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Consequences of loss of the murine dsRNA-dependent protein kinase, PKR, by natural mutation or genetic ablation.

Ninan Abraham

Thesis submitted to the Department of Biochemistry in partial fulfilment of the requirements for the degree of Doctor of Philosophy

University of Ottawa

Ottawa. Ontario. Canada

ABSTRACT

The IFN-induced, dsRNA activated protein kinase PKR, is a member of the family of eIF-2α protein kinases that has well defined roles in anti-viral, anti-proliferative and apoptosis responses. Using a strategy designed to identify phosphotyrosine protein kinases, we have identified PKR as a member of the family of dual specificity kinases since it phosphorylates residues on serine, threonine and autophosphorylates on tyrosine residues. Although this activity has not yet been identified in mammalian expression of PKR, the significance of this novel property raises the possibility of new signaling pathways impinged by PKR.

This work examined the consequences of mutation of PKR, initially examining a mutation identified in a lymphocytic leukemia cell. The putative $mPKR^{del}$ polypeptide encoded by this mutation was cloned and tested in expression studies. Although $mPKR^{del}$ was shown to be catalytically inactive and to bind dsRNA and endogenous PKR, it nonetheless did not demonstrate transdominant inhibition of PKR or alter cell growth when expressed. Instead, no evidence of expression of the mutant polypeptide could be found in the lymphocytic leukemia cell and it is believed the mutation is either silent or exerts an effect not evident in our assays.

A more direct approach to the implications of loss of PKR function was taken using targeted disruption of PKR in the mouse by homologous recombination. Functional ablation of PKR was accomplished by targeting the catalytic domain, in contrast to others who had disrupted PKR at the amino-terminal end. The absence of elevated tumorigenesis, normal anti-vaccinia and influenza responses, intact apoptotic induction and unimpaired cytokine

signaling was unexpected. Although possible that a subtle phenotype has been missed, the major finding of this work is that non-concordance in phenotype compared to the other PKR-null model suggests that loss of PKR can be compensated by previously unappreciated homologs. The unambiguous inactivation of PKR catalytic function has allowed us to determine that rescue of PKR ablation is occurring since phosphorylation of eIF-2α is unaffected. Finally, PKR ablation appears to impair Epo signaling in erythroid CFU suggesting non-redundancy in these cells.

For Carole

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PREFACE

The work presented in this thesis was modified from manuscripts submitted for publication as described below:

Chapter 2

Ninan Abraham, Maria L. Jaramillo, Peter I. Duncan, Nathalie Méthot, Pamela L. Icely, David F. Stojdl, Glen N. Barber, John C. Bell.
The murine PKR tumor suppressor gene is rearranged in a lymphocytic leukemia.
Experimental Cell Research (in press).

Chapter 3

Ninan Abraham, Nathalie Méthot, Andrew R. Cuddihy, David F. Stojdl, Peter I. Duncan, Manon Dubé, Barbara C. Vanderhyden, Harold L. Atkins, Douglas A. Gray, Michael W. McBurney, Antonis E. Koromilas, Earl G. Brown, Nahum Sonenberg, John C. Bell.
Characterization of transgenic mice with targeted disruption of the catalytic domain of PKR.
Manuscript in preparation.

The following people have contributed to the experimental work presented in this thesis:
Nathalie Méthot and Pam Icely isolated the $mPKR^{del}$ cDNA clone. Dr. Maria Jaramillo performed the Northern blot analysis of $mPKR^{del}$ expression. Dr. Douglas A. Gray (Department of Medicine, University of Ottawa) provided the D3 genomic library. Nathalie Méthot assembled the targeting vector. Southern and PCR screening were performed by Simon Ginsberg and Scott Kelso. RT-PCR analysis was performed by David Stojdl. Influenza LD_{50} determination of $PKR^{0/0}$ animals was determined by Dr. Earl G. Brown (Department of Microbiology and Immunology, University of Ottawa). Vaccinia growth curve was done with the technical assistance of Heidi Gruber. ES cell microinjection was performed by Manon Dubé and Dr. Michel Tremblay (Department of Biochemistry, McGill University). eIF-2α phosphorylation was assayed by Andrew R. Cuddihy.

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LIST OF ABBREVIATIONS

bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
dsRBD	Double stranded RNA binding domain
dsRNA	Double stranded ribonucleic acid
DSKs	Dual specificity kinases
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF-2α	Eukaryotic initiation factor-2 alpha subunit
eIF-2B	Eukaryotic initiation factor-2B; guanine nucleotide exchange factor
EMCV	Encephalomyocarditis virus
Epo	Erythropoeitin
ES	Embryonal stem cell
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorter
FIAU	1-(2'-deoxy-2'-fluoro-1-beta-D-arabinofuranosyl)-5-iodouracil
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehyde phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GTP	Guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	Human immunodeficiency virus
hPKR	Human PKR
HRI	Heme regulated inhibitor
HSV	Herpes simplex virus
HSV TK	HSV thymidine kinase
Hyg/TK	Hygromycin phosphotransferase-thymidine kinase fusion
IFN	Interferon
IL-3	Interleukin 3
IRES	Internal ribosome entry site
ISGs	IFN stimulated genes
ISREs	IFN stimulated response element
JAK	Janus kinase
kb	Kilobase
kDa	Kilodalton
KL	Kit ligand
K296R	Lys 296 to Arg mutation
LD_{50}	Lethal dose for 50% of infected animals
LPS	Lipopolysaccharide
MAP	Multiple antigen peptide
MEFs	Mouse embryo fibroblasts

MOI	Multiplicity of infection
mPKR	Murine PKR
$mPKR^{del}$	Predicted mutant mPKR with in frame deletion from L1210 cells
mRNA	Messenger RNA
NDV	Newcastle disease virus
2' 5' OAS	2' 5' oligoadenylate synthetase
ORF	Open reading frame
PARP	Poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
pfu	Plaque forming units
pIC	poly (I) poly (C) synthetic dsRNA
PKI	Inhibitor of PKR in plants: p67 homolog
PKR	DsRNA activated protein kinase
PMSF	Phenylmethylsulfonyl fluoride
pPKR	Plant PKR
PSKs	Protein serine/threonine kinases
PYKs	Protein tyrosine kinases
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src homology 2 domain
STAT	Signal transducers and activators of transcription
TAR	Tat transactivation region
Tik	Anti-phosphotyrosine immunoreactive kinase: mPKR
TNFα	Tumor necrosis factor alpha
TPR	Tetratricopeptide repeat motif
TRBP	TAR binding protein
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
uORFs	Upstream open reading frames
UTR	Untranslated region
VSV	Vesicular stomatitis virus
X	Any amino acid

CHAPTER 1

GENERAL INTRODUCTION

Progress in the understanding of human diseases such as cancer has benefitted from the understanding of the molecular processes of cellular control. Landmarks in the field of cancer research include the recognition that certain tumor causing genes in viruses were of cellular origin (proto-oncogenes) (1-3), that loss of certain other genes (the tumor suppressors, see (4)) in two steps (5-7) could contribute to tumorigenesis ("two-hit" model) and that genetic alterations of multiple targets in cell control pathways was necessary in tumor progression (8). We now understand that cellular control is effected by complex pathways that convey environmental or hormonal cues from the outside of the cell, through the plasma membrane, across the cytoplasm and into the nucleus. Gain of function or loss of function mutations in different molecules in these pathways can result in uncontrolled cell growth. These strides in understanding the molecular basis of cell control provide some means of addressing the issues of deregulated control that results in tumor progression.

Protein kinases and dual-specificity kinases.

Protein kinases are key molecules in regulating signaling pathways by their ability to modulate the activity of specific substrates by the post-translational modification of phosphorylation (9). The most commonly phosphorylated hydroxy amino acid residues are that of serine, threonine and tyrosine, although others have been described (10,11). Two families of protein kinases have conventionally been defined by hydroxy amino acid preference; the protein serine/threonine kinases (PSKs) and the protein tyrosine kinases (PYKs) (12). Although target amino acid preference was previously thought to be predictable by the amino acid sequence of a protein kinase, recent findings have shown that

a third class of protein kinases that bear sequence similarity to the PSKs are however, capable of phosphorylating both serine/threonine and tyrosine residues and therefore, referred to as Dual Specificity Kinases (DSKs) (13,14). In addition, a novel class of dual specificity protein phosphatases has been characterized (15,16). DSKs may, in theory, provide the catalytic function that might otherwise require two different protein kinases of the PSK or PYK families. The finding that DSKs are found in both Metazoan and yeast pathways suggests functional relevance for this new class of protein kinases.

The integration of protein kinases in control of signaling pathways makes apparent the reason for the large number of oncoproteins that are protein kinases. Ten years ago, it was estimated that as many as half the known oncogene products were protein kinases (9,17). Elucidation of some of the molecules involved in the MAPK [Mitogen-Activated Protein Kinase or Extracellular-Regulated Kinase (ERK)] pathway (reviewed in (18-20)), cell cycle control (21,22) and the JAK/STAT (Janus kinase and Signal Transducer and Activators of Transcription) pathways (23,24) illustrate the versatility of protein kinases (and dual specificity kinases) in cellular control. For example, the epidermal growth factor (EGF) receptor is a transmembrane receptor protein tyrosine kinase that activates the MAPK pathway (25,26) by phosphotyrosine-SH2 (Src Homology 2) domain mediated recruitment of signaling partners (27,28). The oncogenic v-erbB and neu variants of EGFR and the related HER2 receptor respectively, bear alterations that activate their protein kinase activity and impinge upon downstream pathways (29,30). In the MAPK pathway, both MEK (MAPK/ERK kinase, MEK-1 and MEK-2) and MAPK (ERK-1 and ERK-2) have DSK activity (31,32). The physiological relevance of this function is best shown in MEK

activation of MAPK by phosphorylation on tyrosine and threonine residues (32,33) which is conserved in yeast, Xenopus and mammals (13,20). The cyclin-dependent kinase cdc2 which is important in cell cycle control is also regulated by tyrosine/threonine phosphorylation. In Schizosaccharomyces pombe, the Wee1 kinase which phosphorylates cdc2 is a DSK, which in turn is regulated by the DSK Nim1 (reviewed in (13)). Evidence that deregulated control of a member of the MAPK pathway may lead to tumorigenesis is seen in the v-raf variant of Raf, a PSK (34,35), as well as oncogenic variants of Ras (see (36) for review). The importance of phosphorylation in cell cycle regulation for normal growth control is seen in the control of the pRb tumor suppressor protein by cyclin dependent kinases (cdks) whereby loss or mutation of the Rb gene causes susceptibility to retinoblastoma in humans (37,38). Finally, the cytokine/JAK pathway is one where direct activation of the STAT proteins by tyrosine phosphorylation by the JAK kinases results in translocation of the STATs to the nucleus, elevation of transcription by these transcription factors (reviewed in (24)) and cellular proliferation. Indeed, a gain of function mutation in Drosophila JAK causes tumors and fly leukemia (39,40).

Work in our laboratory using a screening strategy for PYKs resulted in the cloning of a few protein kinases that have differing degrees of DSK properties (13,14,41-43). One of these, the PKR kinase, had been identified previously as a PSK which is interferon (IFN)-induced and double stranded RNA (dsRNA) dependent with a role in translation control and anti-viral responses. The work in this thesis begins by demonstrating that the protein kinase that others in our laboratory had cloned, Tik (42), was indeed murine PKR. It then describes

my investigation into the role of this protein kinase in cell growth control and anti-viral responses and the consequences of disruption of PKR function.

PKR and nomenclature.

The IFN-induced, dsRNA activated protein kinase PKR has historically borne many names including DAI, dsI, P1 kinase, p68 kinase and Tik, as our laboratory had called it initially. The consensus designation of PKR was selected to indicate the dsRNA dependence of this protein kinase (44). Its ability to phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF-2α) makes it a member of the family of eIF-2α protein kinases (see below).

PKR was first detected as the inhibitor that blocked reticulocyte translation extracts in the presence of low levels of dsRNA and was known as DAI or dsI for dsRNA (activated) inhibitor (45). This inhibition is characterized by biphasic kinetics (46), polysome disaggregation (46), depletion of the 40S ribosomal subunit-Met-tRNA$_i$ complex (47), potentiation by ATP (48,49), reversal by exogenous eukaryotic initiation factor 2 (eIF-2) (50) and activation of a cAMP-independent protein kinase activity that phosphorylates the alpha subunit of eIF-2 (38 kDa) and a 68 kDa polypeptide (49). This latter phosphoprotein was shown to be PKR that undergoes autophosphorylation during activation and which was initially called P1 kinase or p68 kinase by various groups. Phosphoamino acid analysis of PKR had shown it was phosphorylated on serine and threonine residues (42,49,51-54) and therefore, apparently a PSK. Furthermore, when cloned, the amino acid sequence of PKR matched the consensus of PSKs (12,55). As had been previously shown (42) and further

expanded upon later in this thesis (Chapter 2), we had cloned murine PKR (mPKR) using an anti-phosphotyrosine screening strategy and initially named it Tik.

Aside from dsRNA, PKR is activated by polyanionic macromolecules such as heparin, dextran sulfate and chondroitin sulfate (56) but not, however, by tRNA, DNA or DNA/RNA hybrids (56,57). dsRNA activation of PKR is sequence independent and may involve cellular RNA, viral RNA or RNA intermediates or single stranded RNA (ssRNA) with suitable secondary structure (58,59). Low levels (0.1-10 μg/ml) of dsRNA activate PKR whereas higher doses are inhibitory (60,61). Other highly structured viral RNA molecules such as adenovirus VAI RNA act as PKR inhibitors (62) (see below). The IFN inducibility of this kinase was described later when it was observed that IFN treated cells show an enhanced level of dsRNA activated protein kinase activity (51,63-66) and subsequently verified by analysis of the hPKR and mPKR promoters following the genomic cloning of these genes (67) (see below). Most mammalian cells express a low constitutive level of this protein kinase in a latent state which is induced transcriptionally by IFN. PKR is located in cytosolic and ribosome associated fractions (49,68,69) with a small fraction of unknown function reported in the nucleus in proximity to nucleoli (70). Specific targeting to the ribosome (68) has been shown to be mediated by the two RNA binding motifs in the amino terminal region (see below). PKR is also ATP and Mn^{2+} dependent (49,54). The best known substrate of PKR is eIF-2α, which is phosphorylated on residue Ser 51 (71,72) by PKR as well as the other eIF-2α protein kinases, HRI and yeast GCN2. This phosphorylation causes sequestration of the guanine nucleotide exchange factor eIF-2B which inhibits translation initiation (73,74) (discussed later). Other substrates recently

described include IκB. the inhibitor of NFκB (75) and human immunodeficiency virus (HIV)-1 Tat protein (76).

The family of eIF-2α protein kinases:

Human PKR (hPKR) and murine PKR (mPKR).

The human PKR gene was cloned by Meurs et al (55) using an immunological screen of an expression library with a high specificity polyclonal antibody against hPKR. followed by others using polymerase chain reaction (PCR) approaches (77). Most biochemical and molecular characterization of PKR involves human PKR. hPKR is a 551 amino acid polypeptide of 68 kDa apparent molecular weight. The murine PKR gene was cloned more recently by ourselves (42) using an anti-phosphotyrosine immunoscreen and by others (78-80) using low stringency hybridization screens with hPKR, PCR cloning or genomic cloning strategies respectively. mPKR is a 515 amino acid polypeptide of 65 kDa apparent molecular weight. Finally, cloning of rat PKR has been reported (81) using degenerate oligonucleotide primers in PCR cloning. hPKR and mPKR share 62% identity and 75% similarity at the amino acid level. Rat PKR has 76% identity with mPKR and 61% identity with hPKR (81). Comparisons of these three mammalian PKR genes with each other and with other protein kinases and RNA binding proteins allowed identification of conserved motifs in PKR (discussed below) subsequently verified by mutagenesis analysis. Rat and murine PKR also share high conservation (75%) of 5' and 3' UTR regions of its transcript (81) suggesting possible involvement in autoregulation of its translation as has been reported by others (82,83).

Figure 1 depicts the structural features of PKR. The general organization of PKR consists of an amino terminal regulatory region which is more variable in comparison with other protein kinases and a carboxy terminal catalytic region that has the 11 consensus subdomains (I to XI) of protein kinases (12). The regulatory region includes two nearly identical dsRNA binding domains (dsRBD I and II) (84) in the amino terminal end of the polypeptide which are highly conserved in human, mouse and rat (85-88). The specificity of the dsRBD for dsRNA is defined by the molecular recognition of 2'-OH of both strands of RNA along the entire length of the RNA molecule (57). dsRNA activation of PKR is sequence independent and may be activated by RNA duplex of 55 bp or larger but is optimally activated by duplexes of 85 bp or more (89). The minimal duplex that may bind PKR is 28 bp which at high doses may prevent activation of PKR by larger duplexes. RNAse III digestion indicates that together, the RNA binding sites protect about 80 bp of duplex (89). Consistent with dsRNA binding protein recognition of A-form RNA helices (90) which preclude sequence specific recognition (91), footprinting experiments with hPKR indicate protein-dependent protection of the minor groove of dsRNA (57). Interestingly, based on observation of certain dsRNA-binding mutants that are still activated by heparin, it has been suggested that heparin activation of PKR occurs at a different site (92). The 21 residue minimal consensus region of the PKR dsRBD is one which is rich in basic amino acids and is predicted to encode an α-helical structure (85,87):

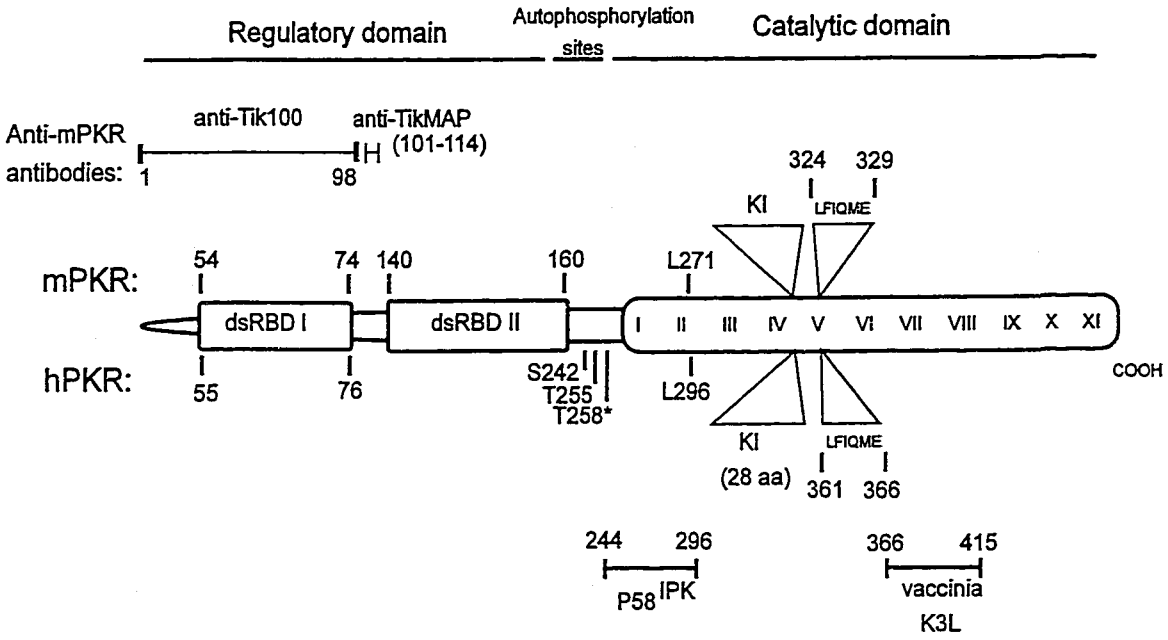
G/A X G* XX K* Q/K E A K/R N/Q X A A K X A V/Y X Q I/K L

This consensus is similar but not identical to that for other dsRBDs from other proteins such as vaccinia E3L, human TAR binding protein, Xenopus RNA-binding protein, Drosophila

Figure 1. Schematic representation of mPKR and hPKR and features of importance.

PKR is drawn showing the dsRNA binding domains (dsRBDs), autophosphorylation sites and catalytic domain. Residues in the 65 kDa mPKR are indicated above the drawing and residues in the 68 kDa hPKR are shown below. Of the three sites marked, only the Thr258 autophosphorylation site (asterixed) is conserved in both hPKR and mPKR. The subdomain II conserved Lys (271 and 296) are marked. The six amino acid motif (LFIQME) required for PKR activation is indicated. Regions in mPKR against which antibodies were raised are shown above the drawing. hPKR sites mapped for $P58^{IPK}$ and vaccinia E3L interaction are indicated below the drawing. Not drawn to scale.

Figure 1


Regulatory domain
Autophosphorylation sites
Catalytic domain
Anti-mPKR antibodies:
anti-Tik100
anti-TikMAP (101-114)
1
98
H
mPKR:
hPKR:
54
74
140
160
L271
324
329
KI
LFIQME
dsRBD I
dsRBD II
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
COOH
55
76
S242
T255
T258*
L296
KI
(28 aa)
LFIQME
361
366
244
296
P58IPK
366
415
vaccinia
K3L

staufen and E. coli RNAse III (85,87). The two asterisked residues Gly 57 and Lys 60 in hPKR are absolutely essential for dsRNA binding (87). These domains have been confirmed by deletion and mutagenesis analysis (78,85,93-95). Studies also show that dsRBD I which has a better match with the dsRBD consensus is more important in binding dsRNA (78,87). Duplication of dsRBD II does not compensate for deletion of dsRBD I although normal RNA binding in PKR with dsRBD I and II swapped in position indicate they cooperate to form the dsRNA binding site (85). This is further supported by the tolerance for only small changes in the spacing between the two motifs. The two dsRBDs are also non-equivalent since point mutations in dsRBD I were more deleterious than in dsRBD II and since duplication of dsRBD I does compensate for deletion of dsRBD II, unlike the converse (78,85). Point mutations in the dsRBDs affect dsRNA and adenovirus VAI RNA binding in a similar fashion indicating inhibitory and activating RNAs bind the same site (85). In mPKR, the two domains are defined to range from residues 54 to 74 and 140 to 160 (87).

In between the dsRNA binding domains and the catalytic domain is a third basic region which bears the sites of autophosphorylation in hPKR. Thr258, Ser242 and Thr255 which are required for hPKR function (96) and inhibition of protein synthesis. Only Thr258 is conserved in mPKR as well. Within the catalytic domain, Lys271 and Lys296 in mPKR and hPKR respectively were predicted as the highly conserved lysine residue in subdomain II required for phosphotransfer reactions in protein kinases. This was demonstrated in mPKR by mutagenesis by our laboratory (see Chapter 2). A 28 amino acid stretch (hPKR residues 323-357) between subdomains IV and V called the kinase insert region which is unique to eIF-2α protein kinases, and specifically Ser 355 was shown to be required for

functional hPKR (97). Subdomain V is a region within the catalytic domain that bears 6 amino acid residues (361- 366; LFIQME) shown to be highly conserved in hPKR, mPKR, HRI and GCN2, thought to interact with their eIF-2α substrate and deletion of which renders hPKR inactive (98). When discussing other regulators of PKR function, other sites of importance on PKR will be noted later (see below). The mode of activation of PKR will also be discussed later in further detail.

Finally, the two rabbit polyclonal antibodies raised in our laboratory against mPKR are directed against His-tagged residues 1-98 (known as anti-Tik100 antiserum) and residues 101-114 on a multiple antigen peptide (MAP) (99) (known as anti-TikMAP antiserum) (Fig. 1).

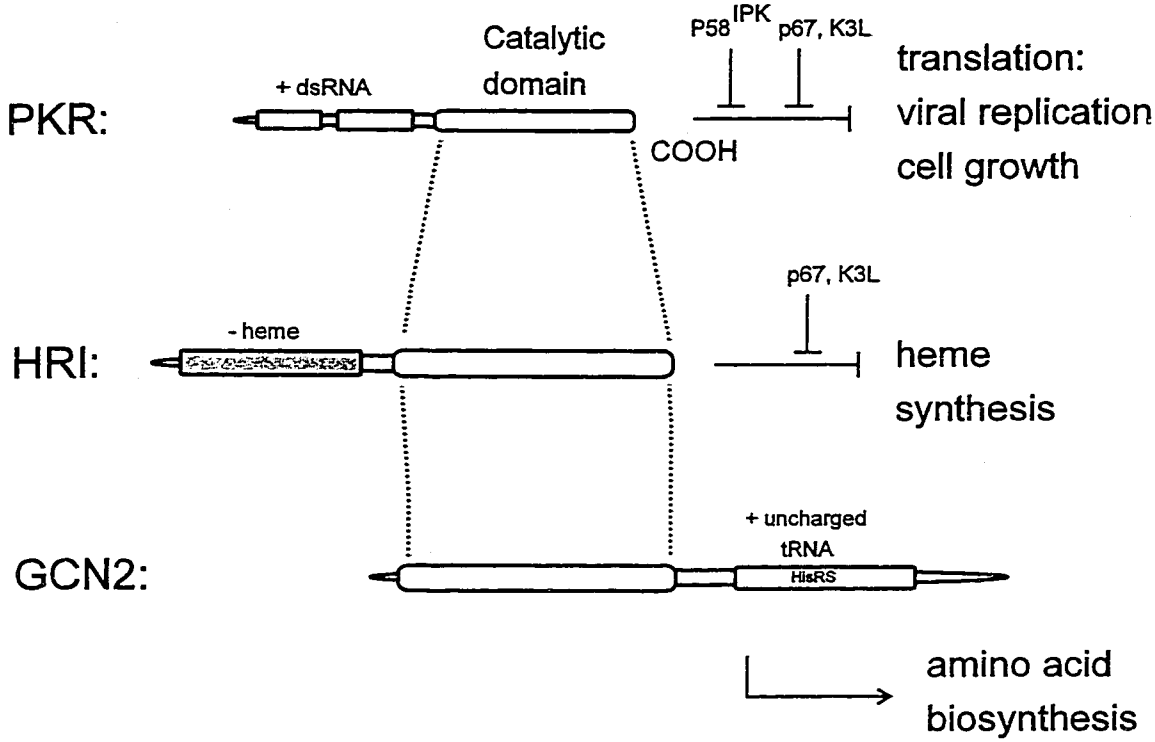
Heme-Regulated Inhibitor (HRI).

At the time of the initiation of this thesis, the only other known mammalian eIF-2α kinase was the Heme-Regulated Inhibitor (HRI) or Heme controlled repressor (HCR). Protein synthesis in reticulocytes or reticulocyte lysates is dependent upon the availability of heme (see (100) for review). Under conditions of heme deficiency, protein (namely, globin) synthesis is inhibited at the level of initiation by activation of HRI, which is reversed in the presence of heme (101,102) (see Fig. 2). HRI and PKR share several characteristics of the mechanism of inhibition including biphasic kinetics (103), polysome disaggregation (104), depletion of the 40S ribosomal subunit-Met-tRNA$_i$ complex (105), potentiation by ATP (48,49), reversal by exogenous eukaryotic initiation factor 2 (eIF-2) (50,106) and activation of a cAMP-independent protein kinase activity that autophosphorylates and phosphorylates the alpha subunit of eIF-2 (38 kDa) (49,107-109). Contrary to PKR where

Figure 2. Known eIF-2α protein kinases and their functional roles.

PKR, HRI and yeast GCN2 are depicted schematically. Highest homology is shared in the catalytic domain with variable regulatory domains that are regulated by different cues indicated. Unlike the other two members, HRI is activated by the absence of regulator, such that heme synthesis is prevented when heme is low. dsRNA activates PKR while uncharged tRNA during amino acid starvation activates GCN2. Nonetheless, PKR and HRI can rescue GCN2 defective yeast. Some inhibitors of PKR and HRI are also shown. $P58^{IPK}$ is specific for PKR, while p67 and vaccinia K3L, the pseudosubstrate inhibitor counter both PKR and HRI.

Figure 2


PKR:
+ dsRNA
Catalytic domain
P58IPK
p67, K3L
COOH
translation:
viral replication
cell growth
HRI:
- heme
p67, K3L
heme
synthesis
GCN2:
+ uncharged tRNA
HisRS
amino acid
biosynthesis

addition of dsRNA activates the protein kinase, addition of heme to HRI inactivates it by binding and promoting intersubunit disulfide bond formation (110) and thereby allowing protein synthesis. HRI is a 92 kDa polypeptide and has been cloned from rabbit reticulocytes (111). Its expression pattern as verified by Northern, Western and RT-PCR analysis is limited to immature erythroid cells, primarily in bone marrow and peripheral blood (112). As such, its major physiological role is to regulate protein synthesis, especially of hemoglobin, according to the concentration of available heme in reticulocytes.

Plant PKR.

Early work with wheat germ extracts led to assumptions of the absence of regulation of protein translation by eIF-2α phosphorylation in plants (see (113)). Recent work, however, has identified a plant PKR (pPKR), a constitutive 68-70 kDa phosphoprotein stimulated by plant virus or viroid infection (114). Various biochemical and immunological analyses suggest pPKR is the dsRNA activated protein kinase in plants. Although this group has not yet cloned pPKR, immunoreactivity with an antibody directed against the conserved dsRBDs of mammalian PKR suggest it has dsRBDs. pPKR is also cytosolic and ribosome associated. Mammalian PKR cDNAs cross hybridize with pPKR transcript (114). The identification of the 42 kDa plant eIF-2α, the conservation of the Ser 51 phosphorylation site in Arabidopsis thaliana and other necessary factors of the eIF-2α phosphorylation pathway suggests that plants can also regulate protein translation by utilizing this pathway (115). This group demonstrated that pPKR can phosphorylate both plant and mammalian eIF-2α and that protein synthesis in wheat germ lysates is inhibited by phosphorylated eIF-2α, dsRNA and hPKR. It was observed that pPKR required higher doses of dsRNA (10 μg/ml)

to completely inhibit protein synthesis, 100-fold higher than for hPKR. Furthermore, they characterized an inhibitor of pPKR, PKI (116) which is the plant homolog of the mammalian p67 inhibitor (see later) which complexes with pPKR. Unlike in mammals, pPKR levels remain constant through the life cycle of the plant. PKI levels however, are temporally regulated during plant growth and development, reaching a peak level in dormant seeds and again during seed development (116). Accordingly, pPKR catalytic activity levels vary widely and correlate inversely with PKI levels. It is thought that PKI inhibition of pPKR may contribute to the rapid elevation of protein synthesis during seed germination. Whether pPKR plays a significant role in plant anti-viral response will be interesting to determine.

Caenorhabditis elegans PKR and Drosophila PKR?

Genome sequencing efforts in various organisms have provided enormous amounts of genetic information in these organisms. Databases of expressed sequence tags (ESTs) and of chromosome sequence allow rapid identification of homologs of transcribed genes of interest in various organisms. These recent advances in molecular genetics allow us to ask what role PKR plays in simpler organisms and whether they may be useful as genetic models. A text based and sequence homology search of C. elegans EST and sequence databases using mPKR show that the nematode does encode a gene that bears homology to PKR, Genbank™ accession number Z68104, locus 1072337. That C. elegans may bear functional PKR is further supported by the observation that an EST encoding a homolog of the endogenous mammalian inhibitor of PKR, $p58^{IPK}$, is also found in nematodes (Genbank™ Accession number U88181, locus 1825700) (see later). The full length cDNAs of either C. elegans PKR or its inhibitor have not been published but have been deduced

from genomic sequence data in the databases mentioned above. The predicted PKR in the nematode is highly similar to mPKR in the catalytic domain but bears a novel amino terminal region with no obvious dsRBDs (Stojdl, D., personal communication). The inhibitor of PKR in C. elegans is conserved with the bovine $p58^{IPK}$ sharing the tetratricopeptide repeats and DNA J domain.

The presence of PKR in nematodes is important since it suggests that the failure to date to identify PKR in Drosophila may be from a lack of trying. This is likely given that the Drosophila homolog to the $p58^{IPK}$ inhibitor of PKR has been identified (Genbank™ Accession number AA536243, EST name LD16733.5prime) (see later). As with the nematode, identification of complete cDNAs of PKR in the fruit fly would give us an indication of the conservation of PKR function in these lower organisms. The nature of its regulatory domain and the activators of these homologs would inform us about the evolutionary significance of being able to respond to dsRNA and inhibit translation. It appears that the evolution of PKR with dsRBDs may not antecede the nematode and arose later in more complex organisms. The presence of dsRBD motifs in plant PKR inferred from immunoreactivity with antibody directed against this domain raises the interesting issue of whether this represents convergent evolution of this domain in the Plant and Animal Kingdoms. Sequence analysis of pPKR once it is cloned would shed light on the evolutionary significance of the dsRBD.

Yeast GCN2.

Along with the two mammalian eIF-2α protein kinases, PKR and HRI, the yeast GCN2 protein kinase is the third member of this family that has been well characterized.

The general control genes of Saccharomyces cerevisiae have been studied by isolating unlinked mutations that do not regulate amino acid biosynthetic genes properly. In gcn mutants, these biosynthetic genes are not inducible, whereas gcd mutants have constitutive induction of these genes. The GCN2 kinase is the yeast eIF-2α protein kinase which regulates the positive regulator GCN4, by translation control. As with HRI, GCN2 shares sequence homology to PKR in the catalytic domain but has a completely different regulatory domain located in the carboxy terminal region (Fig. 2) (111,117). This region bears no dsRBDs but rather, has strong resemblance to the histidyl-tRNA synthetases (118). This and other features of the target GCN4 transcript have been utilized in yeast to enable gene specific translational control of this transcript as elucidated below (see Fig. 3).

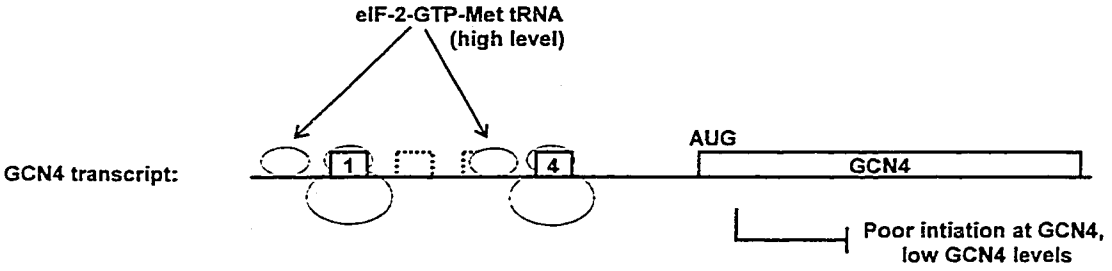
GCN4 is a transcriptional activator of 30-40 genes in 11 different amino acid biosynthetic pathways. In response to amino acid starvation, GCN4 protein level is elevated, increasing the transcript levels of the biosynthetic genes it regulates (reviewed in(119)). The translational regulation of GCN4 requires short upstream open reading frames (uORFs) within its transcript. Such uORFs usually inhibit translation of a downstream ORF, a phenomenon best explained by the ribosome scanning model. The 40S subunit or the assembled ribosome must begin at the 5' leader of a transcript and scan along this leader until it encounters an AUG initiation codon in a favorable context. The presence of an uORF would preferentially favor initiation at this site and preclude translation at the downstream site because reinitiation is an inefficient process. uORFs are rare in mammalian transcripts and occur only in a few percent of yeast transcripts (119). The four uORFs in the GCN4 transcript represent a translation control element that permit ribosomes to reach the

Figure 3. GCN2 regulation of GCN4 translation in response to amino acid levels.

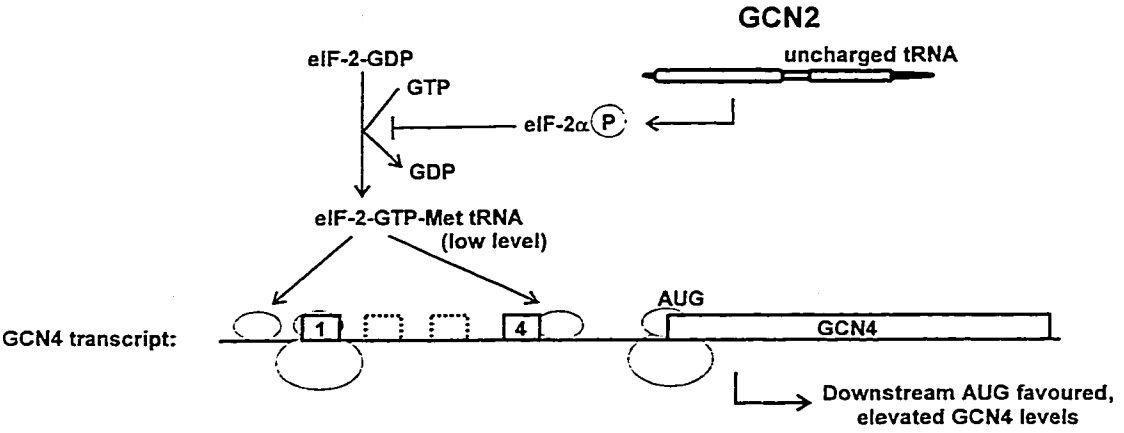
GCN4 transcript is depicted with upstream open reading frames (uORFs, numbered) and GCN4 ORF denoted as boxes. uORF 1 and 4 are essential for GCN4 regulation while uORF 2and 3 (stippled) are not essential. 43S preinitiation complex (see next figure) denoted as small shaded oval. 80S initiation complex shown as two shaded ovals initiating at appropriate initiation codons (AUG) of respective uORF or ORF. Top drawing shows the transcript during conditions of amino acid abundance. In the absence of uncharged tRNA and GCN2 activation, eIF-2α is hypophosphorylated and therefore ternary complex is abundant. This allows efficient reinitiation at uORF4 preventing ribosome scan through to GCN4's AUG and keeping GCN4 levels low. Bottom drawing indicates the change that occurs during amino acid starvation. GCN2 is activated and eIF-2α is phosphorylated, thereby preventing ternary complex recharging with GTP and reducing available levels. This ensures poor reinitiation efficiency such that the ribosome scans through uORF4 and initiates at GCN4 AUG and elevates GCN4 levels.

Figure 3

Amino acid abundance:



Amino acid starvation:



downstream GCN4 ORF only under conditions of amino acid starvation. These uORFs are sufficient to confer GCN4-like regulation when inserted into a heterologous yeast transcript.

Various studies have established that ribosomes translate uORF1, terminate and either reinitiate at uORF4 (under non-starvation conditions) and terminate without reaching the GCN4 ORF or scan past uORF4 and reinitiate at the GCN4 AUG (under amino acid starvation conditions) (see Fig. 3). Thus, GCN4 translation is determined by the efficiency of reinitiation at the latter uORF. This is directly regulated by GCN2 phosphorylation of eIF-2α. Evidence suggests that under starvation conditions, GCN2 kinase activity is stimulated by the binding of uncharged tRNA to the regulatory domain resembling histidyl-tRNA synthetases (118). This activation in amino acid starved cells results in phosphorylation of eIF-2α in a GCN2 dependent manner (120). GCN2 is capable of phosphorylating rabbit or yeast eIF-2α and substitution of Ser 51 on eIF-2α with Ala eliminates such phosphorylation in vitro and in vivo. Furthermore, Ser 51 mutation abolishes GCN4 and amino acid biosynthetic gene induction. The manner in which eIF-2α phosphorylation by GCN2 alters the preferred site of translation reinitiation by ribosomes is based upon the slowing of reacquisition of competence by the ribosome to reinitiate at downstream ORFs, favoring the one further downstream in a position dependent manner. The ability to reinitiate efficiently is dependent on the ability to recycle ternary complex (eIF-2, GTP and Met-tRNA$_i$) which is inhibited when eIF-2α is phosphorylated (Fig. 3, and see next section). Thus, when starved of amino acids, uncharged tRNA binds and activates GCN2 which then phosphorylates eIF-2α. This prevents efficient ribosome reinitiation at uORF4, however the scanning ribosome has sufficient time to bind more ternary complex

before reaching the more distant GCN4 initiation codon. This delay in acquisition of competence to reinitiate by GCN2 activation favors the GCN4 AUG to produce elevated GCN4 levels (120). In this manner, a mechanism that is used in mammalian cells to control total protein synthesis regulates gene-specific translation control in yeast.

The conservation of the catalytic domain in the eIF-2α protein kinases led the Hinnebusch group to also examine whether the mammalian HRI and PKR could functionally substitute for GCN2 in yeast (121). In a yeast strain deficient for GCN2, they established that both these eIF-2α protein kinases could stimulate GCN4 translation and were dependent on an intact Ser 51 site in eIF-2α. Given the divergence of the regulatory regions of these kinases, it was not surprising that this group found no evidence that the mammalian kinases were regulated by levels of uncharged tRNA as for GCN2. It is still uncertain whether their ability to induce GCN4 was due to endogenous activators or simply due to high expression levels. Interestingly, the gene-specific effect of GCN4 regulation was best demonstrated when the protein kinases were expressed at low copy levels. High level induction of HRI or PKR resulted in a slow growth phenotype independent of amino acid levels as described before (117) and which was suppressed by eIF-2α S51A mutation (121). This effect is similar to the effect in mammalian cells of general protein synthesis inhibition by eIF-2α phosphorylation and indicates this may also occur in yeast. This demonstration that mammalian PKR may substitute in GCN4 translation control suggest that perhaps it may also be involved in gene specific control of certain mammalian transcripts which bear uORFs. The work by Marilyn Kozak describing such features in a subset of vertebrate

transcripts provide some grounds to speculate that translation control of such transcripts may be physiologically important (122).

In an interesting aside, unpublished reports from Thomas Dever's laboratory are consistent with our identification of mPKR as a potential DSK by its anti-phosphotyrosine reactivity (see Chapter 2). This group suggests that PKR is capable of phosphorylation of tyrosine when the Ser 51 site of eIF-2α is altered to Tyr (Lu and Dever, unpublished observations). Phosphorylation of S51Y eIF-2α induced GCN4 levels indicating normal in vivo function. No other physiologically relevant tyrosine phosphorylation of a substrate by PKR has been reported.

Human GCN2 homolog?

At the outset of this thesis work, the only known mammalian eIF-2α protein kinases were PKR and HRI. Since HRI is restricted in tissue distribution, there was little evidence of redundancy of PKR function. Recently, searches of ESTs in databases have indicated the partial sequence of a putative human GCN2 homolog (Genbank™ accession numbers R19609 and R19927). These short (372 and 300 bp) segments of transcript bear 63% homology to yeast GCN2 but bear insignificant matches to hPKR and mPKR. No full length cDNA of this human GCN2 homolog has been published to date. The sequence encoded in the human EST transcripts bear homology to the carboxy terminal end of the catalytic domain and the spacer region between the catalytic domain and His-tRNA synthetase regulatory domain of yeast GCN2 (see Fig. 2). As such, we lack a complete picture of the structure of this putative member of the eIF-2α protein kinase family, its tissue distribution

and its likely physiological role. The importance of studying this kinase to understand eIF-2α protein kinase redundancy is paramount.

Translation regulation by eIF-2α phosphorylation.

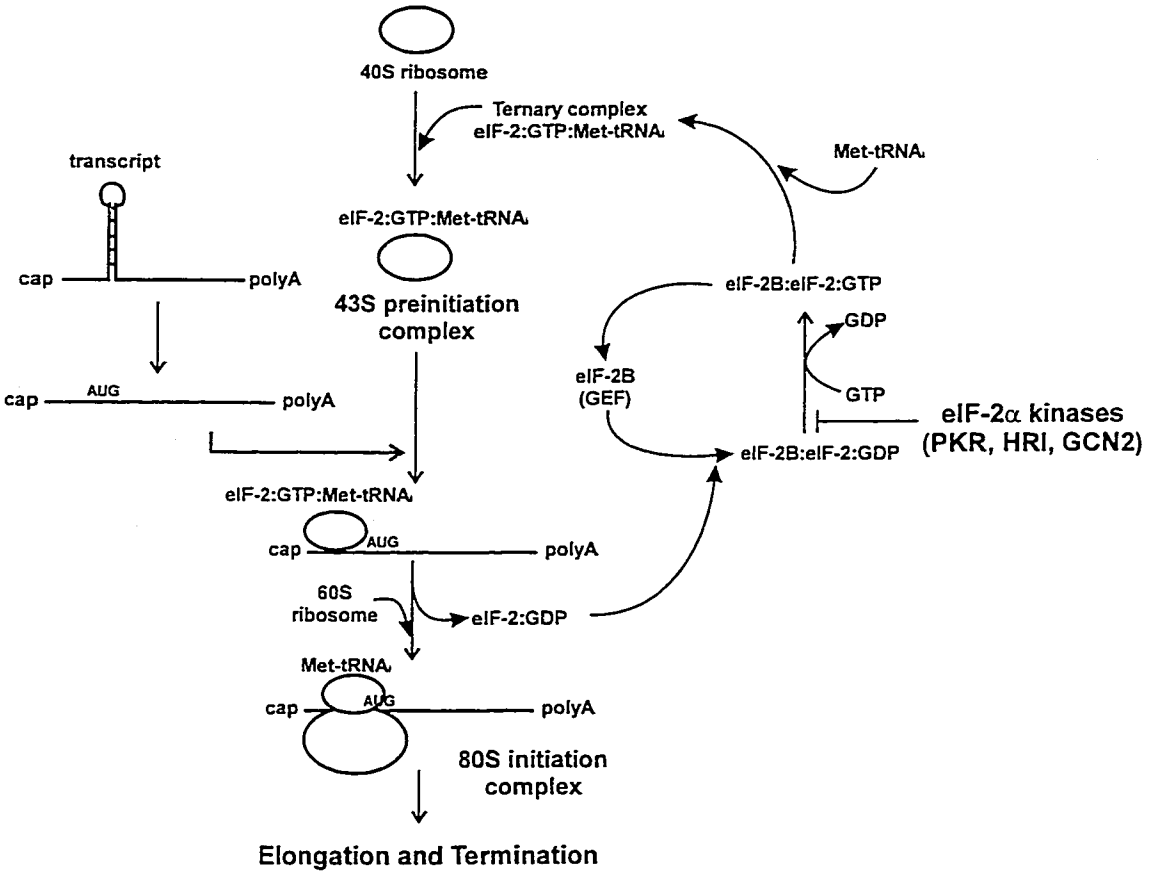
The single common element shared by the family of eIF-2α protein kinases is regulation of translation by phosphorylation of the alpha subunit of eIF-2. This control point of overall protein synthesis in response to a wide range of factors occurs at initiation of translation rather than elongation or termination (123). This section will address the mechanism by which phosphorylation of eIF-2α alters translation initiation efficiency. The phosphorylation site of eIF-2α which was mapped to Ser 51 (72) is highly conserved from yeast to humans. Indeed residues 41-59 of yeast, rat and human eIF-2α are identical (124,125) suggesting strong evolutionary conservation of this site.

Eukaryotic translation initiation involves the association of an mRNA transcript with the 40S and 60S ribosomal subunits along with the initiator tRNA (Met-tRNA$_i$) to form the 80S initiation complex (see Fig. 4). This process is mediated by a series of reactions involving numerous proteins called eukaryotic initiation factors (eIFs). Of these, eIF-2 was determined to play a key role in regulating peptide chain initiation. eIF-2 is a factor that facilitates initiator tRNA binding to free 40S ribosomal subunit by forming a ternary complex consisting of eIF-2, Met-tRNA$_i$ and GTP (reviewed in (123,126)) to form 43S preinitiation complex. eIF-2 itself is a complex of three subunits, eIF-2α (36 kDa in mammals), eIF-2β (38 kDa) and eIF-2γ (52 kDa) (124). Binding of mRNA and 60S ribosomal subunit is dependent on GTP hydrolysis to form viable 80S initiation complex,

Figure 4. eIF-2α protein kinase regulation of translation initiation.

A simplified depiction of the series of reactions that lead to translation initiation and reinitiation. 40S ribosome has to bind ternary complex consisting of eIF-2, GTP and Met-$tRNA_i$ to form the preinitiation complex. The transcript at left is bound by the cap complex (not shown) before it is bound by the 43S preinitiation complex. Hydrolysis of GTP is required, eIF-2 and GDP are released then 60S ribosome subunit binds to make the competent 80S initiation complex. eIF-2:GDP are bound by eIF-2B which catalyzes the exchange of GDP for GTP. Phosphorylation of eIF-2's α subunit by the eIF-2α protein kinases binds and inactivates eIF-2B which is at sub stoichiometric levels to eIF-2α and will limit the recycling of eIF-2 to form new ternary complex with GTP and Met-$tRNA_i$ thereby restricting translation. In the absence of eIF-2α protein kinase activation, regeneration of ternary complex is efficient and translation reinitiation is unimpeded.

Figure 4


40S ribosome
Ternary complex
eIF-2:GTP:Met-tRNAi
Met-tRNAi
transcript
eIF-2:GTP:Met-tRNAi
cap
polyA
43S preinitiation complex
eIF-2B:eIF-2:GTP
GDP
GTP
eIF-2B (GEF)
cap
AUG
polyA
eIF-2α kinases (PKR, HRI, GCN2)
eIF-2B:eIF-2:GDP
eIF-2:GTP:Met-tRNAi
cap
AUG
polyA
60S ribosome
eIF-2:GDP
Met-tRNAi
cap
AUG
polyA
80S initiation complex
Elongation and Termination

and subsequently releases eIF-2-GDP (see Fig. 4). For reinitiation to occur, eIF-2 must exchange the GDP for GTP to catalyze another cycle. This guanine nucleotide exchange is mediated by eIF-2B (or guanine nucleotide exchange factor, GEF). Phosphorylation of eIF-2α on Ser 51 reduces the efficiency of reinitiation by sequestering and inactivating eIF-2B (127), preventing GTP exchange and hence, ternary complex formation. The puzzling observation that only 20-40% of eIF-2α is phosphorylated in fully inhibited lysates is easily explained by this mechanism. Ternary complex formation stops when all free eIF-2B is bound by phosphorylated eIF-2α. The ratio of eIF-2B to eIF-2 has been estimated somewhere between 1:20 and 1:3 (123). Thus, only a fraction of eIF-2α need be phosphorylated to completely shut down translation initiation. Conversely, a fractional decrease in eIF-2α phosphorylation may cause a dramatic increase in translation initiation.

The above mechanism of regulation of translation also explains the properties of activated HRI and PKR to disassemble polysomes, deplete 43S preinitiation complex and be reversed by addition of exogenous eIF-2. All of these are a function of inhibition of ternary complex formation by inactivating eIF-2. It is this elegant regulatory mechanism that is triggered under various cellular stresses such as viral infection (128), heme deprivation (100), amino acid starvation (119), heat shock (129) and Ca^{2+} mobilization (130).

PKR regulation of transcription factors.

Aside from regulating translation, PKR has also been implicated in regulating certain signaling molecules. Early observations suggested that dsRNA treatment of cells could induce the expression of the IFN-β gene (131) and was blocked by 2-aminopurine (2-AP),

a PKR inhibitor. Other studies showed that this was mediated by activation of the transcription factor NF-κB (132). NF-κB is present in the cytoplasm as a multimeric complex of p50, p65 and rel subunits. In unstimulated cells, the latent complex is bound to a specific inhibitor, IκB. Activation of NF-κB requires its release from IκB and translocation to the nucleus to bind target gene promoters. The release by IκB is mediated by phosphorylation events (133) that trigger degradation of IκB by the ubiquitin-proteosome pathway (134-136). Although a ubiquitination-dependent protein kinase activity has now been identified (137), certain groups investigated the possibility that PKR may respond to dsRNA and mediate NF-κB activation. One study showed in vitro evidence of PKR phosphorylation of IκB that mobilized p50 in SVT2 cell lysates to bind a target promoter sequence (75). This group did demonstrate that a subdomain II mutated PKR could reduce dsRNA induction of an NF-κB dependent reporter in vivo by 5 fold. A more convincing study using selective ablation of PKR by 2', 5' oligoadenylate antisense chimeras showed failure to activate NF-κB by dsRNA in HeLa cells (138). A third group determined that pre-B lymphoma 70Z/3 cells expressing an inactive version of PKR (PKRΔ6) inhibited κ gene expression induction by LPS or IFN γ (139). This inhibition however, was independent of NF-κB activation. No change in eIF-2α phosphorylation was observed either, indicating PKR regulates κ gene transcription independently of protein synthesis. They concluded that depending on cell type and stimuli, PKR regulation of transcription could be NF-κB dependent or independent.

As discussed later in Chapter 3, work from a group that generated a PKR knockout mouse by targeted disruption of the amino terminal region (referred to as the Yang-

Weismann PKR-null mouse) describe signaling defects pertaining to IRF-1 and NF-κB (140,141). This group found normal Type I IFN signaling but impaired NF-κB response and Type I IFN induction to dsRNA in mouse embryo fibroblasts (MEFs). Anti-viral response induced by IFN γ and dsRNA was also impaired (140). This defective signaling in IFN γ and dsRNA response was also shown to impair IRF-1 activation (141). Transfection of PKR back into targeted cells restored response. They concluded that PKR acts as a signal transducer for IFN stimulated genes (ISGs, see next section) dependent on NF-κB and IRF-1 activation.

IRF-1 expression can confer an anti-viral state to RNA viruses (VSV, EMCV, NDV) directly, in the absence of IFN production by these cells (142). Furthermore, IRF-1 expression also causes cell growth inhibition and PKR elevation (143). Both IRF-1 mediated IFN β induction and growth inhibition were abolished by catalytically inactive PKR although IRF-1 mediated anti-viral responses apparently were not. Thus, the anti-proliferative and IFN β induction by IRF-1 is mediated in part by PKR, while its anti-viral effect does not require PKR. Consistent with this, IRF-1 is capable of inducing 2' 5' oligoadenylate synthetase (2' 5' OAS) (143).

Less clear is the involvement of PKR in acting as a second messenger or inhibitor in two signal transduction pathways. One study suggests that platelet-derived growth factor (PDGF) stimulation of immediate early genes such as c-fos, c-myc and JE, requires PKR activation, as does dsRNA induction of these same genes (144). Inhibitors of PKR, such as 2-AP and activated Ras (see later), blocked such induction (Fig. 5). Similarly, antisense ablation of PKR also blocked PDGF induction of these genes. Finally, they show PDGF

stimulates PKR autophosphorylation in vivo. The precise mechanism by which PKR regulates the induction of these immediate early genes is not known. The second signaling pathway that PKR is suggested to regulate is the IFN receptor-JAK-STAT1 pathway (145). This study documented physical interaction between PKR and the STAT1 transcription factor which mediates IFN α/β and IFNγ responses (see next section). PKR does not phosphorylate STAT1 in this interaction. STAT1 binding does not require PKR enzymatic activity, but requires the dsRBDs. Instead, it appears that in conditions which activate STAT1 (dsRNA or IFN treatment), PKR and STAT1 interaction diminishes. Indeed, cells expressing PKR defective for dsRNA binding (residues 58-60 mutagenised) show elevated STAT1 activation in response to stimulation. This may suggest a novel function for PKR in modifying IFN transcription responses, one which modulates STAT1 by binding to it in an inverse relation to STAT1 activation.

The IFNs and IFN stimulated genes (ISGs).

The Interferons are a large family of secreted proteins in vertebrates that were discovered and named after their ability to interfere with viral replication (reviewed in (146-148)). In addition to their anti-viral properties, IFNs also demonstrate anti-proliferative and anti-tumor effects on cells (149,150). There are two major types of IFN: Type I IFN (IFN α and IFN β) and Type II IFN (IFN γ). Type I IFNs are produced by most cell types while Type II IFN is produced only by lymphocytes. IFN α and IFN β secretion may be stimulated by various cytokines and growth factors but is most potently induced by dsRNA, frequently encountered as an intermediate during viral infection. Both the IFN α and IFN

β promoters are responsive to the IFN regulatory factor, IRF-1, which is sufficient to induce these genes when overexpressed in cells (151).

The IFNs bind and activate two distinct surface receptors that trigger a signaling response conveyed to the nucleus by JAK/STAT activation. IFN α and IFN β bind the same Type I receptor while IFNγ binds a distinct Type II receptor. Type I and II receptors are present in all or most tissues, respectively, but do not necessarily confer responsiveness to their respective ligands in all cell types, implying the need for accessory molecules. Type I IFN response requires activation of the Janus receptor tyrosine kinases (JAKs) JAK1 and Tyk2 (152-154) whereas Type II IFN receptor requires JAK1 and JAK2 (155,156). Each of these pathways also recruits, phosphorylates and activates in a phosphotyrosine and SH2 domain dependent manner unique members of the signal transducers and activators of transcription (STATs) that hetero- or homodimerize to form transcription factors of specific DNA-binding properties. Thus, IFN α/β treatment results in recruitment of STAT1, STAT2 heterodimers and a third protein p48 to bind a DNA motif (termed an IFN stimulated response element, ISRE) specific to IFN α/β stimulated genes. IFN γ activates the JAKs associated with its receptor which recruit only STAT1 as a homodimer. This translocates to the nucleus and binds a DNA motif (termed gamma activated sequence, GAS) specific for IFN γ stimulated genes.

The IFN stimulated genes (ISGs) are the class of genes upregulated by IFNs that mediate the anti-viral and anti-proliferative effects of these cytokines. Over 30 genes induced by the IFNs have been identified, some exclusively by Type I or II IFN and some by both. Type I IFN induced ISGs include Mx protein, IRF-1 and IRF-2. Type II IFN

induced ISGs include MHC class II. Examples of ISGs induced by both Type I and II IFN are MHC class I, 2' 5' OAS, PKR and guanylate binding protein-1 (GBP-1) (reviewed in (148)).

Along with PKR, 2' 5' OAS is one of the better studied ISGs that exert an anti-viral response. 2' 5' OAS is IFN induced but is in a latent state until activated by dsRNA (see (157)). It utilizes ATP to synthesize oligomers linked by a 2' 5' phosphodiester bond. These oligoadenylate molecules activate the ribonuclease RNAse L which targets cellular and viral RNA for degradation. 2' 5' OAS expression is sufficient to confer resistance to encephalomyocarditis virus (EMCV) (158). PKR induction by IFN α/β and IFN γ has been described (51,63-66). Its anti-viral and anti-proliferative functions will be expanded upon in the following sections. The Mx gene was identified as a mouse locus conferring resistance to influenza virus (157). Mx proteins and the genes encoding them have been identified in mouse, human, rat, fish and even yeast (the Vps1 gene). This protein is normally undetectable in resting cells and its expression is tightly controlled by IFN α/β. The Mx1 and Mx2 genes in the influenza sensitive BalbC and CBA/J mouse strains are either not expressed or mutated respectively (157). The biological activity of Mx1 was demonstrated by high level stable expression in influenza susceptible NIH 3T3 cells. These cell lines survived high dose challenge with influenza virus but not with vesicular stomatitis virus (VSV) or herpes simplex virus (HSV).

The MHC class I antigens are induced by both IFN α/β and IFN γ (see (146,148)) allowing enhanced immune surveillance by cytotoxic T cells. MHC class II antigens are only induced by IFN γ which enhances antigen presentation and immune response to viral

challenge. Interestingly, IFN γ also induces the level of tumor necrosis factor (TNF) receptor, a protein that triggers apoptotic effects in cells (159,160). Finally, among many others, the IFNs also elevate expression of genes such as GBP and ISG-15 of unknown function (146,157).

Conservation of the ISREs controlling IFN α and β production with those regulating ISGs indicate the reason for co-induction of IFN stimulated genes along with the IFN genes. Hence, IRF-1 regulates IFN β and transcription of certain ISGs by binding the ISRE common to these genes (161). This explains how constitutive expression of IRF-1 confers anti-viral properties in the absence of IFN β production, most likely by direct ISG induction (142).

Inhibitors of PKR: viral inhibitors.

No discussion of regulation of PKR function would be complete without a look at the known inhibitors of PKR. The first studies describing mechanisms of inhibition of PKR have come from virology, particularly in understanding how viruses evade the shut off of protein synthesis mediated by PKR activation.

One of the best studied mechanisms of viral inhibition of PKR is that used by adenovirus. Adenovirus is a DNA virus that is insensitive to IFN, a property conferred by a small, virus encoded RNA called VAI RNA (see (126,162). The adenovirus mutant Ad5 dl331 which does not produce VAI RNA, activates PKR and increases eIF-2α phosphorylation thus preventing viral and cellular protein synthesis during late phase infection (163). The source of dsRNA activator from adenovirus replication appears to be symmetrical transcription of the viral genome. The VAI translation enhancement effect is

not restricted to viral mRNAs (164), a function that is better managed by the adenovirus tripartite leader sequence present in its mRNAs (165). VAI is normally produced in large amounts by RNA Polymerase III late in infection. Since VAI RNA is a highly structured molecule of about 160 nucleotides, it was thought that it may act as a short RNA duplex that prevents PKR activation. Consistent with this, VAI has been shown to bind PKR in vitro and in vivo (166,167), inhibit PKR (168) and prevent binding of dsRNA to PKR (169). It is not clear that the imperfect double stranded structure of VAI RNA is responsible for inactivation of PKR since, as with dsRNA, low doses of VAI activate PKR while high doses inactivate it (167). Since adenovirus infection produces extremely large quantities of VAI RNA during late phase infection (162) (greater than 10^8 molecules/cell, in molar equivalence to concentration of ribosomes), VAI transcript functions to inactivate PKR.

Vaccinia virus is a member of the Orthopoxvirus group that also displays resistance to IFN despite producing large amounts of dsRNA late in infection (170). The factors responsible for countering the IFN induced 2' 5' OAS-RNAseL pathway are not known, however, two inhibitors of PKR have been identified. A 25 kDa dsRNA binding protein that co-purifies with and is necessary for PKR inhibition was found to be encoded by the vaccinia E3L gene (171,172). E3L can inhibit PKR in vitro and in vivo and shares close homology with several dsRNA binding proteins, including the dsRBDs of PKR. The second inhibitor of PKR, K3L, was identified by the sequence homology of the 88 amino acid gene product with the amino-terminus of eIF-2α, including, the Ser 51 site of phosphorylation. Virus lacking K3L are IFN sensitive and have a 100-fold drop in virus yields (173). K3L appears to inhibit PKR activation and eIF-2α phosphorylation without itself being

phosphorylated (174). In a study using the same reporter of PKR inactivation, this group showed that E3L inhibited PKR 50-100 fold more efficiently than K3L (175). The mechanism of E3L action was different from K3L in that it interfered with the binding of PKR to dsRNA, whereas K3L did not. Instead, K3L is thought to interfere with PKR interaction with eIF-2α, and indeed consistent with a pseudosubstrate function, has been shown to bind PKR directly (176-178). E3L and K3L did not show synergistic effects on translation or eIF-2α phosphorylation (175). K3L is also capable of inhibiting the HRI (176) suggesting it may serve to map a substrate interaction domain common to the eIF-2α protein kinases. Using deletion mutants of PKR in a yeast two-hybrid assay with K3L, its was found that K3L binds to a region in the catalytic domain of hPKR between residues 366 and 415 (97,179) (Fig. 1). The kinase insert region conserved between the eIF-2α protein kinases was required for catalytic function but failed to bind K3L. It should be noted here that the WR strain of vaccinia used in experiments in Chapter 3 encode E3L, the more potent PKR inhibitor (172).

The HIV-1 retrovirus has also evolved mechanisms to counter PKR mediated protein synthesis shutoff. The transactivation responsive region (TAR) in the 5' untranslated region (UTR) of HIV transcripts is a highly structured RNA moiety that exhibits cis- and trans-inhibitory effects on translation (180,181). TAR has been shown to require its RNA stem structure to bind and activate PKR (58), although studies suggesting TAR inactivates PKR to stimulate protein synthesis have been published (182,183). The potential translation inhibitory effect of TAR appears to be countered by down regulation of PKR. This has been attributed to the regulatory transactivator protein, Tat, since stable expression of Tat in HeLa

cells reduced PKR steady state levels (184). Indeed, it has recently been demonstrated that the single-exon form (but not the two-exon form) of Tat is complexed with PKR and inhibits its activity (76). Furthermore, a host protein TAR binding protein (TRBP), also appears to be capable of inhibiting PKR (see next section).

Reovirus is a virus bearing a dsRNA genome which may activate PKR and inhibit viral mRNA translation (185) and repress its replication (186). This appears to vary by reovirus strain, with some strains showing IFN resistance. Genetic analysis of reovirus strains mapped IFN insensitivity to the S4 gene product, σ3 (187). This protein has been shown to bind dsRNA, inhibit PKR, facilitate translation in transfected cells and can even substitute for vaccinia E3L in recombinant vaccinia (see (188,189)). Strain differences of σ3 mediated host protein synthesis inhibition was shown to be determined by localization of σ3. Strains that had no effect on cellular protein synthesis had σ3 localized throughout the cytoplasm whereas those that shut down host protein synthesis had σ3 restricted to perinuclear viral factories (188). This was determined by σ3 affinity for the capsid protein μ1 and is consistent with a model where efficiency of cellular translation is affected by cytoplasmic σ3 levels not complexed with μ1.

Herpes simplex 1 virus (HSV-1) is a DNA virus that infects neuronal cells but is completely non-neurovirulent when mutated at the γ34.5 gene (190). This gene functions to prevent a host response that terminates protein synthesis after the onset of viral DNA synthesis. Cells infected with HSV-1 activate PKR but do not demonstrate elevated eIF-2α phosphorylation levels unless infected with virus mutated at γ34.5 (191). The carboxy terminus of γ34.5 bears 64 residues which show high homology to the murine MyD116

protein (involved in myeloid differentiation) and the closely related mammalian protein GADD34 (involved in growth arrest and DNA damage). MyD116 is capable of substituting for γ34.5 to preclude premature shutoff of protein synthesis during infection (192). This suggests that MyD116 and GADD34 may have a similar function in preventing translation inhibition in cells undergoing DNA damage, growth arrest or differentiation. γ34.5 and MyD116 appear to prevent translation inhibition by PKR activation by binding to protein phosphatase 1α and directing it to dephosphorylate eIF-2α (193). In a daring approach to determine whether HSV-1 activation of PKR was by a generalized anti-viral sensing mechanism or by discrete activators encoded by viral components, one group asked whether suppressor variants that bypassed the $\gamma 34.5^-$ block in replication could be obtained (194). Several independent viral isolates mapping to a 595 bp region were obtained, presenting the possibility that HSV-1 may encode a discrete PKR activator. This region affects the Us11 and Us 12 genes, of which the Us11 transcript appears to be the most likely candidate for PKR activation. The activation of PKR by an HSV-1 (and other viral) encoded gene(s) along with the necessity of encoding inhibitors such as γ34.5 suggest there may be a selective advantage of viral activation of PKR, that may also involve pathways other than eIF-2α phosphorylation.

Numerous other strategies to avert PKR activation are utilized and will only be mentioned in brief here. Poliovirus relies on cap-independent translation initiation while degrading an essential component of eIF-4F, p220, to favor viral transcripts over cellular transcripts. In addition, protease 2A is also believed to target PKR. PKR levels are dramatically decreased by degradation in poliovirus infected cells, although the remaining

PKR is highly activated and autophosphorylated (195). Another strategy used by simian virus 40 (SV40) rather than block activation or reduce PKR expression is to block effectors downstream of PKR. SV40 large-T antigen was found to antagonize the translation repression of PKR activation in an adenovirus VAI-deficient complementation assay (196). Interestingly, large-T does not prevent PKR activation but acts on a downstream step of PKR, as yet unidentified. Residues 400-600 on large-T confer this function.

Finally, influenza virus encodes a novel mechanism to inactivate PKR, one which it does not encode itself, but rather, recruits from the host. This will be taken up in the following section on endogenous inhibitors of PKR.

Inhibitors of PKR: cellular inhibitors.

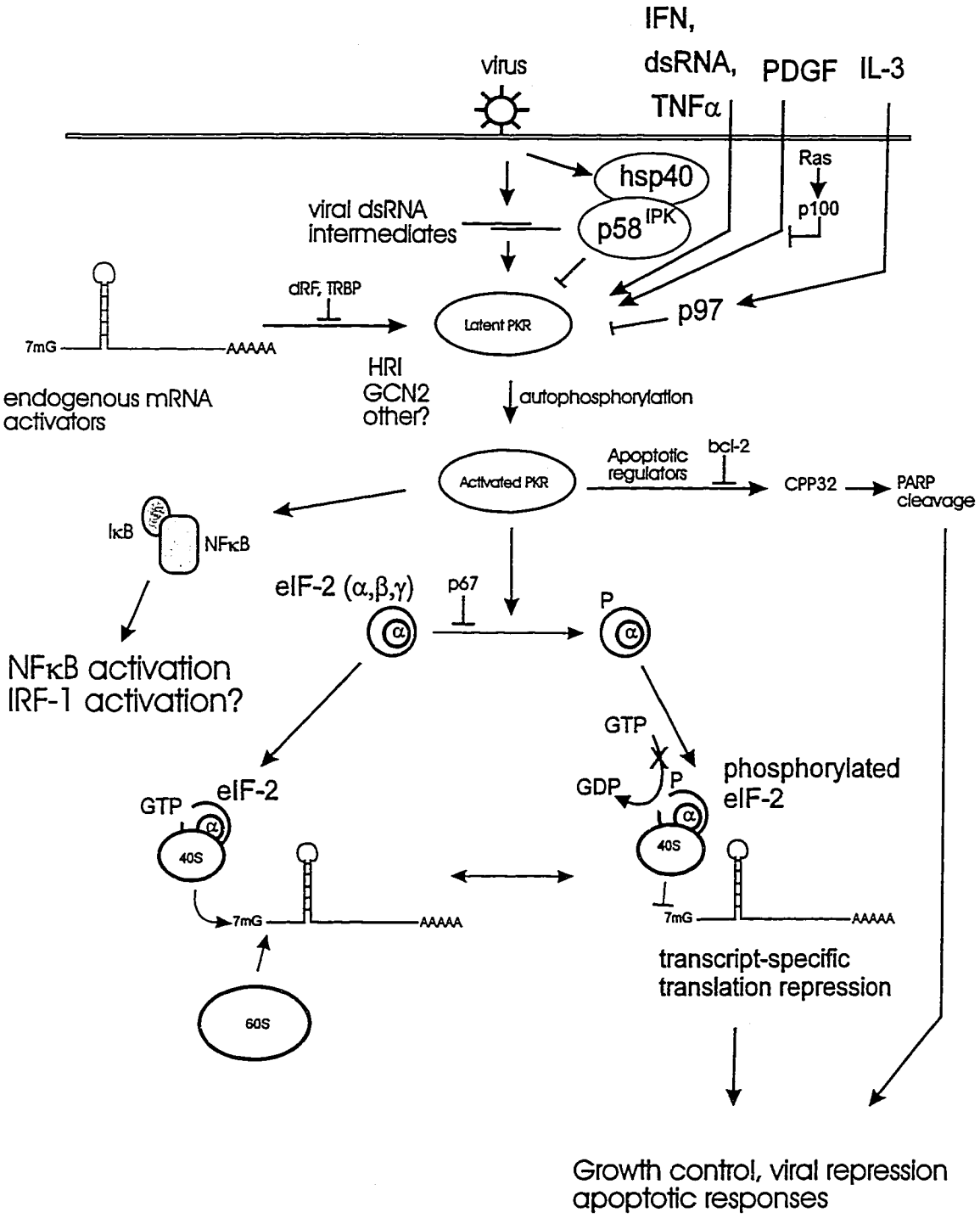
P58^{IPK}:

One of the best studied endogenous PKR inhibitors, P58^{IPK}, was elucidated by studies of influenza virus. Influenza virus is an enveloped virus with a segmented, negative-stranded RNA genome. Upon infection, it favors the translation of viral transcripts by a cap-stealing mechanism, degradation of host mRNA, inhibiting initiation and elongation steps and by activating the cellular PKR inhibitor, P58^{IPK} (197,198) (Fig. 5). This inhibitor is a 58 kDa protein that was purified and cloned. Sequence analysis showed that P58^{IPK} is a member of the tetratricopeptide repeat (TPR) family of proteins, with nine tandemly arranged TPR motifs which can mediate homotypic and heterotypic protein-protein interactions. The carboxy terminus of P58^{IPK} bears a DNA-J motif of the DNA-J heat shock family of proteins which can also mediate protein-protein interactions. The middle of the polypeptide encodes a region with weak homology to eIF-2α (197). P58^{IPK} appears to be highly conserved with

Figure 5. Regulation of PKR and targets of PKR activation.

Schematic depiction of the pathways of cellular function that PKR is thought to be involved in. PKR is activated by dsRNA during viral infection or even from transcripts with secondary structure. Not shown is elevation of PKR transcription and protein levels by IFN during viral infection. Various cellular inhibitors are indicated, no viral inhibitors are shown. $P58^{IPK}$ is a cellular inhibitor bound to hsp40 that is released by influenza infection to inhibit PKR by binding to it. Known targets of PKR include eIF-2α and NF-κB while unknown regulators of apoptosis upstream of bcl-2 action are also affected. Specificity of outcome from these various triggers must involve combinations of localized activation, tissue specific distribution of inhibitors, activators and perhaps, substrates. These stimuli can elicit translation repression causing growth control, viral repression, apoptosis and κ gene regulation.

Figure 5


IFN,
virus
dsRNA,
PDGF
IL-3
TNFα
hsp40
Ras
p58 IPK
p100
viral dsRNA
intermediates
dRF, TRBP
Latent PKR
p97
7mG
AAAAA
endogenous mRNA
activators
HRI
GCN2
other?
autophosphorylation
Activated PKR
Apoptotic
regulators
bcl-2
CPP32
PARP
cleavage
IκB
NFκB
eIF-2 (α,β,γ)
p67
P
NFκB activation
IRF-1 activation?
GTP
GDP
eIF-2
phosphorylated
eIF-2
40S
60S
transcript-specific
translation repression
Growth control, viral repression
apoptotic responses

expression documented in human, monkey, bovine and murine cells. Furthermore searches of EST databases revealed that $P58^{IPK}$ homologs in Drosophila (Genbank™ Accession number AA536243, EST name LD16733.5prime) and C. elegans (Genbank™ Accession number U88181, locus 1825700) have also been cloned. $P58^{IPK}$ is expressed constitutively in mammalian cells (197,199) but its activity is regulated by an inhibitor called $I\text{-}P58^{IPK}$ (198). Influenza is thought to disrupt $I\text{-}P58^{IPK}$ interaction thereby releasing the $P58^{IPK}$ inhibitor to repress PKR function (Fig. 5). $P58^{IPK}$ forms a physical complex with PKR and inhibits its activity in a dose dependent manner (197,200). Furthermore $P58^{IPK}$ appears to inhibit PKR but not HRI (197) (see Fig. 2). Various studies using co-immunoprecipitation, yeast two-hybrid analysis and translation derepression of a reporter gene indicate that $P58^{IPK}$ requires both the C-terminal DNA-J motif and the TPR6 domain within the region of eIF-2α homology to interact with PKR and inhibit it (179,201). The homology with eIF-2α is not essential for $P58^{IPK}$ function indicating theTPR6 motif may serve more as a PKR-$P58^{IPK}$ interaction motif than a pseudosubstrate. The amino terminal TPR motifs are required for $P58^{IPK}$ multimerization. This group also mapped the interaction domain within PKR for the $P58^{IPK}$ to a 52 amino acid segment (residues 244-296) within the ATP-binding region in the PKR catalytic domain (179) (see Fig. 1). This is a distinct site of action from that bound by vaccinia's K3L pseudosubstrate inhibitor of PKR.

It had been observed that antiviral responses mediated by IFN were impaired in heat shocked cells. PKR autophosphorylation in vitro and in vivo is markedly reduced during heat shock despite normal phosphorylation in other proteins and 2' 5' OAS activity (202). Heat shock results in reduced nonionic detergent solubility of PKR and degraded anti-

EMCV response. Recently, it was found that the I-P58^{IPK} repressor of P58^{IPK} had biochemical properties indistinguishable from the molecular chaperone hsp40 (203) and also reacted with hsp40 antibody. This group showed that hsp40 complexes directly with P58^{IPK} and inhibits it in an in vitro functional assay, allowing PKR activation. hsp40 does not activate PKR directly or with P58^{IPK} in the absence of dsRNA. Furthermore, it requires preincubation with P58^{IPK} to block its PKR inhibitory activity but does not induce the degradation of P58^{IPK}. Whereas P58^{IPK} requires equimolar amounts to inhibit PKR, picogram quantities of hsp40 were sufficient to inhibit nanogram amounts of P58^{IPK} (203). Consistent with its role as a molecular chaperone, hsp40 may be able to disrupt secondary, tertiary or even the quarternary structure observed in P58^{IPK} oligomerization (179) such that dramatic effects are seen. These observations suggest a novel intersection between the heat shock and IFN pathways. It appears that the influenza virus has recruited mechanisms in a cellular stress pathway to inhibit PKR function. These findings provide new insights into the mechanisms that regulate PKR during normal cell growth.

p67:

The p67 inhibitor of PKR (and HRI) is a glycoprotein that was identified by its ability to promote protein synthesis in the presence of eIF-2α protein kinases. It co-purifies with eIF-2 and has been isolated and cloned (204). The unglycosylated protein has a molecular mass of 53 kDa and shows sequence identity in the N-terminal region with human eIF-2β. This group had shown that p67 protects the eIF-2α subunit from inhibitory phosphorylation by the eIF-2α protein kinases (205,206) (Fig. 5). Using an in vivo translation reporter, it was demonstrated that transfection of p67 increased protein levels

although not as much as vaccinia K3L (207). p67 action was mediated by reduction of eIF-2α phosphorylation levels, although p67 could not bypass the translation inhibition of a mutated eIF-2α S51D which mimics phosphoserine at residue 51. p67 levels in serum starved cells is markedly reduced by transcriptional control resulting in PKR restriction of protein synthesis (208). This group suggests that PKR and HRI activity levels are constant and modulated by p67 levels, a striking similarity to the plant p67 homolog, PKI (see before) regulation of pPKR during development (116).

p97:

IL-3 stimulation of protein synthesis in an IL-3 dependent cell line is controlled by inhibiting PKR (209). While IL-3 deprivation results in increased PKR autophosphorylation, addition of IL-3 to deprived cells causes rapid PKR and eIF-2α dephosphorylation (Fig. 5). A 97 kDa phosphotyrosine containing protein rapidly and transiently associates with PKR. Evidence that p97 mediates PKR inhibition is indirect whereby its association precedes PKR inactivation and is blocked by genistein inhibition of tyrosine kinases. p97 has not been cloned and its identity is unknown.

The studies that implicated the requirement for PKR in PDGF induction of immediate early genes (see before) also showed that this induction was blocked by activated Ras (144). When analyzed, it was found that oncogenic Ki-v-Ras would block the activity of PKR (210). Activated Ras could induce a 100 kDa inhibitor of PKR that could inhibit in trans (210) (Figure 5). The nature of this inhibitor is unknown as is whether this inhibitor is the same as the p97 inhibitor induced by IL-3.

dRF:

PKR has also been found to be activated during differentiation of preadipocyte 3T3-F442A fibroblasts (211). In non-permissive conditions for differentiation, PKR activity is significantly repressed by an inhibitor called dRF. This reversible inhibitor is a 15 kDa protein that is not a protease or phosphatase, but acts by preventing formation of PKR-dsRNA complexes and prevents ATP binding to PKR (212) (Fig. 5). Various assays also indicated that dRF did not have a direct effect on dsRNA such as degradation, unwinding or direct binding. This suggests that dRF inhibits PKR by binding it directly to inhibit activation.

TAR binding protein (TRBP):

HIV-1 Tat protein is thought to cooperate with cellular factors to bind the TAR region of HIV transcripts. Using a screening strategy to identify TAR-binding proteins in HeLa cells, the TAR binding protein (TRBP) was identified and cloned (213). This cellular factor not only activates the HIV-1 LTR synergistically with Tat, but was also found to bind the Rev-response element (RRE) of HIV-1 (214). TRBP shares significant homology to the dsRBDs of PKR, vaccinia E3L and Drosophila Staufen protein as reported by others (84,214). This study showed that TRBP inhibited PKR autophosphorylation and eIF-2α phosphorylation and enhanced expression of a translation reporter (Fig. 5). TRBP expression in HeLa cells also complemented vaccinia defective for E3L (214). It was also shown by yeast two-hybrid and far-Western blotting analysis that TRBP can homodimerize with itself and heterodimerize with PKR in a manner dependent on dsRNA (178). These data suggest that TRBP is a cellular protein that recognizes specific dsRNA structures to

mediate inhibition of PKR by insulation of such structures from PKR and stimulate translation locally. Exploited by HIV-1, it remains unknown what cellular targets TRBP might recognize and regulate. Transcripts of certain proto-oncogenes and growth factors have secondary structure that may activate PKR and elicit localized translation repression of their products. dsRNA binding proteins like TRBP may bind a subset of transcripts to allow them to evade PKR translation repression in a novel mechanism of cellular and viral regulation.

MyD116/GADD34:

As discussed in the section on HSV-1 inhibition of PKR, MyD116 and GADD34 are endogenous proteins first identified with myeloid differentiation and growth arrest and DNA damage respectively. Their homology with HSV-1 γ34.5 and rescue of γ34.5 function established that they too were capable of preventing premature protein synthesis shutoff. Like γ34.5, at least MyD116 appears to act upon recruitment of a phosphatase to dephosphorylate eIF-2α (193). This implicates the importance of regulation of translation by eIF-2α during myeloid differentiation, DNA damage and growth arrest.

Anti-viral function of PKR.

The overview given above of PKR function and regulation makes apparent one of the key roles of PKR. With induction by IFN and activation by dsRNA predominantly found during viral infection, it is clear that PKR has an anti-viral role. Although the numerous strategies used by viruses to circumvent PKR activation imply the necessity of avoiding PKR restriction, this does not constitute direct proof of PKR effect on viral replication. This

section will address the studies that demonstrate the ability of PKR to limit viral infection (see Fig. 5).

One of the early observations that tied elevated PKR levels and activation with inhibition of virus showed that HeLa cells with high PKR levels inhibited protein synthesis following reovirus infection (215). Similarly, infection with EMCV caused activation of both 2' 5' OAS and PKR with enhanced eIF-2α phosphorylation by 9 hours and increasing at 12 hours post-infection (216). Both viral and host protein synthesis was inhibited. The cloning of hPKR allowed experimental strategies that examined the effect of overexpression of PKR in NIH 3T3 cells on viral sensitivity.

Cells that stably express wild-type hPKR were infected with EMCV at low multiplicity of infection (MOI) (0.1) and virus yields assayed after 10 hours (217). Virus yields from these cells were consistently lower, between 0.94 and 2.1 log-fold decreased, compared to control cells or cells expressing inactivated hPKR. PKR expression alone confers only partial resistance to EMCV since IFN treatment caused between 1.8 to 3.2 log-fold reductions in titre. Viral infection of hPKR expressing cells also resulted in high PKR autophosphorylation and eIF-2α phosphorylation. In this study, PKR anti-viral activity was not evident against VSV. Another study however, suggests that PKR can have inhibitory effects on VSV replication (218). Using co-infection with an inducible vaccinia virus delivery vector, catalytically active PKR was shown to restrict both vaccinia and VSV protein synthesis. Such expression could not, however, inhibit poliovirus protein synthesis. This group had also shown convincing evidence that PKR expression could cause a >90% inhibition of vaccinia virus protein synthesis which correlated with PKR

autophosphorylation (219). Expression of wild-type PKR caused a 100-fold reduction in vaccinia virus yield, whereas the K296R mutated hPKR could not reduce viral replication. The anti-viral effects of PKR expression compare favorably with the protection conferred by human MxA in transgenic mice (220). Embryonic fibroblasts derived from MxA expressing mice yielded 10-40 fold less influenza A virus, 10 fold less VSV infectious particles and completely arrested Thogoto virus growth.

An interesting observation was made in the study of the mechanism of HIV-1 inhibition by PKR. The inhibition of HIV-1 by IFN and PKR was not restricted to translation control but also involved modulation of virus entry and gene expression regulation of NF-κB (221). Cells expressing dominant-negative mutants of PKR showed upregulation of CD4 surface expression and induction of NF-κB activation. Thus, it appears that wild type PKR could down regulate CD4 expression and viral gene expression in HIV-1 infection.

Anti-proliferative function of PKR.

Early in the course of this thesis work, several papers describing a novel aspect of PKR function were published. These findings supported and expanded upon the work described in Chapter 2 investigating the potential anti-proliferative properties of PKR. For the sake of completeness, the larger picture of PKR regulation of growth control elucidated since then will be addressed here.

The first indications of PKR growth regulatory role was seen in yeast. Expression of hPKR in yeast resulted in a slow growth phenotype (117). This has been corroborated by others who found that high copy level expression of hPKR or HRI in yeast caused slow

growth which was due to hyperphosphorylation of eIF-2α and abrogated by the eIF-2α S51A mutation (121).

Conversely, at the time of our own investigation, it was found that expression of catalytically inactive PKR in NIH 3T3 cells caused cellular transformation capable of forming tumors when introduced into nude mice (93,98,222). This suggested a possible tumor suppressor role for PKR (223). The PKR locus in humans has been identified at chromosome 2p21-p22 and in mouse at chromosome 17 E2 (224). Interestingly, the human locus is associated with alterations in malignant lymphomas, myelodysplastic syndromes and acute myeloid leukemias (225). Indeed, PKR is regulated by IRF-1, deletion of which in myelodysplasias is associated with marked reduction in PKR expression (226).

One hPKR variant that showed transforming ability had the conserved LFIQME residues (hPKR 361-366; Δ6 mutant, see Fig. 1) in the catalytic domain deleted which inactivated it in vitro and in whole cell extracts (98). Binding of reovirus dsRNA was shown to be normal in this variant. The second study inactivated hPKR by mutation of the subdomain II conserved Lys 296 residue (K296R mutant) in the catalytic domain which also transformed cells, albeit with an even shorter latency compared to the Δ6 mutant (1 week compared to 2-4 weeks) (222). This group also observed no alteration in eIF-2α phosphorylation in cells expressing mutated PKR. A third study demonstrated that PKR mutated within the regulatory domain by point mutations or deletion of dsRBD I such that these variants could no longer bind dsRNA and only had weak catalytic activity could also act as transdominant inhibitors to transform cells which formed tumors in nude mice (93). PKR dsRBD I mutants reduced eIF-2α phosphorylation levels markedly. Interestingly, this

group was unable to isolate cells expressing PKR dsRBD II mutants, a finding relevant to the work described in Chapter 2. Further analysis of the mechanism of transformation of the PKR K296R mutant and the dsRBD I mutant indicated that although both were transforming, they differed in molecular mechanism (227). Although both can act as dominant negative inhibitors of wild type PKR in vitro to suppress eIF-2α phosphorylation, neither did so by sequestering eIF-2α since the HRI phosphorylation of eIF-2α was unaffected. The K296R catalytic domain mutant appeared to act by sequestering dsRNA since large amounts of dsRNA could reverse its inhibition. Furthermore this mutant could reverse the inhibitory effect of dsRNA on reticulocyte translation and large amounts (10 fold excess) of the polypeptide were required to show a transdominant inhibition of wild type PKR in vitro. In contrast, the dsRBD I mutant which cannot bind dsRNA, inhibited wild type PKR at equimolar concentrations, could not reverse dsRNA inhibition of reticulocyte translation and could not be reversed in in vitro PKR inhibition by addition of more dsRNA activator. Interestingly, the biological consequences manifested in cells expressing the dsRBD I mutant include a higher growth rate and higher growth densities along with a greater suppression of eIF-2α phosphorylation (50 fold) compared to K296R mutants which did not grow at a higher rate but did show elevated growth density and a 2.5 fold decrease in eIF-2α phosphorylation (227). They concluded that dsRBD I mutated PKR acts by inhibiting transdominantly by direct interaction with endogenous PKR to form inactive heterodimers thus reducing eIF-2α phosphorylation, similar to the Δ6 mutant described above. Since the K296R mutant did not alter eIF-2α phosphorylation dramatically, it was postulated that it acts either by sequestering dsRNA activator or alternatively, acts by

inhibiting other non-translational activities of PKR such as NF-κB activation or other unknown functions. It is not clear why the retention of dsRBDs in the K296R mutant preclude it from acting transdominantly by dimerization like the dsRBD I mutant.

During the course of this thesis work, the evidence that PKR mutants may act transdominantly by heterodimerization began to be elucidated. Initially, this was shown by gel filtration analysis, whereby PKR migrated as a 140–160 kDa kinase which resolved to stoichiometric amounts of 66 kDa each indicating dimers of PKR subunits (228). Using differentially tagged PKR variants, others showed that PKR undergoes intermolecular autophosphorylation and autoactivation in a dsRNA dependent manner (229). Work presented in this thesis (see Chapter 2) and by others (178,230) indicates that PKR associates with itself, consistent with models of homodimerization. Whether PKR associates with itself by a specific dimerization motif or indirectly through common association by dsRNA binding is contested. One study that used yeast two-hybrid analysis showed PKR dimerization occurred even with mutants point mutated at K296 or deleted of the catalytic domain (178). Dimerization domains mapped to the dsRBDs whereby deletion of either domain abolished dimerization in their assays. Far-Western analysis confirmed this and also indicated dimerization required dsRNA which could either imply that dimerization is an indirect association mediated by dsRNA binding or that dsRNA is required for PKR monomer to initiate a direct protein-protein dimerization. The same dsRBDs in PKR also allow dimerization with the TRBP inhibitor of PKR which only shares dsRBD homology with PKR. This localization of the dimerization motif within the dsRBDs is at odds with the retention of ability of the dsRBD I deleted mutant described in the previous paragraph (227)

to act transdominantly and transform cells. A third group similarly localized the dimerization motif within the dsRBDs but showed that point mutations that abrogated dsRNA binding had no effect on dimerization indicating that the dimerization motif is nested within the dsRBDs (94,230). Using a translation reporter and in vitro binding assays, they suggest that dominant negative mutants of PKR all require functional dsRNA binding but not dimerization. They found evidence that the dsRBDs may have an inhibitory effect on the catalytic domain, whereby deletion of the entire amino terminal regulatory domain or binding to dsRNA allows acquisition of an active conformation to autophosphorylate in the third basic domain and phosphorylate eIF-2α. dsRNA mediated alteration of conformation allows the dimerization motif to be exposed although no clear function for this motif is given since it is not sufficient or necessary for activation in their assays. This evidence that PKR transdominant mutants required dsRNA binding but not dimerization to inhibit translation was not correspondingly tested in cellular transformation assays which is necessary to fully resolve the conflict with the dsRBD I deletion which is transforming (227).

The consequence of repressing PKR function is underscored by findings that indicate that overexpression of inhibitors of PKR can cause transformation of cells. The $P58^{IPK}$ cellular inhibitor of PKR that is used by influenza to inactivate PKR can transform NIH 3T3 cells when overexpressed in these cells (231). These cells formed tumors when introduced into nude mice with 2-3 week latency, showed decreased doubling time and demonstrated reduced PKR activity and eIF-2α phosphorylation. This suggests that $P58^{IPK}$ may act as an oncogene that acts by down regulation of PKR. Similarly, overexpression of the cellular TRBP inhibitor of PKR also caused morphological transformation with tumors arising when

these cells are introduced into nude mice (232). Contrary to expectations, this study indicates that TRBP inhibition of PKR and binding to PKR is independent of dsRNA and its dsRNA binding domains. Finally, the mechanism of transformation by PKR inactivation has been linked to eIF-2α hypophosphorylation by the finding that expression of non-phosphorylatable eIF-2α mutated at Ser 51 in mammalian cells causes morphological transformation (see Fig. 5). This clearly shows that deregulated translation initiation is tumorigenic and further reinforces the critical nature of maintaining proper control of PKR to regulate growth. This is consistent with the observation that deregulation of the other important step in translation initiation, cap binding, has serious implications. Overexpression of the cap binding protein, eIF-4E, also causes malignant transformation (233).

Apoptotic function of PKR.

In addition to the anti-proliferative effect of PKR, it has also been reported that PKR expression in HeLa cells results in arrested protein synthesis and programmed cell death or apoptosis (234). This group found that expression of the human proto-oncogene, bcl-2, could block PKR induced apoptosis but not PKR repression of translation (235) (see Fig. 5). Bcl-2 expression did not inhibit the ability of PKR to phosphorylate eIF-2α and apparently does not block translation inhibition by PKR. PKR induction of apoptosis did not require dsRBD II but required the basic third domain (hPKR residues 234-272 spanning the autophosphorylation sites, see Fig. 1) and resulted in cleavage of the death substrate poly(ADP-ribose) polymerase (PARP). Thus PKR activation initiates apoptosis upstream

of bcl-2 and activates the cellular protease CPP32 (caspase3) or related interleukin-β converting enzyme (ICE) proteases to cleave PARP. Catalytically inactive PKR expression does not induce apoptosis or PARP cleavage indicating the requirement for functional PKR. Bcl-2 expression blocked PARP cleavage showing that it inhibits early events of apoptosis before proteolytic cascade activation. Another group documented that anti-sense ablation of PKR in U937 cells resulted in resistance to tumor necrosis factor α (TNFα) induced apoptosis (236). Data from the PKR knockout mouse targeted at the amino terminal region indicates that MEFs from these animals are impaired in triggering apoptosis in response to TNFα, dsRNA and lipopolysaccharide (LPS) (237). This appears to be due to defects in activation of IRF-1 and induction of Fas mRNA. These data indicate that PKR is required for apoptotic response to certain stimuli and is a candidate death gene.

The relevance of these observations of PKR having a role in apoptosis becomes clear in two studies that document viral triggers of apoptosis. The first study addressed the mechanism of apoptosis caused in HeLa cells by influenza infection (238). This cell death was preceded by upregulation and activation of the Fas gene which could also be induced by dsRNA and blocked by the PKR inhibitor, 2-AP. This group showed that expression of the catalytically inactive K296R PKR mutant prevented Fas elevation and influenza mediated apoptosis. In vaccinia infection of cells, apoptosis is triggered by dsRNA, a response that is averted by the E3L dsRNA binding protein (239). Vaccinia lacking E3L induces apoptosis in HeLa cells unlike wild type virus. The domains of E3L necessary to prevent cell death map to the dsRNA binding domain of E3L. A temperature sensitive mutant of vaccinia that produces excess dsRNA also induces apoptosis at the restrictive

temperature. Finally, direct transfection of dsRNA into these cells indicates that dsRNA is sufficient to trigger apoptosis. The functional K3L gene in vaccinia deleted for E3L does not appear to be able to rescue the apoptotic phenotype of this virus. Its not clear whether inhibition of eIF-2α phosphorylation by K3L is not sufficient or overcome in E3L deleted virus infected cells. Numerous other examples of viruses triggering apoptosis or encoding inhibitors of apoptosis are known and will be discussed further in Chapter 3. The above studies indicate that PKR may perform its anti-viral function by triggering programmed cell death. This is consistent with its anti-proliferative function and the transforming potential it exhibits when inactivated.

THESIS WORK

A large body of work has contributed to our understanding of the function of the IFN-induced, dsRNA activated protein kinase, PKR. Activated by viral and perhaps cellular RNA moieties and impinging upon translation and transcription regulation, PKR is believed to have an important role to play in anti-viral responses and growth control (Fig. 5). The main objective of the work presented in this thesis was to elucidate the effects upon normal cellular function when PKR is lost or inactivated.

Chapter 2 is centered on the identification and analysis of a naturally occurring mutation of PKR in a lymphocytic leukemia cell. This mutation affects only one allele that is a putative transdominant mutation. This section deals with the test of the hypothesis that the PKR^{del} mutation in this lymphocytic leukemia cell encodes an inactive polypeptide that

acts as a dominant negative inhibitor of PKR to contribute to morphological transformation. The basis of transdominant alleles of PKR is also examined.

Chapter 3 will deal with the organismal approach taken to PKR function in the mouse. The hypothesis that PKR function is critical to anti-proliferative, apoptotic and anti-viral response by regulation of translation or signaling pathways is tested. This was done by examining the consequence of genetic ablation of PKR in the mouse by targeted disruption of the PKR locus at the catalytic domain. Before completion of this work, others had reported targeted disruption of PKR at the amino terminal region. Comparison of data obtained from both these mouse models of PKR ablation provides some new insights on PKR.

Finally, Chapter 4 concludes with a general discussion of the interpretation of my findings with suggestions of experiments that would address the questions that have arisen from this work.

CHAPTER 2

The murine PKR tumor suppressor gene is rearranged in a lymphocytic leukemia.

SUMMARY

The double-stranded RNA-dependent kinase, PKR, is encoded by an interferon inducible gene and is largely responsible for the anti-viral effects of this cytokine. Recent studies have shown that PKR may also play a role in the regulation of normal cellular growth. Although numerous examples of viral strategies for inactivation of PKR exist, there is no evidence of PKR inactivation in tumours. We demonstrate here that the Tik gene, which encodes a dual-specificity kinase is the murine homolog of PKR, the dsRNA-dependent kinase, and has undergone a rearrangement in a murine lymphocytic leukemia cell. We have cloned a cDNA that corresponds to a mutated transcript from the rearranged mPKR gene and show that while the mutated polypeptide retains its ability to dimerize and bind dsRNA, it is catalytically inactive. The possibility that this mutation may act as a dominant negative suppressor of PKR function was examined.

INTRODUCTION

The double-stranded RNA (dsRNA)-activated protein kinase (PKR, p68 kinase, DAI, dsI) is an interferon-inducible phosphoprotein of 68 and 65 kDa in human and mouse cells respectively (55,146,240). PKR has been well-characterized as a critical effector in the host cell antiviral response (184,186,195,215,216,219). It is a serine-threonine protein kinase that is normally found latent in association with ribosomes (146,241). In the presence of dsRNA, PKR undergoes autophosphorylation followed by phosphorylation of its specific substrate, the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) (49,53,60,169). During the process of translation initiation, availability of eIF-2 for ternary complex formation with GTP and initiator Met-tRNA is decreased upon phosphorylation of the α subunit. PKR

phosphorylation of eIF-2α stabilizes the GDP-eIF-2α complex, preventing GTP exchange and subsequent reinitiation of translation (123,242). PKR activation may therefore result in either global inhibition of protein synthesis (164,243) or selective translation of certain classes of mRNA (244-247).

In addition to its anti-viral effects, PKR has been implicated in the anti-proliferative and anti-tumour activities of IFN (146,223,248). In various eukaryotic cells, PKR exerts a growth regulatory effect. In yeast, overexpression of human PKR results in inhibition of polypeptide chain initiation and a marked decrease in cellular proliferation (117). Growth inhibition required PKR catalytic activity and was reversed by co-transfection of either a non-phosphorylatable mutant of eIF-2α or the amino terminal dsRNA-binding domain of PKR. Overexpression of catalytically inactive PKR causes morphological transformation, faster doubling time, higher saturation density and anchorage-independent growth of NIH 3T3 cells (98). In addition, cells expressing mutated PKR cDNAs (Δ6, K296R and ΔdsRBD I PKR) formed tumours in nude mice (93,98,222).

The human PKR gene is located on the short arm of chromosome 2 (224). This locus is associated with abnormalities in myelodysplastic syndromes, malignant lymphomas and acute myeloid leukemias (225). This chapter documents that the Tik kinase (42) is the murine homolog of PKR and is mutated in a murine lymphocytic leukemia cell line. In keeping with accepted convention (44), Tik will be referred to as murine PKR (mPKR). One allele of mPKR appears to have undergone a rearrangement capable of producing an inactive polypeptide. To our knowledge, this is the first documentation of PKR rearrangement in tumours or cell lines.

MATERIALS AND METHODS

Cell culture and IFN induction.

70Z/3, L1210 and COS-1 cells were maintained in a minimal essential medium (MEM) supplemented with 10% calf serum. Murine α and β IFN (Cytimmune) was obtained from Lee Biomolecular Research Laboratories. IFN treatment was routinely performed with 5 x 10^6 cells in 10 ml culture medium at 100 U/ml for 16 hours.

Construction of cDNA expression vectors and tagged proteins.

The inducible bacterial expression vector pET11 was used to express mPKR and human PKR in BL21 bacteria as described before (42,249). mPKR$^-$ and hPKR$^-$ denote catalytic subdomain II phosphotransfer mutants of murine PKR (270L271I) and human PKR (295R). The mPKR$^-$ mutated cDNA was generated by oligonucleotide-directed PCR mutagenesis using the pET11 mPKR$^+$ plasmid and the following 21-mer oligonucleotides: ATTCGCGTTAAATATAACACG and TAAAGCGTATCTCTTTCCATC. Generation of the hPKR$^-$ mutated cDNA has been described before (250).

Normal and mutant mPKR polypeptides were tagged with a six-repeat decameric human Myc epitope at the amino-terminal ends by subcloning the blunted wild-type mPKR and mPKR^{del} cDNAs (Bam H1/Ssp 1 and Bam H1/Sph 1 fragments respectively) into the Sma 1 and blunted Spe 1 sites of the BS KS+ MT6 vector (251). The Myc epitope tag (denoted as M-) was confirmed to be in-frame with the mPKR cDNAs by sequencing. Sal 1/Xba 1 fragments from these constructs bearing the tagged cDNAs were subcloned into the Sal 1/Xba 1 sites of the Pece expression vector (252) to generate Pece M-mPKR and Pece M-mPKR^{del}. The Pece II bicistronic expression vectors were made by removal of the

Bcl1/BamH1fragment encoding the SV40 polyA tail and replacing it with a BamH1 IRES Hyg/TK (internal ribosome entry site and Hygromycin phosphotransferase-Thymidine kinase fusion selectable marker) fragment in all the above plasmids. The Myc epitope is immunoreactive with the mAb 9E10 in immunoprecipitation and immunoblot analysis (251).

Cell extract kinase assays and immunecomplex kinase assays.

Cell extract kinase assays from untreated or IFN treated cells or alternately, transient or stable transfected cells were performed essentially as described before (184). Briefly, cells were lysed in hypotonic lysis buffer (10 mM HEPES (pH 7.5), 10 mM potassium acetate, 1.5 mM magnesium acetate) supplemented with protease and phosphatase inhibitors (2 mM sodium fluoride, 2 mM sodium pyrophosphate, 200 μg/ml PMSF, 500 μM ammonium vanadate, 2 μg/ml aprotinin and 5 μg/ml leupeptin) and cleared by centrifugation at 10,000g for 5 minutes. The protein concentration of the supernatant fraction (S10) was measured by Bio-Rad protein determination assay. 25 μg of whole S10 extracts were incubated with reaction mixes consisting of 2x kinase buffer (40 mM Hepes (pH 7.5), 100 mM potassium chloride, 4 mM magnesium chloride, 4 mM manganese chloride, 4 μM ATP, 10 mM β-mercaptoethanol) with 10 μCi (γ-^{32}P) ATP (Amersham) in the absence or presence of activating levels of reovirus dsRNA (kindly provided by G. Wilson, Harvard Medical School) as indicated for 30 minutes at 25°C. Reactions were arrested with either 2x Laemmli's sample buffer and boiling or terminated with 5 mM EDTA and diluted in Lysis buffer (10 mM Tris (pH 7.5), 150 mM sodium chloride, 5 mM EDTA, 1% Triton X-100) prior to immunoprecipitation. mPKR immunoprecipitation was performed with mPKR-specific rabbit polyclonal antibodies raised against either a multiple antigen peptide (99)

corresponding to mPKR amino acid residues 101-114 (anti-TikMAP2) or purified His-tagged mPKR fusion protein (Qiagen) bearing residues 1-98 (anti-Tik100). This was done by incubation with the anti-mPKR antibody at 1:50 dilution, followed by Protein A-Sepharose. Immunecomplexes were washed four times with Lysis buffer with 500 μM ammonium vanadate. mPKR polypeptide was eluted with Laemmli's sample buffer with β-mercaptoethanol.

Immunecomplex kinase assays were performed by recovery of mPKR from 200 μg of cell extracts prepared as above. Washes were as above followed by one wash with kinase buffer. Reactions were then initiated by addition of reaction mixes of kinase buffer (without unlabelled ATP) with 10 μCi (γ-^{32}P) ATP in the absence or presence of 10 μg/ml reovirus dsRNA as indicated. Reactions were performed at 25°C for 30 minutes, then arrested and protein eluted in sample buffer with 10% β-mercaptoethanol followed by boiling.

Anti-hPKR, anti-phosphotyrosine and anti-Myc immunoblot analysis; dsRNA binding assay.

Lysates from IPTG-induced BL21 bacteria (42,249) were made by resuspension of bacteria in sample buffer, sonication, then supplementing with β-mercaptoethanol. Mammalian cells extracts were derived as for protein kinase assays. Equal amounts of total protein (100 μg) were prepared by addition of equal volume of 2x sample buffer with β-mercaptoethanol. Samples were boiled then resolved on 8% SDS-PAGE and transferred onto nitrocellulose. Blots were blocked in 5% Albumin (Fraction V) in TBST (150 mM sodium chloride, 10 mM Tris (pH 7.5), 0.05% Tween-20).

Anti-hPKR immunoblotting was performed using a rabbit polyclonal antibody raised against purified human PKR (253) at 1:600. Anti-phosphotyrosine immunoblot analysis was done with a mouse monoclonal anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology Inc.) at 1:1000. Ammonium sulfate precipitated culture supernatant of the 9E10 hybridoma was used at 1:10 for anti-Myc immunoblotting. Blots were visualized with I^{125} Protein A or I^{125} sheep anti-mouse for the anti-hPKR and anti-phosphotyrosine analysis respectively followed by autoradiography. Alternatively, anti-hPKR and anti-Myc immunoblots were visualized with horseradish peroxidase conjugated goat anti-rabbit or mouse respectively followed by enhanced chemiluminescence (Amersham) and exposure with Hyperfilm-ECL (Amersham). Binding of PKR to dsRNA was assessed by incubating lysates from transient expressors of M-mPKR and M-mPKR^{del} with poly (I) poly (C) agarose and incubating while mixing for 1.5 hours. Bound polypeptides were washed 4 times with Lysis Buffer then eluted, resolved by SDS-PAGE and visualized by anti-Myc immunoblot.

Northern blots.

Total RNA was prepared from cell lines and tissue and poly A^+ RNA was selected using oligo (dT)-cellulose columns. 2.5 μg of poly A^+ RNA were resolved by formaldehyde-agarose gel electrophoresis, blotted onto Hybond membrane (Amersham), ultraviolet cross-linked and hybridized according to manufacturer's protocols. Blots were washed at high stringency followed by autoradiography. Probe B, the full-length mPKR cDNA probe was obtained by ^{32}P-random-primer labelling of the cDNA. Probe A was generated by random-primer labelling of DNA amplified from mPKR cDNA using the polymerase chain reaction

(PCR) with the primers 5'-CAGACAATGTATGGT-3' and 5'-ATCGACTTGTATCAC-3'. α-tubulin cDNA probe hybridization was used to normalize mRNA loading.

Cloning of mutant cDNA corresponding to aberrant transcripts.

L1210 mRNA was fractionated on a 5-20% sucrose gradient in 70% formamide. After centrifugation at 36000g for 20 hours, fractions were collected and analysed by Northern blotting. Fractions containing 0.5 to 2.0 kb mRNA and which hybridized with the mPKR cDNA probe were used to synthesize an L1210 cDNA library using a cDNA synthesis kit (BRL). The cDNA library was linkered and subcloned into λgt10 phage DNA. Following packaging, recombinant phage was used to infect E. coli C600 hfl bacteria which were screened by plaque filter hybridization. The $mPKR^{del}$ cDNA was cloned from this library by screening with the full-length mPKR cDNA probe.

Genomic Southern analysis.

Genomic DNA was prepared from 70Z/3 and L1210 cells by standard protocols (254). 10 μg of genomic DNA was restricted as indicated and resolved by gel electrophoresis in 0.8% agarose. Gels were treated, transferred to Hybond membranes (Amersham) and hybridized with mPKR cDNA following the manufacturer's protocols. Washes were performed with 0.1x SSC, 0.1% SDS at 42°C followed by autoradiography.

COS-1 transient transfection by electroporation.

Trypsinized COS-1 cells were counted, washed and resuspended in serum-free alpha MEM at 2×10^6 cells in 0.5 ml. 15 μg of the appropriate expression vector DNA were added as indicated. The cells were transferred to 0.4 cm electroporation cuvettes and chilled on ice for 10 minutes. Electroporation was performed on a Gene Pulser apparatus (Bio-Rad) at 220

volts and 960 μF. Cells were left at 25°C for 15 minutes then plated in 10 ml alpha MEM with 10% calf serum. Cells were harvested 24 hours later.

Reticulocyte co-translation and co-immunoprecipitation analysis.

In vitro synthesis of the respective cDNA transcripts was performed with linearized plasmid and either T7 (pET11 series plasmids) or T3 (Bluescript KS series plasmids) RNA Polymerase following the manufacturer's protocol (Promega). RNA was DNAse treated, purified and passed through a G-50 column, precipitated and quantified. 5 μg of RNA was heat denatured and used in reticulocyte extract translations as indicated with ^{35}S-Methionine as a label. Aliquots were saved for analysis and the remainder diluted in Lysis Buffer and immunprecipitated with the anti-Myc 9E10 antibody against the epitope tag used. After recovery and washing five times with Lysis Buffer, samples were eluted and resolved on 10 % SDS-PAGE, enhanced and exposed by autoradiography.

Stable transfection of L cells or NIH 3T3 cells by electroporation.

1 x 10^7 L cells in 1 ml were electroporated as above in the presence of 50 μg of the appropriate linearized Pece expression vector with 5 μg linearized pSV2 Neo selection marker plasmid. After electroporation at 450 volts and 250 μF, cells were plated immediately into culture medium. 24 hours later, one-tenth of cells were re-plated into medium with 750 μg/ml Geneticin (GIBCO). Neomycin^R colonies were picked and screened for stable expression of mPKR by immunoblot analysis. NIH 3T3 stable transfection was performed as above using tagged PKR variants in the Pece II bicistronic expression vector that was linearized. Cells were selected in 400 μg/ml Hygromycin and Hygromycin^R colonies were picked and screened as above.

Growth rate and anchorage independent growth assays.

Doubling time of various NIH 3T3 clones was determined by plating 1 x 10^6 cells in 100mm dishes and performing cell counts exactly 3 days later and calculating the rate at which these cells were doubling [$t_D = \log(2N_0/N_t)t$] (t_D, doubling time; N_0, number of cells plated; N_t, number of cells at time, t; t, time since plating in hours).

eIF-2α phosphorylation in cells.

Lysates from various cells were assayed for eIF-2α phosphorylation in the presence of PKR activators as described previously (255). In brief, following immunoprecipitation of PKR from various cell lysates, kinase reactions were set up with or without pIC or heparin activators and purified eIF-2α. After the reaction was completed as before, samples were eluted, resolved by SDS-PAGE and eIF-2α phosphorylation quantified by scintillation counting.

RESULTS

Tik is the murine homolog of the double stranded RNA-dependent kinase, PKR.

Using an antibody to phosphotyrosine, we have cloned a cDNA encoding a protein kinase called Tik which shows significant sequence homology to the human double stranded RNA (dsRNA)-dependent serine/threonine protein kinase (PKR) (42,77,78,80). We performed a biochemical analysis of the Tik kinase to confirm that it had the properties of the murine PKR. A remarkable feature of the dsRNA-dependent kinase is that autophosphorylation of this enzyme can be detected in total cell extracts, without requiring prior immunoprecipitation (see Materials and Methods). In this assay (referred to in the following as a cell extract kinase assay) PKR is visualized as a 65/68 kDa phosphoprotein

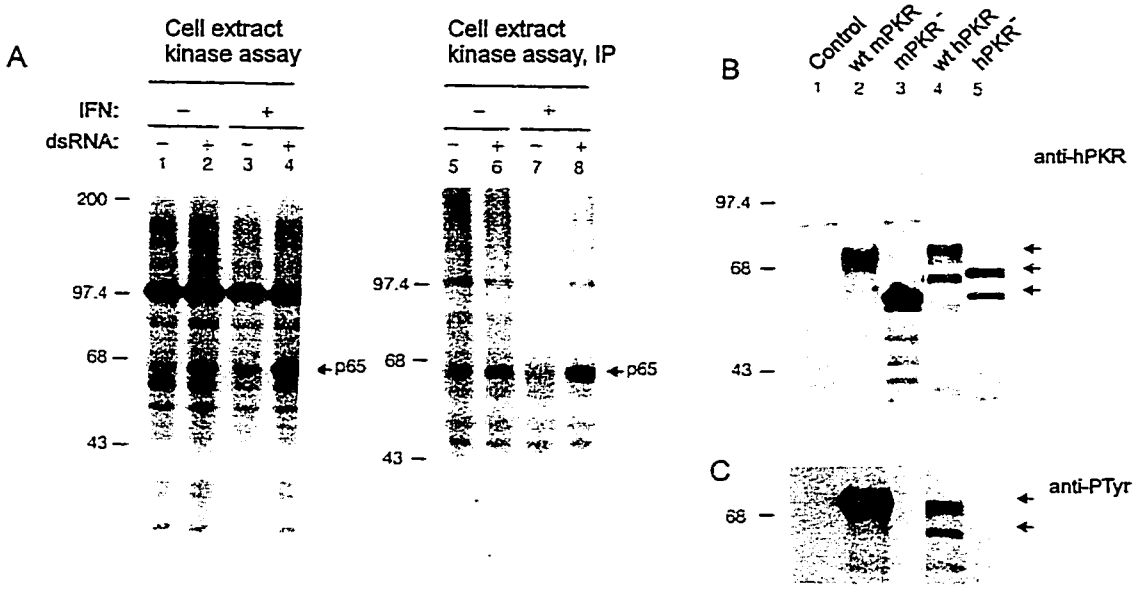
(184). In mouse, there is a 65 kDa protein kinase which is readily detected in the cell extract kinase assay (Fig. 1A, lanes 1-4, ref.(240,256)). The expression of the 65 kDa phosphoprotein is markedly elevated in extracts from IFN-treated cells (lanes 3 and 4) and appears to have greater activity in the presence of dsRNA. An antibody raised against a Tik specific synthetic peptide immunoprecipitates the 65 kDa interferon-inducible, dsRNA-activated kinase (Fig. 1A, lanes 5-8). When expressed in bacteria, the Tik polypeptide is detected with an antibody raised against human PKR (Fig. 1B, lanes 2-5). Bacterially expressed Tik kinase has been shown to be immunoreactive with antibodies to phosphotyrosine (42) (Fig. 1C, lane 2). This property is also a feature of human PKR when it is expressed in bacteria (Fig. 1C, lane 4). The phosphotyrosine epitope is dependent on the catalytic activity of these protein kinases as phosphotransfer mutants are not detected with the antibody to phosphotyrosine (lanes 3 and 5). The difference in electrophoretic mobility between the phosphotransfer mutated polypeptides and the wt Tik and PKR proteins is most likely a phosphorylation dependent phenomenon, as has been observed for the wee1$^-$ kinase (257), and is consistent with the activation-dependent shift in PKR/mPKR mobility observed in mammalian cells ((54), Fig. 8A). These results taken together with the phosphoserine and phosphothreonine content of PKR (42, 49, 51-54) demonstrate that both human PKR and Tik fall into one of the subgroups of the newly described family of dual-specificity kinases (14).

In human cells, interferon treatment results in transcriptional enhancement of the PKR gene (55,77). We have found that the expression of Tik transcripts in the pre-B cell line 70Z/3 is induced several fold following interferon treatment (Fig. 2A). Untreated 70Z/3

Figure 1. Tik is the 65 kDa murine homolog of the dsRNA-dependent protein kinase, PKR.

(A) Immunoprecipitation of the murine p65 dsRNA-dependent protein kinase by anti-Tik antibodies. 70Z/3 cells were untreated or treated with 100 U/ml murine α and β IFN. Cell extract kinase assays were performed for dsRNA-dependent protein kinase autophosphorylation activity as described in Materials and Methods. Lanes: 1-4, cell extract kinase reactions; 5-8, immunoprecipitation of parallel reactions with a Tik-specific rabbit polyclonal antibody (anti-TikMAP2); 1, 3, 5, 7, reactions performed without dsRNA; 2, 4, 6, 8, reactions performed in the presence of 10 μg/ml reovirus dsRNA. The position of the p65 kinase is indicated. The positions of relative molecular mass markers in kDa are indicated on the left. Exposure, 1.5 h (left panel) and 10 h (right panel). (B) Anti-hPKR immunoblot. Lysates from IPTG-induced BL21 bacterial host transformed with pET11 mPKR and hPKR expression constructs were assessed for immunoreactivity to a rabbit polyclonal antibody directed against purified human hPKR (253) by Western analysis using ^{125}I-Protein A for visualization. mPKR$^-$ and hPKR$^-$ denote phosphotransfer mutants of mPKR and hPKR. Lanes: 1, untransformed BL21 host; 2, wild-type mPKR; 3, mPKR$^-$; 4, wild-type hPKR; 5, hPKR$^-$. The positions of mPKR and hPKR are indicated by arrows. The positions of relative molecular mass markers in kDa are indicated on the left. Exposure, 5 h. (C) Anti-phosphotyrosine immunoblot. Activity-dependent immunoreactivity of mPKR and hPKR with anti-phosphotyrosine. Identical lysates as in (B) were blotted with the 4G10 monoclonal anti-phosphotyrosine antibody and ^{125}I sheep anti-mouse. mPKR and hPKR are indicated by arrows. Exposure, 5 h.

Figure 1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Cell extract kinase assay
Cell extract kinase assay, IP
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dsRNA:
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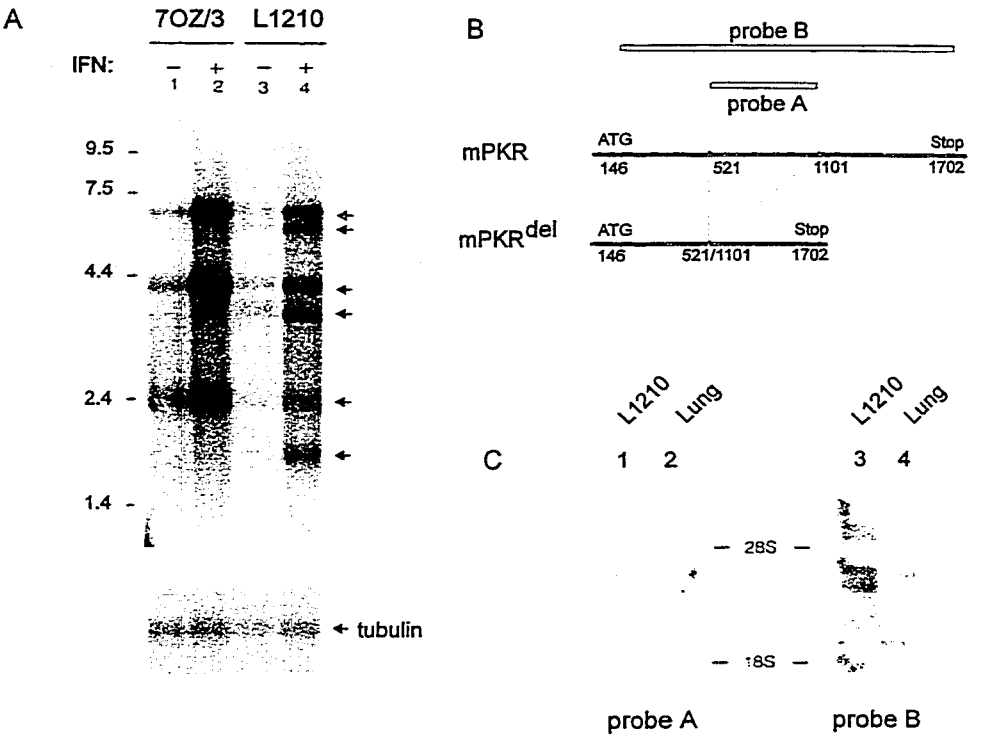
5 6 7 8
200
97.4
68
43
p65
B
Control
wt mPKR
mPKR⁻
wt hPKR
hPKR⁻
1 2 3 4 5
anti-hPKR
C
anti-PTyr

Figure 2. mPKR expression is IFN-inducible in 70Z/3 and L1210 cells.

(A) Northern analysis. Cells were treated or untreated with 100 U/ml IFN as indicated. Poly A^+ RNA purified from these cells show IFN-inducible mPKR transcripts. The leukemic cell line, L1210, demonstrates additional transcripts of altered size (indicated by arrows). Upper panel: Hybridization with full length mPKR cDNA probe. Lower panel: Loading normalization with α-tubulin probe. Molecular weight markers in kb indicated on the left. Exposure, 48 h (upper panel) and 6 h (lower panel). (B) Schematic diagram depicting normal mPKR cDNA (42) and a truncated cDNA, $mPKR^{del}$, cloned from an L1210 cDNA library as described in Materials and Methods. The truncated cDNA bears an in-frame deletion spanning the indicated nucleotides, including the conserved subdomain II lysine residue codon. Probe A: PCR-generated probe corresponding to deleted portion in $mPKR^{del}$; Probe B: full length mPKR cDNA probe. (C) Aberrant L1210 mPKR transcripts bear internal deletion identified in $mPKR^{del}$. Northern blot of poly A^+ RNA from L1210 and murine lung was probed with probe A (left panel), then re-probed with probe B (right panel). The three truncated mPKR transcripts in L1210 seen with the full length probe B, show no hybridization with probe B suggesting deletion of this region. Normal mPKR transcripts from lung show no difference in hybridization to the two probes. Exposure, 24 h (both panels).

Figure 2


A
7OZ/3
L1210
IFN:
– + – +
1 2 3 4
9.5
7.5
4.4
2.4
1.4
tubulin
B
probe B
probe A
mPKR
ATG
Stop
146
521
1101
1702
mPKR del
ATG
Stop
146
521/1101
1702
C
L1210
Lung
L1210
Lung
1 2 3 4
28S
18S
probe A
probe B

cells showed the normal pattern of three Tik transcripts as previously described (42) and IFN treatment resulted in enhanced accumulation of all three transcripts (Fig. 2A, lanes 1 and 2). Thus like the PKR gene, transcripts of the Tik gene are IFN-inducible. The data presented above, taken together with the previously reported homology between Tik and human PKR (62% identity and 75% similarity at the amino acid level,(77-80)) and syntenic chromosomal localization provides strong evidence that Tik is the murine IFN-inducible dsRNA-dependent kinase. We will refer to Tik as murine PKR (mPKR) from here on. Furthermore, both murine PKR and human PKR autophosphorylate on tyrosine residues when expressed in bacteria.

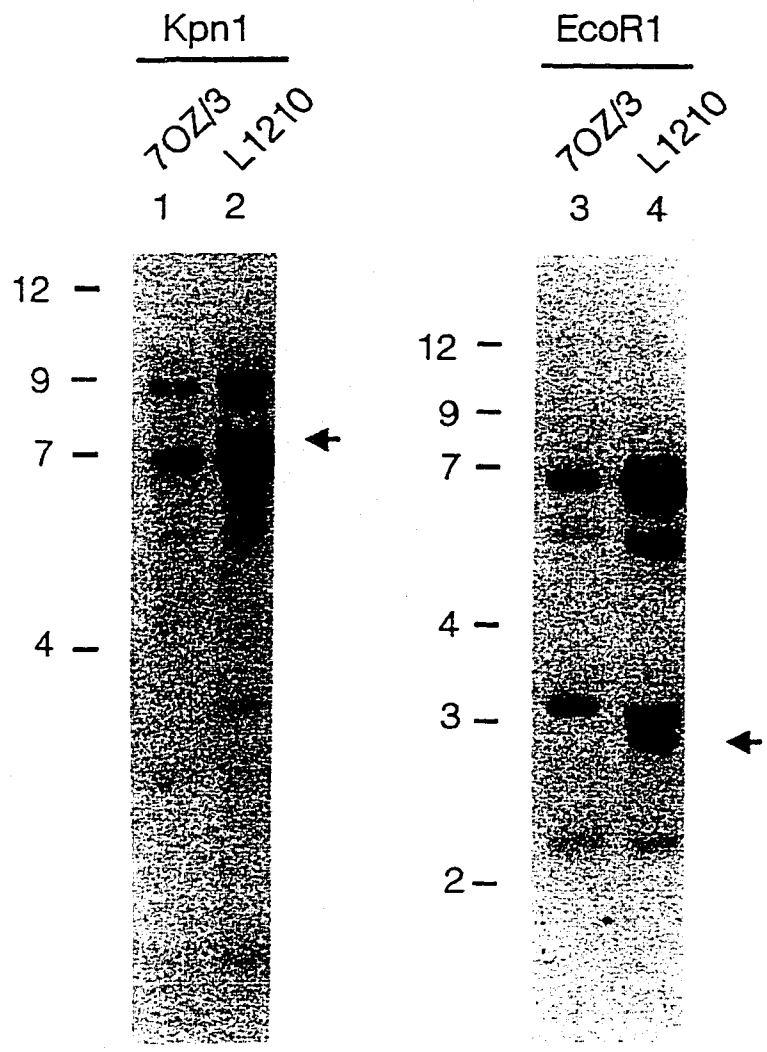
Rearrangement of a mPKR allele in a lymphocytic leukemia cell produces transcripts encoding an inactive mutant polypeptide.

In Northern analysis of various tumour cell lines, we observed that the lymphocytic leukemia cell line L1210, had an abnormal pattern of mPKR transcripts. L1210 cells express not only the wild-type mPKR mRNA species but three additional transcripts (Fig. 2A, lanes 3 and 4). Each of the novel transcripts differs from the normal mPKR mRNAs by approximately 500 base pairs, suggesting an alteration has occurred in one of the mPKR alleles in L1210 resulting in the formation of truncated gene products. To test this idea, genomic DNA from 70Z/3 and L1210 cells (both of these lines were derived from DBA mice) was subjected to Southern blot analysis. When restricted with Kpn1 or EcoR1, DNA from L1210 cells show unique bands (indicated by arrows) which hybridize with a full length cDNA probe (Fig. 3, lanes 2 and 4), in addition to restriction fragments common to 70Z/3 DNA (Fig. 3, lanes 1 and 3) indicating genomic rearrangement in one allele.

Figure 3. Genomic rearrangement of a mPKR allele in L1210 leukemic cells.

Comparison of 70Z/3 and L1210 genomic DNA (both derived from DBA mice) indicates one mPKR allele is rearranged resulting in the appearance of additional bands (marked with arrows) for the restriction digests indicated. Probe used was full length mPKR cDNA. Molecular weight markers in kb indicated on the left. Lanes: 1 and 3, 70Z/3 DNA; 2 and 4, L1210 DNA. Exposure, 7 d.

Figure 3



To determine the nature of the mRNA transcripts produced by the rearranged mPKR allele, a cDNA library generated from size-fractionated L1210 mRNA was screened with a mPKR probe. One clone, called mPKR^{del}, was isolated from this library and found to encode a mutant mPKR cDNA which has a 579 bp in-frame deletion (Fig. 2B) extending from position 521 to 1101 (corresponding to Arg-125 and Tyr-319). This deletion spans the region encoding the dsRBD II domain and Lys-271, the highly conserved lysine residue within the catalytic subdomain II (12) critical in the phosphate transfer reaction (Figure 4). Northern blot analysis of L1210 mRNA using a probe of 550 bp corresponding to this deletion (probe A, Fig. 2B) detects the normal three PKR mRNA species but fails to detect the three mutant transcripts (Fig. 2C, lane 1). When this blot is probed with a full length murine mPKR cDNA (probe B, Fig. 2B), all six L1210 mRNA species are observed (Fig. 2C, lane 3). This analysis proves that the mPKR^{del} cDNA represents a truncated transcript encoded by the rearranged allele. One explanation for the aberrant transcripts is that a genomic alteration affects the splicing of the transcript produced by the rearranged mPKR allele. In favour of this idea, we have sequenced mPKR genomic clones and identified an intron-exon boundary at position 1101 (data not shown).

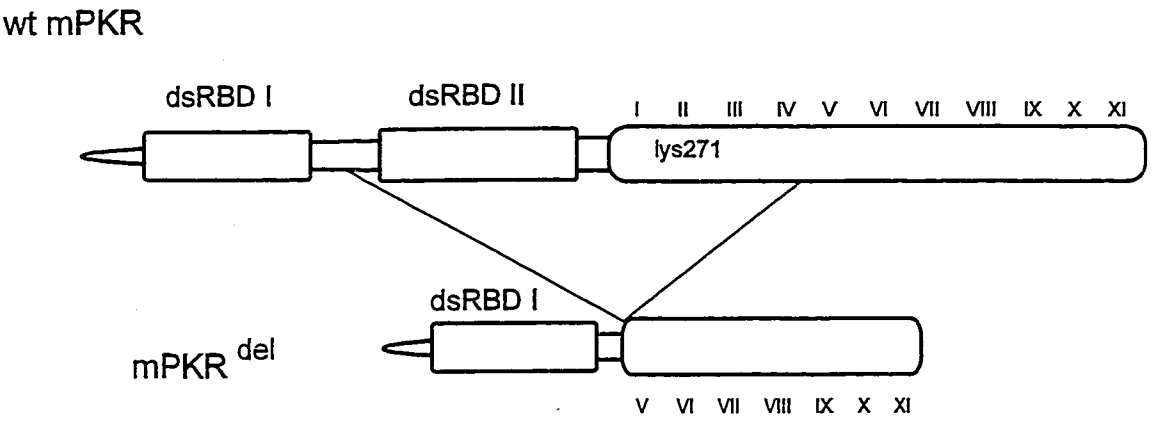
Mutated mPKR^{del} polypeptide forms heterodimers with normal PKR.

The mPKR^{del} cDNA is predicted to encode a 43 kDa truncated polypeptide which lacks an ATP binding fold but still retains part of the catalytic domain and one of the dsRNA-binding domains (Fig. 4, (78,84,85,250)). Synthetic transcripts prepared from the mPKR^{del} cDNA direct the synthesis of a 43 kDa polypeptide in rabbit reticulocyte lysates (data not shown) showing it retained an open reading frame. Anti-hPKR immunoblotting

Figure 4. Schematic representation of the predicted polypeptide, mPKR^{del}.

L1210 cells encode both normal and aberrant transcripts that would encode the indicated polypeptides with the sequence motifs depicted. Not drawn to scale.

Figure 4


wt mPKR
dsRBD I
dsRBD II
I II III IV V VI VII VIII IX X XI
lys271
dsRBD I
mPKR del
V VI VII VIII IX X XI

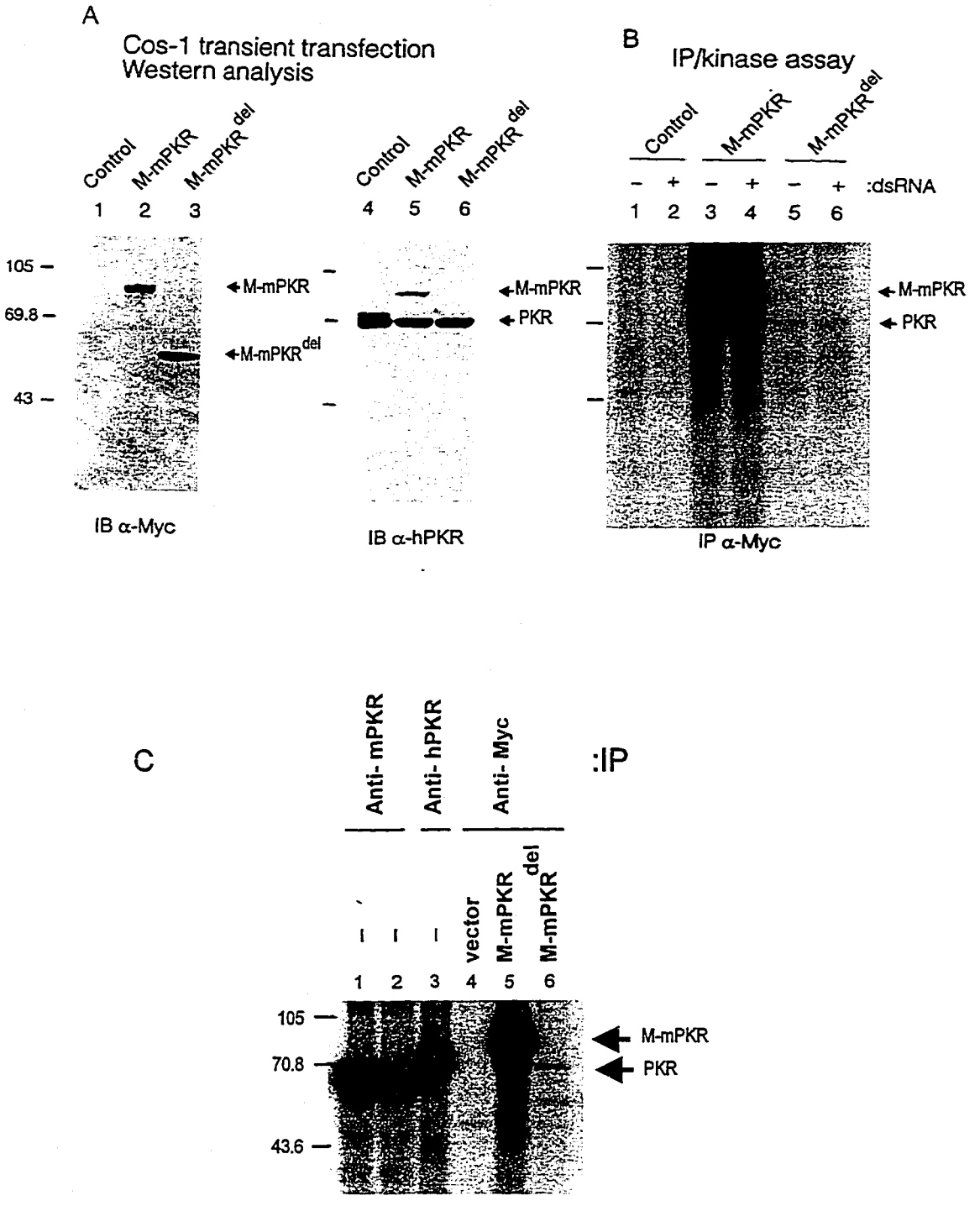
of L1210 lysates, however, fail to show any evidence of the mutated mPKR^{del} polypeptide (Fig. 8C, lanes 3 and 4). We therefore tagged the mPKR^{del} polypeptide with a human Myc epitope and expressed it by transient transfection in COS-1 cells. The Myc epitope tag adds 100 amino acids to the amino-terminal end, thus causing an increase in molecular mass of 12 kDa. COS-1 cells transfected with the PECE vector alone show no proteins detectable by anti-Myc immunoblotting (Fig. 5A, lane 1). Transfection with PECE vectors bearing Myc-tagged mPKR and mPKR^{del} cDNAs demonstrates anti-Myc immunoreactive proteins of approximately 77 kDa and 55 kDa consistent with the tagged normal and mutated mPKR polypeptides respectively (Fig. 5A, lane 2 and 3). When parallel lysates of these transfected cells are blotted with the anti-hPKR antibody, the M-mPKR protein of 77 kDa is specifically detected (Fig. 5A, lane 5) along with the endogenous monkey PKR at 68 kDa (lane 4). Interestingly, the 55 kDa M-mPKR^{del} polypeptide that reacts with the anti-Myc antibody (Fig.5A, lane 3) is not immmunoreactive with this anti-hPKR antibody (lane 6). Since the anti-hPKR polyclonal antibody is immunoreactive with the normal monkey and mouse PKR proteins, it appeared that the failure to detect the 43 kDa mPKR^{del} polypeptide in L1210 lysates by immunoblotting was perhaps due to deletion of epitopes recognized by the anti-hPKR serum.

Anti-Myc immunocomplex kinase assays of transiently transfected COS-1 cells were performed to assess the catalytic activity of normal and mutated mPKR proteins. Cells transfected with the M-mPKR plasmid express a 77 kDa protein kinase activity (Fig. 5B, lanes 3 and 4). We have previously found that immunoprecipitation of mPKR with antibodies to the amino-terminal domain maximally activates the kinase even in the absence

Figure 5. Translation and catalytic activity analysis of tagged mPKR and mPKR^{del} polypeptides.

(A) Immunoblot analysis of COS-1 transient transfectants. Expression vectors bearing Myc epitope-tagged mPKR and mPKR^{del} cDNAs were transiently transfected into COS-1 cells and harvested in Lysis buffer. Immunoblotting and visualization by enhanced chemiluminescence were as described in Materials and Methods. Endogenous PKR , M-mPKR and M-mPKR^{del} denoted on the right. Relative molecular mass markers in kDa are shown on the left. Left panel: anti-Myc immunoblot, right panel: anti-hPKR (253) immunoblot. Lanes: 1, 4, control transfection with Pece vector; 2, 5, Pece M-mPKR transfection; 3, 6, Pece M-mPKR^{del} transfection. Exposure, 30s (left panel) and 15 minutes (right panel). (B) Immunecomplex kinase assays of COS-1 transient transfectants. Parallel lysates of transfectants from (A) were immunoprecipitated with anti-Myc to recover tagged M-mPKR and M-mPKR^{del} and reactions performed as in Materials and Methods. Lanes: 1, 2, control transfection with Pece vector; 3, 4, Pece M-mPKR transfection; 5, 6, Pece M-mPKR^{del} transfection; 1, 3, 5, reactions in the absence of dsRNA; 2, 4, 6, reactions with 10 μg/ml reovirus dsRNA. M-mPKR and co-immunoprecipitated endogenous PKR indicated on the right. Relative molecular mass markers kDa are shown on the left. Exposure, 2 h. (C) Immunecomplex kinase assays of COS-1 transient transfectants and 7OZ/3 cells. A repetition of (B). Lanes 1 and 2, 70Z/3 cells; 3-6, COS-1 cells; 1, reaction in the absence of dsRNA; 2-6, reactions with 10 μg/ml reovirus dsRNA. -, indicates untransfected cell, remainder transfected as indicated. Immunoprecipitation is with antibodies indicated at top. Relative molecular mass markers in kDa are shown on the left. Exposure, 1.5 h.

Figure 5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Cos-1 transient transfection
Western analysis
Control
M-mPKR
M-mPKR del
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4 5 6
105
69.8
43
M-mPKR
M-mPKR del
PKR
IB α-Myc
IB α-hPKR
B
IP/kinase assay
Control
M-mPKR
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– + – + – + :dsRNA
1 2 3 4 5 6
M-mPKR
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Anti- hPKR
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vector
M-mPKR
M-mPKR del
1 2 3 4 5 6
105
70.8
43.6
M-mPKR
PKR

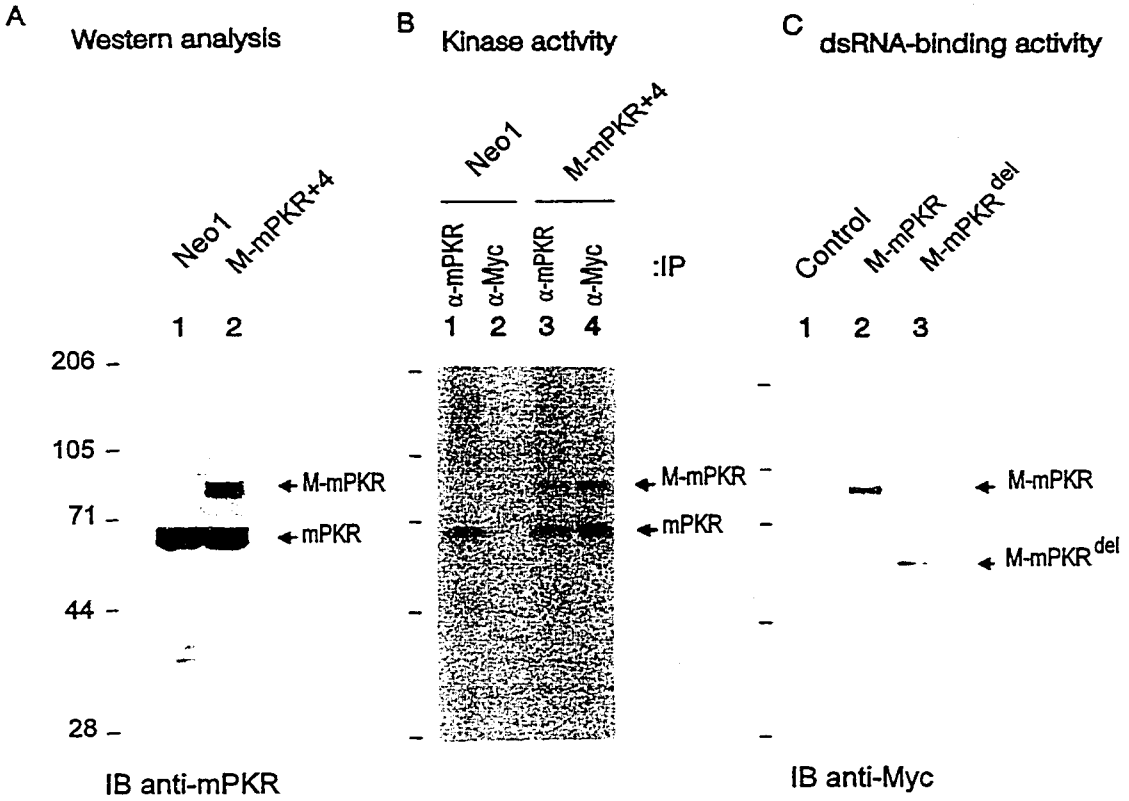
of dsRNA (Fig. 5B, lanes 3 and 4, and Fig. 5C, lanes 1 and 2). Interestingly, we observed the co-immunoprecipitation of the endogenous 68 kDa monkey PKR kinase with the M-mPKR (Fig. 5B, lanes 3 and 4). The identity of this 68 kDa phosphoprotein was confirmed by tryptic analysis (data not shown). Furthermore, visualization of protein by immunoblot analysis after immunoprecipitation was attempted and although extremely high in background, equimolar levels of M-mPKR and mPKR was found in association (data not shown). This association appears to be specific since the 68 kDa PKR is not found in anti-Myc immunoprecipitates from cells transfected with control vector (Fig. 5B, lanes 1 and 2) or in immunoprecipitates from cells expressing the Myc-tagged Sty kinase (data not shown). Although detectable amounts of primate PKR appeared to associate with mPKR, the interaction was less than stoichiometric, possibly due to species differences. To test this idea, mouse cell lines were established using the M-mPKR expression vector. Analysis of one such clone is shown in Figure 6. As expected, mPKR derived from both the transfected (M-mPKR) and endogenous genes (mPKR) was immunoprecipitated with mPKR specific antibodies (Fig. 6B, lane 3). Furthermore, the anti-Myc antibody immunoprecipitated stoichiometric amounts of both mPKR and M-mPKR kinases (Fig. 6B, lane 4) demonstrating that mPKR likely exists as at least a dimer in cells.

We then determined whether the mPKR^{del} mutant was capable of forming dimers. As expected, the 55 kDa M-mPKR^{del} protein lacks detectable kinase activity (Fig. 5B, lanes 5 and 6 and 5C, lane 6). However, some of the COS-1 cell PKR co-precipitated with the mutated polypeptide and is detected as an autophosphorylating band at 68 kDa consistent with mPKR^{del} association with endogenous monkey PKR. This result demonstrates that the

Figure 6. Evidence of mPKR dimerization and dsRNA binding.

(A) Immunoblot analysis of L cell stable transfectants. Expression vectors bearing control and Myc epitope-tagged mPKR cDNAs were stably transfected into L cells and clones screened for expression by immunoblotting and visualization with enhanced chemiluminescence as described in Materials and Methods. Murine endogenous mPKR and M-mPKR are indicated on the right and molecular mass markers in kDa are shown on the left. Lanes: 1, control transfected clone Neo 1; 2, M-mPKR transfected clone M-mPKR +4. Exposure, 15 s. (B) Immunecomplex kinase assays demonstrate co-immunoprecipitation of endogenous mPKR with tagged mPKR. Parallel lysates of cells from (A) were immunoprecipitated with the antibodies indicated and reactions performed as described in Materials and Methods in the presence of 10 μg/ml reovirus dsRNA. Lanes: 1, 2, control clone Neo 1; 3, 4, clone M-mPKR +4. M-mPKR and endogenous mPKR recovered are indicated on the right. Positions of relative molecular mass markers in kDa are shown on the left. Exposure, 2 h. (C) dsRNA binding properties of mPKR and mPKR^{del}. Transient transfections of COS-1 cells were harvested in Lysis buffer and incubated with excess poly (I): poly (C) agarose (Pharmacia), washed as for immunoprecipitates and analyzed by anti-Myc Western analysis. Lanes: 1, control vector transfected cells; 2, M-mPKR transfected cells; 3, M-mPKR^{del} transfected cells. Tagged wt and mutated mPKR^{del} polypeptides recovered with dsRNA are indicated on the right and positions of relative molecular mass markers in kDa are shown on the left. Exposure, 15 s.

Figure 6


A
Western analysis
Neo1
M-mPKR+4
1 2
206
105
71
44
28
M-mPKR
mPKR
IB anti-mPKR
B
Kinase activity
Neo1
M-mPKR+4
α-mPKR
α-Myc
α-mPKR
α-Myc
:IP
1 2 3 4
M-mPKR
mPKR
C
dsRNA-binding activity
Control
M-mPKR
M-mPKR del
1 2 3
M-mPKR
M-mPKR del
IB anti-Myc

in-frame deletion in mPKR^{del} does not eliminate the domain responsible for formation of mPKR dimers. Furthermore, mPKR^{del} is able to bind dsRNA like the wild-type kinase (Fig. 6C, lanes 2 and 3). This dimerization appears dependent on dsRNA as has been suggested previously (178) since it is not reconstituted in RNA-free reticulocyte extracts when mPKR and mPKR^{del} are co-translated (Figure 7, compare lane 6 and lane 12).

L1210 cell extracts lack detectable mPKR kinase activity.

Since both the normal and mutant mPKR alleles are expressed in L1210 cells, we were interested in determining whether the dsRNA-dependent kinase activity in these cells was affected by mPKR^{del} expression. Although L1210 and 70Z/3 cells both express wild type mPKR mRNA (Fig. 2) and protein (Fig. 8C), no IFN-inducible, dsRNA stimulatable kinase activity could be detected in cell extract kinase assays from L1210 cells (Fig. 8A, lanes 5-8) while parallel 70Z/3 cell extracts have readily detectable p65 kinase activity (Fig. 8A, lanes 1-4). Following immunoprecipitation from L1210 lysates however, mPKR kinase activity is detected at levels consistent with the amount of normal mPKR protein present in these lysates (Fig. 8B, lanes 3 and 4 versus lanes 1 and 2). These results suggest that the process of immunoprecipitation overcomes the inhibition of mPKR activity observed in L1210 cells (see discussion) and that the normal mPKR allele bears no other inactivating mutations.

eIF-2α phosphorylation in L1210 cells is marginally inducible.

As another measure of PKR function in L1210 cells, we examined the state of phosphorylation levels of eIF-2α in L1210 cell extracts in the absence or presence of the PKR activators, poly (I) poly (C) (pIC) and heparin (Fig. 9). In the presence of pIC, NIH

Figure 7. RNA-free <u>in vitro</u> translation does not reconstitute mPKR and M-mPKR^{del} dimerization.

Reticulocyte translation of equal amounts of the indicated transcripts was performed as described in Materials and Methods. Lanes 1-6 show efficiency of translation of each transcript. Lanes 7-12 represent polypeptides recovered directly by anti-Myc immunoprecipitation or in association with such polypeptides. Absence of recovery of untagged proteins indicate specificity of recovery. Dimerization between M-mPKR and untagged mPKR is not evident due to smearing or close migration. Association between M-mPKR^{del} and untagged mPKR should be clear due to good resolution. Tagged wt and mutated mPKR^{del} and untagged mPKR polypeptides are indicated on the right and positions of relative molecular mass markers in kDa are shown on the left. Exposure, 16h.

Figure 7

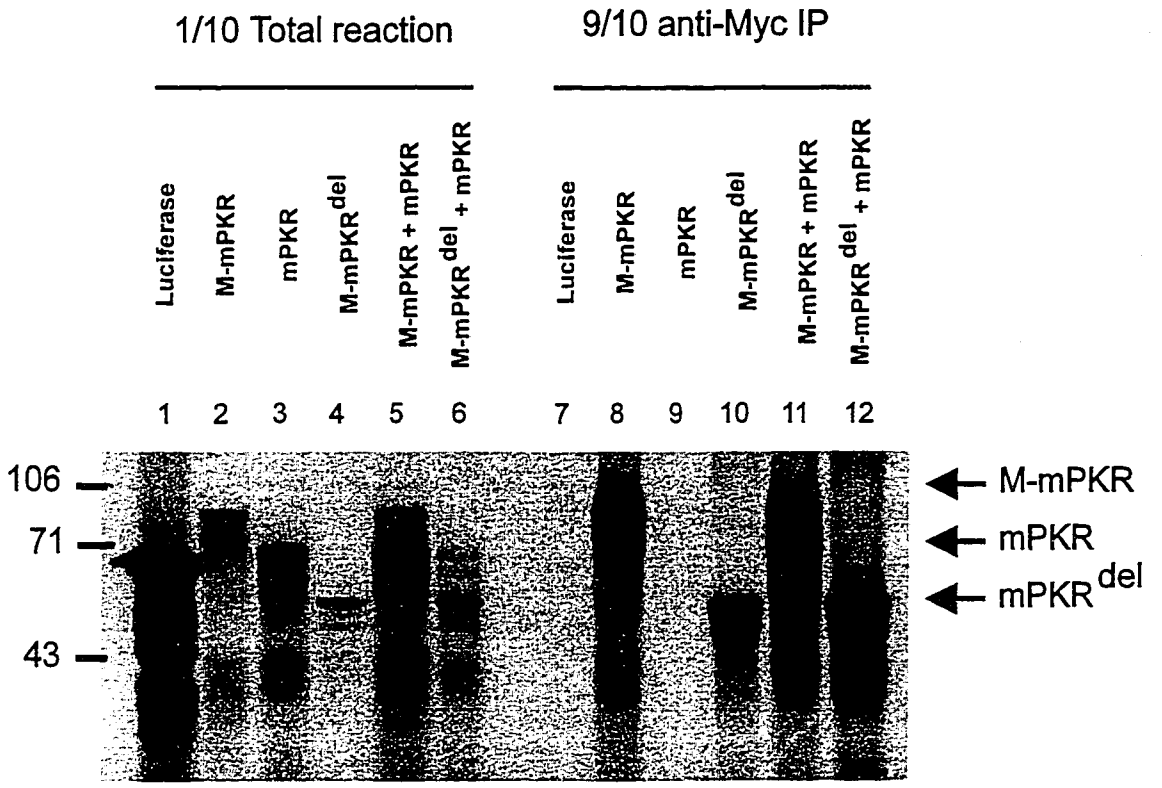

1/10 Total reaction
9/10 anti-Myc IP
Luciferase
M-mPKR
mPKR
M-mPKR del
M-mPKR + mPKR
M-mPKR del + mPKR
1 2 3 4 5 6 7 8 9 10 11 12
106
71
43
M-mPKR
mPKR
mPKR del

Figure 8. Absence of dsRNA-dependent protein kinase activity in L1210 cells.

(A) Cell extract kinase assays of L1210 cells. 70Z/3 and L1210 cells were left untreated or treated with IFN as indicated. Cell extract kinase autophosphorylation assays performed as in Fig. 1A, demonstrate that L1210 cells have no detectable p65 IFN-inducible, dsRNA-dependent protein kinase activity. Lanes: 1-4, 70Z/3 cell lysates; 5-8, L1210 cell lysates; 1, 3, 5, 7, reactions without dsRNA; 2, 4, 6, 8, reactions in the presence of dsRNA. Exposure, 10 h. (B) Immunecomplex kinase assays of L1210 cells. Parallel cell lysates derived in (A) were diluted in Lysis buffer and mPKR polypeptides recovered by immunoprecipitation with anti-mPKR antibody as described in Materials and Methods. Reactions were performed only in the presence of dsRNA. Immunoprecipitation allows detection of mPKR activity in L1210 cells. Lanes: 1, 2, 70Z/3 lysates; 3, 4, L1210 lysates; 1, 3 untreated cells; 2, 4, IFN treated cells. Exposure, 2 h. (C) Immunoblot analysis of mPKR expression in L1210 cells. Parallel lysates from (A) were analyzed by immunoblotting with anti-hPKR antibody and visualized by enhanced chemiluminescence as described in Materials and Methods. Lanes: 1, 2, 70Z/3 lysates; 3, 4, L1210 lysates; 1, 3 untreated cells; 2, 4, IFN treated cells. The relative molecular mass markers in kDa are indicated on the left. The position of mPKR is indicated with an arrow on the right. Exposure, 30s.

Figure 8

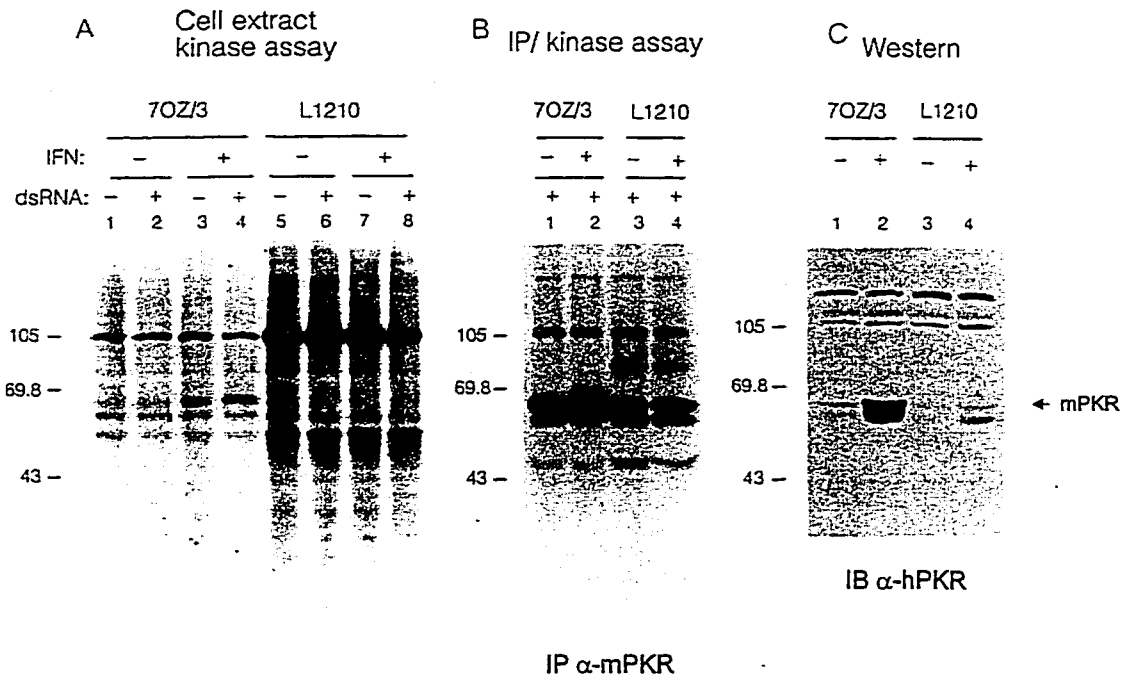
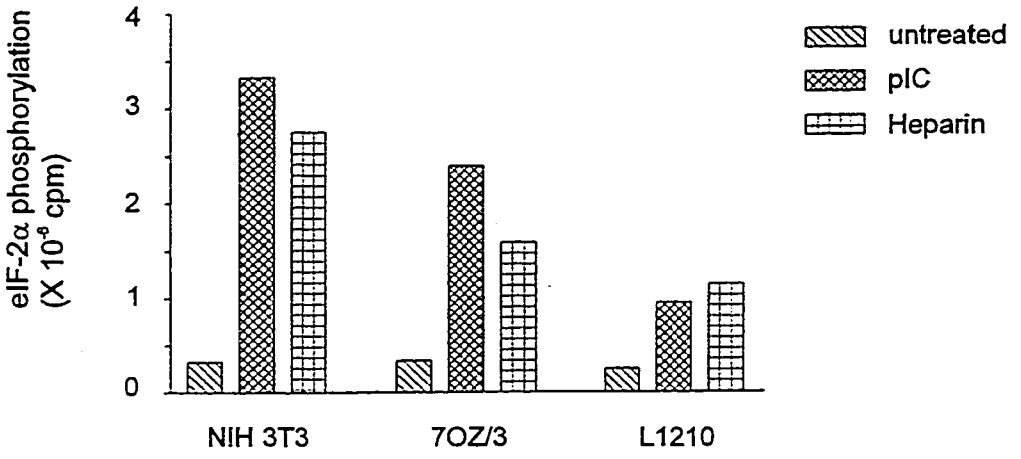

A
Cell extract
kinase assay
7OZ/3
L1210
IFN:
dsRNA:
1 2 3 4 5 6 7 8
105 –
69.8 –
43 –
B
IP/ kinase assay
7OZ/3
L1210
1 2 3 4
105 –
69.8 –
43 –
IP α-mPKR
C
Western
7OZ/3
L1210
1 2 3 4
105 –
69.8 –
43 –
← mPKR
IB α-hPKR

Figure 9. Induction of eIF-2α phosphorylation is aberrant in L1210 cells.

Cell extracts from the indicated cell lines were set up in reaction mixtures with γ-^{32}P ATP and purified eIF-2α. Reactions were done in the absence of activators or with poly (I) poly (C) or heparin. Phosphorylated eIF-2α was resolved on SDS-PAGE and quantitated. pIC, poly (I) poly (C) treatment; heparin, heparin treatment.

Figure 9


eIF-2α phosphorylation
(X 10⁻³ cpm)
4
3
2
1
0
NIH 3T3
7OZ/3
L1210
untreated
pIC
Heparin

3T3 and 70Z/3 cells showed a 10.3-fold and 7.1-fold increase respectively of eIF-2α phosphorylation above that in untreated extracts. Heparin caused an 8.5-fold and 4.7-fold increase in these cells respectively. In contrast, pIC and heparin induced eIF-2α phosphorylation in L1210 cells by only 3.8-fold and 4.6-fold each. Consistent with the cell extract kinase assay of PKR autophosphorylation activity (Fig. 8A), L1210 cells appear to have impaired induction of eIF-2α phosphorylation.

mPKR^{del} fails to express at high levels, demonstrate dominant negative activity or deregulate cell growth control.

In order to directly determine the consequences of mutation of PKR in L1210 cells, stable transfection of mPKR^{del} was attempted in NIH 3T3 cells. After unsuccessfully using conventional expression vectors, a bicistronic expression vector which expressed both the cDNA of interest and a selectable marker was used (Fig. 10). Efficiency of recovery of Hygromycin^{R} clones served as an indicator of potential deleterious effect of cDNA expression. Although expression of tagged wild-type mPKR appeared to produce only a few Hygromycin^{R} colonies, numerous resistant colonies expressing tagged mPKR^{del} were obtained (Fig. 10). Despite this, no clones of NIH 3T3 cells that expressed high levels of tagged mPKR^{del} polypeptide relative to endogenous PKR were obtained (Fig. 11A). In contrast, two clones that had comparable levels of M-mPKR to endogenous mPKR were obtained (M-wt+3 and M-wt+4). In cells that do express low levels of mPKR^{del} (M-del10 and M-del13), no dominant-negative effect on endogenous mPKR was observed (Fig. 11B). Endogenous mPKR in these clones retained inducible mPKR activity. We confirmed this in transient transfection assays in Cos-1 cells expressing mPKR^{del} (Fig. 12). Increasing

Figure 10. Bicistronic expression vectors used for stable transfection and determining consequence of expression.

The Pece expression vectors with various cDNAs were modified (termed Pece II) with the addition of a selectable marker, HygTK (Hygromycin phosphotransferase- Thymidine Kinase fusion) driven by the same SV40 promoter and whose translation is facilitated with the EMCV IRES (internal ribosome entry site). Vectors were linearized with a BamH1 site distal to the HygTK polyA tail and transfected as described in the Materials and Methods. Coupling of cDNA to selectable marker on the same transcript ensures all resistant clones express the cDNA. The number of Hygromycin^R colonies derived from comparable amounts of cells and DNA transfected reflects cDNA expression toxicity.

Figure 10

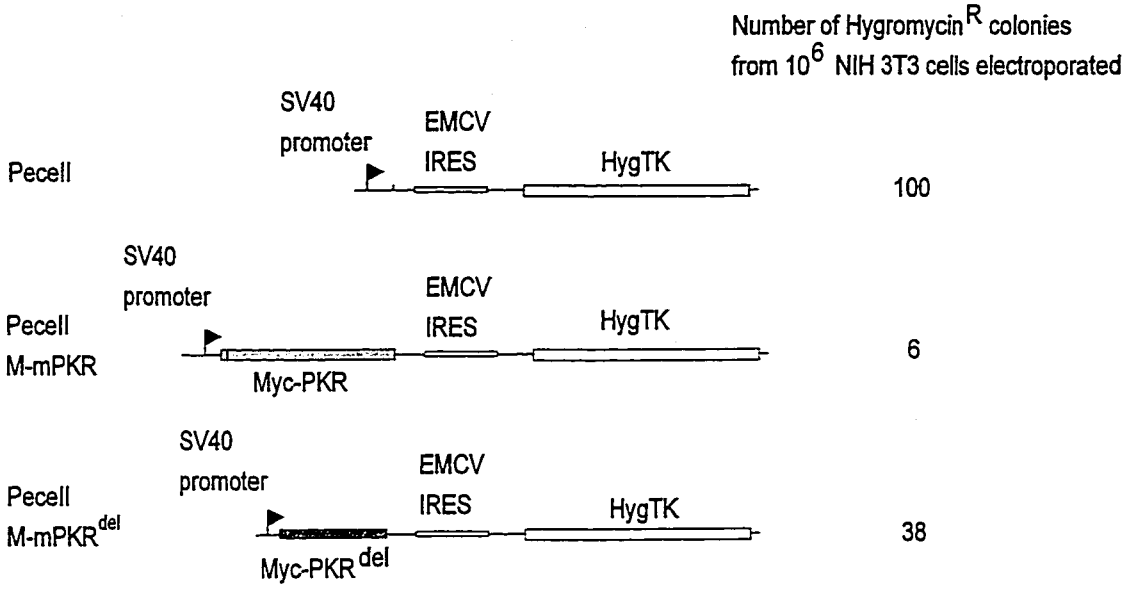
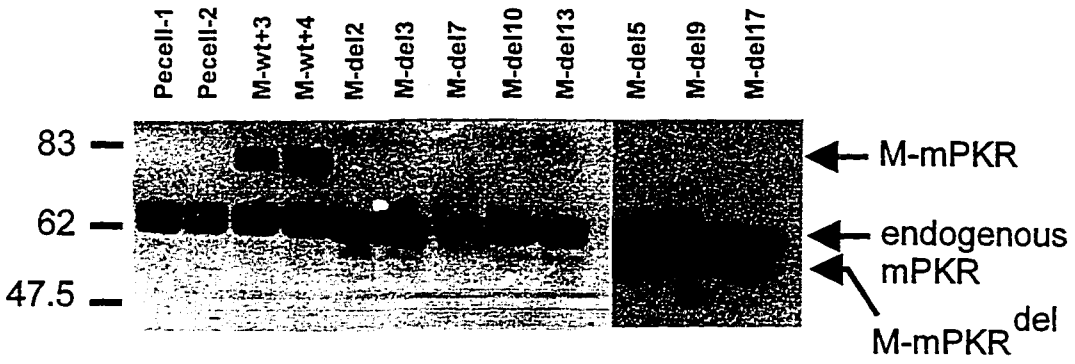

Number of Hygromycin^R colonies
from 10^6 NIH 3T3 cells electroporated
SV40
promoter
EMCV
IRES
HygTK
PecelI
100
SV40
promoter
EMCV
IRES
HygTK
PecelI
M-mPKR
Myc-PKR
6
SV40
promoter
EMCV
IRES
HygTK
PecelI
M-mPKR^{del}
Myc-PKR^{del}
38

Figure 11. mPKR^{del} is not readily expressed to high levels and does not demonstrate dominant negative activity in cell extract kinase assay.

(A) Expression levels of M-mPKR and M-mPKR^{del} in NIH 3T3 stable transfectants. Individual clones were assessed for level of exogenous gene expression by immunoblot analysis of equal amounts of protein lysates. Immunoblot was performed with the anti-Tik100 antibody raised against mPKR residues 1-98. The relative molecular mass markers in kDa are indicated on the left. The position of M-mPKR, endogenous mPKR and M-mPKR^{del} are indicated with an arrow on the right. Exposure, 5s. (B) Cell extract kinase assays of selected NIH 3T3 stable transfectants. Cell extract kinase autophosphorylation assays performed as in Fig. 1A followed by immunoprecipitation of mPKR with anti-Tik100, demonstrate that clones expressing M-mPKR^{del} retain inducible dsRNA-dependent protein kinase activity. PeceII-2, vector transfected clone; M-wt+3 and M-wt+4, PeceII M-mPKR transfected clones; M-del10 and M-del13, PeceII M-mPKR^{del} transfected clones; -, reactions without dsRNA; +, reactions in the presence of 10 μg/ml dsRNA. The relative molecular mass markers in kDa are indicated on the left. The position of M-mPKR, endogenous mPKR are indicated with an arrow on the right. Exposure, 48 h.

Figure 11

A Immunoblot



B Cell extract kinase assay, IP

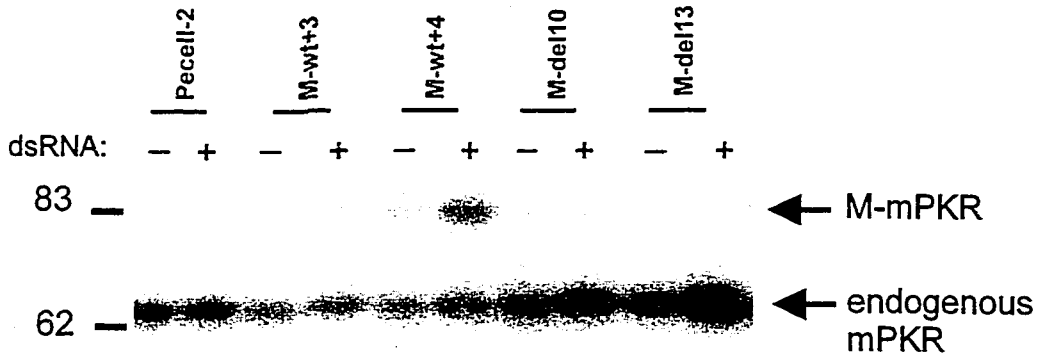
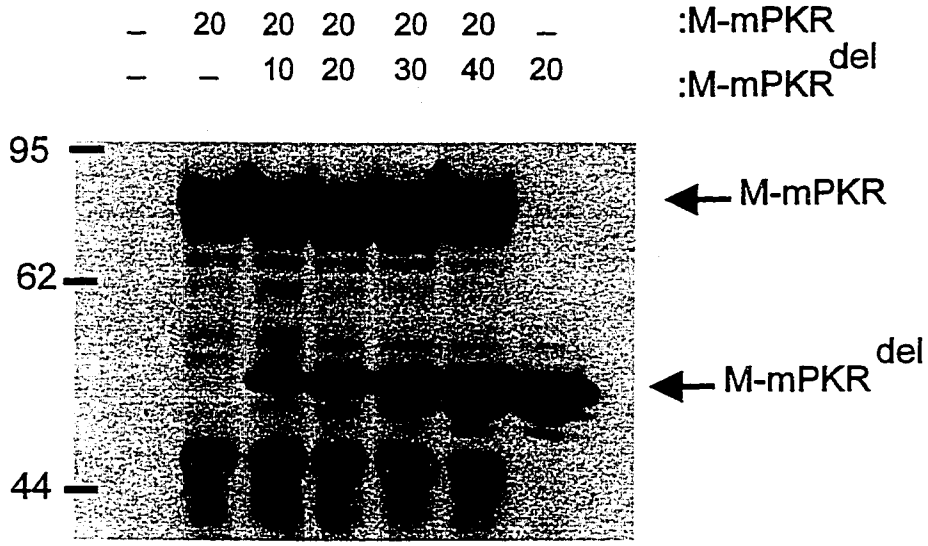


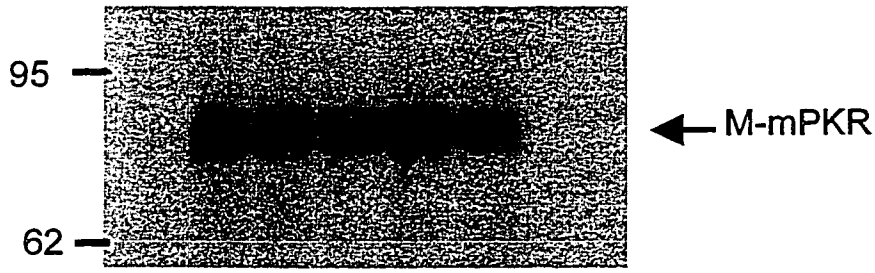
Figure 12. M-mPKR^{del} does not show dominant negative activity in COS-1 transient transfected cell lysates.

A) Immunoblot analysis of expression of tagged mPKR and mPKR^{del} in COS-1 transiently transfected with varying amounts of Pece vectors encoding the indicated cDNAs. Anti-Myc immunoblot detects only the tagged proteins. Figures at top indicate the amount in μg of DNA of each vector transfected. - indicates transfection with filler Pece vector. The relative molecular mass markers in kDa are indicated on the left. The position of M-mPKR and M-mPKR^{del} are indicated with an arrow on the right. Exposure, 15s. (B) Parallel cell extract kinase assay to determine effect of co-expression of M-mPKR^{del} on wild-type mPKR activity. Performed as in Figure 1A followed by anti-Myc immunoprecipitation. Lanes set up as in (A). The relative molecular mass markers in kDa are indicated on the left. The position of M-mPKR is indicated with an arrow on the right. Exposure, 3d.

Figure 12

A Immunoblot





B Cell extract kinase assay, IP

amounts of the mutated PKR in cell extracts did not significantly alter the autophosphorylation of the wild-type mPKR co-transfected.

Finally, we assessed the NIH 3T3 clones for aberrant cell growth. Cells expressing M-mPKR displayed a striking increase in doubling time (Fig. 13) reminiscent of the slow growth phenotype seen in yeast overexpressing PKR. M-mPKR^{del} expressing cells (including M-del17) however, showed no decreases in doubling time compared to controls. In addition these cells do not demonstrate any evidence of elevated morphological transformation (data not shown). Due to the high background level of colonies obtained in soft agar with the control NIH 3T3 cells and the low expression of mPKR^{del}, transfection into lower passage NIH 3T3 cells was performed. No improvement in expressing stoichiometric levels of mPKR^{del} was obtained and further analysis of tumorigenicity was not pursued.

The improvement of detection of M-mPKR^{del} in transfected cells using the anti-Tik100 antibody compared to the anti-hPKR antibody (compare Figure 8C and 11A) allowed us to determine whether we could directly detect the presence of the mPKR^{del} polypeptide in L1210 cells. No evidence of high levels of the predicted 43 kDa mPKR^{del} in L1210 cells was detectable by immunoblot analysis using this antibody (Fig. 14). The smaller immunoreactive protein maybe derived from mPKR^{del} transcript however, fails to show IFN-inducibility as does the transcript (data not shown) and is not expressed at stoichiometric levels to endogenous mPKR.

DISCUSSION

The results presented here and elsewhere (79) demonstrate that the Tik kinase is the murine homolog of the human dsRNA dependent kinase, PKR. The location of hPKR and

Figure 13. Growth rate of NIH 3T3 transfectants shows growth suppression by wild-type mPKR.

The doubling time of individual clones of control, M-mPKR and M-mPKR^{del} transfectants were determined from at least two passages as described in the Materials and Methods. PeceII-1 and -2, vector alone control clones; M-wt+3 and +4, Pece II M-mPKR transfected clones; M-del2, 3, 10, 13 and 17, PeceII M-mPKR^{del} transfected clones. Error bars represent standard error of mean (SEM), sample size of 6 each.

Figure 13

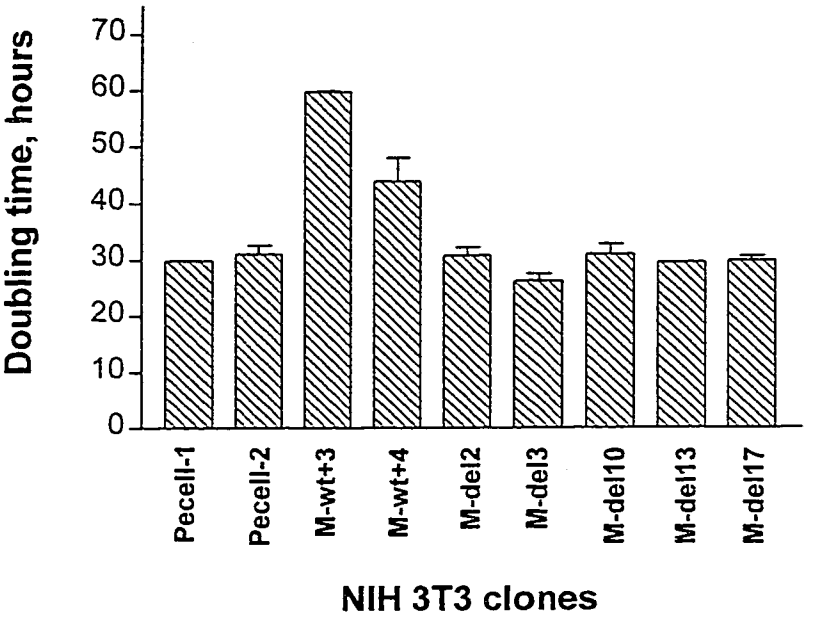
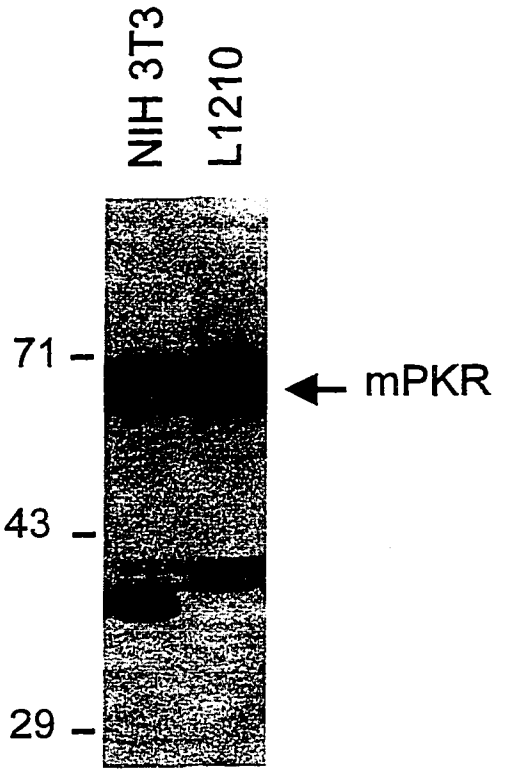

Doubling time, hours
70
60
50
40
30
20
10
0
Pecell-1
Pecell-2
M-wt+3
M-wt+4
M-del2
M-del3
M-del10
M-del13
M-del17
NIH 3T3 clones

Figure 14. Absence of mPKR^{del} polypeptide in L1210 cells.

Immunoblot analysis of L1210 lysates using the anti-Tik100 antibody which detects M-mPKR^{del} (Fig. 11) does not detect the predicted 43 kDa polypeptide. The relative molecular mass markers in kDa are indicated on the left. The position of endogenous mPKR is indicated with an arrow on the right. Exposure, 5s.

Figure 14



Anti-Tik100 immunoblot

Tik on syntenic regions of the respective human and mouse chromosomes supports this as well. For this reason we will refer to the Tik kinase as murine PKR (mPKR).

The strategy of cloning protein tyrosine kinases from bacterial expression libraries using anti-phosphotyrosine antibody screening has identified a new family of enzymes called dual-specificity kinases (DSKs) (14). Here, we show that the human and mouse dsRNA-dependent protein kinases are members of the dual-specificity family of kinases. The significance of this tyrosine kinase activity is currently unknown. Although we and others have been unable to detect phosphotyrosine in PKR expressed in mammalian cells, unpublished reports from Thomas Dever's laboratory suggests that phosphoamino acid analysis of hPKR expressed in yeast does include phosphotyrosine. Furthermore, they show that eIF-2α with Ser 51 altered to Tyr is still phosphorylated on this site and still functional (N. Sonenberg, personal communication). So far, this is the only physiologically relevant substrate of PKR that may be phosphorylated on tyrosine in eukaryotic cells. One attractive hypothesis is that dual-specificity kinases utilize their tyrosine autophosphorylating activity to create docking sites for SH2 containing signaling molecules. Our laboratory and others have recently shown that the two DSKs, Sty and Esk, possess tyrosine residues embedded within a motif of amino acids which resemble SH2 binding sites (Douville, Duncan and Bell unpublished and (28,258)). It remains to be seen if the tyrosine autophosphorylating activity of PKR is involved in its signal transduction properties.

The PKR enzymes have been thought to function as regulators of mRNA translation by phosphorylation of the initiation factor eIF-2α. A role for PKR in other aspects of cellular growth control is beginning to emerge from recent genetic experiments. Koromilas

et al have constructed a six amino acid deletion in the catalytic domain of human PKR and demonstrated that this kinase inactive PKR can cause malignant transformation of NIH 3T3 cells (98). A second mutated form of PKR which is catalytically inactive by virtue of a point mutation in the ATP binding fold is also tumorigenic when introduced into NIH 3T3 cells (222). Furthermore, PKR deleted of dsRBD I is also tumorigenic (93). The identification of a naturally occurring dominant negative mutant of mPKR in a leukemic cell line would be consistent with the idea that the PKR gene product functions as a tumor suppressor. Meurs et al report that their PKR point mutant has no detectable effect on the response of their transformed cells to interferon (222), while we have observed that for L1210 cells and certain human tumor cells, interferon does not inhibit growth but rather is a potent mitogen (data not shown and personal communication, D. Logan). This suggests that the genetically engineered and naturally occurring mutants of PKR may show subtle differences in their impact on cell growth and morphology. Interestingly, Meurs et al found that their mutated kinase induced transformation by a mechanism independent of eIF-2α phosphorylation (222) consistent with the notion that PKR has multiple signaling partners. Deletion analysis of the dsRBDs indicate that loss of dsRBD II allows retention of dsRNA binding with dsRBD I playing a more important role of the two motifs (78,85,87). Thus the predicted $mPKR^{del}$ polypeptide which retains dsRBD I could act as a transdominant mutation. Clones expressing $mPKR^{del}$ however, show no increase in growth rate or enhancement of tumorigenicity as reported for other inactivating PKR mutations (93,98). The absence of IFN-inducible, dsRNA activated PKR activity in L1210 extracts indicated to us that the predicted $mPKR^{del}$ polypeptide may act as a dominant suppressor of wild type mPKR

activity in L1210 cells. However, upon transfection of mPKR^{del} into NIH 3T3 cells, we have been unable to recapitulate the suppression of PKR activity seen in L1210 cells. Although it is entirely possible that this was due to the relatively low level of mPKR^{del} expression in these cells, the absence of effect on mPKR activity is borne out when co-translated in reticulocyte extracts. Similarly, no enhancement of translation of reporter plasmids by mPKR^{del} was observed (data not shown). The inability to detect the mPKR^{del} polypeptide in L1210 cells or to express it stably at significant levels in other cells suggest the possibility that this mutation is silent. Indeed, it has been reported that mutations of hPKR deleting dsRBD II are not readily expressed stably, perhaps due to protein instability (93). Although translated in reticulocyte extracts, expression of mPKR^{del} in transfected cells may have been stabilized by the Myc epitope tag used to facilitate detection. If this is the case, the absence of PKR activity in L1210 cell extracts may have instead been due to altered ratio of PKR inhibitors such as p58^{IPK} (200) to PKR. An increase in this ratio at the mRNA level has been reported in chronic lymphocytic leukemia (CLL) patients (259). It is possible that reduced expression as a result of mutation of one PKR allele is sufficient to decrease PKR activity as shown by the impaired induction of eIF-2α phosphorylation and PKR autophosphorylation. Minor changes in eIF-2α phosphorylation can account for large effects on translation initiation since eIF-2α exceeds eIF-2B in molar ratio which it inhibits by sequestration (123). It is as yet, unclear what the impact of this altered response might have on cellular function.

Through the use of the Myc epitope tag, we have been able to demonstrate that in vivo, the PKR kinase exists as a dimer. This corroborates the work by Langland Jacobs that

showed evidence of PKR dimerization by gel filtration chromatographic analysis (228) and Cosentino et al who showed PKR homodimerization by yeast two-hybrid and far-Western analysis (178). Consistent with work presented by Consentino et al, our data also indicates that mPKR multimerization may be dependent on dsRNA (Fig. 7) although the definitive experiment requires addition of dsRNA to determine if this would reconstitute the ability to dimerize. Thomis and Samuel (229) have demonstrated that when synthesized in vitro, PKR polypeptides are capable of intermolecular cross-phosphorylation and it may be that this is an important step in the activation of the PKR holoenzyme. It seems likely that if co-expressed, mutant:wild type PKR heterodimers form and that these complexes have minimal catalytic activity even in the presence of dsRNA. Following immunoprecipitation from L1210 lysates, mPKR kinase activity is readily detected. It is not clear to us why the process of immunoprecipitation activates the mPKR kinase from L1210 cells although possibilities include an antibody induced conformational change or activation by oligomerization. Indeed, Koromilas et al have similarly shown detectable PKR activity in the presence of inactive mutated PKR following immunoprecipitation (98). Alternatively, immunoprecipitation may liberate PKR from inhibitors, thus revealing its activity. In any case, it appears from these results that the cell extract kinase assay may more closely approximate the in vivo state of PKR activity than immunoprecipitation kinase assays.

Expression of wild-type mPKR has demonstrated several properties worth noting. The number of Hygromycin^R colonies obtained from the bicistronic vector encoding the active mPKR is markedly reduced. Although not examined ourselves, other reports describing PKR induction of apoptosis is a likely explanation for this observation (234-236).

Catalytically inactive PKR is unable to induce apoptosis (235). Clones that do express mPKR to high levels must have acquired mechanisms that counter the death inducing effect of PKR. Indeed, one such clone in L cells appears to have markedly decreased M-mPKR activity (Figure 6B, lane 3). Notwithstanding, two clones that express high levels of M-mPKR in NIH 3T3 cells and which retain dsRNA inducible activity display noticeably slower doubling time. This may be a consequence of global translation repression as seen in yeast (78).

Previous studies have suggested that PKR has a tumor suppressor function, an idea which is consistent with the characterization of the $mPKR^{del}$ mutation in the L1210 leukemia cell line. We report here that mutation of one PKR allele in a murine lymphocytic leukemia may compromise PKR response to certain activators. Significantly, chromosomal aberrations in the area of the PKR locus in humans are most frequently found in neoplasias of the myeloid and lymphoid lineages (225). The $mPKR^{del}$ mutant does not appear to act by transdominant inhibition of PKR activity by the assays designed herein. The biological consequence of this mutation is not clear although other tests designed to elucidate this will be discussed later.

CHAPTER 3

Characterization of knockout mice with targeted disruption of the catalytic domain of PKR.

SUMMARY

The interferon (IFN)-inducible, dsRNA-dependent protein kinase, PKR has been implicated in anti-viral, anti-tumor and apoptotic responses. Others have attempted to examine the requirement of PKR in these roles by targeted disruption at the amino terminus-encoding region of the PKR gene. Using a strategy that aims at disruption of the catalytic domain of PKR, we have generated mice that are genetically ablated for functional PKR. Similar to the other mouse model of PKR disruption, we have observed no consequences of loss of PKR on tumor suppression. Anti-viral response to influenza and vaccinia also appeared to be normal in mice and in cells lacking PKR. Cytokine signalling in the type I IFN pathway is normal but may be compromised in the Erythropoeitin (Epo) pathway in erythroid bone marrow precursors. Contrary to the amino-terminal targeted PKR mouse, TNFα-induced apoptosis is not impaired in catalytic-domain targeted PKR-null cells. Furthermore, the anti-viral apoptosis response to influenza thought to require PKR is still functional. The observation of intact eIF-2α phosphorylation in these PKR-null cells provides proof of rescue by another eIF-2α kinase(s). We postulate that our targeting strategy produces a null allele which permits a PKR homolog to compensate its loss. Such redundancy may be overcome in mouse or cell models with dominant-negative PKR alleles.

INTRODUCTION

The interferon (IFN)-inducible protein kinase PKR is a well characterized effector of anti-viral responses in mammals (128,147). Activation by double stranded RNA (dsRNA) or stem loop RNA structures results in autophosphorylation and subsequent phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF 2α) (49,53,58,167,169,260). This

phosphorylation causes sequestration of the guanine nucleotide exchange factor eIF-2B which prevents the exchange of GDP for GTP on eIF-2 and thereby, inhibits translation initiation (73,74). The decline in protein synthesis rates is deleterious to virus replication and various viral mechanisms exist to circumvent inhibition of translation by PKR. For example, adenovirus inactivates PKR with VAI RNA (166,168), vaccinia encodes a dsRNA binding protein and substrate decoy, E3L and K3L respectively (177), influenza activates an endogenous inhibitor of PKR, $p58^{IPK}$ (198,261) and reovirus sequesters dsRNA with the σ3 protein(262) (for a review see (126,263)). Overexpression of PKR in cells inhibits vaccinia virus (219) and encephalomyocarditis virus (EMCV) and has variable effectiveness against vesicular stomatitis virus (VSV) replication (217,218). Although there has been a considerable focus on PKR's anti-viral role, accumulating evidence suggests an additional role for PKR in regulating other cellular functions such as differentiation (212), transcription (75,138,139,141,145), signal transduction (140), apoptotic response (234,235,238) and growth and proliferation (93,98,117,222).

Observations supporting PKR's growth regulatory role were first made in yeast where expression of PKR results in a slow growth phenotype (117). Expression of catalytically inactive PKR in NIH 3T3 cells resulted in transformation of these cells which formed tumors when introduced into nude mice (93,98,222). The mechanism of action is thought to involve dominant suppression of endogenous PKR function by mutated PKR. Indeed, this is supported by the transforming activity of expression of a non-phosphorylatable variant of eIF2α (264), the $p58^{IPK}$ inhibitor of PKR (231) and the TAR RNA binding protein (TRBP) inhibitor of PKR (232) in NIH 3T3 cells. The importance of translation initiation in control

of cell growth has precedent in the observation of cellular transformation from overexpression of the cap binding protein, eIF-4E (233). The appropriate regulation of translation appears to be critical in control of cellular disposition towards proliferation or programmed cell death. Indeed, PKR expression in HeLa cells induces apoptosis (234) by mechanisms that are inhibitable by bcl-2 (235). Furthermore, anti-sense ablation of PKR conferred resistance to tumor necrosis factor α (TNFα)-induced apoptosis in U937 cells (236) indicating the requirement for PKR in the apoptotic response to TNFα in these cells. It is thought that dsRNA is a trigger for apoptosis in vaccinia virus infected cells (239) and indeed, influenza-mediated apoptosis is suppressed in cells expressing inactivated PKR (238). This raises the possibility that PKR may perform the function of responding to viral infection by mediating programmed cell death responses and preventing further viral spread.

Much of the work elucidating PKR's role in growth control and apoptosis utilized mutated PKR in exogenous expression studies. The use of targeted disruption to genetically ablate a specific gene can be useful to assess directly whether it is essential to cellular function and normal development (265,266). During the course of this work, another group described the targeted disruption of PKR by homologous recombination in mice by interruption of two exons including one which encoded the initiating methionine (140). Surprisingly, analysis of this PKR-defective mouse model showed no evidence of tumors and normal anti-viral responses in untreated animals. The mice were defective in IRF-1 and NF-κB signalling and showed diminished stress-induced apoptotic responses (140,141,237). The absence of any apparent loss of growth control in this mouse model may formally be attributed to the retention of intact sequence encoding the catalytic domain of PKR.

Although no PKR activity was detectable, this group identified splicing variants that could potentially encode a polypeptide lacking only the first 135 amino acids while retaining the carboxy terminal catalytic domain. To examine whether PKR is essential in anti-viral response, anti-proliferative functions of cellular growth control and in apoptotic response to various stimuli, we generated mice devoid of PKR function by targeted disruption of the PKR catalytic domain using homologous recombination that interrupts exon 12. Mice homozygous for PKR disruption ($PKR^{0/0}$) develop normally and are fertile with average sized litters. IFN α and β induction of transcription is intact and the mice show normal hematopoeisis. $PKR^{0/0}$ mice show responses to vaccinia and influenza infection comparable to control animals or cells. Apoptotic response to influenza infection or TNFα was not impaired. Our data indicates that catalytic disruption of PKR is not sufficient to ablate eIF-2α phosphorylation and provides strong evidence that unappreciated members of the eIF-2α kinase family must compensate for loss of PKR function.

MATERIALS AND METHODS

Construction of the PKR gene-targeting vector.

A 28.8 kb region of isogenic DNA encoding murine PKR was isolated and mapped. A 2.9 kb Sac1 fragment which encodes exons 10 and 11 (80) as determined by sequence analysis and a 1.9 kb Xho1/BamH1 fragment which bears intron XII sequence just distal to exon 12 were cloned into the MC1 neo PolyA vector (Stratagene). The herpes simplex virus thymidine kinase expression cassette was introduced into the 3' end of the vector at the Not1 site to produce the PKR catalytic domain replacement vector designated, pTV65TK. Insertion of the neomycin (neo) cassette results in replacement of a 3.7 kb BamH1 to Xho1

fragment of the PKR gene including complete removal of the 180 nucleotide Exon12. The targeting construct was linearized at a unique BstX1 site located in the plasmid vector.

Generation of PKR-deficient mice.

The targeting vector was introduced into the J1 embryonic stem (ES) cell line [strain 129/terSv, (267)] by electroporation and cells selected with neomycin (200μg/ml) and 1-(2'-deoxy-2'-fluoro-1-beta-D-arabinofuranosyl)-5-iodouracil (FIAU) (0.2μM; kindly provided by Dr. Michael A. Rudnicki) as described previously (268). Homologous recombinants were screened by genomic Southern blot analysis. Targeted disruption of the PKR gene was determined by EcoR1 or Pst1 digestion followed by hybridization with probe A or probe B respectively (see Fig. 1). The introduction of novel EcoR1 and Pst1 sites by the targeting vector allows identification of recombinants. To verify that only one copy of each construct was integrated in each targeted ES clone, Xho1 digested DNA was probed with the Neo probe. 3 out of about 750 ES clones screened were true homologous recombinants. ES cells that were homozygous for the targeted PKR allele were obtained by selection of the heterozygous targeted clone 7A8.3 in high neomycin selection (2mg/ml) as previously described (269). Of twenty resistant colonies picked. one clone (7A8.3.7) was homozygous mutant.

Chimeric mice were generated by microinjection of ES cells with a disrupted PKR gene into BalbC donor blastocysts and subsequent implantation into CD-1 foster mothers. Chimeric animals were tested for germ-line transmission of the agouti coat phenotype of 129/Sv-derived ES cells by crossing with BalbC mice. Two independent ES clones (7A8.1 and 34B5) contributed to the germ-line and allowed the establishment of two founder lines.

Heterozygote individuals were identified by genotyping and then intercrossed to generate homozygous lines. Once animals lines were established, genotyping of tail clippings was performed using the Pst1 Southern screen or PCR screening (see Appendix).

Northern blot analysis.

Total RNA was isolated from various ES cells which were untreated or treated with 500 U/ml interferon α and β (Cytimmune) for 16 hours. PolyA$^+$ RNA was selected on an oligo dT-cellulose (GIBCO-BRL) column. 6 μg of mRNA was resolved on an agarose-formaldehyde gel, blotted and hybridized with a murine PKR cDNA Stu1/Ssp1 fragment. The blot was washed at high stringency and imaged on a Molecular Dynamics Phosphoimager. The blot was stripped and reprobed with the mouse GAPDH cDNA, washed and exposed. After stripping the blot again, it was hybridized with a Mlu1/BamH1 fragment of the Neomycin gene, washed and exposed as above. IFN inducibility of 2' 5' oligoadenylate synthetase (OAS) was assessed by rehybridizing this blot with the OAS cDNA.

Derivation of primary mouse embryo fibroblasts (MEFs).

Primary MEF cultures were established from E13.5 embryos as described previously (267). Cells were genotyped as above.

Immunoblot and immunecomplex kinase analysis of PKR null cells.

Wild-type and homozygous-null ES and MEF cells were lysed in Lysis buffer, (10mM Tris (pH7.5), 150mM sodium chloride, 5mM EDTA, 1% Triton X-100), lysates denatured, resolved by SDS-PAGE and blotted onto nitrocellulose membranes. PKR polypeptides were analysed by immunoblotting with antisera (anti-TikMAP2) raised against

a multiple antigen peptide (MAP) bearing mPKR residues 101-114 and visualized by enhanced chemiluminescence (ECL) (Kirkegaard and Perry Laboratories). Enrichment by dsRNA affinity binding was utilized to detect PKR polypeptides. Cell lysates were incubated with poly(I) poly(C) (pIC) agarose (Pharmacia Biotech) at 4°C with rotation to allow binding, washed in lysis buffer, eluted and resolved by SDS-PAGE and blotted. PKR polypeptides able to bind dsRNA were visualized with antisera (anti-Tik100) raised against His tagged mPKR residues 1-98 and ECL.

Immunecomplex kinase reactions to detect PKR activity were performed by incubating lysates from various cells with the anti-Tik100A antisera and recovering the immunecomplex with Protein A Sepharose beads. After three washes followed by a wash in kinase buffer (184), the complexes were resuspended in reaction mixtures of kinase buffer with 5μCi γ ^{32}P-ATP and 10 μg/ml reovirus dsRNA and incubated at 25°C for 30 minutes. Reactions were stopped in sample buffer with 2-mercaptoethanol and eluted polypeptides were boiled and resolved on SDS-PAGE. Autophosphorylation activity of PKR was visualized by phosphoimaging.

Clonogenic assays and hematopoeitic development.

Bone marrow from age matched mice was harvested by flushing tibias under sterile conditions with 1ml PBS using a 25 gauge needle. Bone marrow cells were washed by centrifugation. An aliquot of cells was obtained, erythrocytes removed by lysis in hypotonic solution and nucleated marrow cells were counted using a hemocytometer. Trypan blue exclusion was also used to verify percentage viable cells in each sample. Cells were resuspended in IMDM/methyl cellulose supplemented with 20% fetal calf serum, 0.09 %

BSA and cytokines as noted below. 8 x 10^4 cells were plated in one millilitre of media in 35mm^2 petri dishes and incubated at 37°C and 5% CO_2 for 10 days. Erythroid and myeloid colonies were counted and colony forming units (CFU) were calculated using the cell density per dish and total colonies formed after 10 days. CFU were determined in triplicate from each of six animals of wild-type or PKR null genotypes. Recombinant human Erythropoeitin (Epo) was used at 2U/ml, recombinant murine IL-3 at 10μg/ml, human Kit ligand (KL) from conditioned media at 3 % (v/v) and human G-CSF at 100ng/ml. The hematocrit were determined on heparinized blood in capillary tubes.

Vaccinia virus growth in cell lines.

CV-1 or L-929 control cells or wild-type or PKR-null MEFs were plated in duplicate at 2 X 10^5 cells per well in six well tissue culture plates. The next day, cells were infected with trypsin- treated wild-type vaccinia virus (WR strain) at an MOI of 10 for one hour at 37°C. Following incubation, monolayers were rinsed and the innoculum replaced with media. At 4, 8, 12, 15 and 18 hours after infection, cells were frozen at -80°C. Cells were scraped off, resuspended in 0.3ml serum-free media and freeze-thawed twice before trypsinization and serial dilution in serum containing media. Plaque assays were performed by plating duplicate serial dilutions onto CV-1 monolayers for one hour at 37°C which were then overlayed with 0.6% Noble agar. After two days incubation, plates were fixed, stained with crystal violet and plaques counted to determine the number of pfus/ml of cell lysate as a function of time after infection.

Influenza infection of PKR null mice.

Five week old $PKR^{+/O}$ heterozygous control, Clk1-null (Duncan, P.I. and Bell, J.C. unpublished) and PKR-null mice were anaesthetized with halothane and infected by intra nasal innoculation with 50 µl of serial dilutions of the W29 strain of influenza in PBS. Each group contained 8-12 animals which were monitored daily over a 10 day period. The W29 strain is pneumovirulent for mice due to mutations selected on mouse-adaptation of the prototype human influenza A strain, A/FM/1/47 (270). This W29 influenza virus variant possesses single amino acid replacements in hemagglutinin, controlling virulence, and in the matrix protein, controlling virulence as well as growth. Influenza virus stocks were prepared in chicken embryo allantoic cavity and infectivity titres were assessed by plaque assay on MDCK cells (270). The LD_{50} of the virus in the control and PKR-null animals was assessed by Karber-Spearman analysis. Influenza virus growth in mouse lung was determined from pools of three mice from each group.

Influenza mediated apoptosis in cell lines.

Wild-type and PKR-null MEFs were plated at 2×10^5 cells per well in six well tissue culture plates and either left untreated or treated with 500U/ml IFN α and β overnight. Monolayers were rinsed in PBS then infected with influenza A/HK/1/68 (H3N2) virus in 100 µl PBS at multiplicity of infection (MOI) of 5, 10 or 25 versus mock infected for thirty minutes at 37°C. Following infection, serum containing media was added and cells were left for 24 hours. Monolayers were trypsinized into single cell suspensions, pooled with any cells in the media and cells undergoing apoptosis were determined by Annexin V-FITC staining (Clontech) according to the manufacturer's protocol. After incubation of cells with the Annexin V reagent for ten minutes, stained cells were fixed in 2% paraformaldehyde and

analysed on a Becton-Dickinson FACS instrument for percentage cells with elevated FITC fluorescence indicating apoptosis. Propidium iodide staining for necrotic cell death was utilized only when paraformaldehyde fixation could be omitted. Graphs indicated are representative of experiments done in duplicate.

TNFα, lipopolysaccharide (LPS) and dsRNA mediated apoptosis in PKR null cells.

4 X 10^5 MEFs were plated in 60mm^2 tissue culture plates and treated the next day with 50 to 2500 ng/ml of Actinomycin D for 24 hours to establish minimal cytostatic dose and ensure PKR-null cells responded comparably to control cells. Cell viability was determined by trypsinizing cells and trypan blue staining. Actinomycin D toxicity was not as profound as described previously (237) and was comparable in the two cell types. TNFα, LPS and poly (I) poly (C) synthetic dsRNA (pIC) induction of apoptosis in wild-type MEFs was examined by treating these cells as above with 20ng/ml TNFα, 100ng/ml LPS and 100μg/ml pIC with increasing amounts of Actinomycin D for 24 hours. Cell viability was measured by trypan blue staining and verified by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) assay (Boehringer Mannheim) according to the manufacturer's protocol or morphology with 0.5μg/ml Hoechst staining. TNFα induced apoptosis in these MEFs required Actinomycin D co-treatment. The apoptotic dose response to TNFα was then determined in PKR-null cells by treating cells as above with Actinomycin D (500ng/ml) and between 0.1 to 20 ng/ml TNFα for 24 hours. These experiments were conducted at least in triplicate.

eIF-2α phosphorylation state in PKR-null cells.

Cells were preincubated in phosphate free media with 10% dialyzed serum for 3 hours, then transfected with 0.1 mg/ml pIC in transfection mix (0.5 mg/ml DEAE-Dextran, 50mM Hepes (pH 7.4), DMEM) supplemented with 200 μCi/ml ^{32}P orthophosphate. After 4 hours of incubation at 37 °C, cells were washed and lysed in RIPA (radio immunoprecipitation assay) Buffer with protease and phosphatase inhibitors. After clearing the lysate by centrifugation and preincubation with Protein G Sepharose beads, eIF-2α was immunoprecipitated overnight with a sheep polyclonal anti-eIF-2α antibody, washed and eluted then resolved on a 12% SDS-PAGE. Phosphorylation was determined by autoradiography.

RESULTS

Generation of mice with mutated PKR gene.

Genomic clones encoding PKR were isolated from a phage library of murine genomic DNA using the full length murine PKR cDNA as a probe (42,78,79). Targeted disruption of the PKR gene by homologous recombination of the PKR allele with the targeting vector, pTV65TK, would replace exon 12 (following Kuhen, K.L. et al nomenclature (80)) with an HSV TK gene promoter-driven neomycin resistance cassette in an orientation opposite to the PKR promoter (Fig. 1A). Exon 12 encodes subdomains V and VI of the catalytic domain where removal of only six amino acids ablates the catalytic function of PKR (98). Transfected ES cells were grown in selection media, picked and screened for true homologous recombinants (Fig. 1B and data not shown) by Southern blot analysis. Targeted cell lines showed the predicted sized fragment for single site integration as detected by the internal Neomycin probe (Fig. 1B). Selected ES cells were microinjected

into blastocysts from BalbC mice, implanted into foster mothers and gave rise to chimeric animals. These were bred with wild-type BalbC mice to produce heterozygotes which were then interbred to yield progeny with wild-type, heterozygous and homozygous null genotypes. The frequency of wild-type, heterozygous and homozygous animals was 0.24. 0.57 and 0.19 respectively (Table 1). The apparent depressed frequency of homozygotes was not supported by intercross of homozygotes which were normal in development, behaviour and fertility. Litter sizes of these crosses were comparable to heterozygote crosses (Table 1). As with the amino-terminal targeted PKR mouse (140), no tumours have arisen in homozygous mice targeted at the PKR catalytic domain after a year.

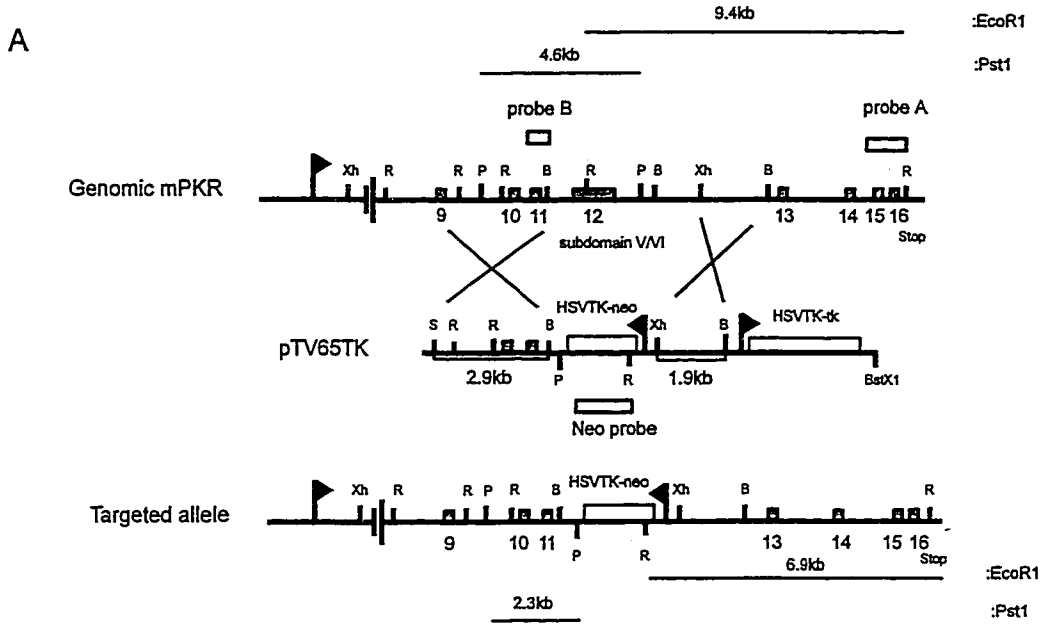
Characterization of targeted disruption of the PKR catalytic domain.

First, we examined whether disruption of PKR using interruption of the catalytic domain results in biochemical inactivation. An ES cell line (7A8.3.7) that was homozygous $PKR^{0/0}$ was selected from the heterozygous mutant cell line, 7A8.3, as previously described (269), using higher dose G418 selection (Fig. 1B). These cell lines and primary mouse embryo fibroblasts (MEFs) derived from homozygous null animals were assessed for PKR activity. Northern blot analysis of $polyA^+$ selected RNA from wild type ES cells showed the normal pattern of murine PKR transcripts of 6 kb, 4 kb and 2.5 kb which are induced in IFN treated cells (Fig. 2, (42)). Heterozygous $PKR^{+/0}$ cells showed similar expression of transcripts. However, homozygous $PKR^{0/0}$ cells completely lacked the fully spliced mature 2.5 kb transcript as well as the incompletely spliced 6 kb transcript (Fig. 2). However, a transcript of about 4 kb and which appeared IFN-inducible was found in all cells. Reprobing

Figure 1. Targeted inactivation of the PKR gene in ES cells and mice.

(A) Schematic drawing of wild-type PKR locus, replacement-type targeting vector pTV65TK, and targeted PKR locus after homologous recombination (not drawn to scale). Shaded boxes represent PKR exons. pTV65TK contains an HSV TK-neo cassette in the opposite transcriptional orientation to the PKR gene and an HSV TK-tk expression cassette on the 3' end of the construct. Homologous recombination results in introduction of new EcoR1 and Pst1 sites in the targeted allele. Probe A was used in initial screens with EcoR1 digestion. Wild-type and recombinant alleles produce 9.4 and 6.9 kb fragments respectively with this screen. Established mouse lines were screened with Pst1 digestion and probe B. This screen produces 4.6 kb and 2.3 kb fragments from wild-type and recombinant alleles respectively. Alternatively, a PCR screen was developed (see Appendix). The Neo probe was used with an Xho1 digest to screen for integration of the targeting vector. R, EcoR1; B, BamH1; S, Sca1; P, Pst1; Xh, Xho1; neo, neomycin expression cassette; HSV TK, herpes simplex virus thymidine kinase promoter; tk, herpes simplex virus thymidine kinase expression cassette. (B) ES cell clones bearing homologous recombinant targeted alleles. Electroporated ES cells colonies were picked and analysed by Southern screening with EcoR1 digest and probe A (left hand panel). 3 in 750 G418-FIAU-resistant cell lines were homologous recombinants. Single copy integration of the targeting vector was checked with a Xho1 digest with the Neo probe showing the predicted 16 kb fragment (right hand panel). The homozygous PKR-null cell line 7A8.3.7 was derived by high dose G418 selection. +/+, wild-type; +/O, heterozygous for PKR-null allele; O/O, homozygous for PKR-null allele. Molecular weight markers in kb indicated on the left of each panel.

Figure 1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probe A
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Stop
subdomain V/VI
HSVTK-neo
HSVTK-tk
pTV65TK
S R R B Xh B
2.9kb P R 1.9kb
BstX1
Neo probe
Targeted allele
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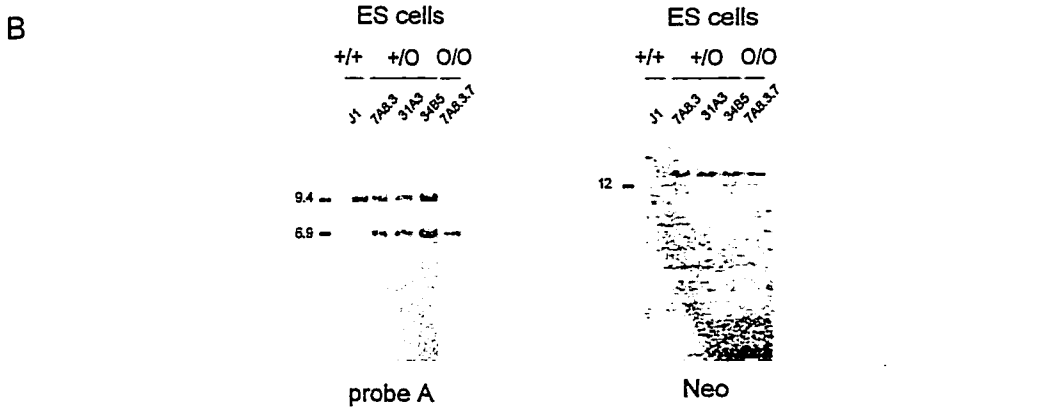

B
ES cells
+/+ +/O O/O
J1 7A8.3 31A3 3485 7A8.3.7
9.4
6.9
probe A
ES cells
+/+ +/O O/O
J1 7A8.3 31A3 3485 7A8.3.7
12
Neo

Table 1. Genotype of progeny of heterozygote and homozygote self crosses.

Several mating pairs of either heterozygotes (upper panel) or homozygotes (lower panel) were bred and their offspring genotyped by Southern analysis. The number of offspring of a particular genotype is denoted above along with the sample size and the average litter size arising from these matings. +/+, wild-type; +/O, heterozygotes; O/O, homozygotes.

Table 1

Genotype of progeny of heterozygote and homozygote self crosses.

Heterozygous-heterozygous crosses

$PKR^{+/+}$	$PKR^{+/0}$	$PKR^{0/0}$	n=	Average litter size
37	88	29	154	7.0

Homozygous-homozygous crosses

$PKR^{0/0}$	n=	Average litter size
61	61	8.7

of this blot with the neomycin probe showed this transcript bore neomycin sequence (Fig. 2, right hand panel) not found in the 4 kb transcript in wild type cells and thus represented a novel transcript that was derived from the targeted allele. Since the 4 kb transcript in wild type cells does not hybridize with the neomycin probe, the transcript seen in $PKR^{0/0}$ cells arises from the targeted allele and coincidentally co-migrates with the 4 kb wild type transcript. The HSV TK promoter-derived neomycin transcript is also detected by the neomycin probe as a smaller transcript of about 900 bp. The IFN-inducibility of the 4 kb novel transcript in $PKR^{0/0}$ cells suggests it is likely PKR promoter-derived with read-through into the neomycin cassette. This was confirmed by RT PCR analysis which detects the proximal intron XI and strand-specific riboprobe hybridization which identifies the distal intron XII in this transcript (data not shown). If splicing variants that splice over the neomycin cassette exist, such transcripts would encounter frame shifts in all downstream exons (80,271). The above observations show that targeted disruption of PKR with our strategy generates alleles that do not produce the normal PKR transcripts and could at most produce message with premature stop codons in the region encoding the catalytic domain of PKR.

Genetic ablation of PKR was verified biochemically using two different antibodies raised against the amino terminal region of PKR. Western blot analysis of cell lysates from wild type and $PKR^{0/0}$ ES cells and primary MEFs using the antibody raised against murine PKR residues 101-114 (anti TikMAP2) show no mature murine PKR of 65 kDa (Fig. 3A). No truncated PKR polypeptide is apparent either, even with eight fold excess loadings (Fig. 3C). We attempted to increase the sensitivity of detection by enriching for PKR

Figure 2. Characterization of the PKR targeted allele.

polyA$^+$ Northern blot analysis of targeted PKR allele. mRNA was isolated from ES cells untreated or treated with IFN α and β as indicated. +/+, wild-type J1 cells; +/O, heterozygous 7A8.3 cells; O/O, homozygous 7A8.3.7 cells. Left hand panel, hybridization with murine PKR (mPKR) cDNA; bottom panel, hybridization with glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. Arrows indicate the 6, 4 and 2.5 kb PKR transcripts. Right hand panel, hybridization with neomycin (Neo) cDNA. Arrows indicate the 4 kb novel transcript and the 0.9 kb HSVTK-neo derived transcript from the targeted allele. 28S and 18S rRNA positions are indicated.

Figure 2

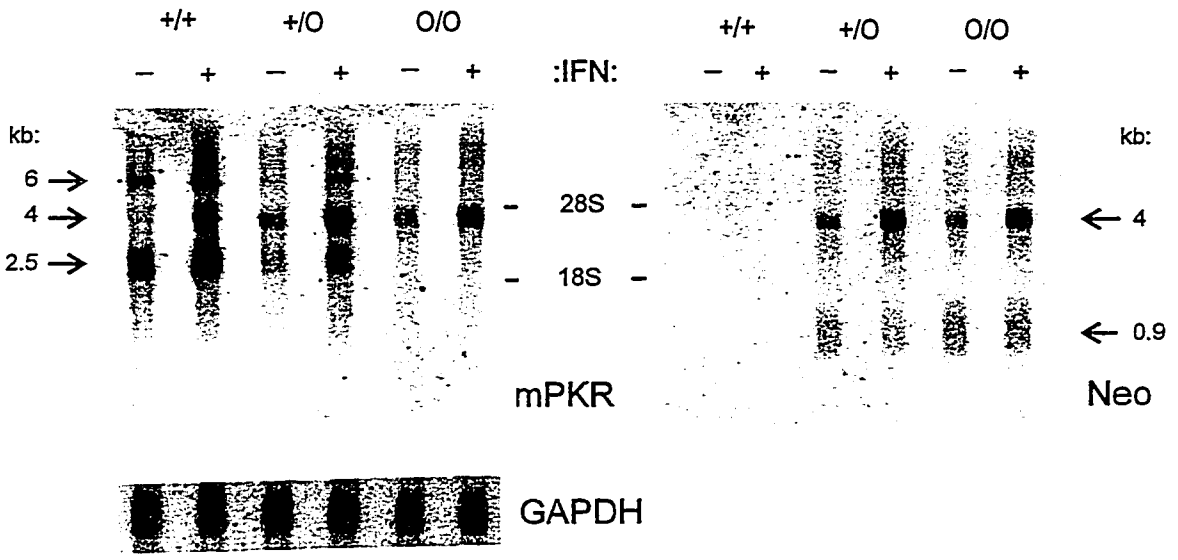
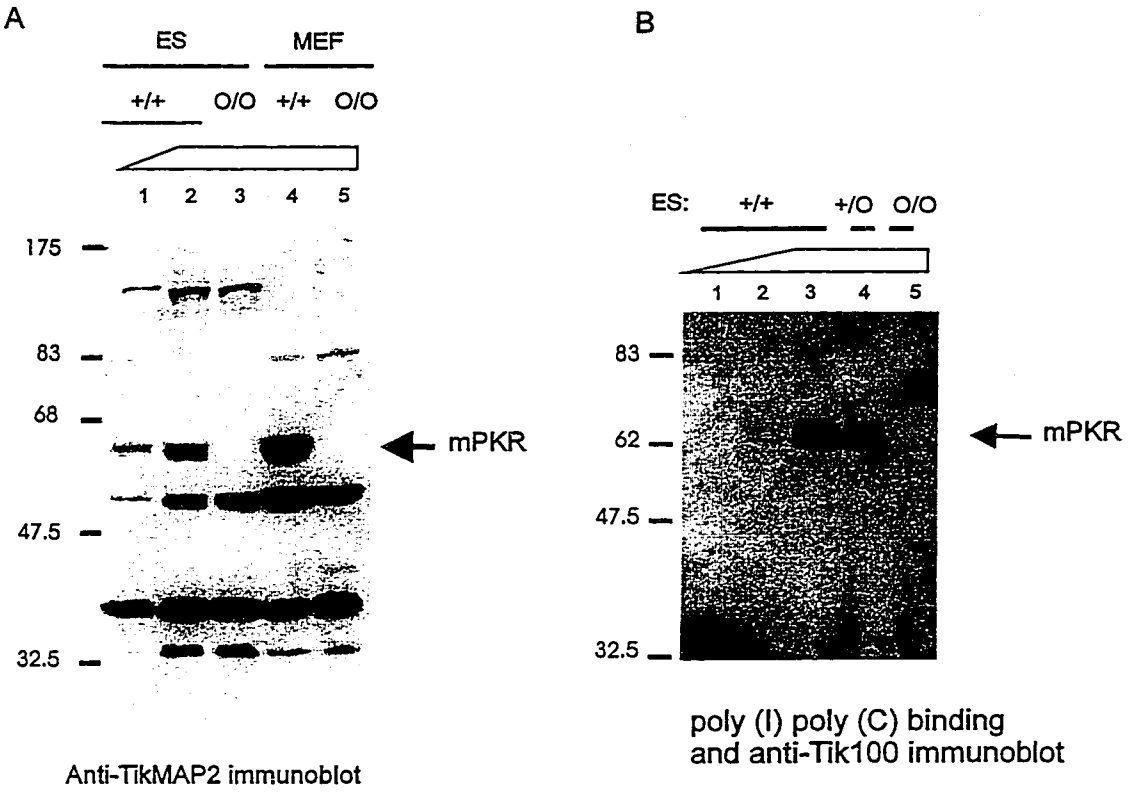


Figure 3. Immunoblot analysis of PKR-null ES and MEF cells demonstrate ablation of PKR protein.

(A) Western analysis. Total cell lysates from wild-type (+/+) or homozygous PKR-null (O/O) cells were extracted, separated by SDS-PAGE, immunoblotted with anti-mPKR antibody (affinity purified anti-TikMAP2 antiserum) directed against residues 101-114 and visualized with ECL. Lane 1 loaded with 25 μg total protein, lanes 2-5 loaded with 100 μg total protein. (B) dsRNA affinity binding enrichment analysis. Total protein from the indicated ES cell lines were incubated with pIC agarose, washed and bound protein analysed by immunoblot analysis. Anti-mPKR antiserum (anti-Tik100A) raised against purified His-mPKR residues 1-98 show mature PKR polypeptide in wild-type (+/+) and heterozygous (+/O) cells but no immunoreactive PKR polypeptides in homozygous (O/O) cells. Lane 1, 1.5 μg total protein, lane 2, 15μg total protein and lanes 3-5, 150 μg total protein incubated with dsRNA. (C) Western analysis. Total cell lysates from wild-type (+/+), heterozygous (+/O) or homozygous PKR-null (O/O) ES cells were extracted, separated by SDS-PAGE. immunoblotted with anti-TikMAP2 antiserum as above and visualized with ECL. -, untreated cells; +, IFN treated cells. Amount of lysate loaded indicated at top in μg protein. The relative molecular mass markers in kDa are indicated on the left and the position of mPKR is indicated with an arrow on the right in each panel.

Figure 3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ES
MEF
+/+
O/O
+/+
O/O
1 2 3 4 5
175
83
68
47.5
32.5
mPKR
Anti-TikMAP2 immunoblot
B
ES: +/+ +/O O/O
1 2 3 4 5
83
62
47.5
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poly (I) poly (C) binding
and anti-Tik100 immunoblot
C
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Anti-TikMAP2 immunoblot

polypeptides with intact RNA binding capacity by incubating lysates with pIC-agarose then immunoblotting for bound PKR polypeptides. Wild type and $PKR^{+/0}$ cells showed murine PKR binding to dsRNA that was detectable by another antibody raised to murine PKR residues 1-98 (anti-Tik100; Fig. 3B). $PKR^{0/0}$ cells, however, have no detectable mature or truncated PKR polypeptides. An immunecomplex kinase autophosphorylation assay using this same antibody was performed on cell extracts from $PKR^{0/0}$ cells. No functional PKR activity was detectable in $PKR^{0/0}$ cells of either ES or MEF origin (Fig. 4, lanes 5 and 8). These results provide convincing evidence that the strategy of catalytic disruption of PKR by targeting exon 12 leads to a null allele with no catalytic function retained.

IFN and cytokine signalling in $PKR^{0/0}$ cells.

Increasing evidence points to a role for PKR in transcription control and cytokine signalling (75,138,139,141,145). PKR is implicated in regulating IRF-1 and NF-κB activation (75,141) and is found complexed with STAT1 in an inverse relation to STAT1 activation (145). We examined the responsiveness of $PKR^{0/0}$ cells to the type I IFNs by assessing the induction of the 2' 5' oligoadenylate synthetase (OAS) transcript in these cells. IFN α/β signalling through STAT1 appears to be unimpaired since induction of 2' 5' OAS was normal in $PKR^{0/0}$ cells (Fig. 5).

Next, we assessed other cytokine signalling pathways by determining whether hematopoeitic development in $PKR^{0/0}$ animals was affected. Bone marrow from animals of wild-type and homozygous null backgrounds were isolated, normalized and plated in colony forming assays in the presence of the indicated cytokines (Fig. 6). Colony forming units (CFU) were scored ten days later for colour and counted to determine the response of each

Figure 4. Immunecomplex kinase assay demonstrates loss of PKR catalytic activity in PKR-null cells.

PKR protein was recovered by incubating lysates from the indicated wild-type and homozygous PKR-null ES and MEF cells with anti-mPKR anti-serum (anti-Tik100A). Immunecomplexes were incubated in reaction buffer with dsRNA and γ ^{32}P-ATP and following autophosphorylation of the kinase, resolved by SDS-PAGE and visualized. Lanes 1,2,4,5,7 and 8, 200 μg total protein, lanes 3 and 6 10 μg total protein. Lane 1, Protein A Sepharose beads alone, lane 2, preimmune anti-serum and Protein A Sepharose beads, lanes 3-8, anti-Tik100A and Protein A Sepharose beads. Molecular weight markers (kDa) are indicated on the left and the 65 kDa murine PKR polypeptide (mPKR) indicated on the right.

Figure 4

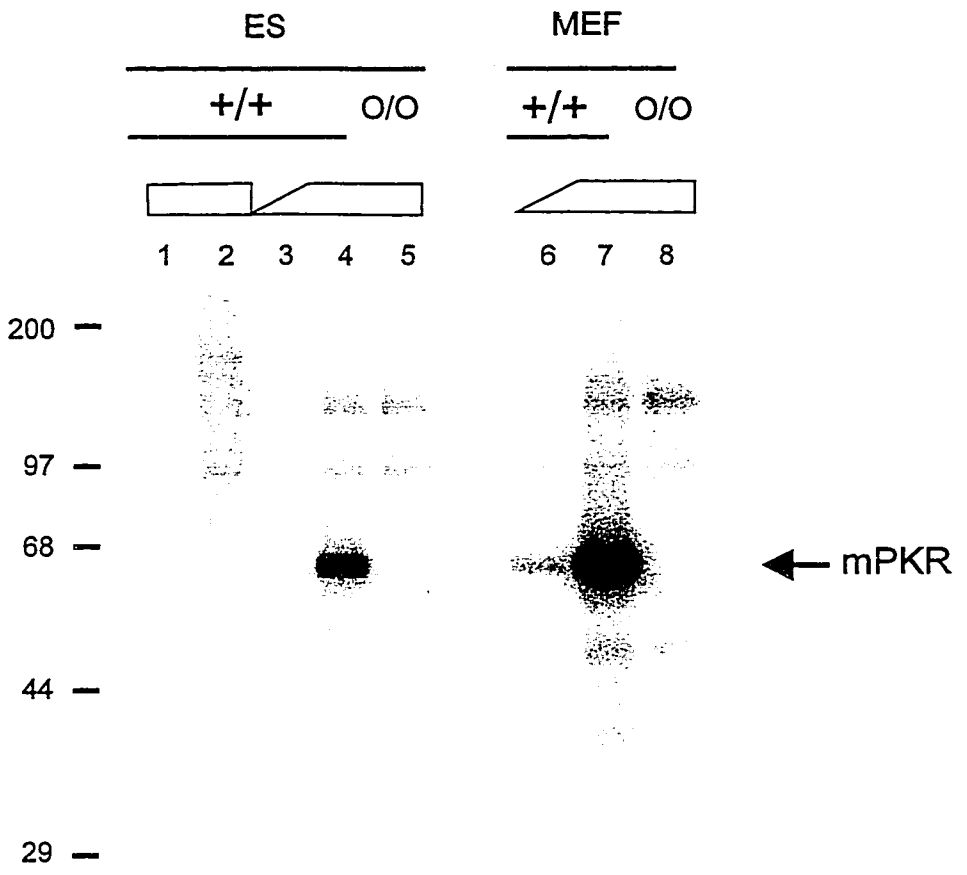


Figure 5. Type I IFN signalling is unimpaired in PKR-null cells.

polyA$^+$ Northern blot analysis of IFN induction of 2' 5' OAS. mRNA was isolated from ES cells untreated or treated with IFN α and β as indicated. +/+, wild-type J1 cells; +/O, heterozygous 7A8.3 cells; O/O, homozygous 7A8.3.7 cells; IFN, interferon α and β treatment. Top panel, hybridization with 2' 5' OAS cDNA; bottom panel, hybridization with GAPDH.

Figure 5

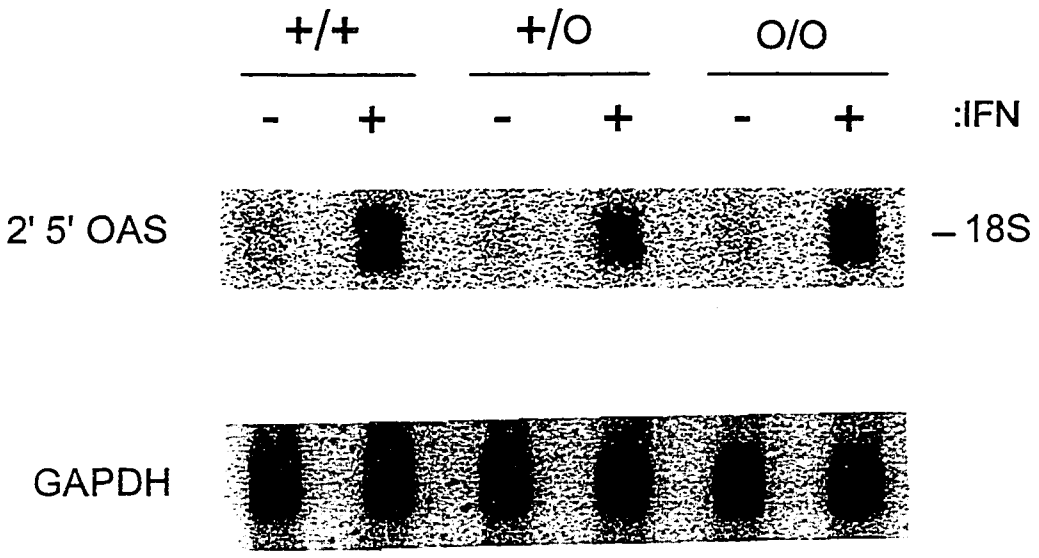
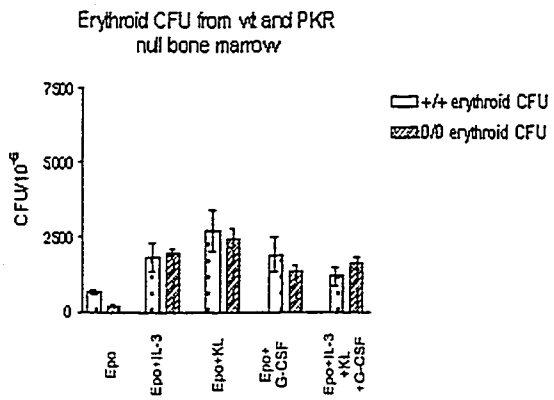


Figure 6. Hematopoeitic development in PKR-null animals appears normal.

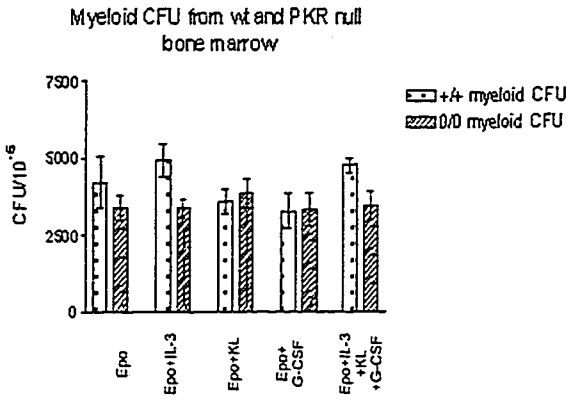
(A) Erythroid bone marrow precursors and (B) Myeloid bone marrow precursors were assessed for cytokine responsiveness by clonogenic assay in the presence of the indicated cytokines. Six animals of each genotype were analysed with triplicate platings of each cytokine treatment. CFU were scored 10 days after plating with cytokine. Error bars represent standard error of the mean (SEM), n=6. Epo, human erythropoeitin; IL-3, murine interleukin-3; KL, human kit ligand; G-CSF, human granulocyte colony stimulating factor.

Figure 6

A


Erythroid CFU from wt and PKR null bone marrow
CFU/10^-5
7500
5000
2500
0
+/+ erythroid CFU
0/0 erythroid CFU
Epo
Epo+IL-3
Epo+KL
Epo+ G-CSF
Epo+IL-3 +KL +G-CSF

B


Myeloid CFU from wt and PKR null bone marrow
CFU/10^-5
7500
5000
2500
0
+/+ myeloid CFU
0/0 myeloid CFU
Epo
Epo+IL-3
Epo+KL
Epo+ G-CSF
Epo+IL-3 +KL +G-CSF

hematopoeitic precursor population to cytokine stimulation. Myeloid bone marrow precursors showed comparable response to IL-3, Kit ligand (KL) and G-CSF treatment (Fig. 6B) although the Epo alone control indicated a high background of myeloid CFU growth even in the absence of other cytokines added. Erythroid CFU growth from $PKR^{0/0}$ animals however, demonstrated a reproducible 3-fold diminished response to Erythropoeitin (Epo). IL-3 or KL co-treatment appear to be able to overcome this PKR-dependent Epo block in erythroid precursors from $PKR^{0/0}$ animals (Fig. 6A). In addition, these precursors show a higher fold stimulation of CFU in response to IL-3 and KL than in wild type bone marrow. This would be consistent with a study implicating PKR in IL-3 stimulation of protein synthesis in an IL-3 dependent cell line (209). This block appears to have little physiological impact since hematocrit volumes from PKR null animals were unaffected (data not shown).

$PKR^{0/0}$ animals retain anti-viral responsiveness.

While $PKR^{0/0}$ animals appeared normal in their cytokine signalling capacities, we wished to determine if loss of PKR would affect their ability to counter viral challenge. Vaccinia maintains resistance to IFN despite producing large amounts of dsRNA in late infection (170). This is thought to be due to the production of K3L and E3L, two PKR inhibitors synthesized by the virus for optimal virus replication (174,175). Induced expression of PKR has nonetheless been shown to inhibit vaccinia replication in a variety of cells by severely restricting viral protein synthesis (219). The growth curve of vaccinia (WR strain) in wild-type and PKR null primary MEF cells in culture was examined (Fig. 7). The production of infectious vaccinia virus particles in control CV-1 and L-929 cells closely matched the kinetics of growth of this virus strain in HeLa cells (219). The virus replicated

Figure 7. Vaccinia growth in PKR-null cells is normal.

Growth curve of vaccinia virus (WR strain) in MEFs and control cells were examined. Cells were infected at MOI 10, washed then monitored over 18 hours for virus production by plaque assay of cell lysates. Error bars represent standard error of the mean (SEM), n=6. Main panel, vaccinia growth in control cells and MEFs; inset, vaccinia growth in wild-type (+/+) and PKR-null (O/O) MEFs.

Figure 7

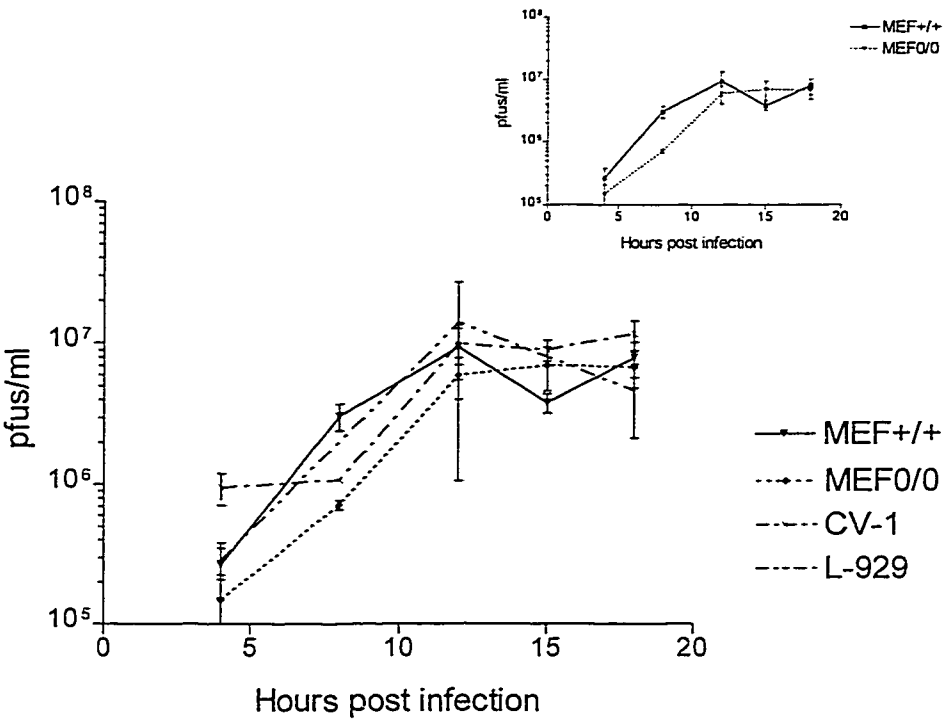


Table 2. Anti-influenza response is unimpaired in $PKR^{0/0}$ animals.

Age matched mice were infected by intra nasal innoculation with serial dilutions of the W29 strain of mouse-adapted influenza virus (270) in groups of 8-12. Mice were monitored over 10 days for lethality and the LD_{50} determined.

Table 2

Influenza LD_{50} in normal and PKR-null animals

	LD_{50}	
$PKR^{-/0}$	$10^{4.3}$	n=27
$PKR^{0/0}$	$10^{4.0}$	n=26
$Clk1^{0/0}$	$10^{4.2}$	n=12
CD-1	$10^{3.1}$	n=45

equally well in wild-type primary MEFs and surprisingly, showed no significant difference in growth curve in PKR-deficient MEFs (Fig. 7 and inset). No enhancement of vaccinia yield was evident in the absence of PKR, in contrast to the repressive effect of over expression of PKR (219). In fact the low virus yield in early infection from $PKR^{0/0}$ cells could imply a requirement for PKR function to maximize viral replication during certain stages as has been suggested for HSV-1 (194).

In order to determine if replication of an RNA virus might be more affected by the loss of PKR, we examined the LD_{50} of the W29 mouse-adapted strain of influenza virus by intranasal infection of control and $PKR^{0/0}$ animals. As shown in Table 2, heterozygous and PKR null animals showed an LD_{50} of $10^{4.3}$ and $10^{4.0}$ infectious particles respectively. This 2 fold difference in apparent susceptibility of $PKR^{0/0}$ animals, comparable to control $Clk1^{0/0}$ and CD-1 mice, does not seem significant when compared to the consequences of loss of STAT1 function upon anti-viral response(154,272). STAT1 deficient mice completely succumbed to doses of VSV challenge that were 10^4 to 10^6 lower than doses that were sublethal to wild-type animals. Furthermore, these animals had elevated susceptibility to a bacterial pathogen, Listeria, and opportunistic infections by murine hepatitis virus (MHV). We also measured the level of influenza growth in mouse lung two days post-infection for pools of three mice from each group. PKR-null mice had 3.8×10^7 pfu/ml versus 9.0×10^7 pfu/ml for control mice. This similarity in viral titres indicates that influenza growth in PKR-null mice is not unchecked. Although the infectivity of other viruses in $PKR^{0/0}$ animals or cells have to be examined, to date, we have found no sign of impaired anti-viral response or opportunistic infections in these animals. Our data demonstrates that PKR is not essential

for IFN type I responses or for countering vaccinia or influenza infections and may be redundant in function.

Virus induced apoptosis in PKR$^{0/0}$ cells is unimpaired.

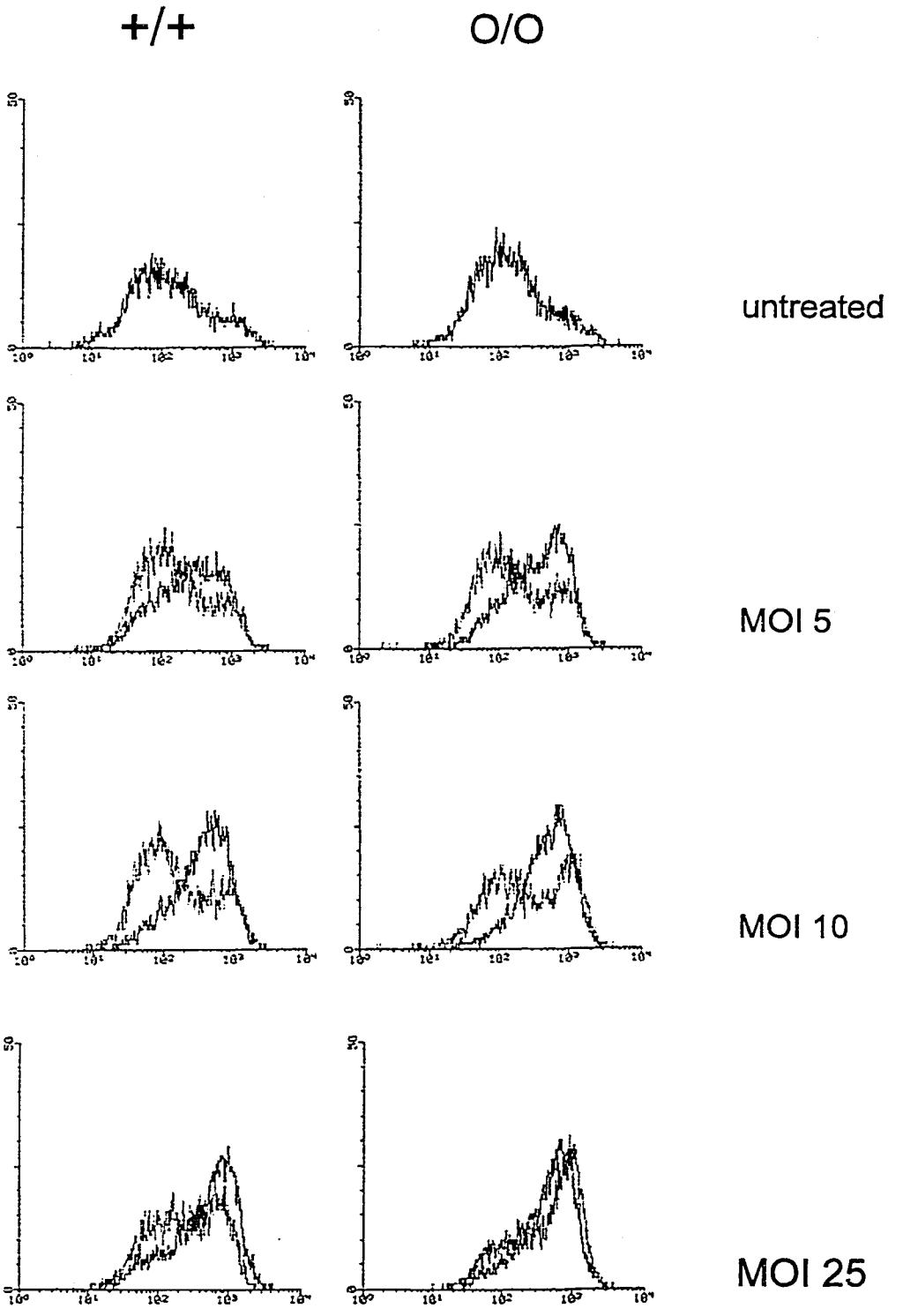
Recent progress in the study of programmed cell death makes it apparent that viruses have evolved strategies to overcome cells responding to infection with apoptosis (reviewed in Wertz and Hanley (273), see discussion). PKR's own role in regulating apoptotic responses has been shown by various groups (234-236,238,239). PKR expression induced apoptosis (234), anti-sense ablation of PKR rendered U937 cells resistant to TNFα induced apoptosis (236) and dominant negative mutants of PKR suppressed Fas induction and cell death induced by influenza infection (238). As such, PKR may have a physiological role to play in responding to viral infection with apoptosis.

To assess whether PKR$^{0/0}$ cells were compromised in their ability to respond to viral infection with apoptotic responses, we challenged MEFs with a strain of influenza A/HK/1/68 (H3N2) (274) at various MOI. Cells undergoing apoptosis were monitored using Annexin V-FITC (Boehringer Mannheim) staining indicating phosphatidyl serine externalization on the plasma membrane, an early apoptosis marker (275). FACS analysis of MEFs infected with virus (Fig. 8) show a dose-dependent increase in cells undergoing apoptosis as determined by the elevated number of FITC positive cells. The percentage of these cells also showing propidium iodide uptake, indicating incidental necrotic death was small (typically 10-20% of total cells) (data not shown). PKR$^{0/0}$ cells are as competent as wild-type cells in triggering apoptosis upon influenza infection. This contrasts with the effect of dominant-negative mutants of PKR which reduced influenza mediated cell death

Figure 8. Apoptotic response to influenza infection is unimpaired in PKR-null cells.

Untreated or IFN pretreated MEFs were mock infected or infected with influenza A/HK/1/68 virus at increasing MOI. After 24 hours, cells were isolated, stained with Annexin V-FITC, fixed and analysed by FACS for cells undergoing apoptosis. Histograms showing distribution of fluorescence of cells of each genotype and treatment are shown. Horizontal axis denotes FITC fluorescence intensity (log scale); vertical axis denotes number of cells (linear scale). Left hand panels, wild-type (+/+) MEFs; right hand panels, PKR-null (O/O) MEFs. Black trace, cells without IFN pretreatment; gray trace, IFN α and β pretreated cells.

Figure 8



(238). IFN α/β pretreatment partially abrogated influenza-induced apoptosis in both normal and PKR null cells up to an MOI of 10 (Fig. 8). At higher MOI, IFN pretreatment was unable to rescue cells from virally induced apoptotic pathways. This data indicates that PKR is not essential for influenza to trigger apoptosis pathways in cells, and that type I IFNs can mount anti-apoptotic responses at low MOI independent of PKR by mechanisms that may occur directly or indirectly through inhibition of viral replication.

Stress induced apoptotic response in $PKR^{0/0}$ cells is normal.

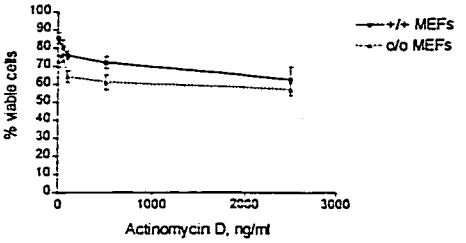
Apoptosis may be induced by a number of signals including cellular stress. PKR itself has been implicated in responses to various forms of stress (276,277). Der et al describe the abrogation of stress-induced apoptosis in the PKR knockout cells they generated (140,237). The cells had impaired apoptotic responses to TNFα, pIC and LPS. We addressed the role of PKR in these pathways by determining whether our PKR-null cells were likewise impaired. We first assessed the dose response of our wild-type and $PKR^{0/0}$ MEFs to actinomycin D (Fig. 9A) which is required to prime cells to respond to these stimuli ((237,278,279) and Fig. 9B). Having found no significant differences, we then optimized TNFα, LPS and pIC treatments of wild-type MEFs to trigger cell death as measured by trypan blue exclusion (Fig. 9B) and verified by TUNEL assay and Hoechst staining. We found that at the cytostatic dose of actinomycin D (50ng/ml) described previously (237), none of these treatments elicited much response from the MEF cells, even at the maximal doses of TNFα, LPS or pIC (Fig. 9B). It required higher doses of co-treatment with actinomycin D (500ng/ml) before any effect was seen on cell viability and this was restricted to TNFα treatment only (Fig. 10 and 11). Untreated, actinomycin D alone treatment or

Figure 9. TNFα-mediated apoptosis is normal in PKR-null cells.

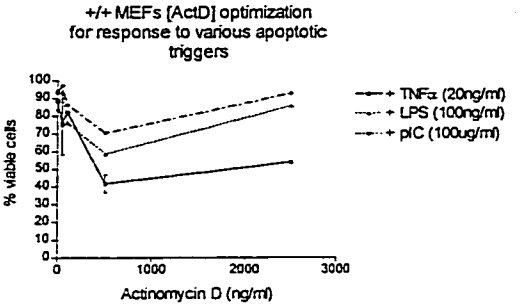
(A) Comparable Actinomycin D dose response in MEFs. Since Actinomycin D co-treatment is required to trigger apoptosis with various stimuli, wild-type (+/+) and PKR-null (O/O) MEFs were first assessed for toxicity response to Actinomycin D. Cells were treated with increasing doses for 24 hours and stained with trypan blue to determine viability. These determinations were performed in triplicate and error bars represent standard error of the mean (SEM). (B) The optimal Actinomycin D dose for various apoptosis triggers was determined. Wild-type MEFs were co-treated with TNFα, LPS or pIC and increasing amounts of Actinomycin D for 24 hours. Cell viability was determined as above. LPS and pIC fail to show apoptosis above Actinomycin D alone levels. Actinomycin D co-treatment is required to potentiate TNFα-induced cell death, and the minimal Actinomycin D dose for such effect is 500ng/ml. (C) TNFα dose response of PKR-null cells indicates unimpaired apoptotic response in the absence of PKR. Wild-type (+/+) and PKR-null (O/O) MEFs were co-treated with Actinomycin D (500ng/ml) and increasing amounts of TNFα for 24 hours. Cell viability was determined as above. TNFα treatment induces cell death in a dose dependent manner that is unaffected by the absence of PKR. Error bars represent standard error of the mean (SEM), n=3. Inset, TNFα dose response plotted on a log scale.

Figure 9

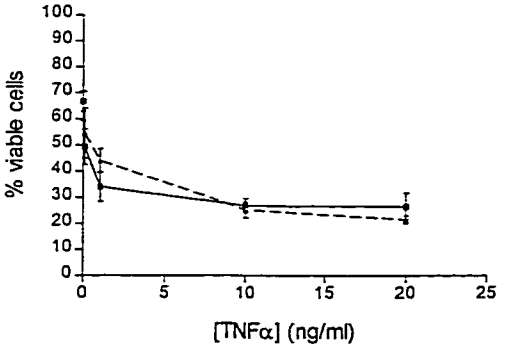
A


Viability of MEFs in ActinomycinD
% viable cells
Actinomycin D, ng/ml
+/+ MEFs
o/o MEFs

B


+/+ MEFs [ActD] optimization for response to various apoptotic triggers
% viable cells
Actinomycin D (ng/ml)
TNFα (20ng/ml)
LPS (100ng/ml)
pIC (100ug/ml)

C


MEF dose response to TNFα
% viable cells
[TNFα] (ng/ml)
+/+ MEFs
o/o MEFs

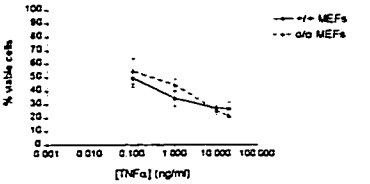


Figure 10. Hoechst and TUNEL assays show normal TNFα-mediated apoptosis in PKR-null cells.

Cells were grown on cover slips, treated as in Figure 9C then fixed, permeabilised and treated with TUNEL FITC reagents as described in the Materials and Methods. This was followed by Hoechst staining and visualization by fluorescence microscopy. Six panels on the left, wild-type MEFs; six panels on the right, $PKR^{0/0}$ MEFs. Panels a-d, untreated cells; e-h, 500 ng/ml Actinomycin D alone treatment; i-l, 500 ng/ml Actinomycin D and 20 ng/ml TNFα treatment. Panels a, c, e, g, i and k, Hoechst visualization; panels b, d, f, h, j and l, FITC visualization.

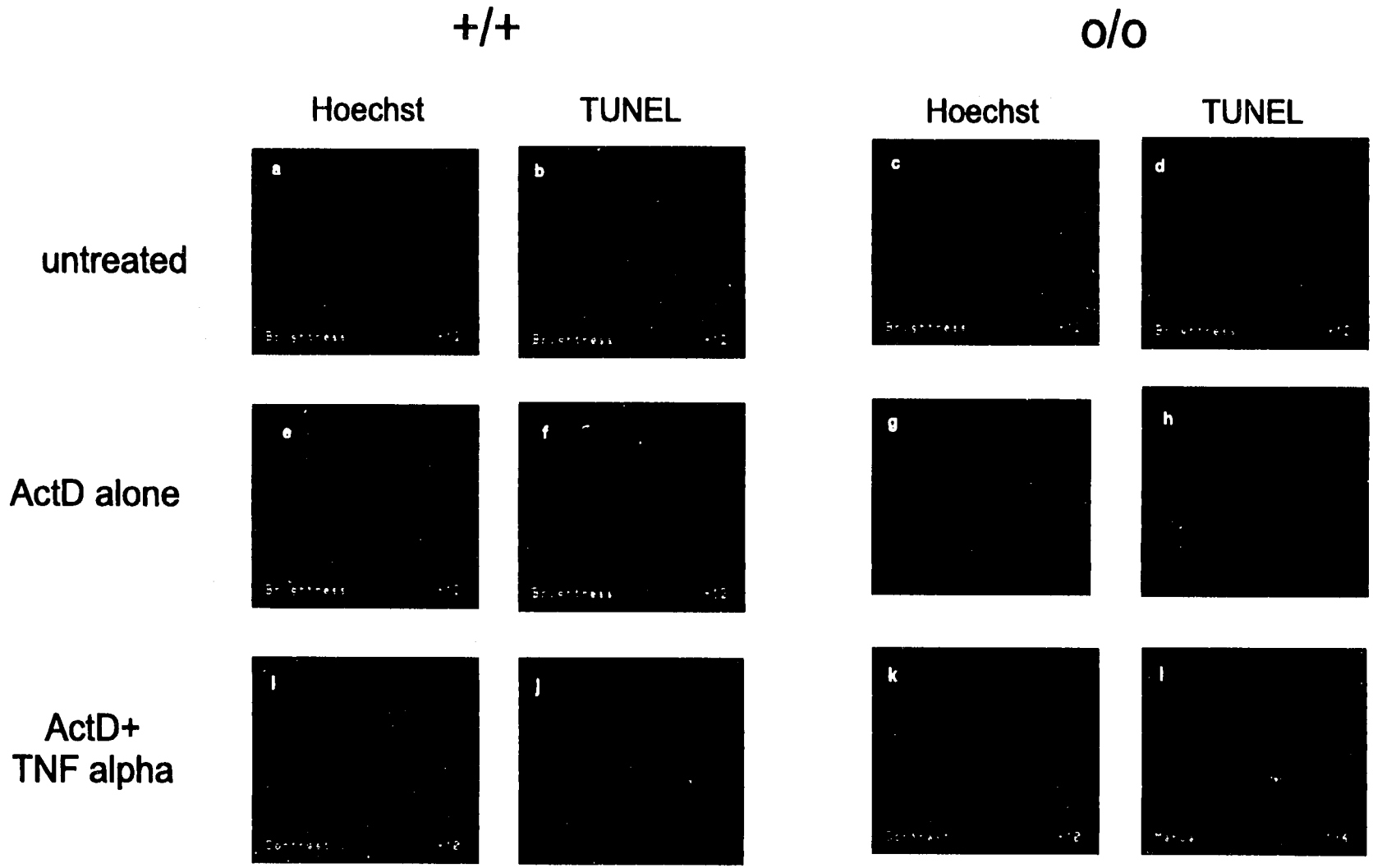

+/+
o/o
Hoechst
TUNEL
Hoechst
TUNEL
untreated
ActD alone
ActD+
TNF alpha
a
b
c
d
e
f
g
h
i
j
k
l

Figure 10

Figure 11. Hoechst and TUNEL assays show no apoptotic response to pIC or LPS treatment.

Cells were grown on cover slips, treated as in Figure 9B with 500 ng/ml Actinomycin D then fixed, permeabilised and treated with TUNEL FITC reagents as described in the Materials and Methods. This was followed by Hoechst staining and visualization by fluorescence microscopy. Six panels on the left, wild-type MEFs; six panels on the right, $PKR^{0/0}$ MEFs. Panels a-d, 500 ng/ml Actinomycin D alone treatment; e-h, 500 ng/ml Actinomycin D and 100 μg/ml pIC treatment; i-l, 500 ng/ml Actinomycin D and 100 ng/ml LPS treatment. Panels a, c, e, g, i and k, Hoechst visualization; panels b, d, f, h, j and l, FITC visualization.

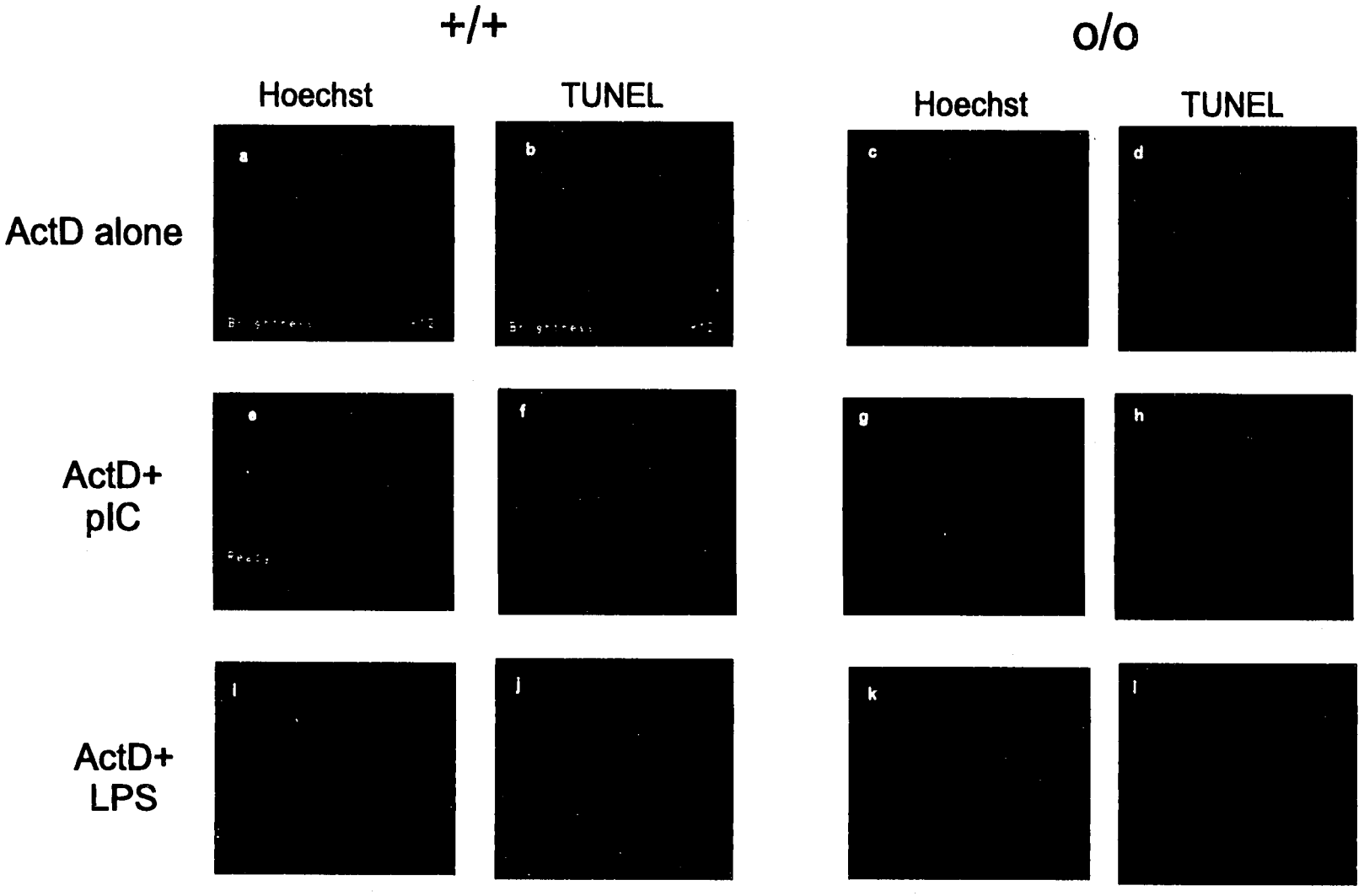

+/+
o/o
Hoechst
TUNEL
Hoechst
TUNEL
ActD alone
ActD+ pIC
ActD+ LPS
a
b
c
d
e
f
g
h
i
j
k
l

Figure 11

actinomycin D co-treatment with pIC or LPS clearly showed many cells with normal morphology with no TUNEL positive signal (Fig. 10, panels b and d, Fig. 11, panels b,d,f,h,j and l). Albeit only a few cells remained adherent in actinomycin D co-treatment with TNFα, intense TUNEL positive signals could be detected in both wild-type and $PKR^{0/0}$ cells remaining (Fig. 10, panels j and l). We are not able to account for the insensitivity to LPS or pIC other than mouse strain or embryo fibroblast differentiation differences. Finally, we determined the dose response of wild-type and PKR-null MEFs to TNFα-induced apoptosis and found that $PKR^{0/0}$ cells with disruption of the catalytic domain had no impairment of cell death response (Fig. 9C) as described in the other PKR-null mouse model (237). This was apparent throughout the dose range of TNFα (0.1-20 ng/ml) used (Fig. 9C and inset). Our data suggests that if indeed PKR has a role to play in apoptotic responses to viral infection or stress triggers such as TNFα as suggested by other studies, that role is non-essential.

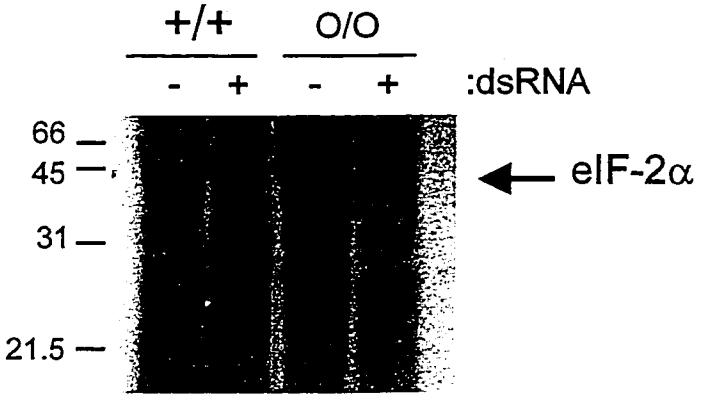
Intact eIF-2α phosphorylation in $PKR^{0/0}$ cells may indicate the presence of a PKR homolog.

The best known mammalian PKR homolog, the heme-regulated inhibitor (HRI), is restricted to cells of erythroid lineage (112) and restricts globin synthesis in these cells when activated by the absence of heme (100,280). The only other homolog of PKR is the yeast GCN2 kinase which is activated by uncharged tRNA and regulates amino acid biosynthesis by control of translation of the GCN4 transcript (281). Despite apparent specialization in function with unique regulatory domains, both mammalian eIF-2α kinases, HRI and PKR can rescue GCN2 defective yeast (121). The absence of a clear phenotype in mice targeted at the PKR locus at the catalytic domain raised the question of whether this targeting strategy

Figure 12. Retention of eIF-2α phosphorylation in $PKR^{0/0}$ cells clearly indicates PKR redundancy.

In vivo labelling of wild-type (+/+) and PKR catalytic domain targeted MEFs (O/O) with either transfection (+) or no transfection (-) of dsRNA. Recovery of eIF-2α by immunoprecipitation indicates it is still phosphorylated suggesting the presence of compensatory homolog(s).

Figure 12



IP anti-eIF-2α

produces a null allele that is rescued by redundancy of the eIF-2α kinases. Since this strategy effectively renders the PKR catalytic domain inactive, any eIF-2α phosphorylation in homozygous null cells could be attributed to such redundancy. Preliminary examination of the state of phosphorylation of eIF-2α in $PKR^{0/0}$ cells surprisingly showed that it was unimpaired (Fig. 12). This finding is consistent with the absence of any phenotype in anti-proliferative, anti-viral and apoptotic responses, since at least one of the PKR substrates, eIF-2α, is clearly still regulated by an eIF-2α kinase.

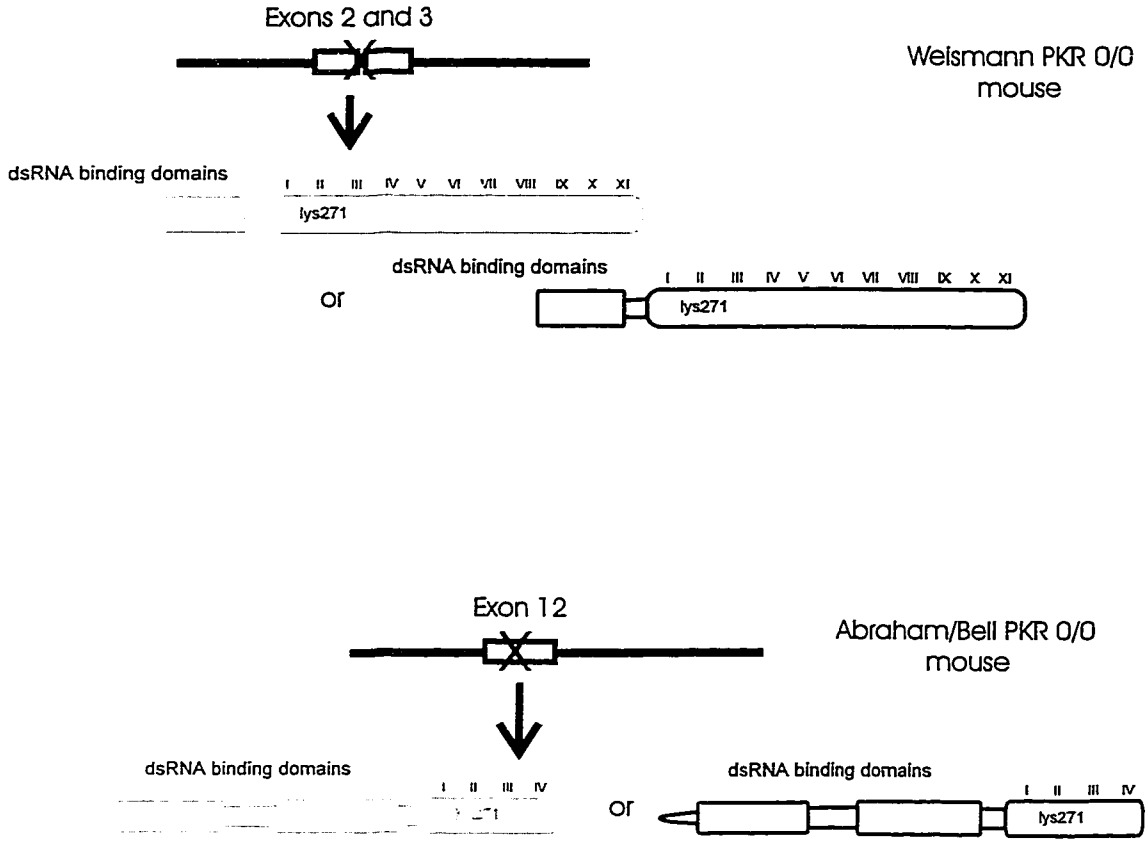
DISCUSSION

Targeted disruption of PKR at the catalytic domain was performed to fully explore the consequences of loss of PKR function at the organismal level. The absence of any evidence of elevated tumorigenesis in the PKR null mouse generated by Yang et al (140) which targeted amino terminal domains of PKR and had documented splicing over of the targeted exons raised the issue of whether the retention of catalytic domain integrity might somehow maintain PKR function by an undetected polypeptide (Fig. 13). Indeed, all three mPKR transcripts were detected with slight mobility alteration representing splicing deletion in Northern analysis of the Yang-Weismann $PKR^{0/0}$ mouse (compare Fig. 2). Our targeting strategy inactivates the catalytic domain by replacing in entirety an exon which encodes sixty amino acid residues including six amino acids which are required for catalytic function (98) (Fig. 13). Furthermore, if any splicing variants exist that bypass the disrupted exon and splice into any of the distal exons 13-16 (282), all such transcripts will encounter frame shifts that terminate prematurely within the catalytic domain. Analysis of the single transcript derived from this targeted allele indicates that it contains intron sequence and anti-

Figure 13. Comparison of targeting strategy in the two PKR-null mouse models and their likely outcomes.

This schematic representation shows the region of the PKR gene targeted by the Yang-Weismann study (140) and by our work presented here. The Wesimann $PKR^{0/0}$ mouse disrupts Exons 2 and 3, but has shown splicing variants that bypass the disrupted exons to splice into the splice acceptor site in Exon 4. This could allow targeted alleles to encode a polypeptide beginning at residue 136 (140), leaving only dsRBD I deleted which may act as a transdominant mutation. Alternatively, since no polypeptide or catalytic activity is detectable, the transcript or product may be unstable and therefore, absent (gray outline). Our targeting strategy replaces Exon 12 which produces a transcript which retains introns flanking the disrupted exon. Furthermore, all distal splice acceptor sites alter the reading frame, effectively disrupting the transcript at this position. In principle, this allele could encode polypeptide with residues 1-318. Alternatively, with no detectable protein or activity, the polypeptide could be unstable (gray outline) thus producing a null allele.

Figure 13


Exons 2 and 3
Weismann PKR 0/0 mouse
dsRNA binding domains
I II III IV V VI VII VIII IX X XI
lys271
dsRNA binding domains
or
I II III IV V VI VII VIII IX X XI
lys271
Exon 12
Abraham/Bell PKR 0/0 mouse
dsRNA binding domains
I II III IV
dsRNA binding domains
or
I II III IV
lys271

sense neomycin sequence which would terminate the reading frame. No mature or truncated polypeptide was observed in $PKR^{0/0}$ cells and PKR catalytic activity was undetectable. Mice homozygous for this PKR disruption were bred and appeared to have no obvious defects in gross anatomy and were fertile with average sized litters. No evidence of susceptibility to opportunistic infections were seen and no signs of increased tumorigenesis have been observed similar to the Yang-Weismann mouse.

The maintenance of proper control of translation is understood to be critical to cellular growth control. A subset of transcripts is thought to exist with certain features in sequence or structure that tightly control their translation and whose deregulation results in aberrant growth control. Indeed, it has been reported that translation restrictive elements are found in a disproportionate number of the untranslated regions of transcripts of proto-oncogenes, growth factors, hormone receptors and transcription factors (122). The absence of any tumorigenic phenotype in either our PKR-null mice or that of Yang et al was puzzling given the evidence of the consequences of translation deregulation. When the restriction of translation at the cap binding step is circumvented by over-expression of the cap binding protein, eIF-4E, cellular transformation occurs (233). In yeast, the control of efficiency of reinitiation by phosphorylation of eIF-2α is important in control of growth (283). The ubiquitous dsRNA activated eIF-2α protein kinase PKR restricts growth when expressed in yeast and causes morphological transformation when inactive mutated versions are expressed in mammalian cells. Evidence that the mechanism of this transformation may occur by deregulated translation due to the hypophosphorylation of eIF-2α is provided by the observation that expression of non-phosphorylatable eIF-2α mutated at the Serine-51 target

of PKR also transforms cells (264). The identification and cloning of inhibitors of PKR, $p58^{IPK}$, a member of the tetratricopeptide repeat family (197,200) and TAR binding protein (TRBP) has similarly elucidated the transforming effect of repressing PKR function by over-expression of these inhibitors (231,232). Given this data, the absence of tumors in PKR-null mice suggests a redundancy of function that is not compromised in transgenic mice with null alleles but may be revealed by dominant negative alleles.

The anti-viral function of PKR has been studied with analyses of virus mutants lacking anti-PKR inhibitors as well as over expression of PKR and its effect on viral growth. Strategies utilized by viruses to overcome PKR activation are varied ranging from producing inactivating RNAs, pseudosubstrates, endogenous inhibitor mobilization, PKR expression down regulation and dsRNA sequestration (see Chapter 1). Certain viruses have also developed means to prevent cell death responses to infection (273). For example, Cowpox crmA encodes an inhibitor of the ICE protease (284), baculovirus IAP (inhibitor of apoptosis) permits escape from premature cell death and impaired replication (285), adenovirus E1B opposes the apoptotic function of E1A (286) by inactivating the p53 pathway (287,288) and Epstein-Barr virus upregulates bcl-2 expression and encodes a bcl-2 homolog, BHRF1 (289,290). A few studies now implicate PKR in controlling apoptosis in response to some triggers. PKR is itself a candidate "death gene" where overexpression causes apoptosis (234) and requires the third basic region involved in autoregulatory regulation (96). PKR is located upstream of bcl-2 function since bcl-2 abrogates PKR-mediated apoptosis (235). Furthermore, expression of inactivated PKR suppresses influenza mediated apoptosis involving upregulation of Fas (238) and anti-sense ablation of PKR

provides immunity to TNFα (236). This is a function consistent with PKR's role of monitoring cellular integrity and responding to viral infection to inhibit replication. The absence of anti-viral response defects in PKR catalytic-domain targeted null mice is most surprising. Both viral growth and virus-induced apoptosis is normal in $PKR^{0/0}$ cells. This is indicative of either the effectiveness of other anti-viral mechanisms or of redundancy of PKR function.

Contrary to the mouse generated by Yang et al (140), we did not find any evidence of defective cytokine signalling (141) or apoptotic response to TNFα (237) as well as to influenza infection. Type I IFN induction of 2' 5' OAS was normal in $PKR^{0/0}$ cells. This also contrasts with the observation that cells expressing the catalytically inactive PKRΔ6 mutant are unable to induce a STAT1 inducible gene in response to IFN α/β (145). The defect in Epo induced erythroid CFU development may indicate one developmental signalling pathway that requires PKR, although such defect appears readily compensated by other cytokines in vitro and in vivo. We attempted to assess our knockout model for a phenotype in stress induced apoptosis using exactly the same assays conditions and cell lineage as in the Yang-Weismann mouse (237) to best be able to determine what differences existed between the two PKR-null models.

The possibility that the difference between our results and Yang et al (140) can be accounted for by mouse strain differences cannot be ruled out. The mice with targeted PKR-deletion in exons 2 and 3 have a 129/Sv(ev) X C57BL/6J background (140) whereas our PKR-null mice have a 129/terSv X BalbC background. Strain differences in p53 targeted mice (291,292) have been shown to account for phenotypic differences. It is also possible

that the assays that might reveal a defect in our knockout mouse have not been worked out yet. Alternatively, the absence of any signs of tumor susceptibility, impaired anti-viral or apoptotic responses in these mice disrupted at the catalytic domain of PKR may be informative. The apparent discrepancy in observations with the two PKR null mouse models can be resolved if we assume PKR redundancy exists in a homolog that rescues its function when it is absent and that this redundancy may be affected differently by each null allele. Knockout strategies that produce null alleles will be compensated whereas those that produce dominant negative polypeptides that can suppress the homolog will demonstrate some phenotype. As such, we speculate that our knockout strategy has produced a null allele whereas the Yang-Weismann knockout strategy has yielded a transdominant allele, albeit not detected by their antibodies (Fig. 13).

The observation of intact eIF-2α phosphorylation in cells derived from our mice with PKR targeted at the catalytic domain is convincing evidence of this redundancy and the absence of dominant negative effect of this targeted allele's products, at least upon phosphorylation of eIF-2α. It would be of interest to determine the in vivo state of eIF-2α phosphorylation in cells from the Yang-Weismann PKR-null model to see if, as would be predicted, this allele acts to produce a dominant-negative mutated PKR that inhibits eIF-2α phosphorylation and results in the phenotypes described (140,141,237). Alternatively, loss of PKR may have less impact on eIF-2α than on other substrates such as NF-κB where differing disruptions may impact with varying effectiveness on these other pathways. The former model also explains the clear transformation phenotypes associated with ectopic expression of PKR mutants or inhibitors or substrates. Any mutation that can also suppress

the action of the putative homolog will demonstrate the consequences of loss of PKR activity. The putative polypeptide that could be encoded by the Yang-Weismann PKR knockout mouse would be strikingly similar to the dsRBD I deletion (93) which is a stable, transdominant mutation that dramatically reduces eIF-2α phosphorylation and can cause tumorigenesis. It is still unclear why Yang et al do not see tumors in their mice despite showing impaired IRF-1 and IFNγ signalling and apoptotic responses. As a test of our model of rescue by redundancy, transgenic or knockout mice expressing inactivating mutations of PKR that act as dominant negative mutations would be expected to show impairment of these pathways as well as tumor susceptibility when crossed with our mice.

The nature of PKR redundancy is not known and could be attributed to ectopic expression of the known mammalian homolog, HRI, normally restricted in expression to erythroid cells (112) or alternatively, could be due to a previously unappreciated, more widely expressed mammalian member of this family of kinases. It is interesting that the presence of other non-erythroid eIF-2α kinases has been described (293,294) and indeed the human homolog of yeast GCN2 has been recently described. The disruption of PKR at the catalytic domain allows a definitive demonstration that other eIF-2α kinases can compensate for PKR. Studying the mouse or human GCN2 homolog would allow further elucidation of the importance of the family of eIF-2α protein kinases, and their role in control of cell growth and apoptosis.

CHAPTER 4

CONCLUSION

CONCLUSION

The IFN-induced, double stranded RNA activated protein kinase, PKR, has a role in the control of key pathways in response to several stimuli. These triggers may vary from normal control of certain transcripts and their translation to responses to cellular stress such as heat shock or viral infection. The body of work elucidating the workings of this protein kinase is large and progress in our understanding of its function is vigorous. Our laboratory came to study PKR having cloned it when screening for PYKs and discovering a number of DSKs. This thesis began by reconciling this apparently novel DSK-like feature of PKR, then focused entirely on the consequence of loss of PKR in normal cellular function. This was done by examining two models of PKR inactivation, one that occurred in a cell line and the other by genetic ablation in the mouse.

The first finding of this thesis is that notwithstanding its appearance at the amino acid sequence level of a PSK, the dsRNA dependent protein kinase, PKR bears the novel property of being detected by anti-phosphotyrosine antibodies when expressed in bacteria. This is true of both mPKR and hPKR and requires PKR to be catalytically functional indicating it is not due to phosphorylation by another PYK. This finding indicates that caution is necessary when we classify protein kinases based on amino acid sequence or even by phosphoamino acid analysis since sensitivity of detection, the ability to protect sensitive sites during detection or even the ability to label stable sites of phosphorylation can alter our conclusion. In support of our findings, others have also reported detection of phosphotyrosine by phosphoamino acid analysis of hPKR expressed in yeast cells and the ability of S51Y mutations of eIF-2α to be regulated by PKR in yeast (Lu and Dever,

unpublished results). This is of importance precisely to assess the possibility of involvement of PKR in other pathways that may require phosphotyrosine-SH2 recruitment. Thus, we propose that PKR is a member of the DSK kinases, which is schematically depicted in the model of PKR action (Fig. 1). In vivo tyrosine phosphorylation of PKR in mammalian cells has not been found yet and its physiological significance is still unknown.

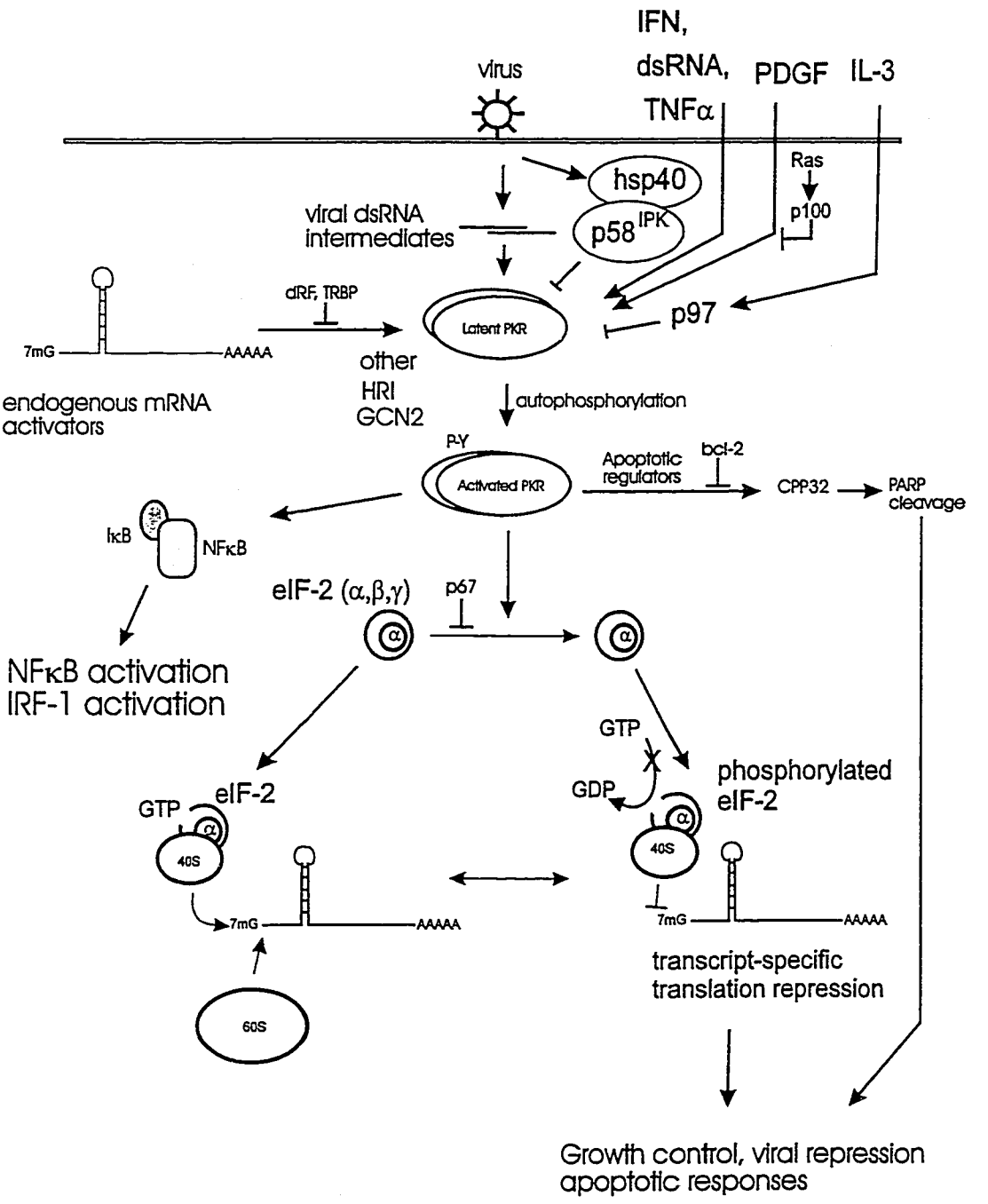
The thesis work then deals with the significance of a mutation of mPKR detected in a lymphocytic leukemia. In the context of studies showing cellular transformation by inactivated mutants of PKR and that the PKR locus in humans was associated with abnormalities in acute myeloid leukemias, malignant lymphomas and myelodyplastic syndromes, PKR was believed to have a role as a tumor suppressor. Thus, we examined the hypothesis that the mutation of PKR in L1210 cells can give rise to an inactive polypeptide termed $mPKR^{del}$ that can act as a transdominant inhibitor of PKR function and contribute to cellular transformation. Initially, we lacked the antibody reagents developed during the course of this work and found no detectable $mPKR^{del}$ in L1210 lysates even though sequence analysis of $mPKR^{del}$ cDNA and in vitro translation analysis indicated the mutation retained an open reading frame that was translatable. Myc epitope tagged $mPKR^{del}$ was detected when expressed transiently and confirmed the prediction that this polypeptide was catalytically inactive.

These studies provided the second major finding that mPKR was associated with itself in mammalian cells, perhaps as a dimer as also reported by others using less direct detection methods (178,228). Detection of protein by immunoblot analysis after immunoprecipitation indicated that equimolar levels of tagged mPKR and mPKR was found

Figure 1. PKR is present as a dimer in mammalian cells and has cryptic DSK properties.

Update of the schematic from Chapter 1, Fig. 5, showing new findings on PKR. Dimerization shown in this work and by others explains one mechanism of transdominant inhibition by PKR mutants. The other mechanism involves binding of dsRNA in the cell to prevent wild-type PKR binding and activation. $mPKR^{del}$ when expressed, is thought to act by binding dsRNA and may also dimerize with wild-type PKR. It is however, not detected stably expressed. PKR was shown to have DSK properties in bacteria and yeast, with the ability to autophosphorylate on tyrosine residues (denoted P-Y) and even eIF-2α S51Y when present. PKR phosphorylation of tyrosine in mammalian cells is still speculative but may indicate novel functions.

Figure 1


IFN,
dsRNA,
TNFα
virus
PDGF
IL-3
Ras
hsp40
p58 IPK
p100
viral dsRNA
intermediates
dRF, TRBP
Latent PKR
p97
7mG
AAAAA
endogenous mRNA
activators
other
HRI
GCN2
autophosphorylation
P-Y
Activated PKR
Apoptotic
regulators
bcl-2
CPP32
PARP
cleavage
IκB
NFκB
eIF-2 (α,β,γ)
p67
NFκB activation
IRF-1 activation
GTP
eIF-2
GDP
phosphorylated
eIF-2
40S
7mG
AAAAA
transcript-specific
translation repression
60S
Growth control, viral repression
apoptotic responses

in association. This finding which is essential to our understanding of the effect of PKR mutants is also depicted in our schematic model (Fig. 1). Furthermore, it appeared that mPKR^{del} was similarly capable of association with wild-type mPKR although the activity based assay used did not provide a clear idea of the stoichiometry of this association. It is thought that mPKR complexed with inactive mutants is suppressed since activation requires transphosphorylation (229) and incidentally, will not reflect the level of protein heterodimerization in our assay. Alternative models suggest that some dominant negative mutants of PKR act by binding dsRNA to prevent PKR activation (93,94). We have found that mPKR^{del} which lacks dsRBD II but retains dsRBD I is also capable of binding dsRNA. Interestingly, Barber et al demonstrate that PKR mutants lacking dsRBD I likely act as transdominant mutants by dimerization since they do not bind dsRNA effectively whereas K296R point mutated PKR appears to act by binding dsRNA and not by dimerization (93). This latter mechanism is reported to be less efficient and requires a greater fold excess to inhibit PKR. In this respect, mPKR^{del} in our transient expression studies appears to resemble the K296R mutant in mechanism of action since it showed no inhibition of wild-type PKR when expressed at near stoichiometric levels and may require greater fold excess to show any effect. Since it retains dsRBD I like the K296R mutant, it too may act by dsRNA sequestration rather than dimerization. This can be tested by assessing whether mPKR^{del}, like the K296R mutant, is able to reverse dsRNA inhibition of reticulocyte translation. Rescue of translation inhibition by mPKR^{del} should also be reversed by further addition of excess dsRNA (227). We also established that, at least specifically with respect to mPKR^{del} dimerization with mPKR, this appears dependent on dsRNA since RNA-free co-translation

did not reconstitute the co-immunoprecipitation seen in COS-1 cells which supports findings by others (178).

We moved the analyses into a defined cell background by expressing $mPKR^{del}$ exogenously and asked if it exerted an effect on endogenous mPKR activity and growth control. No evidence of dominant negative function was seen in NIH 3T3 cells expressing $mPKR^{del}$ by activity assays or in growth control assays. These analyses were based on assumptions of $mPKR^{del}$ expression in L1210 cells that was not detected due to shortcomings in antibodies available early on (Chapter 2, Fig. 5A) but could be verified by in vitro translation analysis. The development of antibodies that could detect tagged $mPKR^{del}$ in transient or stable transfection but not untagged $mPKR^{del}$ in L1210 cells showed us that perhaps the tagged protein was stabilized but nonetheless was not readily expressed at high levels. This is supported by the observation made by Barber et al that PKR mutants deleted for dsRBD II much like $mPKR^{del}$ are not readily expressed stably in mammalian cells (93). The absence then, of detectable PKR activity in whole cell extract kinase assays of L1210 cells could be attributable to inhibitors other than $mPKR^{del}$ many of which are now identified.

We concluded that the mutation in L1210 cell is likely a silent mutation that is not stable and exerts little effect on wild-type PKR when it is translated. It remains formally possible that we have not developed assays capable of detecting the effects of the $mPKR^{del}$ mutation in L1210 cells. Measurement of eIF-2α phosphorylation by L1210 extracts shows a reduced induction with activators of PKR. This may be a significant decrease to exert an effect on translation since minor changes in eIF-2α phosphorylation can have large impact (see Chapter 1). It still remains unknown given the uncertainty of comparing two different

cell lines whether this is due to the mutation of one PKR allele (dosage effect or suppression) or is due to differing levels of a PKR inhibitor in these cells. Future approaches to this are discussed in the next section. The third significant observation made was the slow growth phenotype exerted by wild-type mPKR expression in mammalian cells. This supports the findings in yeast and the model of PKR as a tumor suppressor (Fig. 1).

The second stage of this thesis examined the hypothesis that functional PKR is essential in anti-proliferative, anti-viral and apoptotic responses in the mouse by assessing the consequence of genetic ablation of PKR. The strategy devised involved targeted disruption by homologous recombination of the PKR gene within the region encoding the catalytic domain. After having begun the project, others reported the generation of a mouse model with disruption of PKR at the 5' end of the gene (140) (see Chapter 3, Fig. 13) referred to here as the Yang-Weismann mouse. The production by this targeted allele of transcripts that could encode a putative PKR polypeptide that retained an intact catalytic domain while deleted of only the dsRBD I motif was documented, although no detection of such a truncated protein was reported. This suggested to us the necessity of determining the consequence of PKR ablation which disrupted any possible activity such as would be achieved by our strategy.

This work documents that we have accomplished functional inactivation of PKR, with no evidence of catalytic activity or truncated polypeptides in $PKR^{0/0}$ cells. This was performed using two antibodies directed at the amino-terminal region common to normal mPKR and the putative truncated polypeptide that could be encoded (see Chapter 1, Fig. 1 and Chapter 3, Fig. 13) although it remains formally possible these may not detect a

truncated polypeptide. Catalytic ablation of PKR does not appear to have any impact on development or growth regulation. The only phenotypic observation made that is shared by the two PKR knockout mouse models is the absence of increased tumorigenesis that was predicted from the loss of this tumor suppressor. The Yang-Weismann mouse is reported to demonstrate defects in signaling responses to dsRNA and IFNγ which was traced to impaired NF-κB and IRF-1 activation (140,141). Furthermore, this mouse model is reported to have defective response to stress induced apoptosis by TNFα, dsRNA and LPS (237). Analysis of our knockout mouse for the ability to signal in response to Type I IFN (which requires STAT1 and IRF-1 activation) as well as other cytokines showed no defects in these pathways other than a diminished stimulation of erythroid CFU in response to Epo. IFNγ signaling itself was not assessed which perhaps should be done. Stress induced apoptosis, specifically in response to TNFα was also unperturbed in our $PKR^{0/0}$ cells. Furthermore, we extended this analysis to influenza-mediated apoptosis which has been shown to be blocked by mutants of PKR (238). This response was also normal in our PKR-null model. Finally, the anti-viral response of $PKR^{0/0}$ cells or animals to vaccinia or influenza challenge is surprisingly normal compared to control samples. The numerous viral encoded mechanisms to counteract PKR restriction (as is the case with the WR strain of vaccinia used which encodes E3L) as well as other IFN induced responses may account for the non-essential PKR role in anti-viral response. It is not clear how PKR's role in translation and growth control, so readily compromised in transformation assays with inactive mutants, is compensated for on our genetically altered mouse model.

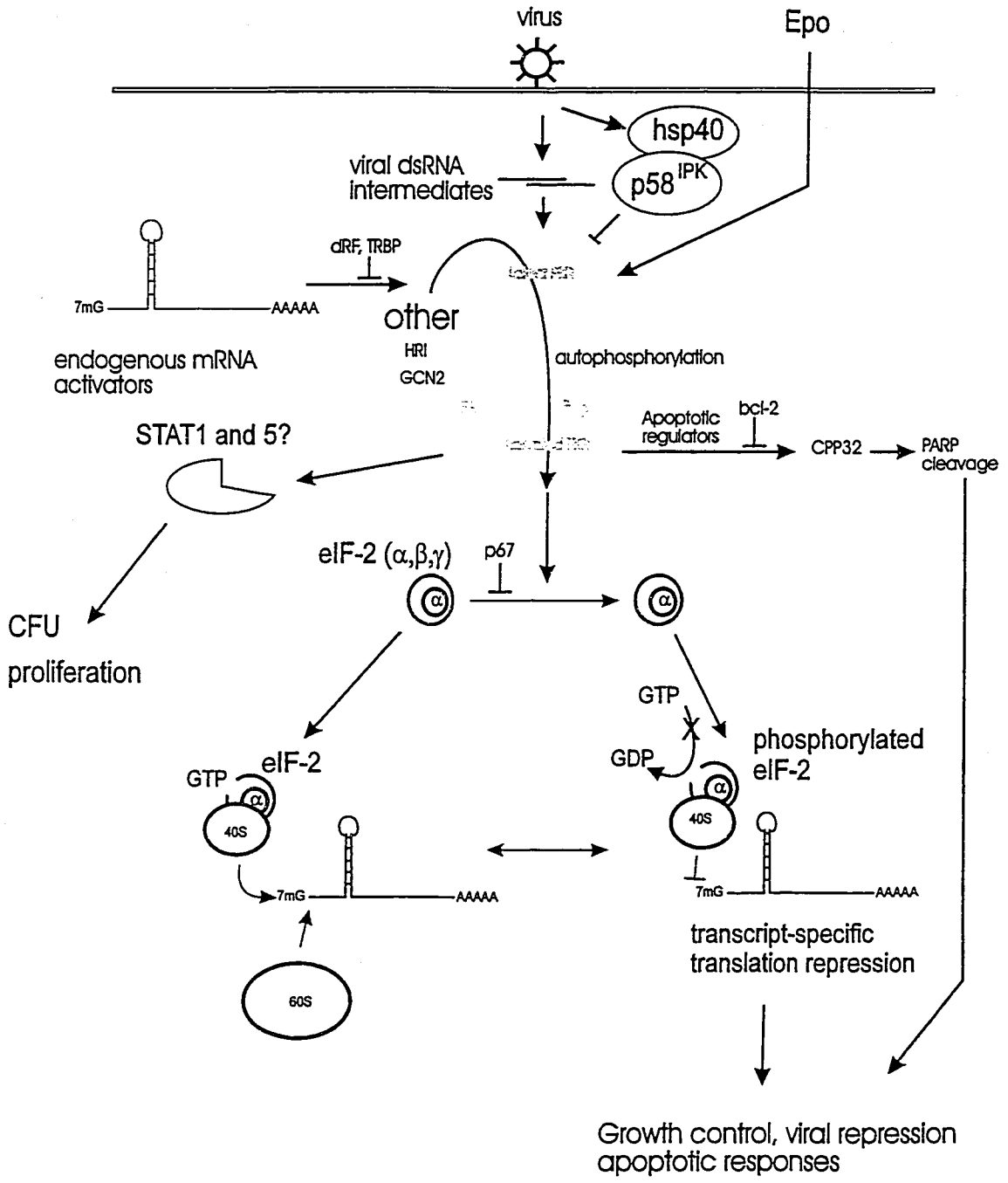
One possibility that we cannot absolutely rule out is that the two targeted alleles may impact different pathways in different ways and that future analysis may yet yield subtle defects in our knockout mouse. Furthermore, it is possible that strain differences accounts for the differing outcomes. This can only be resolved by repeating the knockout in a matching strain. Alternatively, the apparent non-concordance in phenotype between the two PKR knockout mouse models may be informative of PKR function. The current evidence suggests that PKR function is rescued by previously unappreciated homologs (Fig. 2). As pointed out before, no other ubiquitous mammalian eIF-2α protein kinase member has been described, since the HRI is restricted to reticulocytes. The recently identified human homolog of GCN2 may be just such a homolog of PKR. Analysis of eIF-2α phosphorylation has shown convincing evidence that other eIF-2α protein kinases continue to exert regulation of at least one of PKR's known substrates. The disruption of PKR at the catalytic domain including six essential amino acids in this region allows us to clearly rule out any possible activity from the targeted PKR (Fig. 2).

The large body of evidence of the impact of PKR mutation on growth control, viral or stress induced apoptosis or translation control in cells or even the Yang-Weismann mouse can be reconciled with the absence of such effects in our mouse model by attribution to dominant negative suppression of any compensatory homologs by these mutated PKR. In order to explain the discrepancy between the phenotype observable in the Yang-Weismann mice and our mice, we postulate that our strategy has produced a true null allele whereas the Yang-Weismann strategy produces a stable dominant negative polypeptide that has gone undetected (Chapter 3, Fig. 13). This is likely given their cloning of a translatable transcript

Figure 2. A PKR-null allele allows rescue by compensatory protein kinase(s).

Targeted disruption of PKR is denoted by stippled PKR. The absence of phenotype implies PKR function can be compensated. No ubiquitous mammalian eIF-2α protein kinases are known. Identification of a human GCN2 may provide one candidate. The complementation of eIF-2α phosphorylation in yeast may provide a more direct way of cloning PKR homologs.

Figure 2


virus
Epo
hsp40
p58 IPK
viral dsRNA intermediates
dRF, TRBP
7mG
AAAAA
other
HRI
GCN2
endogenous mRNA activators
autophosphorylation
STAT1 and 5?
Apoptotic regulators
bcl-2
CPP32
PARP cleavage
eIF-2 (α,β,γ)
p67
CFU
proliferation
GTP
GDP
phosphorylated eIF-2
eIF-2
40S
transcript-specific translation repression
60S
Growth control, viral repression apoptotic responses

from their targeted allele which would initiate polypeptide synthesis at residue 136 leaving out only dsRBD I, a protein remarkably similar to the transdominant dsRBD I deletion mutant reported by Barber et al which is transforming (93). It is still not clear why the Yang-Weismann PKR mutation which impacts the apoptotic and signaling pathways regulated by PKR does not show elevated tumorigenicity. Some have suggested this argues against any tumor suppressor function for PKR, however it is also possible that this specific mutation merely indicates the multiplicity of PKR action which impacts various pathways differently with normal regulation of growth retained. Transdominant mutations might be expected to exert their effect even in heterozygote animals which has not been reported. This may be due to inadequate stoichiometry of the mutated polypeptide to wild-type protein including that of homologs. Tests of this model of redundancy rescuing our knockout will be described in the next section. The major contribution of this work is that it shows unambiguously that a null of PKR can be compensated, indicating rescue by homologs. The Yang-Weismann mouse did not suggest this since a phenotype was observed. The absence of this phenotype assayed in exactly the same way in the same cell lineages in our knockout model hints at a larger picture of PKR function. Our findings should direct new interest in establishing what protein kinase(s) may replace PKR in its many functions.

The impairment of Epo signaling in erythroid cells is another important finding which may indicate non-redundancy of PKR in this specific pathway in these cells (Fig. 2). In fact, the presence of HRI in this cell lineage may indicate that it is not a candidate homolog to rescue PKR function in the animal. This defect may be compensated by other cytokines since no evidence of anemia is seen in $PKR^{0/0}$ animals. Epo is known to activate

the STAT5. Based on the reported regulation of STAT1 by PKR, it is important to establish whether PKR is required to regulate STAT5 signaling also.

Analysis of the impact of PKR mutation in cell culture has provided valuable insight into the pathways that PKR may be involved in. Cellular control of viral infection, of general protein synthesis, perhaps of specific transcript translation and commitment to programmed cell death can all pivot on PKR activation. Loss of PKR in spontaneous mutations or by genetic ablation have the potential to show us more about its role. It is apparent from this work that PKR function is complex involving dsRNA recognition, dimerization, and then activation and recruitment to various downstream pathways. Its functional role is sufficiently important to be evolutionarily conserved as well as rendered redundant such that its loss can be compensated. Not much effort had been made previously in identifying PKR homologs. A strategy to do so will be described in the next section.

FUTURE EXPERIMENTS.

The possibility that the L1210 PKR mutation produces a polypeptide that has altered stability should be examined by determination of the half-life of $mPKR^{del}$ compared to mPKR. This may be done using pulse-chase metabolic labeling of stable or transient expressors of untagged $mPKR^{del}$ and mPKR recovered with our anti-Tik100 antibody that detects both polypeptides. Alternatively, an <u>in vivo</u> biological test of $mPKR^{del}$ suppression of mPKR can be designed based on the observation of reduced $Hygromycin^{R}$ in cells transfected stably with Pece II encoded wild-type mPKR (Chapter 2, Fig. 10). This could first be established as the apoptotic consequence of mPKR expression. Co-expression of

mPKR^{del} using a different selectable marker should allow recovery of more Hygromycin^{R} colonies if it has any inhibitory effect on mPKR. If the protein is unstable or neutral in action, no change would be observed from mPKR alone transfected cells. Finally, the requirement for dsRNA for dimerization of mPKR^{del} to PKR may be assessed using reticulocyte extract co-translation. Addition of dsRNA to the extract after synthesis and before immunoprecipitation should determine whether co-association of mPKR^{del} is reconstituted in this system.

Further experiments suggested by this study of a PKR-null mouse include eliminating the possibility that HRI rescues PKR function by determining if in the absence of PKR, ectopic expression of HRI is detected in non-erythroid tissue. This may be done by immunoblot analysis of various tissue for HRI expression or RT-PCR analysis. The impaired erythroid CFU response to Epo could be analyzed by determining if Epo-STAT5 activation is normal in PKR$^{0/0}$ erythroid precursor cells. STAT5 phosphorylation on tyrosine and DNA binding to its target sequence in response to Epo could be examined.

To test our model of rescue by compensating homologs, transgenic or knockout mice expressing dominant negative mutants of PKR which exhibit impairment of various pathways requiring PKR could be utilized. The Yang-Weismann mice crossed with our knockout may show phenotype as heterozygotes with our targeted allele when they would not otherwise due to stoichiometry of transdominant activity required to suppress rescuing homologs. A better demonstration would be to use a dominant negative PKR mutant expressed from another locus such as from a heterologous transgenic promoter-cDNA construct which has an identifiable phenotype. This would allow us to retain PKR$^{0/0}$

homozygosity in a cross. Such transgenic mice crossed with our PKR-null mice should demonstrate phenotype showing suppression of rescuing homologs. Dosage compensation may also rescue transgenic mice expressing wild-type PKR which are runty (G. Barber, personal communication) when crossed with our PKR-null mouse. Finally, the knockout of PKR inhibitor TRBP which is also runty (Robert Braun, personal communication) would be informative when crossed with our PKR-null mice. Rescue of the phenotype would indicate PKR was necessary to cause the TRBP-null phenotype. Failure to rescue would confirm that PKR is redundant and that its homolog(s) can effect its action.

A genetic strategy to identify PKR homologs may be designed assuming common action upon eIF-2α. I propose we can utilize the yeast GCN4 regulation by the eIF-2α protein kinases (121) to identify new members of this family that rescue the gcn2Δ mutant. Growth in derepressing (or GCN4 stimulating) conditions is performed by histidine starvation by growth on 3-AT in synthetic dextrose media. The gcn2Δ mutant is 3-AT sensitive since reinitiation is favored at the uORF4 (see Chapter 1, Fig. 3) not the GCN4 ORF. Introduction of a library of mammalian genes into a gcn2Δ mutant under the control of a suitable promoter with low expression (since high expression can cause growth arrest) followed by selection in 3-AT would allow identification of clones that regulate eIF-2α. This screen should identify PKR, HRI and any novel homologs present. This would allow us to expand our analysis of the family of eIF-2α protein kinases and their roles in growth control, cell death and viral responses in mammalian cells.

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APPENDIX

PKR-knockout genomic PCR screening.

Oligonucleotides used:

For WT allele: NA-31: GGA ACT TTG GAG CAA TGG A (Tm=56°C)

NA-32: TGC CAA TCA CAA ATC TAA AAC (Tm=56°C)

For targeted allele: NA-33: TGT TCT GTG GCT ATC AGG G (Tm=58°C)

MCNeo2: TGA CGA GTT CTT CTG AGG G (Tm=58°C).

PCR screen yields (Fig. 1):

WT amplicon (of exon 12)= 225 bp

PKR Knockout amplicon (between intron X and Neo cassette)= 432 bp

1) Prepare genomic DNA from tail clippings of mice (may use phenol-chloroform extracted samples also). Quantitate if desired. Will require around 300 ng per reaction to type.

2) Set up PCR reaction master mix (as below) with negative (water) and positive controls (parent strains).

3) Make up each reaction tube with enough reaction mix, water and finally, genomic DNA in a final volume of 49.5 µl.

4) Add 0.5 µl of Taq polymerase (no hot start required) per tube, seal tubes and cycle as follows:

5) 94°C, 30s denaturation
56°C, 30s annealing
72°C, 30s extension, 30 cycles,

72°C, 10' extension.

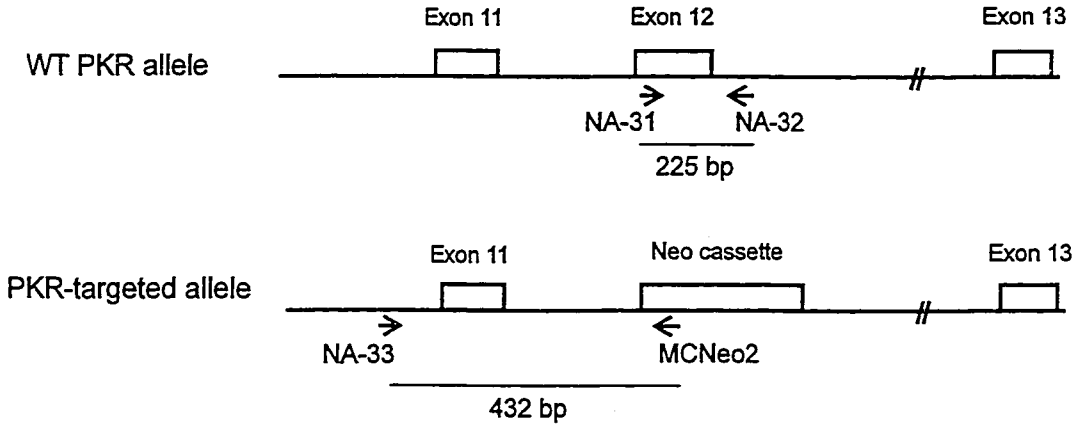
6) Save half of reaction (may be used for second round PCR if no products are seen), remaining half has loading dye added and analyzed on 1.2% agarose gel.

7) Genotyping is usually based on repeated PCR analysis.

Appendix Figure 1. PCR genotyping of PKR locus targeted at the catalytic domain.

Schematic of PKR locus with boxes indicating exons or selectable cassette insertion and solid lines representing introns. Oligonucleotides are indicated by arrows. Amplicons are shown below each locus with expected size in basepairs indicated.

Appendix, Figure 1



PCR reaction mix: (final concentrations)

1X PCR buffer minus Mg^{2-} (Gibco-BRL)
20 pmol NA-31
20 pmol NA-32
20 pmol NA-33
20 pmol MCNeo2
200 μM dNTPs
2.5 mM Mg^{2+} supplement
~300 ng genomic DNA
2.5U Taq polymerase (GIBCO-BRL)

CURRICULUM VITAE

Mr. Ninan Abraham, M.Sc.

Cancer Research Laboratory,
3rd Floor,
Ottawa Regional Cancer Centre
501, Smyth Road,
Ottawa, Ontario
Canada
K1H 8L6

Education

1991-1998: Ph.D (Biochemistry), University of Ottawa, Ottawa, Ontario, Canada.

1988-1991: M.Sc. (Experimental Medicine), McGill University, Montreal, Quebec, Canada.

1984-1988: B.Sc.Hons. (Biochemistry/Microbiology), Dalhousie University, Halifax, Nova Scotia, Canada.

Awards

National Cancer Institute of Canada Studentship, 1994-1997.

Medical Research Council Studentship, 1992-1994.

Cancer Research Society Inc. Studentship, 1990-1992.

Multiple Sclerosis Society Studentship, 1989.

Department of Medicine Award, McGill University, 1988.

Robert Bruce Scholarship, Dalhousie University, 1985.

Arts and Science Scholarship, Dalhousie University, 1985.

Burgess McKittrick Prize, Department of Physics, Dalhousie University, 1985.

Publications

1. Abraham, N. and Veillette, A. (1990). Mol Cell Biol 10: 5197-5206. Activation of $p56^{lck}$ through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation.

2. Abraham, N., Miceli, C.M., Parnes, J.R. and Veillette, A. (1991). Nature 350: 62-66. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$.

3. Abraham, N. and Veillette, A. (1991). Cancer Invest 9: 455-463. The lymphocyte-specific tyrosine protein kinase $p56^{lck}$.

4. Veillette, A., Abraham, N., Caron, L. and Davidson, D. (1991). Semin Immunol 3: 143-152. The lymphocyte-specific tyrosine protein kinase $p56^{lck}$.

5. Ellis, C., Liu, X., Anderson, D., Abraham, N., Veillette, A. and Pawson, T. (1991). Oncogene 6:895-901. Tyrosine phosphorylation of GAP and GAP-associated proteins in lymphoid and fibroblast cells expressing *lck*.

6. Caron, L., Abraham, N., Pawson, T. and Veillette, A. (1992). Mol Cell Biol 12: 2720-2729.
Structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$.

7. Douville, E., Duncan, P.I., Abraham , N. and Bell, J.C. (1994). Cancer Metastasis Rev 13: 1-7. Dual specificity kinases--a new family of signal transducers.

8. Jaramillo, M.L., Abraham, N. and Bell, J.C. (1995). Cancer Invest 13: 327-338.
The interferon system: a review with emphasis on the role of PKR in growth control.

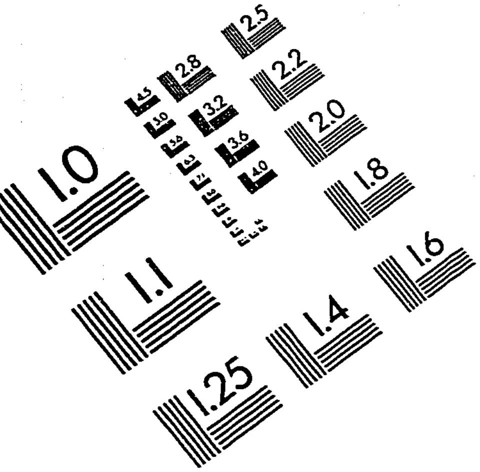
9. Abraham, N. and Bell, J.C. (1995). The Protein Kinase Factsbook: Protein-Serine Kinases. Academic Press. London. pp317-318.

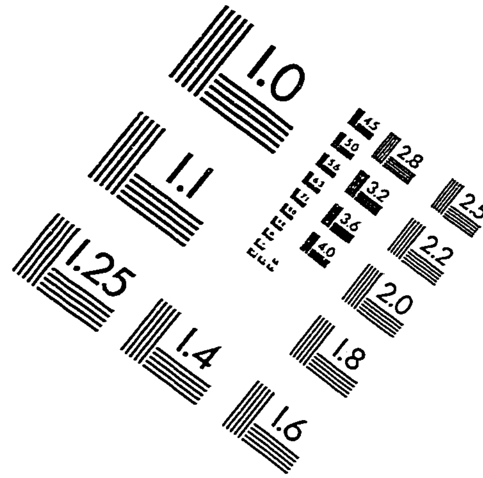
10. Abraham, N., Jaramillo, M.L., Duncan, P.I., Méthot, N., Icely, P.L., Stojdl, D.F., Barber, G.N., Bell, J.C.. The murine PKR tumor suppressor gene is rearranged in a lymphocytic leukemia. Exp. Cell Res. (in press).

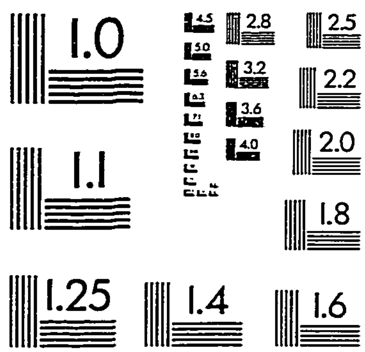
Manuscript submitted

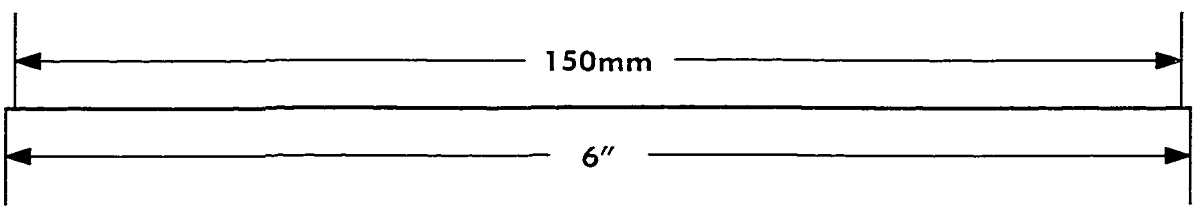
1. Ninan Abraham, Nathalie Méthot, Andrew R. Cuddihy, David F. Stojdl, Peter I. Duncan, Manon Dubé, Barbara C. Vanderhyden, Harold L. Atkins, Douglas A. Gray, Michael W. McBurney, Antonis E. Koromilas, Earl G. Brown, Nahum Sonenberg, John C. Bell. Characterization of transgenic mice with targeted disruption of the catalytic domain of PKR.

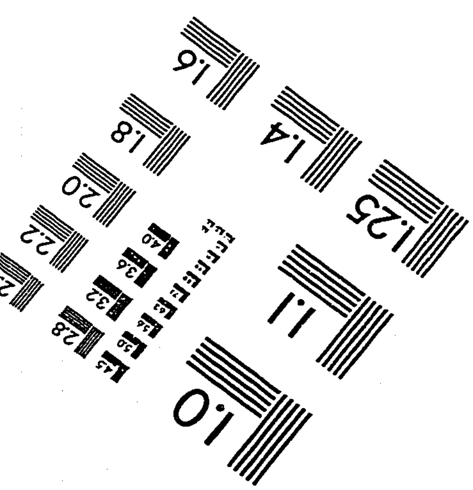
IMAGE EVALUATION TEST TARGET (QA-3)

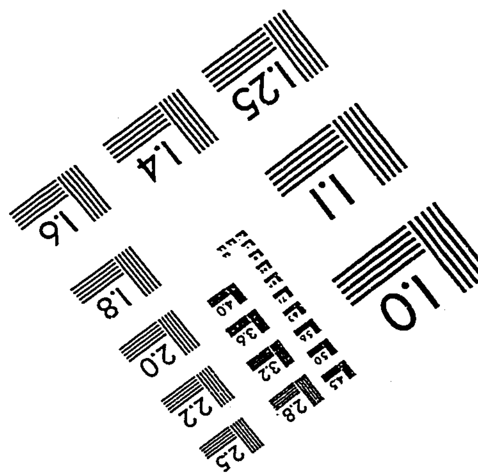












APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989