α phosphorylation in the cell lines were determined by vertical slab isoelectric focusing and immunoblotting as previously described (Savinova

and Jagus 1997) with the following modifications: (1) no BPA-1000 treatment was used during the harvest of the cells, (2) immunoprecipitation of eIF-2 α from the cell lysate prior to gel loading was not performed, (3) IEF gels were transferred to nitrocellulose and blocked in a PBS solution containing 10% w/v nonfat dried milk and 0.2% Tween 20, (4) all antibody incubations were done in PBS containing 0.1-0.2% Tween 20.

Injection of nude mice

4-6 week old athymic mice (Balb/c nu⁻/nu⁻ from Taconic Farms, Germantown, NY) were injected subcutaneously in the right inguinal area with 2×10^6 cells in 500 μ l of DMEM. The nude mice were housed in a specific pathogen-free environment.

Extraction and propagation of tumors

Tumors were extracted from euthanized mice under aseptic conditions; washed 4 times in PBS to remove excess blood, fat and necrotic tissue; and minced into small pieces. The tumor pieces were then added to DMEM containing 0.5% collagenase/dispase (Sigma, St. Louis, MO); homogenized with a Dounce vessel; and incubated for 2 hr at 37 $^{\circ}$ C, with gentle stirring. Dissociated cells were washed once with complete DMEM and seeded into plates for the generation of tumor cell lines.

Cell growth, soft agar and tumorigenicity assays

Assays were done as described in figure 4.4 and Table III.

Electrophoretic mobility shift assay (EMSA) for NF- κ B activity

80% confluent cells (grown in 10cm dishes) were washed and serum-starved for 18 hours, after which they were washed again in serum-free medium and stimulated with either 100 μ g/ml poly(rI):poly(rC) (Sigma) or 10ng/ml tumor necrosis alpha (Boehringer Mannheim, Indianapolis, IN). Whole cell extracts were then prepared as previously described (Haque and Williams 1994) with the exception that the 15,000 x g supernatant material was not subject to dialysis. Detection of activated NF- κ B was performed by incubation of 5 μ g of cell extract with 0.2ng of γ -³²P-end labelled PRDII oligonucleotide in binding buffer (20 mM HEPES, 50 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, 0.6 mM EDTA, and 1.0 μ g/ml poly(dI):poly(dC)) for 20 min at room temperature. Complexes were analyzed using native 5% polyacrylamide gel electrophoresis in 0.5X TBE buffer. The gels were dried and complexes were visualized by autoradiography.

TUNEL assay for apoptosis

Cells (5×10^5 /sample) were treated with poly(dI):poly(dC) (0.1 to 100 μ g/ml) together with actinomycin D (50 ng/ml) for 16 h. Apoptosis-induced DNA fragmentation was detected by *in situ* labeling of DNA strand breaks with terminal deoxynucleotidyltransferase-mediated incorporation of fluorescein dUTP as described by the manufacturer (Boehringer Mannheim). Fluorescein incorporation was analyzed using a FACStar Flow Cytometer (Immunocytometry System, Becton Dickinson).

Cell viability and DNA fragmentation assays

Cells (10^6) were cultured in 6-well plates overnight prior to treatment with tumor necrosis factor-alpha (TNF- α ; Boehringer Mannheim, Indianapolis, IN) alone or in combination with actinomycin D (Sigma). After 18 hours, cells were trypsinized and viability assessed by trypan blue exclusion. In parallel experiments, the total cells from each well were collected and low molecular weight DNA was isolated to assay for the presence of a characteristic apoptotic DNA fragmentation ladder. In brief, cells were pelleted and incubated in lysis buffer (20mM Tris, pH 8.0, 10 mM EDTA and 1% Triton-X-100) on ice for 20 min. The samples were then extracted with phenol-chloroform and DNA was precipitated by addition of sodium acetate (0.3M) and ethanol. DNA was resuspended in TE containing RNase A (0.1 mg/ml), incubated at 37°C for 30 min and analyzed by 2% agarose gel electrophoresis.

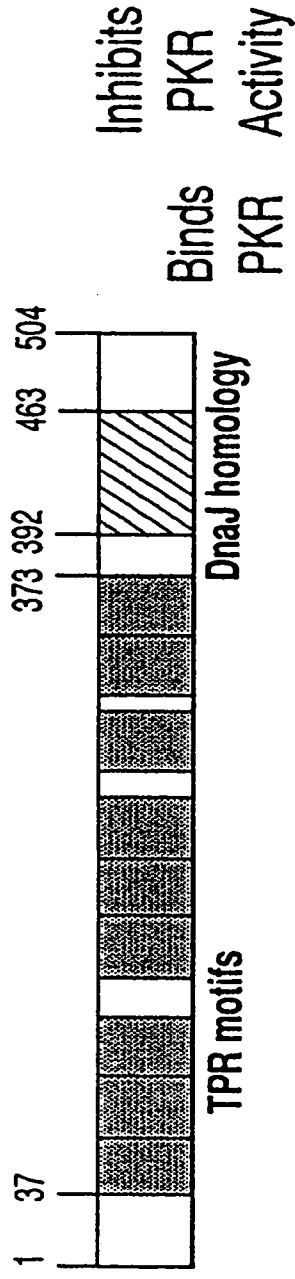
Results

NIH 3T3 cell lines expressing Δ TPR6 display a transformed phenotype

Previously, we performed a structure/function study on the P58^{IPK} molecule to determine which regions were required to inhibit PKR activity. Using an *in vitro* approach, we demonstrated that the sixth TPR motif (TPR6) of P58^{IPK} is required for inhibition of PKR phosphorylation of substrate (Tang et al. 1996). In addition, this same

region is required to stimulate reporter gene protein synthesis initiation *in vivo* (Tang et al. 1996). Using the yeast two hybrid system, our lab found that a P58^{IPK} variant lacking TPR6 (Δ TPR6) is incapable of interacting with PKR (Gale, Jr. et al. 1996). These observations led us to hypothesize that the expression of Δ TPR6 in mouse cells would have no effect on PKR activity. Therefore, to determine whether a P58^{IPK} mutant molecule that does not inhibit PKR activity can transform cells, we stably transfected NIH 3T3 fibroblasts with pcDNAI/Neo expression plasmids containing cDNA encoding Δ TPR6 (Figure 4.1). As a positive control for cellular transformation, NIH 3T3 cells were transfected with pcDNAI/Neo expression plasmids containing cDNA encoding bovine, wild type P58^{IPK}. For a negative control, we used empty pcDNAI/Neo expression plasmids (Vector/Ctl) for the transfection. After selecting for neomycin resistance, 16 random individual clones from each transfection were selected for immunoblot analysis. Representative clones are shown in figure 4.2A. Using a P58^{IPK} polyclonal antibody that does not recognize the endogenous mouse species, detectable levels of Δ TPR6 proteins were found in all three of the Δ TPR6 clones. The three independent clones also displayed varying levels of Δ TPR6 protein expression. Cell lines expressing P58^{IPK} also showed varying levels of P58^{IPK} protein expression (Figure 4.2A). Because of its small deletion, Δ TPR6 migrated at approximately the same position as P58^{IPK} in the 12% acrylamide gel shown in Figure 4.2A. The identity of Δ TPR6 was confirmed using 10-20% acrylamide gradient gels which distinguished between the 58kDa P58^{IPK} protein and the 54kDa

Figure 4.1. Schematic of domain structures of wild type and mutant P58^{IPK} deletion mutant constructs. Wild type P58^{IPK} (wtP58^{IPK}) contains nine tandemly arranged 34-amino acid repeats termed TPR motifs. P58^{IPK} amino acids 207-303 are homologous to the amino-terminal region of eIF-2 α (amino acids 26-120). The DnaJ homology region resides at the carboxyl-terminus (amino acids 392-463). The Δ TPR6 mutant is an in-frame deletion of the sixth TPR motif. A summary of the ability of the P58^{IPK} constructs to interact with PKR is indicated to the *right* along with their abilities to block PKR phosphorylation activity as determined by previous *in vitro* and *in vivo* assays (*far right*).



P58^{IPK}

ΔTPR6 222 235

Figure 4.2. Expression of bovine wild type and mutant P58^{IPK} in stably transfected NIH 3T3 cells (A), tumors (B) and cell lines derived from tumors (C). Western blot analyses were performed on cytoplasmic extracts prepared from the following sources. Panel A, two independent wild type (wtP58^{IPK}-1 and -2) and three mutant (Δ TPR6-1,-2,-3) P58^{IPK}-expressing cell lines were analyzed using a polyclonal antibody which recognizes the transfected bovine P58^{IPK} but not the endogenous mouse species (see *Materials and Methods* section). Panel B, one tumor from nude mice injected with the wtP58^{IPK}-1 cell line (P58^{IPK}-1.1) and four tumors (two from mice injected with the Δ TPR6-1 cell line, designated Δ TPR6-1.1 and Δ TPR6-1.2, and two from mice injected with the Δ TPR6-2 cell line, designated Δ TPR6-2.1 and Δ TPR6-2.2) were also analyzed using the 2F8-58 monoclonal antibody previously described (Barber et al. 1994). This P58^{IPK} monoclonal also does not recognize the endogenous mouse species. Panel C, cell lines derived from the tumors described in panel B were analyzed using the P58^{IPK} polyclonal antibody. In all three panels, cell extracts prepared from MDBK cells were used as a source of bovine P58^{IPK} protein, and thus served as a positive control. In addition, cell extracts prepared from NIH 3T3 cell lines expressing the empty pcDNA1/Neo vector were analyzed in parallel as a negative control. All sample lanes contained approximately 100 μ g of protein.

A. Cell Lines

MDBK
Vector/Ctl
P58^{IPK-2}
P58^{IPK-1}
ΔTPR6-1
ΔTPR6-2
ΔTPR6-3

B. Tumors

MDBK
Vector/Ctl
ΔTPR6-1.1
ΔTPR6-1.2
ΔTPR6-2.1
ΔTPR6-2.2
P58^{IPK-1}

C. Tumor Cell Lines

MDBK
Vector/Ctl
ΔTPR6-1.1
ΔTPR6-1.2
ΔTPR6-2.1
ΔTPR6-2.2
P58^{IPK-1}

P58^{IPK} →



Δ TPR6 protein (data not shown).

For further characterization, we focused on two clones of Δ TPR6-expressing cells: a high-expressing clone (Δ TPR6-1), and a low-expressing clone (Δ TPR6-2). For comparison, we also chose one high-expressing clone (P58^{IPK}-1) and one low-expressing clone (P58^{IPK}-2) from the P58^{IPK} transfected cells. One Vector/Ctl cell line was selected as a negative control for comparison and further characterization. When we examined exponentially growing cells under the microscope, we saw morphological changes in both Δ TPR6 cell lines. Like P58^{IPK}-expressing cells, Δ TPR6-expressing cells exhibited spindle-shaped morphology and increased refractility (Figure 4.3, *B* and *C*). In contrast, Vector/Ctl cells had an appearance similar to that of parental, untransfected NIH 3T3 cells (Figure 4.3A). Consistent with the morphological changes, we noticed that transformed foci readily formed on top of cell monolayers that expressed either Δ TPR6 or P58^{IPK}, but not Vector/Ctl (Figure 4.3, *D-F*). Furthermore, Δ TPR6 and P58^{IPK} cell lines grew faster (Figure 4.4, Table III) and to a higher saturation density than the Vector/Ctl cell line (Table III). In concordance with their transformed appearance and altered growth properties, both Δ TPR6- and wtP58^{IPK}-expressing cells formed colonies in soft agar, indicating anchorage-independent growth (Figure 4.3, *H* and *I*). None of the Vector/Ctl cells were capable of growth in soft agar (Figure 4.3G and Table III). These results demonstrate that the expression of Δ TPR6, like P58^{IPK}, can transform NIH 3T3

Figure 4.3. Morphological characteristics of P58^{IPK}-expressing cells. Stably transfected NIH 3T3 cells expressing the empty pcDNA1/Neo vector control (*A, D, and G*) wild type P58^{IPK} (*B, E, and H*) and Δ TPR6 mutant (*C, F, and I*) were plated at 1X10⁵ cells per 100mm dish in DMEM containing 10% fetal bovine serum and 400 μ g/ml G418. Panels *A-C*, logarithmically growing cells. Panels *D-F*, cells maintained in culture 3 days after reaching confluency. Panels *G-I*, anchorage-independent growth of cells in soft agar. Optical magnification (x100).

Vector/Ctl

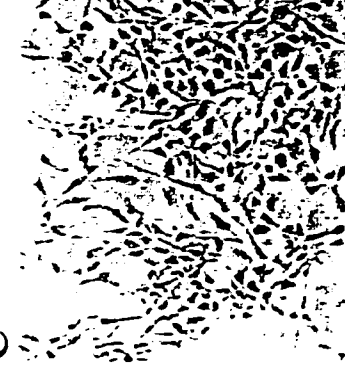
P58^{IPK}

Δ TPR6

A

B

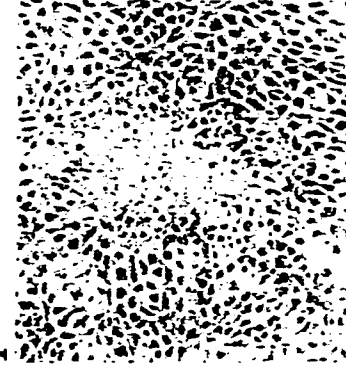
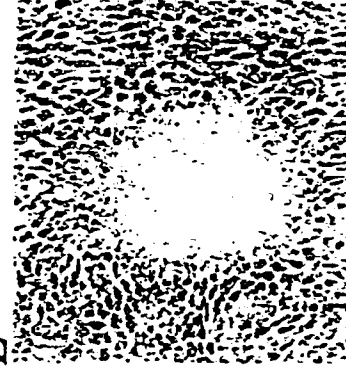
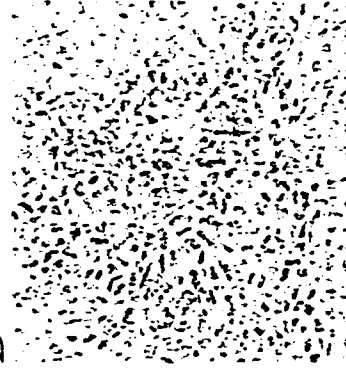
C



D

E

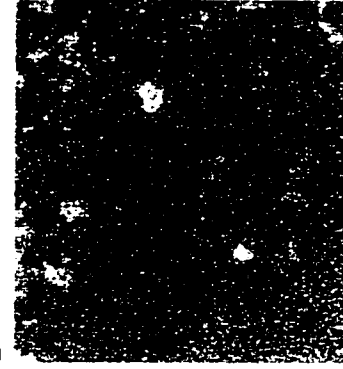
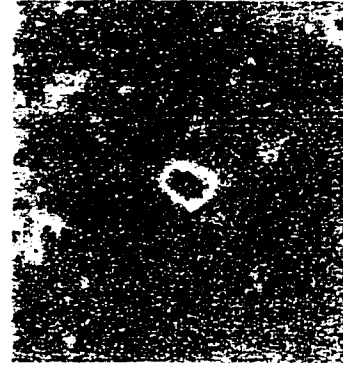
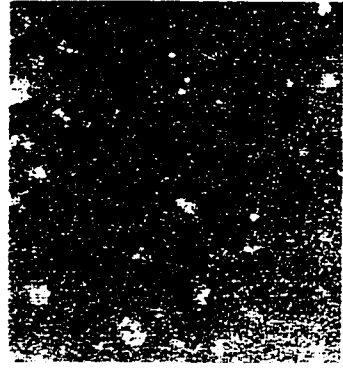
F



G

H

I



**Log
Growth**

**4d Post-
Confluency**

**Soft
Agar**

Figure 4.4. NIH 3T3 cells overexpressing wild type P58^{IPK} or Δ TPR6 mutant grow faster than vector control cells. Cells were grown in DMEM supplemented with 10% FBS and 400 μ g/ml G418. The medium was changed every 24 hrs and the cell numbers determined using a hemacytometer.

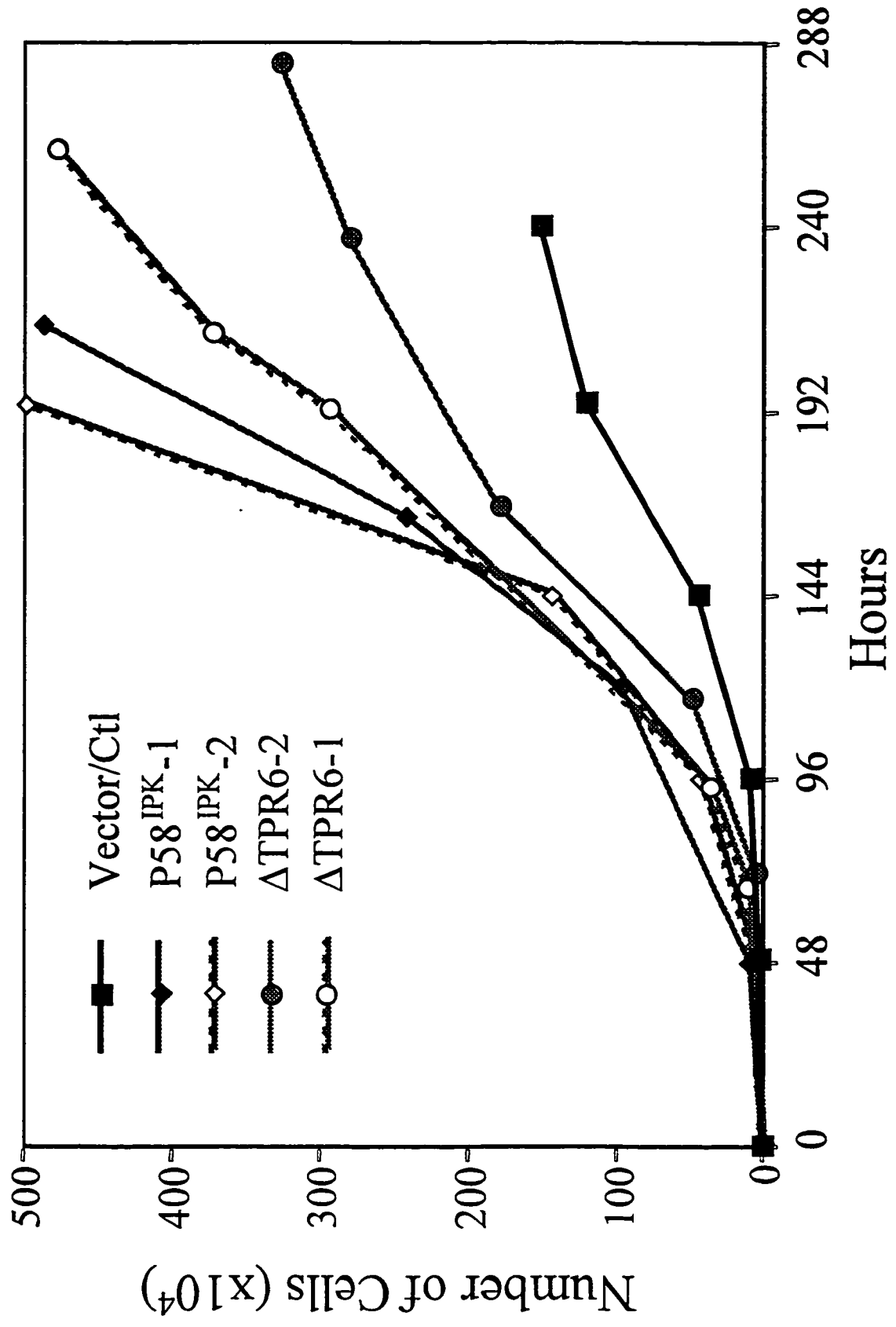


Table III. Growth properties and tumorigenicity of cells expressing wild type and mutant P58^{IPK}. The growth in monolayers was measured by seeding 2×10^4 cells in DMEM supplemented with 10% FBS and G418. Medium was changed every 3 days. *Doubling time* was determined by counting cells every 2 days and calculating the growth rate of exponentially growing cells. *Saturation density* is defined as the total number of cells in culture 4 days after reaching confluency. Numbers represent the average of 2 experiments. To determine the *cloning efficiency*, 1×10^4 cells were suspended in 0.35% agarose solution and the colonies were scored 2-4 weeks after plating. *Cloning efficiency* is the # of colonies multiplied by 100 and divided by the # of cells plated. A colony is defined as a cell cluster consisting of 4 or more cells. The numbers shown here represent the average of 2 experiments. Latency is the number of days required to produce tumors of ≥ 2 mm. Tumor cell lines (*TCL*) were derived from tumors and selected for their ability to grow in G418-containing culture medium.

Table III. Growth Properties of Cells expressing wt or mutant P58^{IPK}

Clone	Growth in monolayers			Tumorigenicity		
	Doubling time (hrs)	Saturation density (10 ⁶ cells)	Cloning efficiency (%)	Animals with tumors/animals injected	Latency (days)	
Vector/Ctl	32.7±0.4	1.4±0.1	0	0/5	>112	
P58 ^{IPK} -1	21.9±0.2	4.9±0.3	15.2±3.8	5/5	14-16	
P58 ^{IPK} -2	23.3±1.3	3.1±0.4	13.3±5.3	2/2	14-16	
ΔTPR6-1	22.8±0.9	4.8±0.2	3.1±0.4	5/5	29-35	
ΔTPR6-2	25.7±0.2	3.3±0.4	7.0±0.1	5/5	29-51	
ΔTPR6-1 (TCL)	ND	ND	ND	6/6	4	
ΔTPR6-2 (TCL)	ND	ND	ND	6/6	4	

fibroblasts.

Δ TPR6 cell lines are tumorigenic in nude mice

To test whether the Δ TPR6 cell lines could form tumors in nude mice, we injected Balb/c nu⁻/nu⁻ mice subcutaneously in the right inguinal area with 2×10^6 Δ TPR6-expressing cells per mouse. Five athymic mice were injected with each Δ TPR6 cell clone. In 10/10 mice injected with the two independent Δ TPR6 clones, a visible tumor of ≥ 2 mm appeared in 4-5 weeks (Table III). By comparison, mice injected with the Vector/Ctl cell line did not form tumors during the 16 week observation period. Tumors were also seen in mice injected with P58^{IPK}-expressing cells. These tumors appeared two weeks after injection. To verify that the tumors expressed either the introduced Δ TPR6 or P58^{IPK}, protein extracts were prepared from tumor homogenates and analyzed by immunoblotting using the previously described 2F8-58 monoclonal which does not recognize mouse P58^{IPK} (Barber et al. 1994). As shown in Figure 4.2B, all of the Δ TPR6 tumors analyzed showed high level expression of Δ TPR6 protein. Tumors extracted from P58^{IPK}-injected mice also showed a high level of bovine P58^{IPK} protein expression.

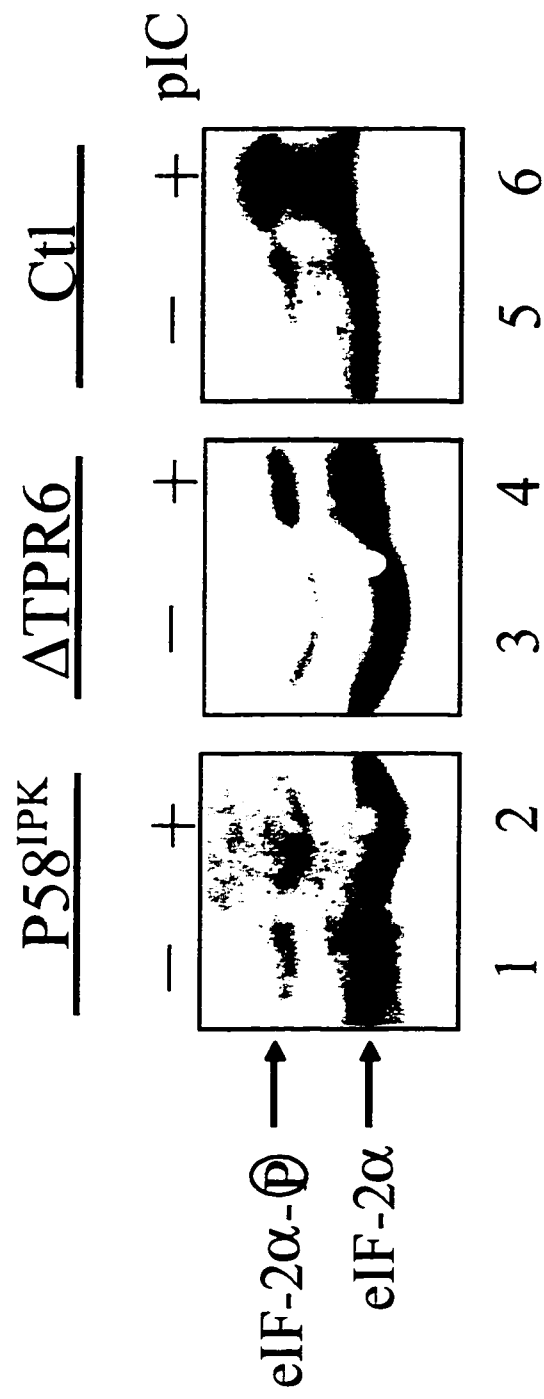
As additional confirmation that Δ TPR6 was responsible for tumor induction, we cultured the tumors and derived cell lines from them. Cells derived from tumors were selected for their ability to grow in medium containing G418 and analyzed by immunoblotting. Consistent with earlier results (Barber et al. 1994), cell lines derived

from P58^{IPK} tumors all showed high level protein expression. In contrast, cell lines derived from Δ TPR6 tumors showed low level Δ TPR6 expression (Figure 4.2C). These differences may reflect an intrinsic instability of the Δ TPR6 protein or may be a consequence of a downregulation of Δ TPR6 expression during cell culture conditions. Nevertheless, the cell lines derived from Δ TPR6 tumors were capable of further tumor formation when injected into a second round of nude mice. As shown in Table III, 12/12 mice injected with Δ TPR6 tumor cell lines developed tumors in 4 days. This tumor latency period is similar to that reported by Barber and colleagues for cell lines derived from P58^{IPK} tumors (Barber et al. 1994). These results clearly demonstrate that the overexpression of Δ TPR6, like P58^{IPK}, is tumorigenic in nude mice.

Cells expressing Δ TPR6 do not show reduced eIF-2 α phosphorylation

We have previously established in several systems that Δ TPR6 is an inactive P58^{IPK} mutant that does not inhibit PKR activity. However, it was formally possible that Δ TPR6 is active in the NIH 3T3 cell lines. To confirm that Δ TPR6-induced oncogenicity was not a result of the inhibition of PKR activity, we examined the level of eIF-2 α phosphorylation in the Δ TPR6-expressing cells and compared it to the levels in P58^{IPK}- and Vector/Ctl cells. As illustrated in figure 4.5 (-pIC, lanes 1, 3 and 5), logarithmically growing cells exhibited very low levels of eIF-2 α phosphorylation. However, when treated with poly(rI):poly(rC) (pIC) to activate PKR, both the Vector/Ctl

Figure 4.5. eIF-2 α phosphorylation is inhibited in NIH 3T3 cells overexpressing wild type P58^{IPK} but not the Δ TPR6 mutant. Exponentially growing wild type P58^{IPK}-, Δ TPR6 mutant- or Vector control-overexpressing cells were incubated with complete medium (DMEM containing 10% calf serum and 400 μ g/ml G418) \pm poly(rI):poly(rC) (pIC) for 20 hrs. The cells were harvested, lysed and 20 μ g of cellular extracts was subjected to vertical slab isoelectric focusing. The separated polypeptides were transferred to nitrocellulose and the blot probed with an eIF-2 α monoclonal antibody (Henshaw lab). A representative experiment is shown here.



and Δ TPR6-expressing cells demonstrated a significant increase in eIF-2 α phosphorylation (Figure 4.5, lanes 4 and 6). In contrast, P58^{PK}-expressing cells showed no change in the level of eIF-2 α phosphorylation (Figure 4.5, lane 2), indicating loss of PKR activity. These findings demonstrate that Δ TPR6-mediated oncogenicity is not through the inhibition of PKR phosphorylation of eIF-2 α .

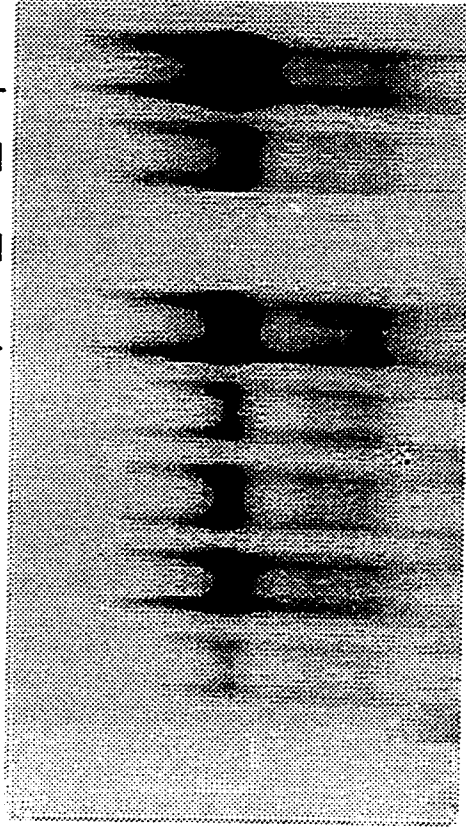
Δ TPR6 cell lines do not inhibit pIC-induced PKR activation of NF- κ B

In addition to the phosphorylation of eIF-2 α , previous studies have shown that PKR is required for the induction of nuclear factor-kappa B (NF- κ B) activity in the presence of pIC (Maran et al. 1994; Der et al. 1997). Therefore, to further evaluate whether PKR is active in the Δ TPR6-expressing NIH 3T3 cell lines, we prepared whole cell extracts from pIC-stimulated and unstimulated cells. The cellular extracts were incubated with γ -³²P-labelled PRDII (a promoter sequence in the human interferon β gene, called Positive Regulatory Domain II, which is known to bind NF- κ B). Activated NF- κ B was detected by the upward shift of the radiolabelled PRDII DNA sequence. As shown in figure 4.6, when Vector/Ctl cells were treated with pIC, there was a marked increase in NF- κ B activity (compare lane 1 to lane 2). Similar to Vector/Ctl cells, cells expressing Δ TPR6 also showed an induction of NF- κ B activity (compare lane 7 to lane 8). In contrast, P58^{PK}-expressing cells exhibited no induction of NF- κ B activity in the presence of pIC inducer (compare lane 4 to lane 5). As a positive control for NF- κ B

Figure 4.6. NF- κ B binding activities in P58^{IPK} and Δ TTPR6 cell lines. Electrophoretic mobility shift assay of whole cell extracts prepared from P58^{IPK} (lanes 4-6), Δ TTPR6 (lanes 7-9) and Vector/Ctl cell lines (lanes 1-3). The cell lines were stimulated with medium alone ("Media"), medium containing 100 μ g/ml poly(rI):poly(rC) ("pIC"), or medium containing 10ng/ml TNF- α (TNF- α). NF- κ B activation was detected using a γ -³²P-end labelled PRDII oligonucleotide probe. NF- κ B-specific complexes are designated by the arrow.

Ctl P58^{IPK} ΔTPR6

Media PIC TNF-α Media PIC TNF-α Media PIC TNF-α



← NF-κB

1 2 3 4 5 6 7 8 9

activation that is PKR-independent, we treated the P58^{IPK}-expressing cells with TNF- α . When stimulated with TNF- α , P58^{IPK}-expressing cells showed a further increase in NF- κ B binding activity (compare *lane 4* to *lane 6*). These results are similar to those reported in previous studies (Yang et al. 1995; Der et al. 1997; Maran et al. 1994) showing that the loss of PKR (through gene knockout or targeted mRNA ablation) results in the specific loss of pIC-induced NF- κ B activation and has no effect on NF- κ B activation induced by TNF- α . Thus, the data presented here support the idea that Δ TPR6-induced oncogenesis is not via the inhibition of PKR-mediated activation of NF- κ B.

Curiously, cells expressing P58^{IPK} showed an unusually high level of basal NF- κ B activity in the absence of inducer (Figure 4.6, *lane 4*). This constitutive activation of NF- κ B was seen in three independent clones of P58^{IPK} (data not shown). Because such an activity is not seen in cells deleted for PKR (Maran et al. 1994; Der et al. 1997), this observation suggests that P58^{IPK} can activate NF- κ B independent of PKR. Thus, we may have stumbled upon a novel function for P58^{IPK} in the transcriptional regulation of cellular gene expression through a PKR-independent activation of NF- κ B.

Cells expressing Δ TPR6 are not resistant to pIC-induced apoptosis

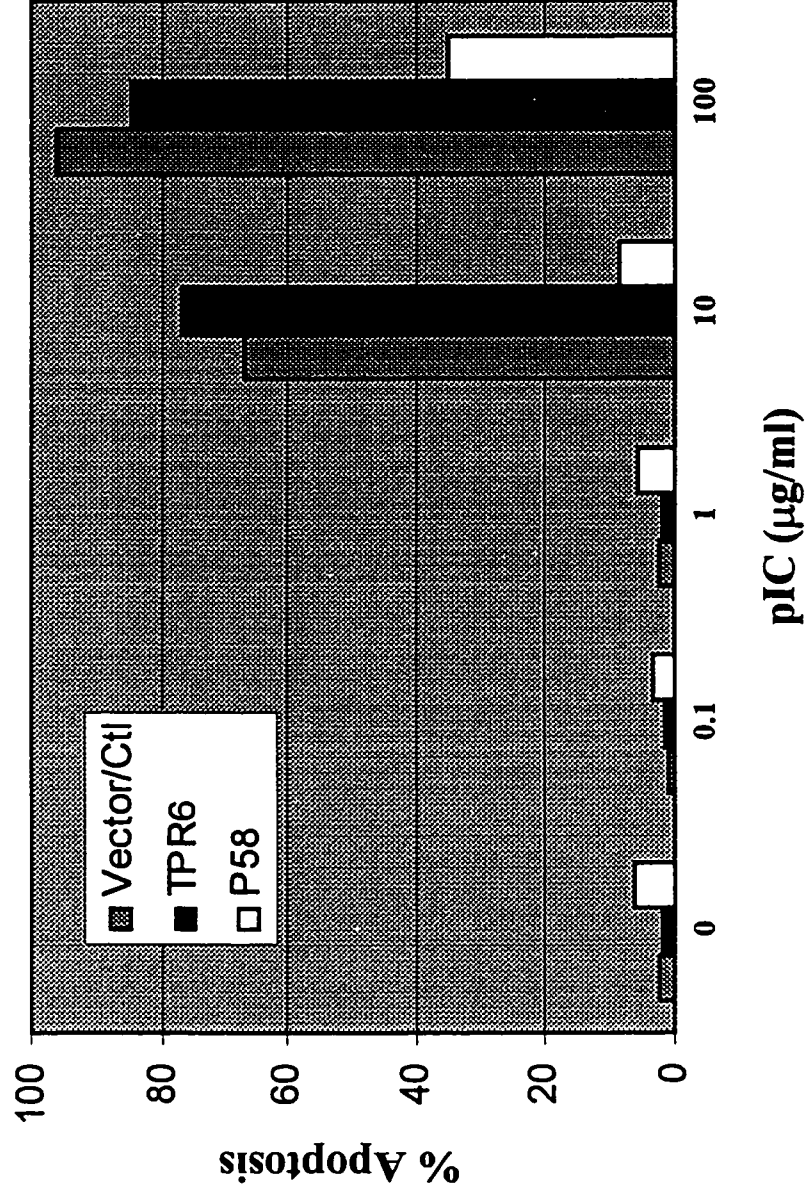
PKR has also been shown to be required for pIC-induced apoptosis in several systems, including fibroblasts (Lee and Esteban 1994; Yeung et al. 1996; Der et

al. 1997). To evaluate whether P58^{IPK}-induced oncogenesis may involve the inhibition of such a pathway, we treated the mouse cell lines to varying concentrations of pIC in the presence of 50ng/ml actinomycin D, after which we performed TUNEL assays (Boehringer Mannheim) to quantify the number of apoptotic cells. As shown in figure 4.7, Vector/Ctl cells and Δ TPR6 cells exhibited marked apoptotic cell death when treated with 10 μ g/ml or 100 μ g/ml of pIC. In contrast, cells expressing P58^{IPK} resisted the pIC-induced apoptosis at these concentrations. These results illustrate, once again, that PKR is active in the Δ TPR6-expressing cells and inactive in the P58^{IPK}-expressing cells. Likewise, these data demonstrate that Δ TPR6-induced tumorigenesis is not through the inhibition of pIC-induced, PKR-mediated programmed cell death.

Both P58^{IPK} and Δ TPR6 cell lines show resistance to TNF- α -induced apoptosis

It has long been known that TNF- α treatment, along with low concentrations of actinomycin D, can trigger apoptotic cell death in certain cell types, including fibroblasts (Grooten et al. 1993). Recently, it has been shown that PKR is required for TNF- α -induced apoptosis in fibroblasts and promonocytic cells (Yeung et al. 1996; Der et al. 1997). Thus, to further characterize the PKR activities in the P58^{IPK} and Δ TPR6 mouse fibroblast cell lines, we examined the effect of TNF- α treatment on the viability of these cells. As shown in figure 4.8 *left* series of panels, Vector/Ctl cells treated with 0.2ng/ml TNF- α in the presence of 100ng/ml actinomycin D showed extensive cell death. Under these same conditions, both P58^{IPK}- and Δ TPR6-expressing

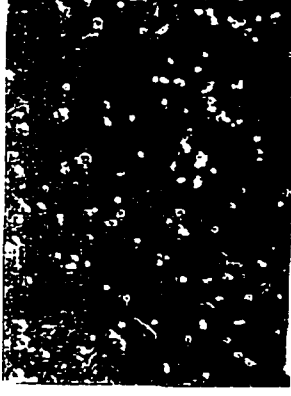
Figure 4.7. Analysis of pIC-induced apoptosis in the P58^{IPK} and Δ TPR6 cell lines. 5 x 10⁵ cells expressing either P58^{IPK}, Δ TPR6 or Vector/Ctl were treated 50ng/ml actinomycin D along with varying concentrations of pIC as indicated for 16 hours. TUNEL assays (Boehringer Mannheim) were performed to quantify the total number of apoptotic cells. Incorporation of fluorescein-conjugated dUTP into DNA strand breaks was analyzed using a FACStar Flow Cytometer (Immunocytometry System, Becton Dickinson). The % *Apoptosis* is defined as the number of apoptotic cells counted divided by 5 x 10⁵ cells times 100.



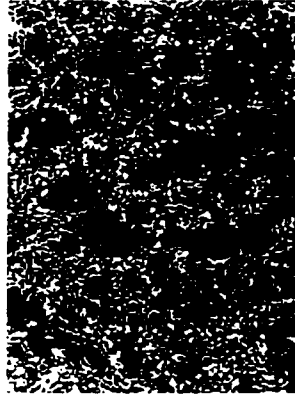
cells resisted TNF- α -induced cell death. Using trypan blue dye exclusion technique, we quantified the Vector/Ctl cells to be less than 20% viable. In contrast, P58^{IPK} cells and Δ TPR6 cells maintained greater than 75% cell viability. Interestingly, when the TNF- α concentration was increased to 1.0ng/ml, Δ TPR6-expressing cells displayed a phenotype intermediate to that of P58^{IPK}- and Vector/Ctl cells (Figure 4.8, *Right*). By trypan blue quantification, Δ TPR6-expressing cells exhibited 44% cell viability, while P58^{IPK} and Vector/Ctl cells exhibited 75% and 17% viability, respectively. The intermediate protection from programmed cell death in the cells expressing Δ TPR6 suggests that TNF- α induces apoptosis via both PKR-dependent and PKR-independent pathways. This idea is consistent with our observation that expression of P58^{IPK} does not completely (only up to 75%) rescue cells from TNF- α -induced death. Moreover, Der and colleagues reported a similar partial protection of ~72% cell viability in Pkr^{0/0} mouse embryo fibroblast cells (Der et al. 1997).

To verify that the expression of P58^{IPK} or Δ TPR6 was suppressing cell death by an apoptotic mechanism, agarose gel electrophoresis was performed on the cellular nucleic acid to look for DNA fragmentation, one of the hallmarks of apoptosis. As shown in Figure 4.9, Vector/Ctl cells displayed extensive DNA fragmentation in the presence of TNF- α (*lanes 4 and 5*). In contrast, P58^{IPK} cells showed no degradation (*lanes 12 and 13*) and Δ TPR6 cells showed little or no fragmentation (*lanes 8 and 9*). These differences were not due to varying sensitivities of the cell lines to actinomycin D drug

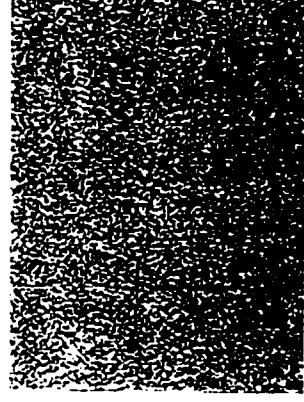
Figure 4.8. P58^{IPK} and Δ TPR6 cell lines are resistant to TNF- α -induced apoptosis. Microscopic examination (x100) of NIH 3T3 cell lines treated with 100 μ g/ml actinomycin D and either 0.2ng/ml (Left) or 1.0ng/ml TNF- α (Right).



Vector/Ctl



ΔTPR6



P58IPK

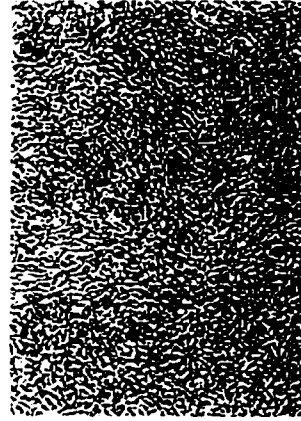
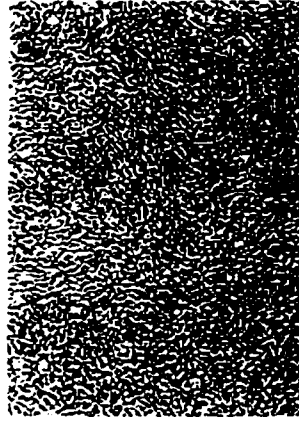
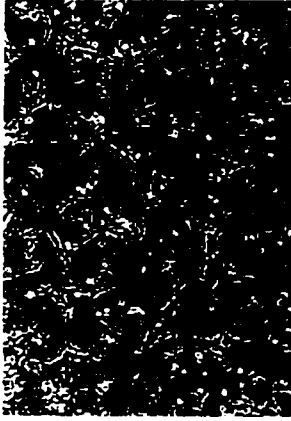
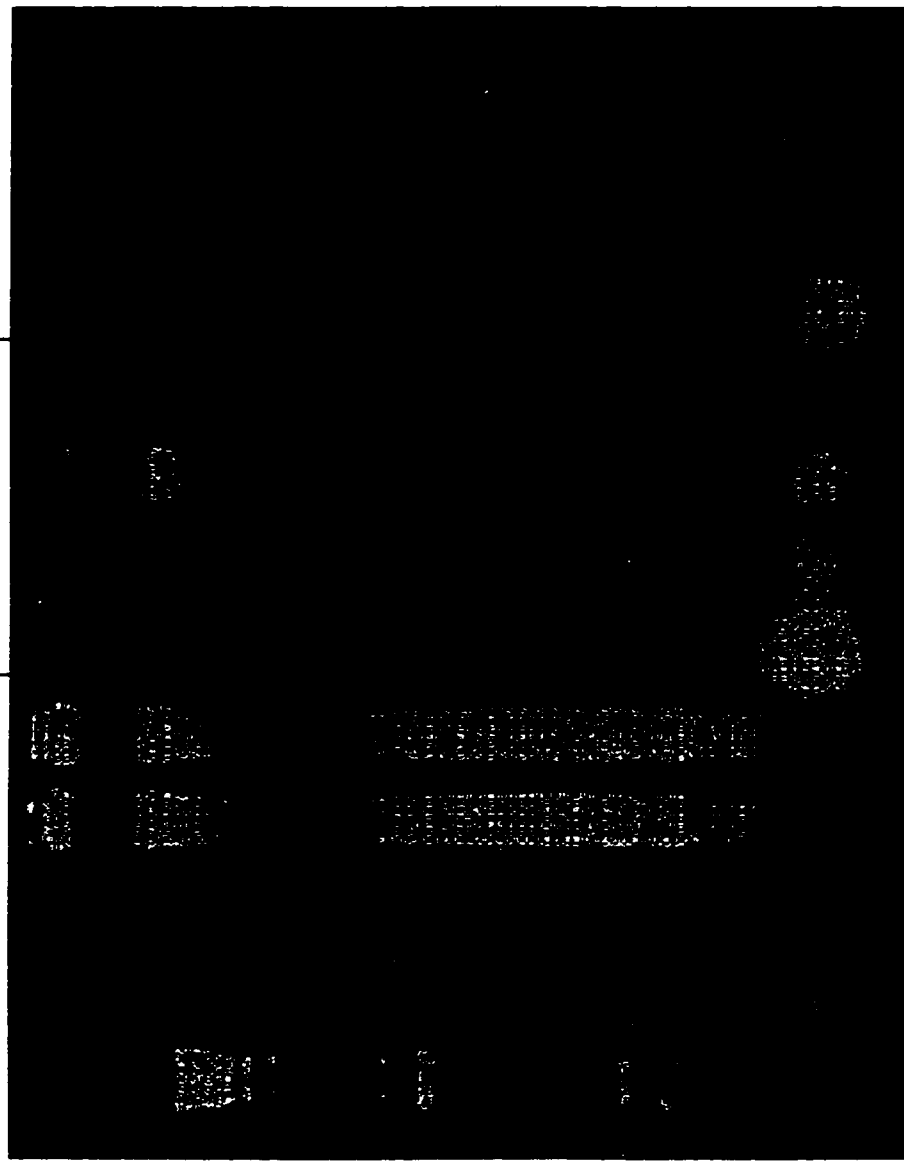


Figure 4.9. Agarose gel electrophoresis to examine DNA laddering in NIH 3T3 cell lines. Agarose gel electrophoresis of low molecular weight DNA isolated from cells treated with varying concentrations of TNF- α in the presence of 50ng/ml actinomycin D. *Lane 1*, DNA molecular weight markers (100 bp DNA ladder; Gibco, Gaithersburg, MD) in 100 base pairs increments ranging in size from 100 base pairs (bottom band) to 2,072 base pairs (upper band).

	Vector/Ctl			ΔTPR6			P58IPK					
	∅	+	+	∅	+	+	∅	+	+	+	+	+
MW	∅	∅	0.1	∅	∅	0.1	∅	∅	0.1	∅	∅	1.0
			1.0			1.0			1.0			TNF-α (ng/ml)



1 2 3 4 5 6 7 8 9 10 11 12 13

treatment since all three of the cell lines, in the absence of TNF- α , maintained full DNA integrity (Figure 4.9, *lanes* 3, 7 & 11). Moreover, in the absence of TNF- α , the three cell lines maintained 100% cell viability (data not shown). Because both P58^{IPK} and Δ TPR6 cells showed activation of NF- κ B in the presence of TNF- α (Figure 4.6, *lanes* 6 and 9), the high cell viability observed for the P58^{IPK} and Δ TPR6 cells did not reflect a lack of response of these cells to TNF- α treatment. The observation that cells expressing Δ TPR6 resist TNF- α -induced apoptosis suggests that P58^{IPK} may induce oncogenesis by suppressing programmed cell death through a PKR-independent mechanism.

Discussion

Inhibition of PKR and oncogenesis

Prior to this study, only one function for P58^{IPK} had been identified: the inhibition of PKR phosphorylation activity. Even though P58^{IPK} had previously been shown to malignantly transform cells (Barber et al. 1994), it was thought that P58^{IPK}-induced oncogenesis occurred through the inhibition of PKR activity. Moreover, it was thought that P58^{IPK} specifically blocked PKR-mediated eIF-2 α phosphorylation, and the reduction in eIF-2 α phosphorylation led to malignant transformation. This hypothesis

was based on observations linking functional inactivations of PKR to eIF-2 α hypophosphorylation and malignant transformation. The results presented in this study, with Δ TPR6, clearly demonstrate that oncogenesis can occur without decreases in eIF-2 α phosphorylation. Such decoupling of tumorigenesis to eIF-2 α hypophosphorylation was seen once earlier with the K296R transdominant PKR mutant (Meurs et al. 1993; Barber et al. 1995). Interestingly, NIH 3T3 cells expressing K296R, although able to form tumors in nude mice, do not display hallmarks of *in vitro* cellular transformation (Donzé et al. 1995). This observation suggests that fulminant neoplastic transformation requires both the suppression of PKR-mediated eIF-2 α phosphorylation and the activation of one or more tumorigenic pathways. Such may be the case also for P58^{IPK}-induced oncogenesis. In this study, we noticed several instances whereby Δ TPR6 displayed reduced oncogenicity relative to P58^{IPK}. First, when compared to P58^{IPK}-expressing cells, Δ TPR6-expressing cells appeared to form smaller and fewer colonies in soft agar (Figure 4.3, *H* and *I*, Table III). Second, at high concentrations of inducer, cells expressing Δ TPR6 appeared less resistant to TNF- α -induced apoptosis than P58^{IPK}-expressing cells (Figures 4.8 and 4.99). Third, mice injected with Δ TPR6 cell lines took twice as long to form tumors than mice injected with P58^{IPK} cell lines (Table III). Although these assays were not meant to be precisely quantitative, these findings do suggest that the inhibition of PKR activity may also contribute to P58^{IPK} tumorigenicity. To more directly address this issue, the expression of P58^{IPK} would need to be examined in a Pkr⁰⁰ background cell.

A PKR-independent mechanism of P58^{IPK}-induced oncogenesis

Although P58^{IPK} can induce oncogenesis via what may be a PKR-independent mechanism, the molecular details of such a PKR-independent pathway are still unknown. Based on the results of this study, one possible mechanism is the constitutive activation of NF- κ B and the subsequent inhibition of apoptosis. NF- κ B was first suggested to have an anti-apoptotic function because mice lacking *Rel A*, the gene that encodes the p65 subunit of NF- κ B, die from extensive apoptosis within the liver (Beg et al. 1995). Since then, several groups have demonstrated that cells lacking functional NF- κ B (as a result of overexpression of a mutant I κ B α super-repressor or homozygous deletion of *RelA*) are more sensitive to apoptotic cell death induced by TNF- α or chemotherapeutic agents (Van Antwerp et al. 1996; Wang et al. 1996; Beg and Baltimore 1996). That the constitutive activation of NF- κ B and the subsequent suppression of apoptosis participates in oncogenesis was recently demonstrated for Ras-induced malignant transformation. In the case of Ras, functional inactivation (through the overexpression of the mutant I κ B α super repressor (Wang et al. 1996; Van Antwerp et al. 1996) of this oncoprotein leads to both the reduction of foci formation and a p53-independent apoptotic cell death (Finco et al. 1997; Mayo et al. 1997). These studies validate the notion that oncogenesis can occur through the activation of NF- κ B and the subsequent suppression of programmed cell death. In our current study, we found that P58^{IPK}, like Ras, also constitutively activated NF- κ B. This P58^{IPK} activity does not

involve the inhibition of PKR since cells deleted for PKR do not show this phenotype. We also found, in this study, that P58^{IPK} suppressed TNF- α -induced apoptosis. Because Δ TPR6-expressing cells also showed a similar phenotype, the suppression of apoptosis by P58^{IPK} may be at least partly PKR-independent. By demonstrating that P58^{IPK} can function independently of PKR to activate NF- κ B and suppress apoptotic cell death, we have identified two novel functions for P58^{IPK}. Moreover, we may have uncovered a PKR-independent mechanism by which P58^{IPK} induces malignant transformation.

Role of DnaJ in cellular transformation

So far, we have suggested two possible mechanisms by which P58^{IPK} could induce malignant transformation: one PKR-dependent; the other, PKR-independent. However, P58^{IPK} tumorigenicity may be even more complex since P58^{IPK} contains homology to the J-domain of bacterial DnaJ protein and such a domain has been shown to mediate oncogenesis. In studies of simian virus 40 (SV-40) transformed cells, it has been shown that the J-domain present in the large T antigen of SV-40 (TAg) is required for virus-induced oncogenesis (Stubdal et al. 1997; Srinivasan et al. 1997; Peden and Pipas 1992). The J-domain of TAg and other polyomavirus large T antigens is also required for binding to and inactivation of the retinoblastoma protein (pRb) (Stubdal et al. 1997; Sheng et al. 1997). Lastly, the J-domain of TAg stimulates the ATPase activity of members of the heat shock 70 (hsp70) family of proteins (Campbell et al. 1997; Srinivasan et al. 1997). These observations have led to the hypothesis that one

mechanism by which SV-40 induces transformation is via the J-domain of TAg which acts to direct the association of hsp70 to the E2F-pRB complex, leading to the release of active E2F transcription factor and the subsequent expression of genes which promote entry of cells into S phase (Sheng et al. 1997). A similar scenario may be envisioned for P58^{IPK} whereby the J-domain present at the carboxyl terminus of P58^{IPK} directs hsp70 to a tumor suppressor protein, leading to the subsequent inactivation of the tumor suppressor protein and the induction of oncogenesis. Such a tumor suppressor protein may be PKR or some other unidentified P58^{IPK}-interacting protein. In support of such a pathway for P58^{IPK}-induced oncogenesis, our lab has evidence showing that P58^{IPK} interacts with and modulates the activity of hsp70 (Melville et al, unpublished). The J-domain at the carboxyl terminus of P58^{IPK} will, likely, play an important role in P58^{IPK} regulation of cell growth since loss of this domain results in loss of P58^{IPK} ability to stimulate protein synthesis activity in mammalian cells (Tang et al. 1996). Moreover, we observed that expression of such a P58^{IPK} mutant in mouse cells was toxic (Tang et al, unpublished). Thus, through its J-domain, P58^{IPK} may interact with the hsp70 molecular chaperone to modulate the activity of proteins which regulate cell viability, growth and tumorigenesis.

TPR motifs and P58^{IPK}-protein interactions

It is also possible that additional domains present in P58^{IPK}, such as TPR motifs, may play a role in tumorigenesis. Spanning the P58^{IPK} molecule are nine tandemly arranged TPR motifs. Each TPR consists of 34 amino acids which form an amphipathic alpha helix that can participate in protein-protein interactions (Lamb et al.

1995). Through these protein-protein interactions, TPR family members have been implicated in a variety of cellular functions, including mitosis, protein transport, RNA synthesis, steroid receptor signaling and neurogenesis (Goebel and Yanagida 1991; Chen et al. 1996). One human TPR protein, called IEF SSP 3521, has been implicated in oncogenesis since it was found to be upregulated in SV-40 transformed fibroblasts (Honore et al. 1992). Another TPR protein, H-NUC, is a human homolog of the yeast *nuc2⁺* protein, which is required for proper mitotic chromosome disjunction (Chen et al. 1995; Hirano et al. 1990). H-NUC has been suggested to play a role in the regulation of cell growth because, via its c-terminal TPR motifs, H-NUC binds the hypophosphorylated form of pRB *in vitro* (Chen et al. 1995). Recently, a protein-serine phosphatase, designated PP5, has been shown to bind hsp90 via its TPR motif to regulate glucocorticoid receptor activation (Chen et al. 1996). In the case of P58^{IPK}, we have found at least two TPR motifs which mediate P58^{IPK} interaction with other cellular proteins. From our previous studies, we found that the sixth TPR motif is the recognition domain for PKR and facilitates inhibition of PKR activity by P58^{IPK} (Tang et al. 1996; Gale, Jr. et al. 1996). Very recently, our lab found that a novel hsp90-like protein, called P52^{rIPK}, interacted with P58^{IPK} and blocked the ability of P58^{IPK} to inhibit PKR phosphorylation activity (Gale, Jr. et al. 1998). The interaction domain for P52^{rIPK} on P58^{IPK} is TPR7 (Gale et al, unpublished). In addition to TPRs 6 and 7, P58^{IPK} contains seven other TPR motifs, each of which could potentially mediate P58^{IPK} interaction with other cellular proteins. Our lab previously demonstrated that the molecular chaperone

hsp40 binds P58^{IPK} and inhibits P58^{IPK} function (Melville et al. 1997). Although the hsp70-interacting region on P58^{IPK} has not yet been identified, we have found that an in-frame deletion of the fifth TPR motif leads to an increase in P58^{IPK} activity (Tang et al. 1996), suggesting that a negative regulator of P58^{IPK}, such as hsp40, may bind to this site. Because members of the heat shock family of proteins have been associated with a variety of disease states (Morimoto et al. 1994) and because expression of hsp70 suppresses heat-induced apoptotic cell death (Mosser et al. 1997), it is, likely, that P58^{IPK} induces neoplastic transformation by modulating the activity of the various chaperone proteins. Such interactions between P58^{IPK} and molecular chaperones could occur via the simultaneous participation of both TPR motifs and the J-domain present in P58^{IPK}. In support of this idea, we previously found that both TPR6 and the J-domain are required for P58^{IPK} protein synthesis activity in mammalian cells (Tang et al. 1996). In addition, both of these domains may be required for full P58^{IPK} tumorigenicity and cell viability (this study). These observations suggest that the coordinate action of the different functional domains of P58^{IPK} may be required to effect complex, global cellular changes, such as malignant transformation.

P58^{IPK}-induced neoplastic transformation is, likely, to be a complex phenomenon in which multiple oncogenic pathways are simultaneously activated through the various functional domains of P58^{IPK}. Some of these pathways may involve molecular chaperones, while others may involve PKR or some other protein yet to be identified. Together, these P58^{IPK}-protein interactions result in changes in protein activity, as well as

altered gene expression at both the transcriptional and translational levels. The molecular details on how P58^{IPK} effect these cellular changes will need to be elucidated. At the present moment, it appears that P58^{IPK}-induced neoplastic transformation encompasses the intersection of stress, interferon, cell death and growth-regulatory pathways.

Chapter 5: Summary And Future Directions

Summary

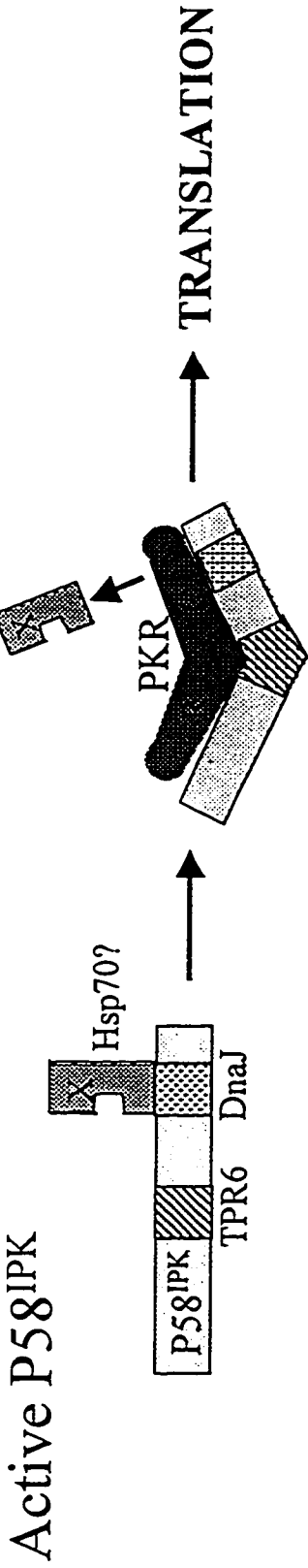
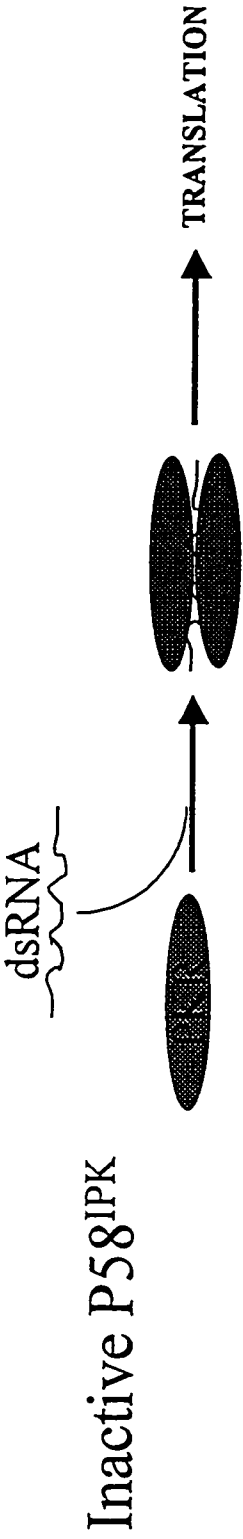
PKR is an interferon-induced, dsRNA-activated serine/threonine kinase that regulates protein synthesis in the cell through the phosphorylation of the initiation factor eIF-2. PKR is best studied for its role in translational control and antiviral defense. In addition to these roles, PKR is implicated in a variety of cellular processes, including growth control and tumor suppression. In the course of studying translational control during influenza virus infection, it was discovered that a cellular protein, P58^{IPK}, was activated during influenza virus infection to block the deleterious effects of PKR activity on viral protein synthesis. P58^{IPK} was subsequently cloned from MDBK cells and shown to inhibit both PKR autophosphorylation and the phosphorylation of exogenous eIF-2 α substrate *in vitro* (Lee et al. 1994). In addition, P58^{IPK} was shown to possess oncogenic properties (Barber et al. 1994). The mechanism by which P58^{IPK} inhibits PKR phosphorylation activity is unknown. Furthermore, the physiologic role of P58^{IPK} is undetermined. The work presented in this thesis was initiated to elucidate the mechanisms by which P58^{IPK} inhibits PKR activity in the cell and induces malignant cellular transformation.

Through mutagenic studies of P58^{IPK}, it was found that the sixth TPR motif

(TPR6) in the middle region of the P58^{IPK} molecule was required for P58^{IPK} inhibition of PKR activity *in vitro*. Furthermore, it was found that P58^{IPK} inhibitory activity *in vitro* correlates with P58^{IPK} binding, i.e. those P58^{IPK} constructs that inhibit kinase activity *in vitro* also bind to PKR. These data demonstrate that P58^{IPK} inhibits PKR through a direct interaction, requiring TPR6. Using *in vivo* approaches, it was shown that increases in P58^{IPK} can stimulate cellular protein synthesis. This *in vivo* function reflects the ability of P58^{IPK} to block PKR-mediated downregulation of protein synthesis and requires, not only TPR6, but also the carboxyl terminus of P58^{IPK} (which includes the DnaJ similarity region). It is hypothesized that the DnaJ similarity region at the COOH terminus interacts with other cellular factors to either 1) target P58^{IPK} to PKR in the cell, or 2) induce proper folding of P58^{IPK} such that it can interact with PKR. The requirement for both the TPR6 and the DnaJ domains in the stimulation of protein synthesis correlates with the same requirements for P58^{IPK}-mediated prevention of PKR dimerization (Tan et al, unpublished). Since PKR dimerization may be required for kinase function (see chapter 1 for details), the findings presented in this thesis suggest the following model for the regulation of PKR activity and mRNA translation by P58^{IPK} in the cell. As depicted in figure 5.1, when P58^{IPK} is inactive in the cell, dsRNA can activate PKR by bridging two kinase monomers to form an active PKR dimer molecule. Active PKR then phosphorylates eIF-2 α to downregulate protein synthesis initiation in the cell. In contrast, when P58^{IPK} is activated in the cell (perhaps through dissociation from its own inhibitor, Hsp40), it then interacts, via its DnaJ domain, with a cellular factor ("X") which

Figure 5.1. Model for the regulation of PKR activity and mRNA translation by the cellular inhibitor P58^{IPK}. (*Upper panel*), When P58^{IPK} is inactive in the cell, dsRNA can activate PKR by bridging two kinase monomers to form an active PKR dimer molecule. Active PKR then phosphorylates eIF-2 α to downregulate protein synthesis initiation in the cell.. (*Lower panel*), In contrast, when P58^{IPK} is activated in the cell, it interacts, via its DnaJ domain, with a cellular factor (“X”) which promotes P58^{IPK} binding to PKR (either through localization or conformational change of P58^{IPK}). Through TPR6, P58^{IPK} binds PKR to prevent kinase dimerization and activation. Because inactive PKR can not phosphorylate eIF-2 α , protein synthesis initiation is not blocked and mRNA translation is not compromised.

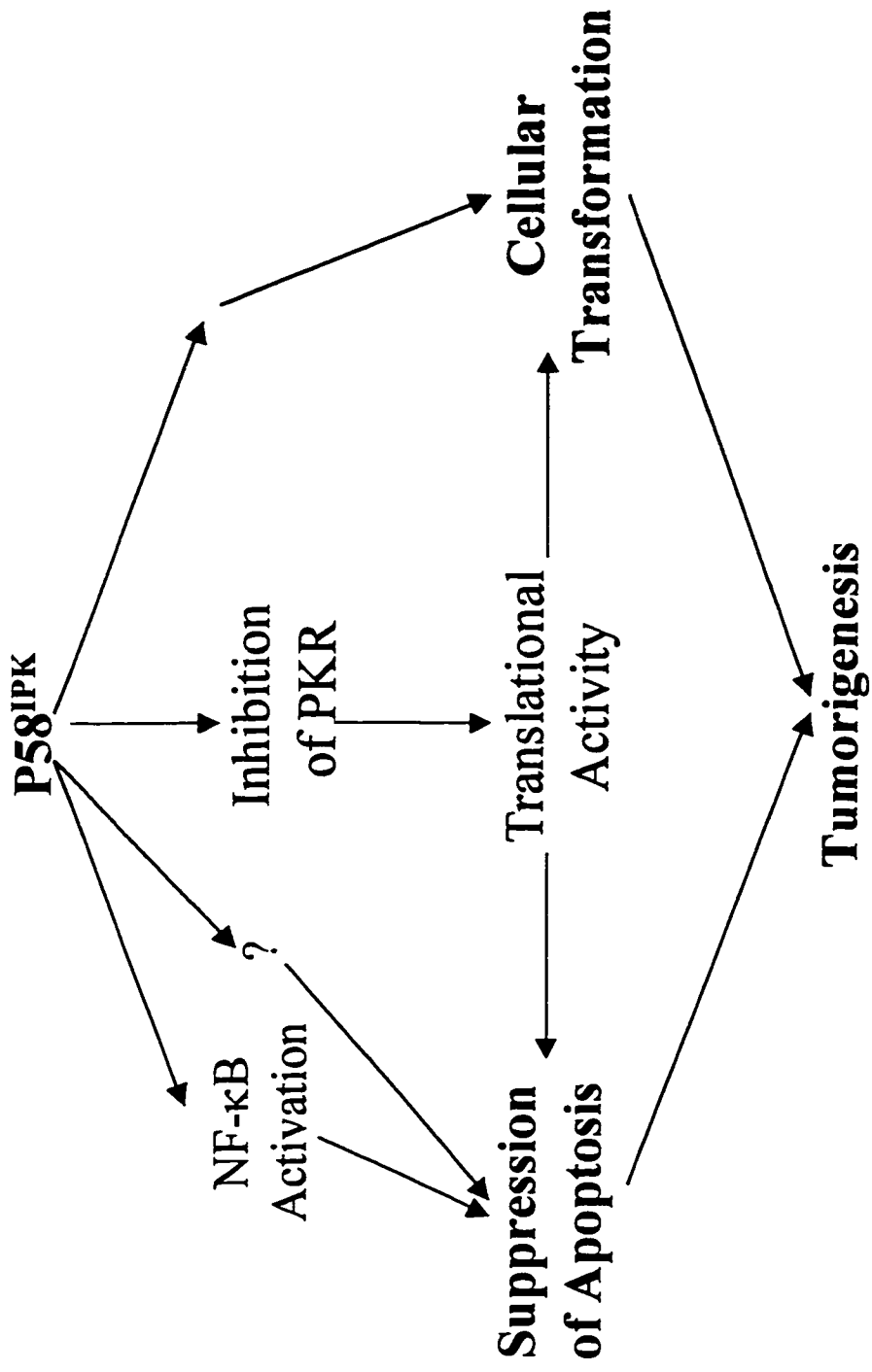
Model For the Regulation of PKR Activity and mRNA Translation By the Cellular Inhibitor P58^{IPK}



promotes P58^{IPK} binding to PKR (either through localization or conformational change of P58^{IPK}). Through TPR6, P58^{IPK} binds PKR to prevent kinase dimerization and activation. Because inactive PKR can not phosphorylate eIF-2 α , protein synthesis initiation is not blocked and mRNA translation is not compromised.

In addition to its role in the regulation of PKR activity and mRNA translation, P58^{IPK} was shown to induce oncogenesis via a mechanism independent of PKR inhibition. P58^{IPK} induction of oncogenesis correlates with the activation of NF- κ B and the suppression of apoptosis. Although PKR inhibition and subsequent decreases in eIF-2 α phosphorylation are not essential for P58^{IPK}-induced oncogenesis, it does appear to facilitate fulminant neoplastic transformation by enhancing both cellular transformation (altered morphology, shorter doubling time, loss of contact inhibition and anchorage-independence) and the suppression of apoptotic cell death. Thus, translational activity may act concordantly with apoptotic suppression and cellular transformation to perturb tissue homeostasis, leading to tumorigenesis. In the model depicted in figure 5.2 for the regulation of cell growth and tumorigenesis by P58^{IPK}, P58^{IPK} is hypothesized to induce oncogenesis via two major pathways. The first pathway (illustrated on the left side of the figure) involves the suppression of apoptosis which may involve both the activation of NF- κ B and an as yet unknown mechanism which also protects cells from the deleterious effects of TNF- α . The inability of the cell to undergo programmed cell death can lead to tumorigenesis due to the abnormal accumulation of cells. The second pathway

Figure 5.2. Model for the regulation of cell growth and tumorigenesis by P58^{IPK}. P58^{IPK} is hypothesized to induce oncogenesis via two distinct pathways. The first pathway (illustrated on the *left*) involves the suppression of apoptosis which may involve both the activation of NF- κ B and an as yet unknown mechanism which also protects cells from the deleterious effects of TNF- α . The inability of the cell to undergo programmed cell death can lead to tumorigenesis due to the abnormal accumulation of cells. The second pathway (illustrated on the *right*) shows P58^{IPK} to induce cellular transformation, resulting in deregulated growth and proliferation. Such aberrant growth and proliferation can also lead to increases in cell number and cancer. Both P58^{IPK}-mediated suppression of apoptosis and cellular transformation is facilitated by the inhibition of PKR activity, which leads to increases in protein synthetic rates (*middle* pathway of figure). Depending on the growth stage of the cell, it is likely that activation of one or the other pathway, or both, can lead to tumorigenesis.



(illustrated on the right side of the figure) shows P58^{IPK} to induce cellular transformation, resulting in deregulated growth and proliferation. Such aberrant growth and proliferation can also lead to increases in cell number and cancer. Both P58^{IPK}-mediated suppression of apoptosis and cellular transformation are facilitated by the inhibition of PKR activity, which leads to increases in protein synthetic rates (middle pathway of figure). Depending on the growth stage of the cell, it is likely that activation of one or the other pathway, or both, can lead to tumorigenesis. The model depicted in figure 5.2 indicates that increases in translational activity alone do not lead to tumorigenesis. This is consistent with the observation that reductions in eIF-2 α phosphorylation do not correlate with neoplastic transformation. The model is also consistent with the findings of numerous studies showing that protein synthesis blockers, such as cycloheximide, increase cell sensitivity to apoptosis. The molecular mechanism by which P58^{IPK} induces cellular transformation and deregulates cell growth is unknown and yet to be identified.

In summary, the findings presented in this thesis demonstrate that P58^{IPK} functions in the cell to regulate protein synthesis through the inhibition of PKR. P58^{IPK}'s ability to inhibit PKR and stimulate mRNA translation requires both the TPR6 domain and the DnaJ domain at the COOH terminus. The mechanism of kinase inhibition by P58^{IPK} is through the direct binding of P58^{IPK} to PKR, which blocks both kinase autophosphorylation and the phosphorylation of substrate. P58^{IPK} also functions in the cell, independent of PKR, to regulate gene expression at the transcriptional level through

the activation of NF- κ B. Both of these functions may contribute to P58^{IPK}'s biologic effects of tumor induction and suppression of apoptosis.

Future Directions

This thesis describes the regulation of PKR by P58^{IPK}. It remains to be determined how P58^{IPK}, itself, is regulated in the cell. In chapter 2, it was mentioned that the P58^{IPK} cDNA sequence contains multiple sites which can be potentially phosphorylated by various protein kinases. Moreover, there is preliminary evidence that P58^{IPK} may be a phosphoprotein. It was found that radiolabeled P58^{IPK} proteins were immunoprecipitated from cell lysates treated with ³²P-ortho-phosphate using a P58^{IPK}-specific monoclonal antibody (Melville and Katze, unpublished). In addition, when purified, histidine-tagged, wild type or mutant P58^{IPK} fusion proteins were incubated *in vitro* with purified, *E. coli*-expressed PKR and [γ -³²P]ATP, both wild type and mutant P58^{IPK} fusion proteins were visualized as radiolabeled proteins by autoradiography (Tang and Katze, unpublished). When this identical *in vitro* experiment was performed using purified, mammalian cell-expressed PKR, no phosphorylation of wild type or mutant P58^{IPK} fusion protein was observed, suggesting that a contaminating protein kinase from the *E. coli* extracts copurified with PKR to phosphorylate P58^{IPK} *in vitro*. It remains to be confirmed, through phosphomapping of tryptic peptides, whether P58^{IPK}, is a

phosphoprotein. If P58^{IPK}, indeed, is a phosphoprotein, the kinase that phosphorylates it will need to be identified.

P58^{IPK} may also be regulated in the cell at the level of protein dimerization. Using the yeast two hybrid assay, it was shown that P58^{IPK} can interact with itself via its amino-terminus. Moreover, P58^{IPK} mutant proteins lacking the amino terminus, such as 9-1, do not interact with PKR in yeast (Gale, Jr. et al. 1996). These *in vivo* results in yeast do not correlate with earlier *in vitro* data showing that the 9-1 mutant can complex with PKR and inhibit kinase phosphorylation activity (Polyak et al. 1996). Although there are obvious differences between the yeast system and the *in vitro* assays, it remains to be clarified whether P58^{IPK} dimerization is required for P58^{IPK} function in the cell. One possible experiment to clarify this issue and show P58^{IPK} homodimerization is to express different forms of P58^{IPK} in mammalian cells and show their heterocomplex formation. For instance, FLAG-epitope-tagged P58^{IPK} and HA-epitope-tagged P58^{IPK} DNA constructs can be transiently transfected into COS-1 cells (or HeLa cells), after which cell lysates are prepared and analyzed by immunoprecipitation with FLAG antibodies and HA antibodies. Coimmunoprecipitation of HA-tagged P58^{IPK} with antibodies to the FLAG epitope, or vice versa (coimmunoprecipitation of FLAG-tagged P58^{IPK} with antibodies to HA), would suggest P58^{IPK} homodimerization *in vivo*. Another possible experiment is to correlate homo and heterodimerization of P58^{IPK} mutant proteins with their functional activities *in vivo*, such as the stimulation of reporter gene protein synthesis, NF- κ B

activation, induction of oncogenesis and suppression of TNF- α -induced apoptosis.

Another question related to P58^{IPK} homodimerization is whether inactive mutants of P58^{IPK}, can heterodimerize with wild type P58^{IPK} to functionally inactivate P58^{IPK} in the cell. In preliminary experiments using an *in vitro* kinase assay, it was observed that when purified GST-P58^{IPK} was added to purified PKR, there was a slight inhibition of PKR's ability to both autophosphorylate and phosphorylate histone substrate (Tang and Katze, unpublished). This inhibition was relieved, in a dose-dependent manner, when purified GST- Δ TPR6 was preincubated with purified GST-P58^{IPK} prior to the addition of purified kinase. Thus, Δ TPR6 may act in a transdominant manner to inhibit the ability of P58^{IPK} to regulate PKR activity. Because expression of the 8-3 mutant in NIH 3T3 cells leads to toxicity (chapter 4), it may turn out that 8-3 acts in a transdominant manner to block P58^{IPK}-mediated cell growth and proliferation. It will be interesting to determine whether Δ TPR6 and 8-3 are, indeed, dominant negative mutants. If they are, these mutants can serve as valuable reagents in the dissection of molecular pathways to cellular oncogenesis. Furthermore, these mutants can serve to further elucidate the roles of P58^{IPK} and PKR in both influenza virus-infected and uninfected cells. Earlier in this thesis, it was alluded to that P58^{IPK} interacts with a cellular factor, protein "X", which promotes P58^{IPK} interaction with PKR, either through localization of P58^{IPK} or through proper P58^{IPK} protein folding. To further understand the regulation of P58^{IPK}, it would be interesting to find protein "X" using coprecipitation studies or the

yeast two hybrid assay. These approaches may also detect additional proteins which interact with P58^{IPK} to regulate its various cellular activities.

On a broader level, it remains to be determined whether the activity of P58^{IPK} promotes influenza virus replication in the host cell. Since influenza virus infects mainly human cells, HeLa cell lines can be created which overexpress P58^{IPK}. These cell lines could then be used to test whether overexpression of P58^{IPK} can rescue adenovirus *d1331* mutant virus replication. Assuming that Δ TPR6 is a transdominant mutant for inhibition of PKR activity, another test for the importance of P58^{IPK} in influenza virus infection is to assay for infectious virus in HeLa cell lines stably expressing Δ TPR6.

These are just a few examples of studies which can be performed to further elucidate the P58^{IPK} pathway. Obviously, other experimental approaches can be taken to address these same questions, as well as many other questions remain unanswered regarding P58^{IPK}, PKR and the P58^{IPK}-PKR regulatory pathway. Although the above experiments will elucidate P58^{IPK} functions and mechanisms in the cell, definitive proof of its physiologic role(s) will need to come from examinations of mice bearing gene knockouts of P58^{IPK}. Bearing in mind that the creation of gene knockout mice requires considerable time and expense, alternative reagents/tools could be generated and used to more precisely examine P58^{IPK} function in the cell. For example, cell lines expressing P58^{IPK} under an inducible promoter could be created to determine whether phenotypes or functions previously attributed to P58^{IPK} is actually specific to P58^{IPK}. In addition, these

cell lines could be valuable in allowing variable and incremental expression of P58^{IPK}. Thus, one may use these cell lines to correlate certain biologic and/or cellular effects to the degree or level of P58^{IPK} expression in the cell. An alternative to the inducible promoter is the use of the Lox/Cre system (Fukushige and Sauer 1992; Bergemann et al. 1995; Choulika et al. 1996; Bethke and Sauer 1997). In the Lox/Cre system, P58^{IPK} is cloned into a retrovirus vector containing one Lox site in the retroviral 3'-LTR (Long Terminal Repeat). Upon retroviral transduction of mammalian cells, the retrovirus vector is incorporated into the host genome generating a provirus sequence with two copies of the LOX site: one in the 5'-LTR and one in the 3'-LTR, and each flanking the P58^{IPK} transgene. The value of this system is that the P58^{IPK} phenotypes that were observed in these stable cell lines can be confirmed through the subsequent transduction of a second retroviral vector which expresses the Cre protein. Cre is a recombinase which recognizes and binds specifically to the two LOX sites present in the viral LTRs, causing recombination and excision of the cloned P58^{IPK} gene. This recombination event is over 95% efficient. Thus, a reversal of a specific P58^{IPK}-induced phenotype can be achieved.

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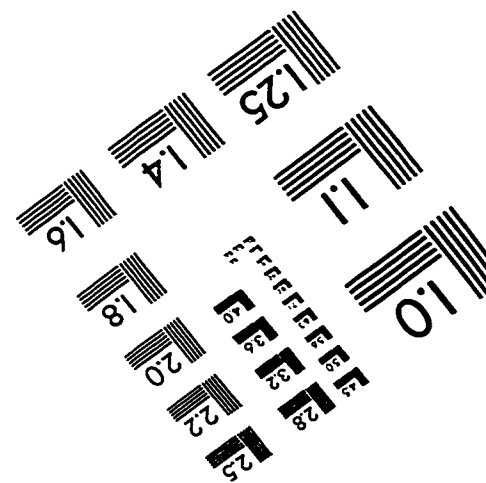
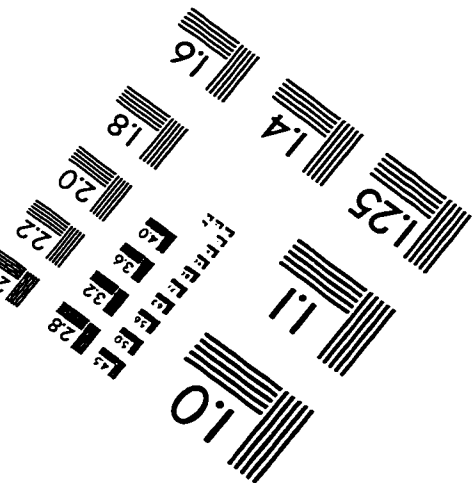
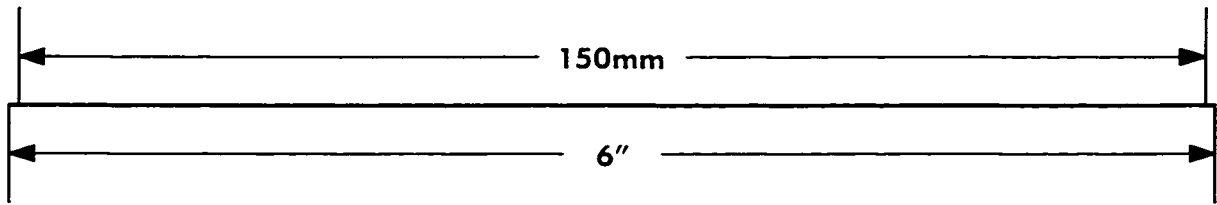
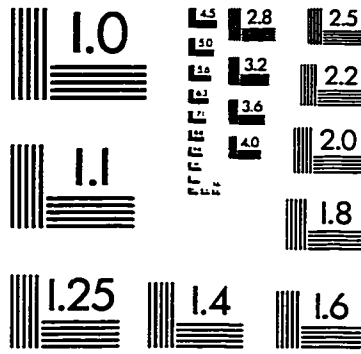
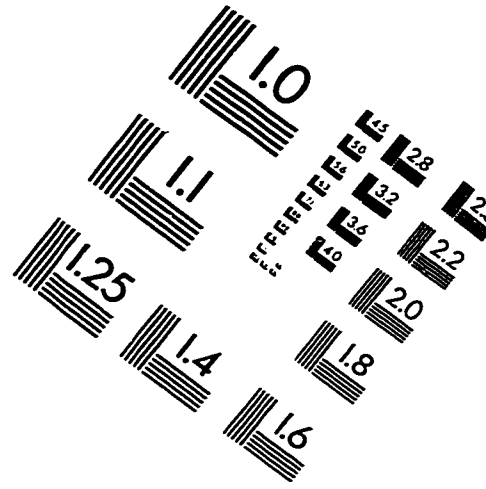
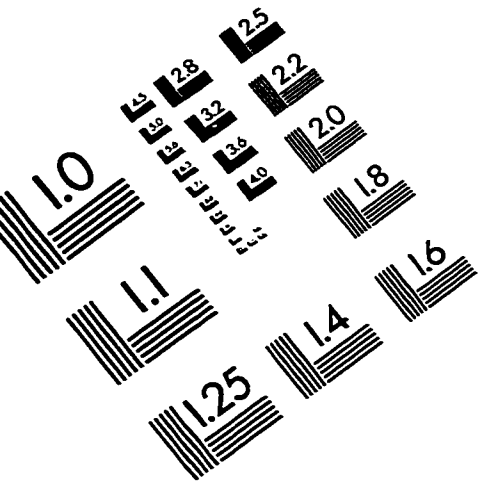
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IMAGE EVALUATION TEST TARGET (QA-3)



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