REGULATION OF THE STAT1 TRANSCRIPTION FACTOR BY THE INTERFERON-INDUCIBLE DOUBLE-STRANDED RNA-DEPENDENT PROTEIN KINASE PKR

by

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To Mom and Dad,
for your love that knows no earthly bounds

To Joseph, my "little brother",
for being my big brother in so many ways

To Alice,
for loyal friendship and spiritual guidance

And to Nicolas,
for filling my life with so much happiness

ABSTRACT

The interferon (IFN)-inducible protein kinase PKR is thought to mediate the biological effects of IFNs through its ability to phosphorylate eukaryotic initiation factor 2 and to inhibit protein synthesis. Additional reports have implicated PKR in transcriptional regulation. In this study, a role for PKR in the regulation of signal transduction pathways that are required for IFN action was examined. It is demonstrated that PKR negatively regulates signaling pathways induced by IFNs and dsRNA. This regulatory effect is attributed to a protein-protein interaction between PKR and STAT1, the signaling component common to these pathways, that impairs STAT1 transcriptional function. The interaction between PKR and STAT1 is not a kinase-substrate relationship since PKR does not phosphorylate STAT1 and both wildtype and catalytically inactive PKR can interact with STAT1. It is also shown that PKR regulates STAT1 protein expression. The regulatory effect does not occur at the transcriptional or post-transcriptional level since STAT1 mRNA transcription and stability are unaffected by PKR. Regulation of STAT1 expression is specific for STAT1 since expression of other STATs is not affected by PKR. The ability of PKR to regulate STAT1 function and expression has important implications in the control of gene expression, viral immunity, and cell growth.

ABRÉGÉ

La protéine-kinase PKR induite par les interférons (IFN) régule les effets biologiques des IFNs grâce à la phosphorylation du facteur d'initiation eIF-2 et à l'inhibition de la synthèse protéique. De nombreux auteurs ont impliqué PKR dans la régulation de la transcription. Dans cette étude, nous avons examiné le rôle de PKR dans le régulation des voies de transmission du signal intracellulaire requit pour l'action d'IFN. Nous avons observé une régulation négative de PKR sur la voies de transmission du signal induites par les IFNs et l'ARN double brin. Cette régulation est attribuée à une interaction entre le protéines PKR et STAT1, qui abolit l'activité transcriptionnelle de STAT1. Cette interaction n'est pas une relation kinase-substrat puisque PKR ne phosphoryle pas STAT1, de plus une protéine PKR n'ayant plus d'activité catalytique peut tout de même intéragir avec STAT1. Nous avons aussi démontré que PKR régule l'expression de la protéine STAT1. Cette régulation ne s'effectue pas au niveau transcriptionnel ou post-transcriptionnel puisque lex niveaux et la stabilité de l'ARNm de STAT1 ne sont pas affectés par PKR. Cette régulation est spécifique à STAT1 puisque l'expression des autres membres de la famille STAT n'est pas affectée par PKR. La capacité de PKR de réguler la fonction et l'expression de PKR a d'importantes implications dans le contrôle de l'expression des gènes, dans l'immunité virale, et dans la croissance cellulaire.

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PREFACE

All the work presented in this thesis was performed by the candidate with the exception of the following: immortalization of primary $PKR^{+/+}$ and $PKR^{-/-}$ mouse embryonic fibroblasts, generation of NIH 3T3 cell lines stably expressing PKRΔ6 or PKR LS4, and generation of the Hela S3 cell line stably expressing PKRΔ6HA, which was performed by Dr. Antonis Koromilas; construction of the PKRΔ6HA cDNA, which was performed by Luc Chadonnet; and Western blot analysis of STAT1 in NIH 3T3 cells expressing PKRΔ6 or PKR LS4 which was performed by Andrew Wong.

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Part of the data presented in this thesis can be found in the following publication and presentation of the data herein has been agreed to by the first co-author Andrew Hoi-Tao Wong:

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

1. *Interferons and immunity to viral infection*

The primary function of the immune system is to provide protection to a host in the event of pathogenic invasion. Among the complex network of cell types and molecules that comprise this elaborate system are cytokines, protein hormones that are secreted by the cells of the immune system and that coordinate innate and acquired immune responses. One particular group of cytokines, known as interferons (IFNs), plays a key protective role during the course of viral infection.

1.1 General features of IFNs

IFNs encompass a large family of secreted proteins that are found in vertebrates and can be synthesized by almost all cell types *in vitro* and *in vivo* (Lengyel, 1982; Pestka *et al.*, 1987). One of the primary functions of this class of cytokines is to establish a state of host resistance to viral infection; under these conditions, collectively termed the anti-viral state, infected cells serve as a poor host for viral replication (Baron and Dianzani, 1994). Although a small number of cell types express IFNs constitutively, these biological compounds are usually present at undetectable levels in most organs and cells in culture. Inducers of IFN expression include certain bacteria and protozoa, endotoxins, polysaccharides, mitogens, and antigens in previously sensitized hosts. However, the most potent inducers of IFN expression are most types of viruses and natural or synthetic double-stranded (ds) RNA.

IFNs can be classified into two broad categories based on the ability to bind to different receptors. Type I IFN is comprised of two groups of serologically distinct proteins. The first group, termed leukocyte IFN or IFN–α, represents a family of about 20 independently encoded yet structurally related polypeptides of approximately 18 kDa. Type I IFN species share roughly 70% homology at the amino acid level, and the primary sources of this type of IFN *in vivo* are

leukocytes and monocytes. The second group of type I IFNs consists of a single 20 kDa glycoprotein, IFN–β or fibroblast IFN, and is isolated primarily from fibroblasts cultured *in vitro*. Although IFN–α and IFN–β (hereafter referred to as IFN–α/β) share little structural similarity, they bind to the same cell surface receptor and appear to induce the same set of cellular responses. Type II IFN, otherwise known as IFN–γ or the immune interferon, is a homodimeric glycoprotein ranging between 21 to 24 kDa; the range in its molecular weight is attributed to a variable degree of glycosylation. IFN–γ is predominantly produced by T-lymphocytes in response to antigenic or mitogenic stimulation.

1.2 Biological activities of interferons

The most studied function of IFNs is their ability to convert virally-infected cells to an anti-viral state (Lengyel, 1982; Pestka *et al.*, 1987; Baron and Dianzani, 1994). IFNs are able to do so by inducing the expression of proteins that can inhibit viral DNA or RNA replication, a process that requires active RNA transcription and protein synthesis. The anti-viral state is transient as it will dissipate within a few days after cells cease to be exposed to IFNs (Lengyel, 1982).

IFNs have also been implicated in the inhibition of cell growth (Lengyel, 1993). Presently, it is not entirely clear how IFNs inhibit cell growth although their anti-proliferative activity has been attributed in part to the induction of proteins that downregulate protein synthesis (Pestka *et al.*, 1987). Many current applications of IFNs in anti-tumour therapy have been based on empirical evidence indicating which cell types are sensitive to the anti-proliferative effects of IFNs. In this regard, IFN therapy has proven to be beneficial in the treatment of a number of cancers such as Kaposi's sarcoma, chronic myelogenous leukemia, non-Hodgkin's lymphoma, and hairy cell leukemia (Baron *et al.*, 1991).

Another major role of IFNs in coordinating the immune response during viral infection is the stimulation of certain cells of the immune system (Pestka *et al.*, 1987). It has been shown that both IFN-α/β and IFN-γ upregulate the expression of the class I major histocompatibility complex (MHC) molecules, thereby potentiating cytotoxic T-lymphocyte-mediated killing of intracellular pathogens. However, IFN-γ exhibits several properties that distinguish it from IFN-α/β. Importantly, IFN-γ increases the expression of not only class I but also class II MHC molecules and thus enhances antigen presentation to helper T-lymphocytes. In addition, IFN-γ is a potent activator of macrophages, stimulating both non-specific cytotoxic, inflammatory, and tissue repair activities as well as antibody-dependent cell cytotoxicity via the induced expression of cell surface immunoglobulin Fc receptors.

2. *Interferon signal transduction*

2.1 Interferon receptors

The biological effects of IFNs are initiated by binding of IFN to its cognate receptor. The receptors for IFN-α/β and IFN-γ together with the interleukin-10 receptor comprise the class II cytokine receptor family (Pestka, 1997; Williams and Haque, 1997; Haque and Williams, 1998). Class II cytokine receptors possess a conserved motif containing four conserved cysteine residues in their ligand binding domains, and signaling through these receptors is dependent upon ligand-dependent oligomerization of receptor subunits. Unlike growth factors receptors that have intrinsic catalytic activity for intracellular signaling, class II cytokine receptors lack catalytic motifs in their cytoplasmic domains. Instead, these receptors are coupled to kinases that are involved in intracellular signaling.

The IFN-α/β receptor serves as the common receptor for both IFN-α and IFN-β. It is a single transmembrane spanning protein that is made up of three

polypeptide components. Two of these components, IFNAR-1 and IFNAR-2, have been cloned and shown to be homologous in their extracellular domains (Uze *et al.*, 1990; Novick *et al.*, 1994; Domanski *et al.*, 1995). IFNAR-1 was the first subunit cloned and is a 550 amino acid glycoprotein. IFNAR-2 exists as three isoforms that arise from alternative splicing and multiple polyadenylation sites; the longest 515 amino acid form binds IFN-α/β. Studies have indicated that both IFNAR-1 and IFNAR-2 are capable of ligand binding, but only IFNAR-2 can support IFN-induced signaling in cells deficient in this response (Lutfalla *et al.*, 1995).

The IFN-γ receptor is also a single transmembrane spanning protein and is comprised of two components. Both IFNGR-1 and IFNGR-2 have been cloned (Aguet *et al.*, 1988; Soh *et al.*, 1994; Hemmi *et al.*, 1994), and mutational analyses of IFNGR-1 have indicated structural requirements for IFN-γ-induced signaling. An amino acid stretch spanning residues 439 to 444 appears to be required for IFN-induced transcription (Greenlund *et al.*, 1994 and 1995; Heim *et al.*, 1995). In addition, phosphorylation of tyrosine 440 is required for IFN-γ signal transduction and is dependent upon the presence of IFNGR-2 (Greenlund *et al.*, 1994).

2.2 *The JAK/STAT paradigm*

Studies of IFN-induced transcription have led to the elucidation of the biochemical pathway by which IFNs mediate cell surface-to-nuclear events. It is now known as the JAK/STAT signal transduction pathway (Darnell, 1997; Horvath and Darnell, 1997; Pelligrini and Dusanter-Fourt, 1997; O'Shea, 1997; Leonard and O'Shea, 1998; Decker and Meinke, 1998; Liu *et al.*, 1998). Although the JAK/STAT pathway was initially identified in the context of IFN-mediated signal transduction, it has since served as a signaling paradigm for numerous other cytokines, growth factors, and hormones.

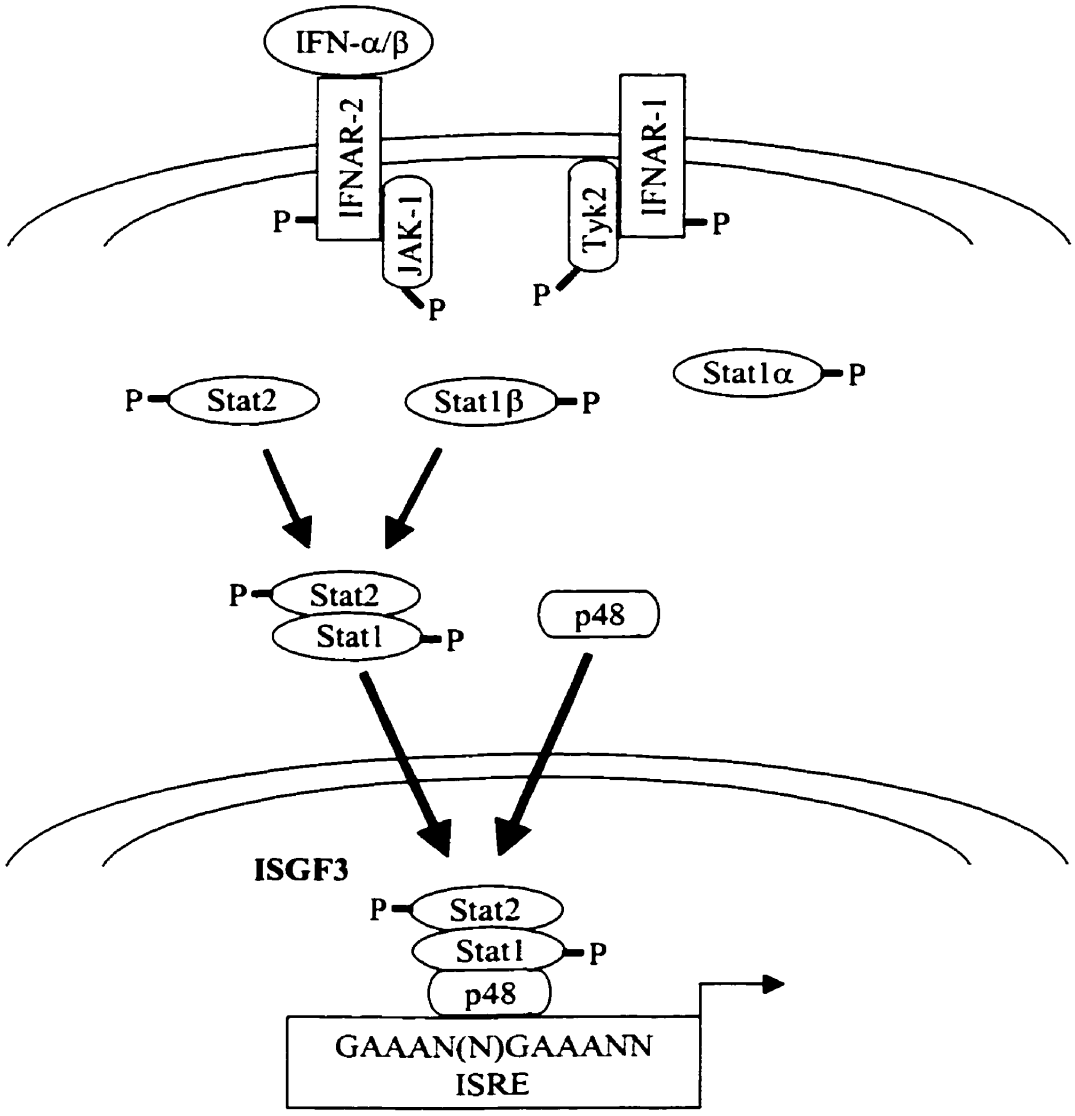
In this signaling pathway, a transmembrane receptor is associated with one or more members of the Janus family of tyrosine kinases (JAKs). Upon ligand engagement, the receptor undergoes oligomerization of receptor components, and consequently the associated JAKs are reciprocally tyrosine phosphorylated and activated. Activated JAKs proceed to phosphorylate specific tyrosine residues located in the cytoplasmic tail of the receptor that then serve as docking sites for a family of latent cytoplasmic transcription factors known as signal transducers and activators of transcription (STATs). Upon recruitment to the receptor-kinase complex, STATs are tyrosine and possibly serine phosphorylated. Activated STATs then oligomerize, translocate to the nucleus, and bind specific DNA sequences to transactivate specific gene expression.

In IFN-α/β signal transduction (Gilmour and Reich, 1995; Jaramillo *et al.*, 1995; Williams and Haque, 1997; Haque and Williams, 1998), two members of the JAK family are associated with the IFN-α/β receptor. Tyk2 is coupled to IFNAR-1, and JAK1 with IFNAR-2. Upon binding of IFN-α/β to its receptor, these JAKs are transphosphorylated and activated. Latent STAT2 and STAT1 are then sequentially recruited into the proximity of the receptor-kinase complex and tyrosine phosphorylated. Activated STAT1 and STAT2 form heterodimers and combine with a 48 kDa DNA-binding protein called interferon-stimulated gene factor 3 gamma (ISGF-3γ) to form the ISGF-3 transcriptional complex. ISGF-3 then binds an IFN-stimulated response element (ISRE; nucleotide sequence: AGTTTN$_3$TTTCC) that is found upstream of select IFN-stimulated genes (ISGs) and activates their transcription (Figure 1). Consistent with this model, studies of IFN-α/β-mediated signal transduction have indicated that the transcriptional response to IFN-α/β does not require *de novo* protein synthesis (Friedman *et al.*, 1984).

It has been shown that the subset of genes transcriptionally activated by IFN-α/β can also be induced in an IFN-α/β-independent manner. In this regard, it has been found that transcription of these genes can be stimulated by infection of

Figure 1

Schematic representation of IFN-α/β signal transduction events. Binding of IFN-α/β to the IFN-α/β receptor results in the activation of the receptor and subsequent transphosphorylation and activation of associated tyk2 and JAK1 kinases. Tyk2 then phosphorylates IFNAR1, creating a docking site for STAT2 and inducing its phosphorylation. Phosphorylated STAT2 in turn acts as a docking site for STAT1 which is phosphorylated at tyrosine residue 701. This is followed by formation of STAT1:STAT2 heterodimers which associate with the p48 DNA-binding subunit to form the transcriptionally active ISGF-3 complex. ISGF-3 transactivates gene expression from the ISRE located upstream of ISGs. (adapted from Haque and Williams, 1998)


IFN-α/β
IFNAR-2
IFNAR-1
JAK-1
Tyk2
P
Stat2
Stat1β
Stat1α
Stat1
p48
ISGF3
GAAAN(N)GAAANN
ISRE

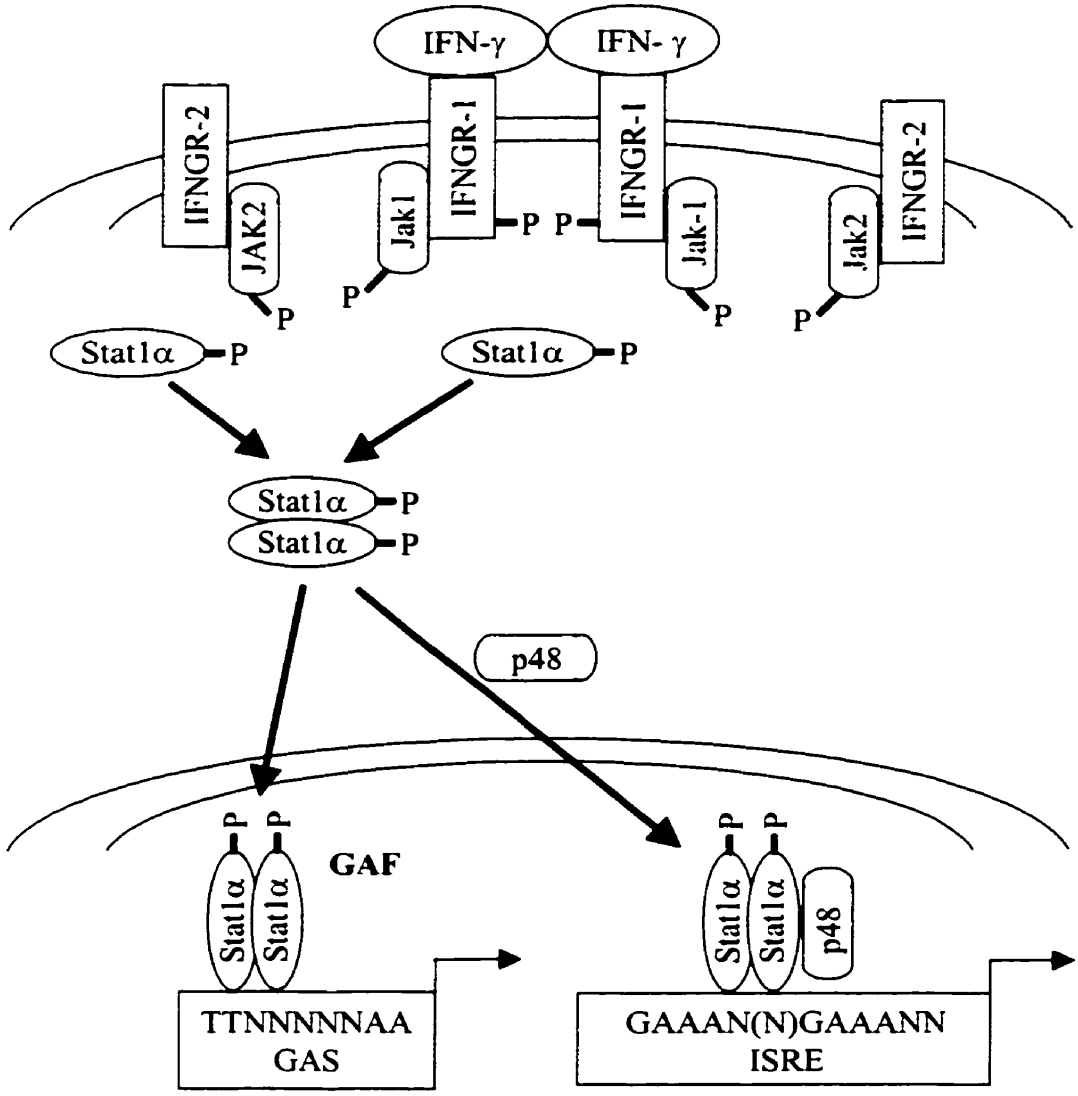
cells with virus or by transfection of cells with dsRNA (Wathelet *et al.*, 1992; Bazzigher *et al.*, 1992; Decker, 1992). Transcriptional activation is also mediated through the ISRE but through the activity of different transcription factors known as dsRNA activated factors (DRAFs; Daly and Reich, 1993). Like ISGF-3, the activation of DRAFs occurs independently of *de novo* protein synthesis and requires tyrosine phosphorylation (Daly and Reich, 1993). It has been proposed that activation of DRAFs takes place during the early stages of viral infection, thereby providing host protection prior to IFN production.

In the case of IFN-γ-mediated signaling (Gilmour and Reich, 1995; Jaramillo *et al.*, 1995; Williams and Haque, 1997; Haque and Williams, 1998), ligand engagement of the IFN-γ receptor results in multimerization of individual receptors and leads to the reciprocal phosphorylation of associated JAK1 and JAK2. These kinases are complexed to IFNGR-1 and IFNGR-2 respectively. Phosphorylation of STAT1 follows, and activated STAT1 molecules then form dimers to yield the transcriptionally active gamma-activated factor (GAF) complex. GAF proceeds to bind a DNA response element termed gamma activation sequence (GAS; nucleotide sequence: TTN_5AA) located upstream of certain IFN-inducible genes and activates their expression. Alternatively, GAF can bind the ISRE in conjunction with the ISGF-3γ DNA-binding subunit (Bluyssen *et al.*, 1995; Figure 2).

The genes transactivated by IFN-γ appear to fall into one of two categories. In some cases, the pattern of transcription is similar to the one observed in IFN-α/β signaling. That is, these genes are transcribed independently of *de novo* protein synthesis; in contrast to IFN-α/β, however, the transcriptional induction of these genes occurs more rapidly, even within a few minutes of IFN-γ stimulation (Lew *et al.*, 1989). In other cases, the pattern of transcription induced by IFN-γ requires new protein synthesis. As such, the transcription of these genes appears to be a secondary response to IFN-γ and to be dependent on the synthesis of an intermediary transcription factor (Blanar *et*

Figure 2

Schematic representation of signal transduction events leading to expression of IFN-γ-inducible genes. Dimeric IFN–γ binds to two IFNGR-1 molecules and induces multimerization with IFNGR-2 subunits. Ligand-induced activation of the IFN–γ receptor leads to transphosphorylation and activation of associated JAK1 and JAK2 kinases. Phosphorylation of IFNGR1 follows, creating a docking site for STAT1. STAT1 is phosphorylated at tyrosine residue 701 and released from the receptor. Phosphorylated STAT1α molecules then form homodimers and translocate to the nucleus to bind the GAS element found upstream of ISGs. Alternatively, STAT1 homodimers can bind ISREs of certain ISGs in conjunction with the p48 DNA-binding subunit. (adapted from Haque and Williams, 1998)


IFN-γ
IFN- γ
IFNGR-2
IFNGR-1
IFNGR-1
IFNGR-2
JAK2
Jak1
Jak-1
Jak2
P
Stat1α
Stat1α
p48
GAF
TTNNNNNAA
GAS
GAAAN(N)GAAANN
ISRE

al., 1988). There is a limited degree of overlap between the subsets of genes activated by IFN-α/β and IFN-γ (Lew *et al.*, 1989 and 1991; Levy, 1990).

2.3 *The STAT family of transcription factors*

The STAT proteins are defined as a family of transcription factors for regions of shared amino acid sequence similarities (Schindler and Darnell, 1995; Ihle, 1996; Darnell, 1997; Horvath and Darnell, 1997; Pelligrini and Dusanter-Fourt, 1997; O'Shea, 1997; Leonard and O'Shea, 1998). All STATs share a conserved tyrosine residue that is critical for cytoplasmic activation and in their activated forms bind DNA in a sequence-specific manner. The STATs have been shown to be activated by more than 35 signaling peptides and, consequently, have been implicated in a spectrum of biological responses. At present, seven STAT family members have been identified in mammals (Schindler *et al.*, 1992; Fu *et al.*, 1992; Akira *et al.*, 1994; Zhong *et al.*, 1994a and 1994b; Yakamoto *et al.*, 1994; Wakao *et al.*, 1994; Quelle *et al.*, 1995). In addition, STATs have been identified in *Drosophila* (Yan *et al.*, 1996; Hou *et al.*, 1996) and *Dicytostelium discoideum* (Kawata *et al.*, 1997). In terms of chromosomal localization, the genes encoding STATs 1 and 4 map closely to a region in mouse chromosome 1; STATs 3, 5A, and 5B are localized to mouse chromosome 11; and STATs 2 and 6 map to mouse chromosome 10 (Copeland *et al.*, 1995). The chromosomal distribution of mammalian STATs suggests that the family may have arisen as a result of tandem duplication events of a single primordial gene. Indeed, the identification of STAT members in invertebrates (D-STAT/marelle) and plants (DIF) indicates that STATs have been conserved throughout evolution and suggest a potential role in developmental processes.

As members of a family, STATs share a common structural organization. STATs are approximately 750-850 amino acids long, and at the amino-terminus of these proteins is a conserved region of approximately 50 amino acids that has been implicated in the regulation of STAT activity. A DNA recognition domain

has been localized within an absolutely conserved region extending from amino acid residue 250 to residue 480; mutations in this DNA-binding domain result in dominant negative forms of STAT1, STAT3, and STAT6. An SH3-like domain is found between residues 500 to 600, but the role of this domain in STAT function has not yet been determined. A highly conserved SH2 domain is present from residues 600 to 700, and mutational analyses of this functional domain have demonstrated that it mediates STAT dimerization. Although the carboxy termini of the STATs represent the most divergent regions of the proteins, phosphorylation of a conserved tyrosine residue in the proximity of residue 700 is essential for STAT activation.

The identification of the precise biological roles of STATs has been confounded by two key factors. First, with the exception of STAT4, STATs are ubiquitiously expressed in almost all cell types and tissues (Schindler and Darnell, 1995). Second, the majority of STATs can be activated by a number of different signaling ligands, an observation that would suggest that the STATs may be functionally redundant. Thus, it has only been with the generation of STAT "knockouts" that the biological roles of STATs have been ascertained. Strikingly, targeted disruption of many of the STAT genes results in highly specific phenotypes *in vivo* that correspond to their functions identified *in vitro*. STAT-deficient mice exhibit defects in either innate immunity (STAT1; Meraz *et al.*, 1996; Durbin *et al.*, 1996) or acquired immunity (STAT4 and STAT6; Kaplan *et al.*, 1996; Thierfelder *et al.*, 1996; Takeda *et al.*, 1996; Shimoda *et al.*, 1996). In addition, STAT5 knockout mice exhibit defects in breast development and lactation (Liu *et al.*, 1997; Udy *et al.*, 1997). As well, STAT2 and STAT3 appear to be important in embryogenesis as targeted disruption of either of these genes results in embryonic lethality (Darnell, 1997; Takeda *et al.*, 1997).

2.4 STAT1

Initially identified for its role in IFN signal transduction, STAT1 has since been shown to be activated by a large number of ligands, including interleukins, growth factors, and hormones (Schindler and Darnell, 1995). The STAT1 protein occurs as two spliced variants that are derived from the same STAT1 gene. The STAT1α and STAT1β isoforms, 91 kDa and 84 kDa respectively, differ in the last 38 amino acids of their carboxy-termini (Schindler *et al.*, 1992). Whereas STAT1α is capable of DNA-binding and transcriptional activities, STAT1β is deficient in transactivation function (Meuller *et al.*, 1993). Consistent with this finding are reports that STAT1α homodimers and STAT1α:STAT1β heterodimers, but not STAT1β homodimers, can support IFN-induced transcription (Shuai *et al.*, 1993; Bromberg *et al.*, 1996; Horvath *et al.*, 1996).

Mutational analyses of STAT1 have indicated a number of structural features that are essential for optimal STAT1 function. A requirement for phosphorylation has been previously discussed. The difference in transcriptional function between STAT1α and STAT1β has been attributed to a requirement for phosphorylation at both tyrosine 701 and serine 727 for maximal transcriptional activity; serine 727 is not present in the STAT1β isoform (Shuai *et al.*, 1992 and 1993; Zhang *et al.*, 1995; Wen *et al.*, 1995 and 1997). In addition to phosphorylation, an intact SH2 domain is required for STAT1 function; mutation of arginine 602, which is located in the floor of the "SH2 pocket", abrogates the ability of STAT1 to be activated and to form dimers (Improta *et al.*, 1994; Shuai *et al.*, 1994; Gupta *et al.*, 1996). The SH2 domain of STAT1 also appears to confer specificity of phosphorylation since chimeric STAT1 molecules that contain the SH2 domain of STAT2 fail to be phosphorylated in response to IFN–γ (Heim *et al.*, 1995). Additional structural alterations that disrupt STAT1 transcriptional function include deletions in the amino-terminus and removal of heptad leucine repeats that render the protein non-phosphorylable (Improta *et al.*, 1994). In addition, STAT1 activity has been reported to be regulated by mutations within

the first 60 amino acids of the amino-terminus (Shuai *et al.*, 1996), phosphatase activity (Igarishi *et al.*, 1993; David *et al.*, 1993; Haque *et al.*, 1995; Haspel *et al.*, 1996; Shuai *et al.*, 1996), and proteasome activity (Kim and Maniatis, 1995).

The most widely studied function of STAT1 is its role as a transcription factor. In this capacity, STAT1 has been shown to undergo homo- and heterodimerization with other transcription factors such as STATs and the ISGF-3γ member of the interferon regulatory factor family, members of which regulate the expression of IFN genes. As well, it has been shown that STAT1 can also interact with transcriptional co-activators, namely, CBP/p300 (Zhang *et al.*, 1996; Horvai *et al.*, 1997). It has been suggested that STAT1 can compete for binding to these co-activators with the AP-1 and ets transcription factors and antagonize their activities (Horvai *et al.*, 1997). Collectively, protein-protein interactions account for the ability of STAT1 to be recruited by a range of signaling cytokines, growth factors, and hormones (Schindler and Darnell, 1995).

Studies of mice with a targeted deletion of the STAT1 gene have provided further insight into the biological role of the protein. Studies of STAT1 "knockout" mice have indicated a centrol role for the protein in immunity since these mice are exquisitely sensitive to microbial infection (Meraz *et al.*, 1996; Durbin et al., 1996). The basis for this biological effect becomes apparent in experiments using embryonic fibroblasts derived from mice lacking STAT1; in the absence of the gene, IFN-induced transcription cannot be supported (Meraz *et al.*, 1996; Durbin *et al.*, 1996).

In addition to the role of STAT1 in IFN-mediated signal transduction and viral immunity, there have been reports that STAT1 can also regulate the expression of genes involved in the control of cell proliferation and apoptosis (Schindler, 1998), namely, $p21^{CIP/WAF1}$, caspase-1, Fas, and the Fas ligand, (Chin *et al.*, 1996 and 1997; Xu *et al.*, 1998). Interestingly, a function in tumour

necrosis factor (TNF) α-induced apoptosis has also been ascribed to STAT1 that appears to be independent of its transcriptional function (Kumar *et al.*, 1997).

3. Interferon-inducible genes

3.1 Overview

The effects of IFNs are initiated by signal transduction events that result in specific gene transcription. However, the biological actions of IFNs, particularly in the context of viral infection, are mediated by the activity of IFN-inducible gene products (Lengyel , 1982; Pestka *et al.*, 1987). IFN treatment of a variety of cell types results in the induction of approximately a dozen proteins at the transcriptional level. The accumulation of these IFN-inducible mRNAs can be observed anywhere from five minutes to two hours after IFN treatment. Although the identity of most of these IFN-inducible mRNAs is still unknown, cDNAs corresponding to a number of them have been isolated and identified. The list of IFN-inducible proteins include the 2',5' oligoadenylate (2-5A) synthetase, metallothionien-II, thymosin B4, the major histocompatibility antigens, and the Mx protein. In addition, IFNs induce the expression of the "200 family" of proteins, a member of which has been shown to be important in transcriptional regulation (Choubey *et al.*, 1996; Min *et al.*, 1996; Datta *et al.*, 1996).

3.2 PKR

One of the best characterized IFN-inducible gene products is the IFN-inducible dsRNA-activated protein kinase PKR. PKR was initially identified for its ability to inhibit protein synthesis in a cell-free system (Levin and London, 1978; Levin *et al.*, 1980). Since that time, PKR has been shown to exhibit anti-viral, anti-proliferative, and tumour suppressor activities (Proud, 1995; Williams, 1995 and 1997; Clemens, 1997; Clemens and Elia, 1997). To date, the cDNAs

encoding the mouse, rat, and human forms of PKR have been isolated and characterized (Meurs *et al.*, 1990; Baier *et al.*, 1993; Mellor *et al.*, 1994). In addition, a homologue of PKR has been identified in plants (Langland *et al.*, 1995 and 1996).

PKR is a serine/threonine kinase that is expressed at low levels by most cell types, but its expression can be induced by IFN (Samuel, 1979). PKR displays a constitutive level of basal activity and is activated by natural or synthetic dsRNA species. The kinase can also be activated by other polyanions such as heparin, dextran sulphate, chondroitin sulphate, and poly (L–glutamine) (Hovanessian, 1989), by exposure to calcium ionophores (Srivastava *et al.*, 1995; Prostko *et al.*, 1995), and by stress inducers such as sodium arsenite (Bromstom *et al.*, 1996). Upon activation, PKR displays two distinct catalytic activities. First, it undergoes extensive autophosphorylation (Taylor *et al.*, 1996; Romano *et al.*, 1998). Second, it phosphorylates the eukaryotic initiation factor–2α (Samuel, 1993; Wek, 1994; De Haro *et al.*, 1995); phosphorylation of the inhibitor of the NF-κB transcription factor IκB and the tumour suppressor p53 has also been reported *in vitro* (Kumar *et al.*, 1994; Cuddihy *et al.*, in press)

The predicted structure of PKR indicates a 551 amino acid protein that contains a number of discrete functional motifs. The amino-terminal half of the protein contains two dsRNA-binding domains located between residues 11-77 and 101-167 (Clemens and Elia, 1997; Nanduri *et al.*, 1998). The first domain has been shown to be sufficient to mediate dsRNA-binding activity (Clemens and Elia, 1997). The role of the second domain in PKR activation is less clear; some reports indicate that it is not essential for dsRNA binding activity whereas other studies suggest that is may be as important as the first domain (Clemens and Elia, 1997). In addition, PKR activation is influenced by the concentration of dsRNA as it displays a bell-shaped activation curve (Clemens and Elia, 1997). That is, low concentrations of dsRNA activate the kinase whereas high concentrations of dsRNA actually inhibit its activation. In addition to the dsRNA

binding domains, there are eleven catalytic motifs that are conserved among all protein kinases present in the carboxy-terminal region of the protein (Meurs *et al.*, 1990). A domain termed the kinase insert domain has also been identified in this region of the protein and is believed to play a role in the activation of the kinase (Craig *et al.*, 1995).

Analysis of the promoter region of mouse and human PKR genes suggests that PKR expression may be regulated by a variety of signaling pathways. Both the ISRE and GAS transcriptional elements (Tanaka and Samuel, 1994; Kuhen and Samuel, 1997) are present, and IFN-induced transcription of PKR appears to proceed through the ISRE (Kuhen and Samuel, 1997). In addition, the KCS (kinase conserved sequence) element is present in both the mouse and human PKR genes and enhances IFN-mediated transcriptional induction of PKR through the ISRE (Kuhen and Samuel, 1997). Other putative regulatory elements found within the promoter region include the IL-6 sensitive APRF, NF-κB, and NF-IL-6 elements (Tanaka and Samuel, 1994; Kuhen and Samuel, 1997).

The most extensively studied biological role of PKR is its ability to inhibit protein synthesis. In this regard, PKR belongs to a family of eIF-2α kinases (Samuel, 1993; Wek, 1994; De Haro *et al.*, 1995). The other members of this group include the heme control repressor that is present in erythrocytes and involved in the regulation of globin synthesis during periods of heme deficiency (Chen *et al.*, 1991). This family also contains the *Sacchromyces cerevisiae* GCN2 that regulates the expression of the GCN4 transcripton factor under amino acid starvation conditions (Dever *et al.*, 1992). All these kinases phosphorylate a common substrate, the α subunit of eIF-2, at serine 51. This modification prevents the recycling of eIF-2 mediated by a guanine nucleotide exchange reaction catalyzed by eIF-2B and thereby inhibits protein synthesis at the level of initiation (Hershey, 1989 and 1991; Rhoads, 1993).

The role of PKR in translational regulation is believed to contribute to the anti-viral state induced by IFNs in infected cells (Gale and Katze, 1998). It is suggested that during the course of viral infection, PKR can be activated by viral species of dsRNA or ssRNA with extensive secondary structure. Activated PKR in turn phosphorylates eIF-2α and inhibits protein synthesis, effectively preventing viral replication and the spread of infection. However, different viruses vary in their susceptibility to PKR. This effect is largely attributed to the activity of virally-encoded inhibitors that allow viruses to circumvent PKR-mediated translational regulation (Gale and Katze, 1998).

Although the role of PKR in the biological effects of IFN has been well studied, a growing area of investigation concerns the role of the kinase in cell growth and differentiation (Clemens, 1992; Lengyel, 1993; Proud, 1995; Jaramillo *et al.*, 1995; Clemens and Elia, 1997). These studies extend initial observations that PKR activity varies according to the state of growth of mammalian cells in culture (Petryshyn and London, 1984; Petryshyn *et al.*, 1988). The ability of PKR to inhibit cell growth was first demonstrated by the suppression of yeast growth by the expression of wildtype PKR but not a dominant negative form of the kinase (Chong *et al.*, 1992). Subsequent studies have reported that expression of wildtype PKR can also inhibit the growth of mammalian cells (Koromilas *et al.*, 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). In addition to regulating cell proliferation, several groups have reported a role for PKR in cellular differentiation processes induced by cytokines and growth factors. In this regard, preliminary evidence has implicated PKR in the biological effects of platelet derived growth factor (Mundschau and Faller, 1995), interleukin-3 (Ito *et al.*, 1994), and transforming growth factor-β (Salzerg *et al.*, 1995).

Consistent with a role in cell growth and proliferation, it has been proposed that PKR may function as a tumour suppressor (Clemens, 1992; Lengyel, 1993; Proud, 1995; Jaramillo *et al.*, 1995; Clemens and Elia, 1997). This hypothesis is based predominantly on studies that have measured the ability of various PKR

dominant negative mutants to induce transformation *in vitro* and tumourigenesis *in vivo* (Koromilas *et al.*, 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). Further studies have also suggested a role for PKR in apoptosis. The expression of dominant negative PKR mutants cells in mammalian cell lines results in reduced susceptibility to apoptosis induced by viruses (Lee and Esteban, 1993 and 1994; Takizawa *et al.*, 1994; Kibler *et al.*, 1997) and stress (Yeung *et al.*, 1996; Der *et al.*, 1997; Srivastava *et al.*, 1998; Yeung and Lau, 1998).

The putative roles ascribed to PKR in the regulation of normal cell growth and proliferation have been largely attributed to its ability to phosphorylate eIF-2α. This conclusion would appear to be corroborated by studies indicating that a non-phosphorylable form of eIF-2α increases the rate of growth of mammalian cells and induces tumour formation in mice (Donzé *et al.*, 1995). However, mice with a targeted deletion of the *pkr* gene do not exhibit a higher incidence of tumourigenesis compared to their wildtype littermates even though phosphorylation of eIF-2α is deficient (Yang *et al.*, 1995). These findings suggest that the role of PKR in cell growth may be redundant and can be complemented by another factor. An alternative explanation is that dominant negative PKR mutants may interfere with other pathways that regulate cell growth which are not affected in the absence of PKR.

In addition to a role in translational regulation, several reports indicate a role for PKR in the regulation of transcription (Clemens and Elia, 1997; Williams, 1995, 1997). To date PKR, has been implicated in a number of signaling pathways involved in the regulation of cell growth. PKR is required for the induction of several growth related genes, namely, the *c-fos*, *c-myc*, and JE genes, since a chemical inhibitor of PKR blocks the induction of these genes by platelet derived growth factor (Mundschau and Faller, 1995). PKR is also involved in signal transduction events mediated by the transcription factor interferon regulatory factor 1 (IRF-1) since NIH 3T3 cells expressing PKR dominant negative mutants are resistant to the growth inhibitory effects of IRF-1

(Kirchhoff *et al.*, 1995). PKR has also been implicated in the regulation of the transcription factor NF-κB, which regulates a large number of genes involved in cell growth. PKR has been shown to phosphorylate an inhibitor of NFκB, IκB, *in vitro* (Kumar *et al.*, 1994), and selective ablation of PKR mRNA abrogates NFκB transcriptional activity (Maran *et al.*, 1994). In addition, there are reports that indicate a requirement for PKR in transcriptional events that are important in adaptive immune responses. These include induction of immunoglobulin κ light chain expression (Koromilas *et al.*, 1992) and IgE class switching (Rager *et al.*, 1998) in B-lymphocytes and the regulation of the expression of the CD4 surface molecule that is important in antigen presentation to T-lymphocytes and HIV pathogenesis (Nagai *et al.*, 1997).

4. Scientific rationale and research objectives

The biological effects of IFNs have been attributed to the activity of proteins that are upregulated at the transcriptional level. Of these IFN-inducible proteins, the p202 protein of the "p200 family" has been well studied and has provided gainful insight into the biochemical events that underlie the effects of IFNs. It has been demonstrated that this protein interacts with numerous transcription factors including NF-κB, c-fos, c-Jun, E2F, and p53 which regulate gene expression during various immune responses and cell proliferation (Choubey *et al.*, 1996; Min *et al.*, 1996; Datta *et al.*, 1996). These protein-protein interactions have been shown to be responsible for regulating the activity of these transcription factors and have been proposed to mediate, in part, the pattern of gene expression induced by IFNs.

Based on studies describing the action of the interferon-inducible protein p202 and others implicating PKR in transcriptional regulation, a study was carried out to investigate whether, PKR, another IFN-inducible protein, might also exhibit a similar role in IFN action in addition to its demonstrated role in translational regulation. The objective of the work presented in this thesis was to examine the

role of PKR in signal transduction pathways that mediate the biological effects of IFNs and that are important in viral immunity. The goals of the project were (1) to examine the role of PKR in IFN-α/β, IFN-γ, and dsRNA signaling pathways; (2) to identify the signaling component(s) regulated by PKR; and (3) to determine the mechanism(s) employed by PKR to regulate the component(s).

MATERIALS AND METHODS

1. *Cells lines and culture conditions*

Mouse embryonic fibroblasts (MEFs), NIH 3T3 (ATCC CRL-1658), and HeLa S3 (ATCC CCL-2.2) cells were maintained as monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.), supplemented with 10% fetal calf serum, 2mM L-glutamine, penicillin (100 units/ ml), and streptomycin (100 units/ml). All cells were maintained at 37°C with 5% CO_2.

$PKR^{+/+}$ and $PKR^{-/-}$ immortalized polyclonal populations were established according to the method described elsewhere for spontaneous immortilization of primary murine fibroblasts on a 3T3 schedule (Todaro and Green 1963). NIH 3T3 clones expressing mutants of PKR were generated by stable transfection of PKRΔ6 (Koromilas *et al.*, 1992) or PKRLS4 (Green and Matthews, 1992) cDNA and selection in G418 (400 μg/ml). HeLa S3 clones expressing the catalytic mutant PKRΔ6 were generated by stable transfection of the hemagglutinin (HA)-tagged PKRΔ6 (PKRΔ6HA) cDNA and selection in G418 (400 μg/ml).

2. *Cell treatment*

2.1 IFN stimulation

Cells were grown to 70-80% confluency prior to treatment. Cells were then incubated with 1000 IU/ml of recombinant murine IFN-α/β (Lee Biomolecules) or 100 IU/ml IFN-γ (Cedarlane) in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ ml), and streptomycin (100 units/ml).

2.2 DsRNA transfection

Cells were grown to 70-80% confluency prior to treatment. DsRNA transfections were conducted in a DEAE-dextran dependent manner (Yang *et al.*, 1995) with 100 μg/ml poly(rI)-poly(rC) (Pharmacia) that had been previously denatured at 50°C for 1 hr and renatured at room temperature. DsRNA transfections were performed in DMEM supplemented with 2 mM L-glutamine and in the presence of Type I IFN monoclonal antibody (Kirchhoff *et al.*, 1993).

2.3 Actinomycin D treatment

Cells were grown to 70-80% confluency prior to treatment and then incubated with actinomycin D (ICN) at a final concentration of 10 μg/ml. Treatment was performed in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ ml), and streptomycin (100 units/ml).

2.4 Cycloheximide treatment

Cells were grown to 70-80% confluency prior to treatment and then incubated with cycloheximide (Sigma) at a final concentration of 50 μg/ml. Treatment was performed in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ ml), and streptomycin (100 units/ml).

3. Reagents

3.1 Plasmids and restriction enzymes

The pcDNA3.0/STAT1 construct was generated by subcloning of the HpaI-EcoRV fragment of the human STAT1 cDNA (Schindler *et al.*, 1992) into the EcoRV site of pcDNA3.0/neomycin expression vector (Invitrogen). The

pcDNA3.1/PKR constructs were created by subcloning the HindIII-BamHI fragment of the human wildtype PKR cDNA or the XbaI-BamHI fragment of the PKR K296R cDNA (Katze *et al.*, 1992) into the corresponding sites within the pcDNA3.1/zeocin expression vector (Invitrogen). The pcDNA3.0/PKR constructs were generated by introduction of the HindIII-BamHI fragments into the corresponding sites of the pcDNA3.0/neomycin expression vector (Invitrogen). The PKRΔ6HA cDNA was generated from the PKRHA cDNA by replacing the 330 bp HindIII/StuI fragment of the PKRHA cDNA (Chong *et al.*, 1992), which contains the HA epitope within the unique NcoI site of the human PKR cDNA. Restriction enzymes used were obtained from Boehringer Mannheim.

3.2 Antisera

The following antisera were used in these studies: mouse monoclonal anti-human PKR antibodies (13B8-F9 and E8), mouse monoclonal anti-human STAT1α antibody (aa 613-739; Santa Cruz), rabbit antiserum to human STAT1α/β (aa 598-705), rabbit polyclonal anti-human STAT2 antibody (aa 671-806; UBI), rabbit polyclonal anti-mouse STAT3 antibody (aa 750-769; Santa Cruz), rabbit antiserum to STAT5, anti-HA antibody (clone 12CA5; Boehringer Mannheim), and mouse monoclonal anti-actin antibody (clone C4; ICN).

4. Preparation of cell extracts

4.1 Whole cell extracts

For preparation of whole cell extracts, cells were harvested in 1 X phosphate buffered saline [PBS; 140 mM NaCl, 15 mM KH_2PO_4 (pH 7.2), and 2.7 mM KCl] – 1 mM EDTA, washed with ice-cold 1 X PBS, and lysed in 1 X Nonidet P-40 lysis buffer [50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5% Nonidet-P40, 10% glycerol, 150 mM NaCl, and 50 mM NaF] supplemented with 1 X protease inhibitors [3 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mM

phenylmethylsulfonyl fluoride (PMSF)] and 1 mM dithiothreitol (DTT). Lysates were incubated at 0°C for 15 min and centrifuged at 10,000 X g for 15 min; supernatants were collected and stored at –85°C.

4.2 Cytoplasmic extracts

For preparation of cytoplasmic cell extracts, cells were harvested in 1 X PBS-1 mM EDTA, washed with ice-cold 1 X PBS, and lysed in 1 X Triton X-100 lysis buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, and 1% Triton X-100] supplemented with 1 X protease inhibitors and 1 mM DTT. Lysates were incubated at 0°C for 15 min and centrifuged at 10,000 X g for 15 min; supernatants were collected and stored at –85°C.

5. Electrophoretic mobility shift assay

For electrophoretic mobility shift assays, the following double-stranded oligonucleotides were used (Sheldon Biotechnology Centre, Montreal, Canada): the ISG-15 ISRE (5'-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3'; Reich and Darnell, 1989) and the IFP-53 GAS (5'-GATCCAGATTCTCAGAAA-3'; Strehlow *et al.*, 1993).

To measure the kinetics of factors binding to the ISRE and/or GAS elements following treatment with IFN or dsRNA, whole cell extract (10 μg) was added to [α–^{32}P]-dGTP-labeled dsDNA oligonucleotide (0.5-2.0 ng), containing ~2 x 10^5 c.p.m. Binding reactions were contained in a buffer with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.9), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, poly (dI)-(dC) (250 ng/ml), 4 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF. Protein-DNA complexes formed during a 30 min incubation at room temperature were subsequently electrophoresed on a 6% non-denaturing polyacrylamide gel in 0.2 X TBE at 400 V at 4°C. To ensure the specificity of

interactions, a 200-fold excess of unlabeled dsDNA oligonucleotides was used in cold competition reactions. Induced DNA-protein complexes were visualized by autoradiography (Kodak).

6. RNA isolation and Northern blot analysis

Total RNA was isolated according to the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Northern blotting was performed by denaturing RNA (10 or 20 μg) with glyoxal and dimethylsulfoxide at 50°C for 1 hr (Sambrook *et al.*). RNA was then electrophoresed at 70V on a 1% agarose-10mM sodium phosphate (pH 7.0) in 10 mM sodium phosphate at 4°C. RNA was transferred to nylon membrane (BioTrans, ICN) in 20 X SSC [3 M NaCl and 300 mM sodium citrate (pH 7.0)] overnight at 4°C. STAT1 RNA levels were measured by hybridization at 65°C for 16 hours with an [α-^{32}P]-dATP-labeled randomly-primed probe corresponding to the 3.0 kbp HpaI-EcoRV fragment of the STAT1 cDNA (Schindler *et al.*, 1992). Equal loading of RNA was confirmed by hybridization with an [α-^{32}P]-dATP-labeled sequence containing the mouse β-actin cDNA. After hybridization the filters were washed three times with 2 X SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min and two washings with 0.1 X SSC and 0.1% SDS at 50°C for 15 min and were analyzed by autoradiography (Kodak).

7. Western blot analysis

For immunoblot analyses, 10 to 50 μg of protein from cytoplasmic or whole cell extracts were measured by Bradford assay (BioRad) and fractionated on SDS 8% or 10% polyacrylamide gels. Separated proteins were then electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore). Membranes were incubated in blocking solution (5% non-fat milk and 0.5% Triton X-100 in 1 X PBX) for 1 hr at room temperature. Primary antibody incubation was performed in blocking solution for 2 hr at room temperature

followed by three washings with wash buffer (0.5% Triton X-100 in 1 X PBS) for 10 min at room temperature. After incubation with an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Amersham Corp.) in blocking solution, membranes were rinsed in wash buffer three times for 10 min at room temperature. Proteins were visualized using the enhanced chemiluminesence (ECL) detection system (Amersham Corp.) according to manufacturer's specifications.

8. *In vitro* PKR phosphorylation assay

One hundred μg of protein extracts from untreated HeLa S3 cells or HeLa S3 that had been treated with human IFN-β for 18 h (1000 IU/ml; Lee Biomolecules) were suspended in a kinase reaction buffer [10 mM Tris-HCl (pH 7.7), 50 mM KCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF] and 20 μCi of [γ-^{32}P]-ATP (ICN). Reovirus dsRNA was added to a final concentration of 0.1 μg/ml. After incubation at 30°C for 30 min, the reaction was split into two fractions. Immunoprecipitations were performed with antibodies to PKR (13B8-F9) and STAT1α/β and Protein G-Sepharose (Pharmacia). Immunoprecipitates were washed three times with cold 1 X RIPA [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 1% SDS] supplemented with 1 M NaCl, 1 X protease inhibitors, and 1 mM DTT and three times with cold 1 X RIPA supplemented with 1 X protease inhibitors and 1 mM DTT. Immunoprecipitates were fractionated on SDS 7% polyacrylamide gels and visualized by autoradiography (Kodak).

9. *In vitro* transcription and translation

Human PKR and human STAT1α proteins were synthesized from the human PKR and STAT1α cDNAs, cloned into pcDNA3.1/zeo or pcDNA3.0/neo respectively under the control of the T7 promoter, using the TNT SP6/T7 *in vitro*

transcription and translation system (Promega) and [^{35}S]-methionine (>1000 Ci/mmol; Amersham) according to manufacturer's specifications. Equal counts of *in vitro* transcribed and translated proteins, determined by Cherenkov counting of trichloracetic acid precipitates, were mixed and immunoprecipitated with an anti-STAT1α antibody. Immunoprecipitates were captured with Protein G-Sepharose (Pharmacia) and washed with 1 X RIPA buffer. Immunoprecipitates were electrophoresed on SDS 8% polyacrylamide gels that were treated with EN^{3}HANCE (Dupont) according to manufacturer's specifications before visualization by fluorography.

10. In vivo labeling and immunoprecipitation

For pulse-chase analysis, cells were incubated in methionine-free DMEM (Life Technologies Inc.), supplemented with 10% dialyzed FCS and [^{35}S]-methionine (10 μCi/10^6 cells; ICN) for 2 h at 37°C. Cells were washed with 1 X PBS and incubated in DMEM plus 10% FCS for an additional 2 h at 37°C. Radiolabeled proteins were collected by washing cells twice with ice-cold 1 X PBS containing 100 mM NaF, 20 mM β–glycerophosphate, and 20 mM Na_2MdO_4 and lysing in 1 X RIPA lysis buffer containing 1 X protease inhibitors and 1 mM DTT and measured by Cherenkov counting of 10% trichloracetic acid precipitates. Equal counts from supernatants were pre-cleared with rabbit or mouse pre-immune serum (Sigma, MO) and then incubated with mouse monoclonal anti-STAT1α antibody in the presence of Protein G-Sepharose (Pharmacia) at 4°C under rotation. Immunoprecipitates were washed three times with cold 1 X RIPA supplemented with 1 M NaCl, 1 X protease inhibitors, and 1 mM DTT and three times with cold 1 X RIPA supplemented with 1 X protease inhibitors and 1 mM DTT. Proteins were fractionated on SDS 10% polyacrylamide gels and visualized by autoradiography (Kodak).

RESULTS

1. Regulation of STAT1 function by PKR

The effects of IFNs have been largely attributed to the transcriptional induction of proteins that perform effector functions. In this regard, the IFN-inducible p202 protein has been shown to interact with a number of transcription factors and thereby to affect a variety of signal transduction pathways (Min *et al.*, 1996; Choubey *et al.*, 1996; Datta *et al.*, 1996). To examine a possible role for PKR, another IFN-inducible protein, in signaling pathways required for IFN action, cells lacking PKR, namely, embryonic fibroblasts derived from $PKR^{-/-}$ mice (Yang *et al.*, 1995) were used as an experimental system.

1.1 DNA-binding activity induced by IFNs or dsRNA is upregulated in cells lacking PKR

$PKR^{+/+}$ and $PKR^{-/-}$ mouse embryonic fibroblasts (MEFs) were first tested for their relative abilities to respond to different extracellular stimuli known to elicit patterns of IFN-induced transcription. To do so, $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were treated with these stimuli, and the transcriptional response of these cells was measured by electrophoretic mobility shift assay (EMSA), otherwise known as gel shift analysis.

$PKR^{+/+}$ and $PKR^{-/-}$ MEFs were first treated with IFN-α/β, and the ability of the ISGF-3 transcriptional complex to bind DNA was determined by EMSA using a radiolabeled ISRE from the IFN-α/β-inducible ISG-15 gene. As shown in Figure 3A, ISGF-3 DNA-binding is observed in both cell types in response to IFN. However, there is a 3- to 5-fold increase in ISGF-3 DNA-binding activity in $PKR^{-/-}$ MEFs compared to $PKR^{+/+}$ MEFs (compare lanes 2-4 and 7 -9). Supershift analysis with a STAT1 antibody indicated the presence of STAT1 in the induced transcriptional complex (Figure 3B, lane 2).

The ability of $PKR^{+/+}$ and $PKR^{-/-}$ MEFs to respond to IFN–γ was tested next. To determine the activity of the GAF transcription factor, $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were incubated with IFN–γ, and gel shift analysis was performed with a radiolabeled GAS derived from the IFN–γ-inducible IFP-53 gene. In a manner similar to IFN–α/β treatment, both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs displayed induction of GAF DNA-binding activity in response to IFN–γ (Figure 4A). As well, $PKR^{-/-}$ MEFs exhibit elevated GAF DNA-binding activity relative to $PKR^{+/+}$ MEFs (compare lanes 2-4 with 7-9). Supershift analysis with a STAT1 antibody verified the identity of the transcriptional complex as GAF (Figure 4B, lane 2).

In addition to transcriptional events mediated through the GAS element, IFN–γ has been shown to stimulate transcription through the ISRE of certain genes. It does so by inducing the formation of a transcriptional complex containing GAF and the ISGF-3γ DNA binding subunit (Bluyssen *et al.*, 1995). To determine whether this aspect of IFN–γ signaling pathway was also affected by PKR, $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were stimulated with IFN–γ, and EMSA was carried out with the ISG-15 ISRE. The results of this experiment indicate that both alternate pathways of IFN–γ signaling are affected by PKR as an increase in IFN-γ-inducible ISRE-binding activity is seen in $PKR^{-/-}$ MEFs compared to $PKR^{+/+}$ MEFs (Figure 5, compare lanes 2-4 with 7-9).

In addition to IFNs, the transcriptional induction of certain IFN-inducible genes has been shown to occur upon viral infection or dsRNA treatment and to be mediated through the ISRE (Decker, 1992; Daly and Reich, 1993). It has also been previously shown that the one of the DRAFs that binds the ISRE in response to dsRNA treatment contains STAT1 (Bandhyopadhyay *et al.*, 1995). Considering the effect of PKR on DNA-binding activity stimulated by IFN–α/β and IFN–γ, whether PKR might also affect DNA-binding mediated by dsRNA was examined. To this end, the ability of dsRNA to stimulate the IFN–α/β-inducible gene ISG-15 was tested by gel shift analysis using the ISG-15 ISRE. Upon dsRNA transfection, it was observed that $PKR^{-/-}$ MEFs have a higher induction of

ISRE DNA-binding compared to $PKR^{+/+}$ MEFs (Figure 6A, compare lanes 2-4 with 7-9). Supershift analysis with a STAT1 antibody indicated that the transcriptional complex examined contained STAT1 (Figure 6B, lane 2). These results together with previous data suggest that PKR is involved in the regulation of signal transduction pathways that mediate IFN activity.

1.2. PKR interacts with STAT1

The increase in ISGF-3, GAF, GAF/ISGF-3γ, and DRAF DNA binding activities observed in $PKR^{-/-}$ MEFs relative to $PKR^{+/+}$ MEFs suggested a possible PKR-mediated regulation of STAT1, the common component of these transcriptional complexes. Considering that the regulatory effects of the p202 protein on transcription are mediated through protein-protein interactions (Min *et al.*, 1996; Choubey *et al.*, 1996; Datta *et al.*, 1996), one potential mechanism by which PKR could regulate transcriptional responses that require STAT1 may have involved an interaction between the two proteins. To pursue this possibility, the ability of PKR and STAT1 to associate was tested *in vitro*. Human STAT1 and PKR were transcribed and translated *in vitro* with [^{35}S]-methionine. Equal counts of ^{35}S-labeled PKR and STAT1 proteins were combined, and the mixture was immunoprecipitated with an anti-PKR antibody (Figure 7). In this experiment, approximately 10% of STAT1 was able to co-immunoprecipitate with wildtype PKR (Figure 7, lane 6), thereby demonstrating an interaction between the two proteins.

Considering that one type of protein-protein interaction is the transient association between a kinase and its substrate, whether the ability of PKR to associate with STAT1 was dependent upon its catalytic activity was tested. In the same experiment using wildtype PKR, equal amounts of [^{35}S]-labeled STAT1 and PKR K296R (Katze *et al.*, 1991), a dominant negative catalytic mutant of PKR, were combined, and the resulting mixture was immunoprecipitated with an anti-PKR antibody. In a manner similar to wildltype PKR, PKR K296R was able

to associate with STAT1 (Figure 7, lane 8). This result indicates that the catalytic activity of PKR is not required for the association between the two proteins.

1.3 PKR does not phosphorylate STAT1

To verify that the association between PKR and STAT1 did not represent a kinase-substrate interaction, the ability of PKR to phosphorylate STAT1 was tested. To do so, an *in vitro* kinase assay was performed using HeLa S3 cell extracts in which PKR was activated by autophosphorylation in the presence of activator reovirus dsRNA and [γ-^{32}P]-ATP (Figure 8). After incubation, one-half of the reaction was subjected to PKR immunoprecipitation and the other half to STAT1 immunoprecipitation. Immunoprecipitation with an anti-PKR antibody indicated that PKR was autophosphorylated and thus catalytically active (Figure 8, lanes 1, 3, 5, and 7). Phosphorylation of PKR was shown to occur in the presence of dsRNA and correlated to the amount of PKR protein (Figure 8, lanes 5 and 7). Immunoprecipitation with an anti-STAT1 antibody, however, did not precipitate a phosphorylated protein the size of STAT1α (Figure 8, lanes 2, 4, 6, and 8). However, we noted that STAT1α could co-precipitate with a phosphoprotein corresponding to the size of PKR. These results confirm the interaction between the two proteins and indicate that PKR does not regulate STAT1 activity through phosphorylation.

1.4 Additional studies of PKR-mediated regulation of STAT1 function

While this study of PKR-mediated regulation of STAT1 function was being performed in PKR$^{-/-}$ cells, a parallel similar study was being conducted in NIH 3T3 cells stably expressing dominant negative mutants of PKR (Tam/Wong *et al.*, 1997). In summary, the following are the major findings: (1) STAT1 DNA-binding activity is downregulated in cells overexpressing PKR catalytic mutants; (2) STAT1 DNA-binding activity is upregulated in cells overexpressing a dsRNA-binding defective mutant of PKR; (3) STAT1 can interact with PKR catalytic

mutants but not with the dsRNA-binding defective mutant of PKR *in vivo*; and (4) the interaction between PKR and STAT1 is disrupted by IFN or dsRNA treatment.

2. *Regulation of STAT1 expression by PKR*

2.1 STAT1 expression is enhanced in the absence of PKR

During the study of PKR-mediated regulation of STAT1 function, it was noticed that STAT1 expression was upregulated in cells lacking PKR. Specifically, STAT1 protein levels were ~3 fold higher in $PKR^{-/-}$ MEFs relative to $PKR^{+/+}$ MEFs as judged by Western blot analysis with an anti-STAT1α antibody (Figure 9, compare lanes 1-3 with 4-6).

2.2 PKR is not involved in STAT1 transcriptional or post-transcriptional regulation.

To determine whether the enhanced STAT1 expression observed in $PKR^{-/-}$ MEFs could be attributed to an increase in STAT1 transcription, STAT1 RNA levels were examined in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs before and after IFN-α/β treatment. As shown in Figure 10A, Northern blot analysis indicated no significant differences in either the basal (first row, lanes 1 and 3) or inducible levels of STAT1 mRNA between $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (first row, lanes 2 and 4) although both STAT1α and STAT1β protein levels were elevated in $PKR^{-/-}$ MEFs relative to $PKR^{+/+}$ MEFs (third row). The detection of three bands in the Northern blot was most likely due to the expression of different STAT1 isoforms in mouse cells (Schindler *et al.*, 1992). These results indicate that STAT1 upregulation in $PKR^{-/-}$ MEFs is not transcriptional in nature.

The possibility that PKR may regulate STAT1 at the post-transcriptional level was examined next. To determine whether STAT1 mRNA stability might be

affected by PKR, $PKR^{+/+}$ and $PKR^{-/-}$ cells were treated with actinomycin D for increasing periods of time followed by Northern blot analysis with a STAT1 cDNA probe (Figure 10B). It appeared that the STAT1 mRNA was a fairly stable species and that the rates of STAT1 mRNA turnover were not significantly different between $PKR^{+/+}$ and $PKR^{-/-}$ cells (top row, compare lanes 1-4 with 5-8).

2.3 STAT1 protein stability is altered in the absence of PKR.

Considering the role of PKR in protein synthesis, it was reasoned that the observed increase in STAT1 could be due to changes in STAT1 translation. At the same time, since PKR could interact with STAT1, it was also possible that the interaction between the PKR and STAT1 might have an effect on the stability of the STAT1 protein. To distinguish between these two possibilities, $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were radiolabeled with [^{35}S]-methionine, and STAT1 protein turnover was examined by pulse-chase analysis and quantified by densitometry. As seen in Figure 11A, in $PKR^{+/+}$ MEFs the level of STAT1 protein decreased to approximately 50% at roughly 100 min of the chase period (lanes 1-4). In $PKR^{-/-}$ MEFs, however, the expression of STAT1 appeared to remain relatively constant throughout the course of the chase period (lanes 5-8).

To examine regulation of STAT1 stability more closely, another experiment to measure STAT1 protein turnover was performed. In this case, $PKR^{+/+}$ and $PKR^{-/-}$ 3T3-like cells were treated with cycloheximide (CHX) for various lengths of time. STAT1 protein turnover was then assessed by Western blot analysis and quantified by densitometry (Figure 11B). In a manner similar to the pulse-chase experiment, STAT1 protein levels reached 50% at approximately 100 min after CHX treatment in $PKR^{+/+}$ cells (lanes 1-4) whereas they were relatively stable in $PKR^{-/-}$ cells (lanes 5-8) during the same period.

2.4 STAT1 expression is regulated by PKR dominant negative mutants.

To test whether the increase in STAT1 expression observed in cells lacking PKR might require the enzymatic activity of PKR, NIH 3T3 cells stably expressing either the catalytic mutant PKRΔ6 (Koromilas *et al.*, 1992) or a dsRNA-binding defective mutant of PKR, PKRLS4 (Green and Mathews, 1992), were assessed for STAT1 protein expression (Figure 12). The relative levels of expression of the PKR mutants were analyzed by Western blotting (Figure 12, second row) with an antibody which recognized human but not murine PKR (Koromilias *et al.*, 1995). The differences in the levels of the PKR mutants were most likely due to the varying degrees with which the mutants affect their own translation as previously reported (Thomis and Samuel, 1992; Barber *et al.*, 1993). It was observed that, similar to $PKR^{-/-}$ cells, STAT1 protein was upregulated ~ 3 fold in NIH 3T3 cells expressing PKR mutants relative to control cells (Figure 12, first row). These results suggested that STAT1 expression could be regulated by PKR mutants that function in a dominant negative manner in PKR-mediated translational regulation.

2.5 *PKR regulates expression of STAT1 and not other STATs.*

Since STAT1 is a member of a family of transcription factors, whether the effect of PKR on STAT1 was specific or general for other STAT family members was tested. To do so, the expression of various STATs was examined in $PKR^{+/+}$ and $PKR^{-/-}$ 3T3-like cells (Figure 13A). Although STAT1 expression was increased in $PKR^{-/-}$ cells compared to $PKR^{+/+}$ cells (top row), the expression of STAT3 (middle row) or STAT5 (bottom row) did not differ between the cell lines. In addition to murine cells, STAT expression was analyzed in human HeLa S3 cells expressing the PKRΔ6HA mutant (Figure 13B). Consistent with the data obtained from murine cell lines, STAT1 expression was increased in PKRΔ6HA-expressing cells relative to control cells (first row). In addition, similar effects on STAT3 (third row) and STAT5 (fourth row) expression as well as STAT2 (second row) were observed; that is, there were no significant differences in the expression of these proteins in HeLa S3 cells expressing PKRΔ6HA relative to

control cells. These data indicate that PKR regulates the expression of specifically STAT1 and not of other STATs.

Figure 3

(A) Analysis of IFN-α/β-inducible DNA-binding activity in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. MEFs were untreated (lanes 1 and 6) or treated with 1000 IU/ml IFN-α/β for 30 (lanes 2 and 7), 60 (lanes 3 and 8), and 120 min (lanes 4, 5, 9, and 10). EMSA was performed with 10 μg of whole cell protein extracts and the ISRE from the ISG-15 gene. A 200-fold excess of unlabelled ISG-15 ISRE was used in cold competition reactions (lanes 5 and 10). (B) Identification of ISGF-3 transcriptional complex. For supershift analysis, 10 μg of whole cell extract from $PKR^{+/+}$ MEFs treated with IFN-α/β for 120 min were incubated with 3 μg of mouse IgG (lane 1), anti-STAT1α antibody (lane 2), or 200-fold excess of unlabelled ISG-15 ISRE (lane 3).

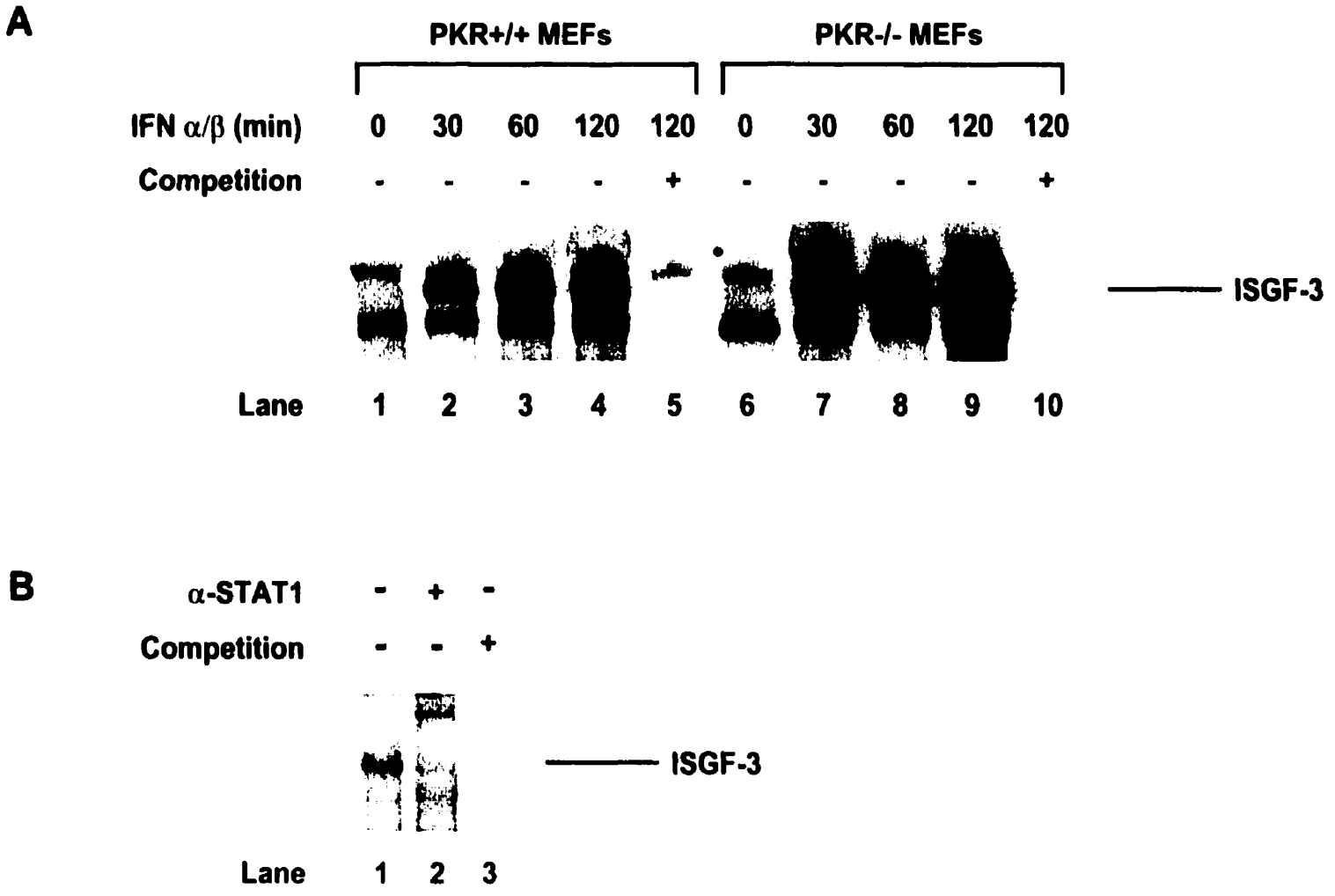

A
PKR+/+ MEFs
PKR-/- MEFs
IFN α/β (min)
0 30 60 120 120 0 30 60 120 120
Competition
- - - - + - - - - +
ISGF-3
Lane
1 2 3 4 5 6 7 8 9 10
B
α-STAT1
- + -
Competition
- - +
ISGF-3
Lane
1 2 3

Figure 4

(A) Analysis of IFN–γ-inducible DNA-binding activity in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. MEFs were untreated (lanes 1 and 6) or treated with 100 IU/ml IFN–γ for 30 (lanes 2 and 7), 60 (lanes 3 and 8), and 120 min (lanes 4, 5, 9, and 10). EMSA was performed with 10 μg of whole cell protein extracts and the GAS from the IFP-53 gene. A 200-fold excess of unlabelled IFP-53 GAS was used in cold competition reactions (lanes 5 and 10). (B) Identification of GAF transcriptional complex. For supershift analysis, 10 μg of whole cell extract from $PKR^{+/+}$ MEFs treated with IFN–γ for 120 min were incubated with 3 μg of mouse IgG (lane 1), anti-STAT1α antibody (lane 2), or 200-fold excess of unlabelled IFP-53 GAS (lane 3).

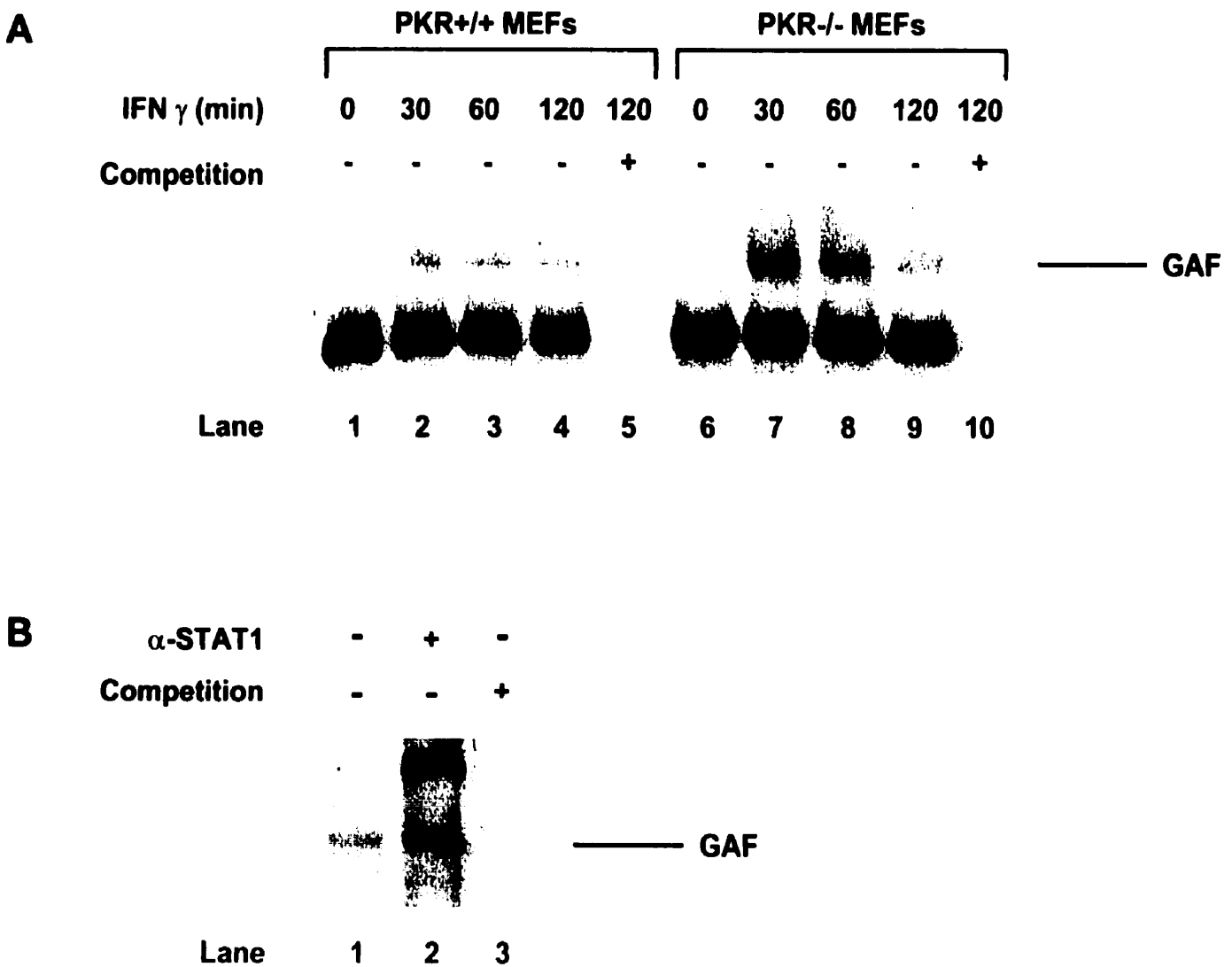

A
PKR+/+ MEFs
PKR-/- MEFs
IFN γ (min)
0 30 60 120 120 0 30 60 120 120
Competition
- - - - + - - - - +
GAF
Lane
1 2 3 4 5 6 7 8 9 10
B
α-STAT1
- + -
Competition
- - +
GAF
Lane
1 2 3

Figure 5

Analysis of GAF/ISGF-3γ DNA-binding activity in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. MEFs were untreated (lanes 1 and 6) or treated with 100 IU/ml IFN-γ for 30 (lanes 2 and 7), 60 (lanes 3 and 8), and 120 min (lanes 4, 5, 9, and 10). EMSA was performed with 10 μg of whole cell protein extracts and the GAS from the IFP-53 gene. A 200-fold excess of unlabelled IFP-53 GAS was used in cold competition reactions (lanes 5 and 10).

PKR+/+ MEFs
PKR-/- MEFs
IFN γ (min)
0 30 60 120 120 0 30 60 120 120
Competition
- - - - + - - - - +
Lane
1 2 3 4 5 6 7 8 9 10
GAF/ISGF-3γ

Figure 6

(A) Analysis of dsRNA-inducible DNA-binding activity in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. MEFs were untreated (lanes 1 and 6) or treated with 100 μg/ml poly(rI)-(rC) for 2 (lanes 2 and 7), 4 (lanes 3 and 8), and 6 h (lanes 4, 5, 9, and 10). EMSA was performed with 10 μg of whole cell protein extracts and the ISRE from the ISG-15 gene. A 200-fold excess of unlabelled ISG-15 ISRE was used in cold competition reactions (lanes 5 and 10). (B) Identification of STAT1-containing transcriptional complex. For supershift analysis, 10 μg of whole cell extract from $PKR^{+/+}$ MEFs treated with dsRNA for 6 h were incubated with 3 μg of mouse IgG (lane 1), anti-STAT1α antibody (lane 2), or 200-fold excess of unlabelled ISG-15 ISRE (lane 3).

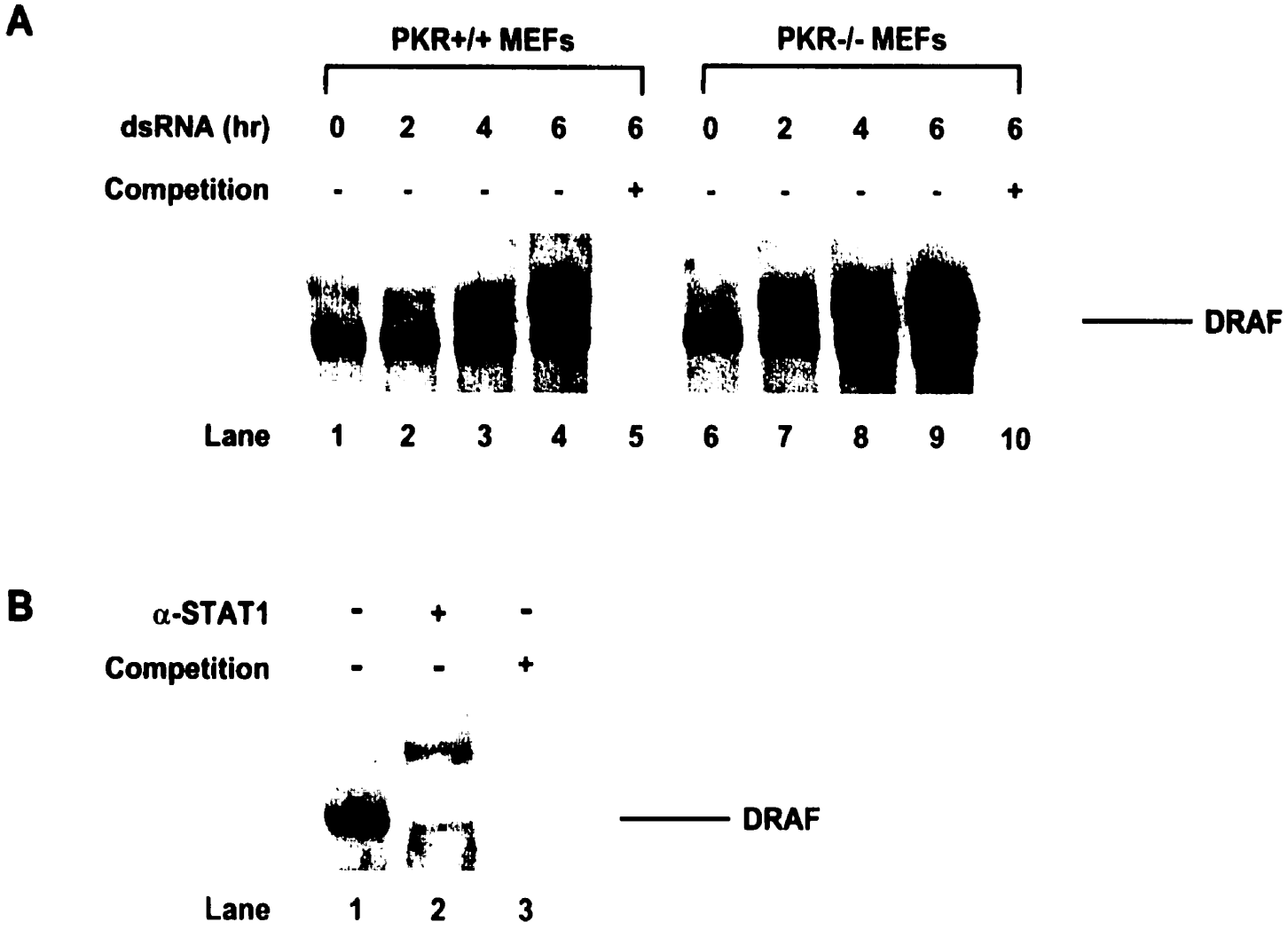

A
PKR+/+ MEFs
PKR-/- MEFs
dsRNA (hr)
0 2 4 6 6 0 2 4 6 6
Competition
- - - - + - - - - +
DRAF
Lane
1 2 3 4 5 6 7 8 9 10
B
α-STAT1
- + -
Competition
- - +
DRAF
Lane
1 2 3

Figure 7

Interaction between PKR and STAT1. [^{35}S]-labeled human STAT1α, wildtype PKR, and PKR K296R were synthesized *in vitro* (lanes 2, 3 and 4). [^{35}S]-labeled STAT1 was immunoprecipitated with anti-STAT1α antibody (lanes 5 and 7) or incubated with [^{35}S]-labeled PKR and immunoprecipitated with anti-PKR antibody (lanes 6 and 8). Reactions were subjected to SDS PAGE and fluorography.

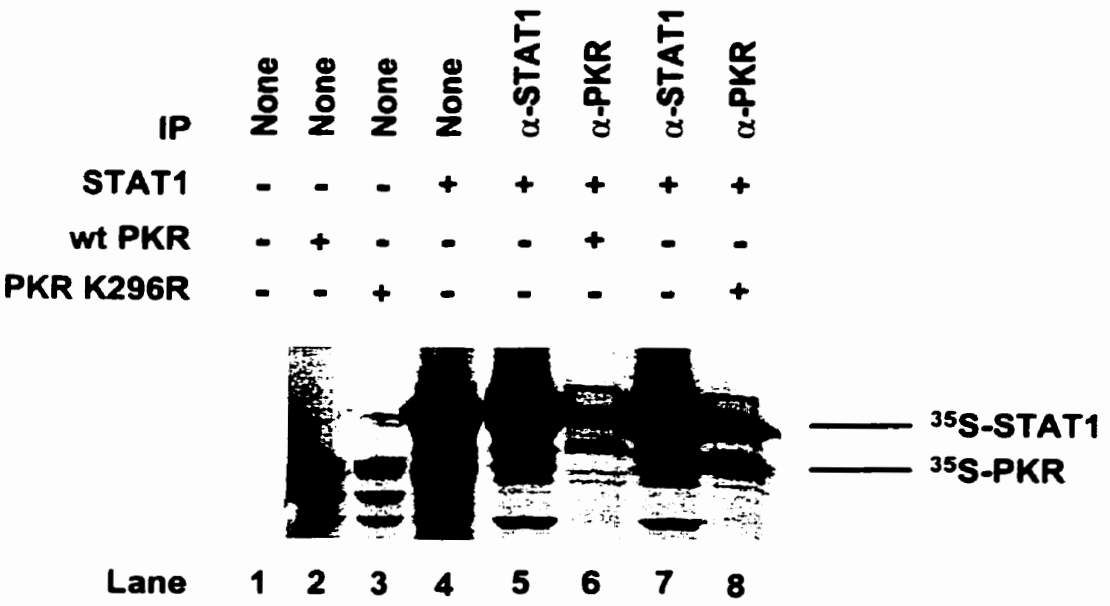

IP
None
None
None
None
α-STAT1
α-PKR
α-STAT1
α-PKR
STAT1 - - - + + + + +
wt PKR - + - - - + - -
PKR K296R - - + - - - - +
35S-STAT1
35S-PKR
Lane 1 2 3 4 5 6 7 8

Figure 8

PKR *in vitro* phosphorylation assay with STAT1. Untreated (lanes 1, 2, 3 and 4) or IFN-β-treated (1000 IU/ml for 18 h; lanes 5, 6, 7, and 8) HeLa S3 extracts were incubated with [γ-^{32}P]-ATP in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of activator reovirus dsRNA (0.1 μg/ml). Reactions were immunoprecipitated with anti-PKR (lanes 1, 3, 5, and 7) or anti-STAT1α (lanes 2, 4, 6, and 8) antibody and subjected to SDS PAGE.

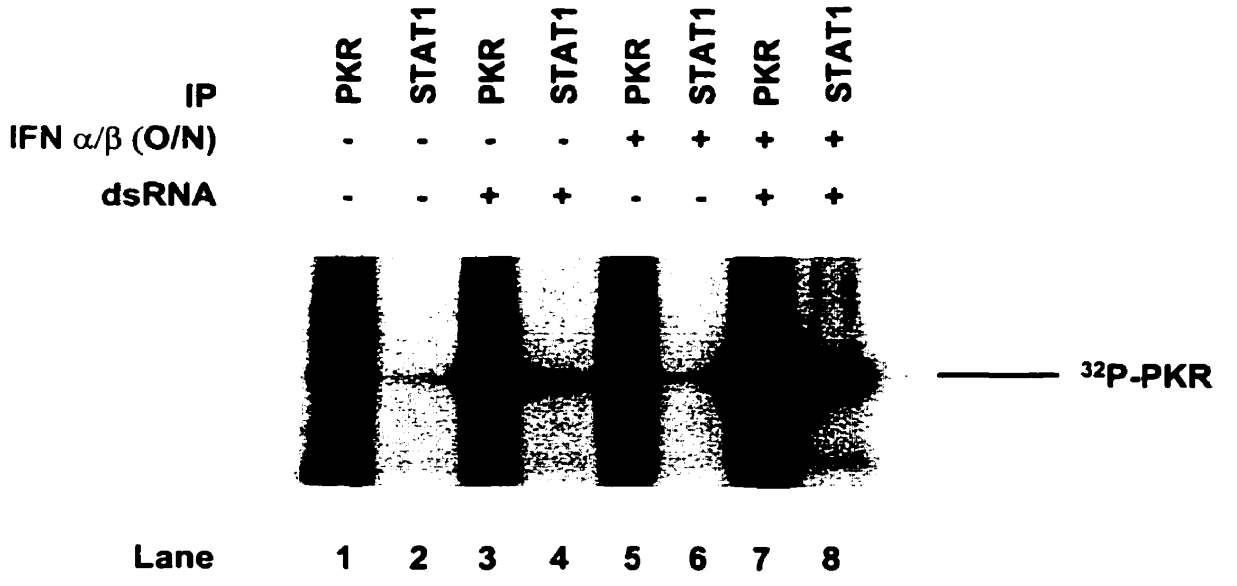

IP
PKR
STAT1
PKR
STAT1
PKR
STAT1
PKR
STAT1
IFN α/β (O/N)
- - - - + + + +
dsRNA
- - + + - - + +
32P-PKR
Lane
1 2 3 4 5 6 7 8

Figure 9

STAT1 protein expression in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. Ten, 20, and 50 μg of cytoplasmic cell extracts from $PKR^{+/+}$ (lanes 1, 2 and 3) and $PKR^{-/-}$ MEFs (lanes 4, 5, and 6) were tested for STAT1 (top row) and actin (bottom row) protein levels by Western blot analysis with an anti-STAT1α and anti-actin antibody respectively.

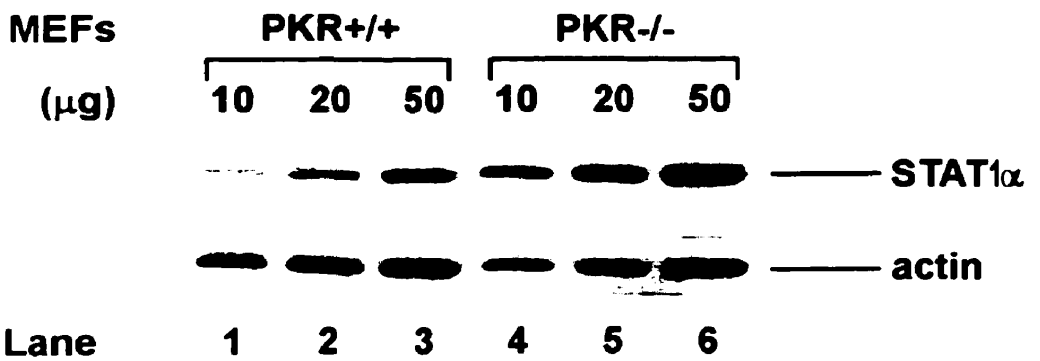
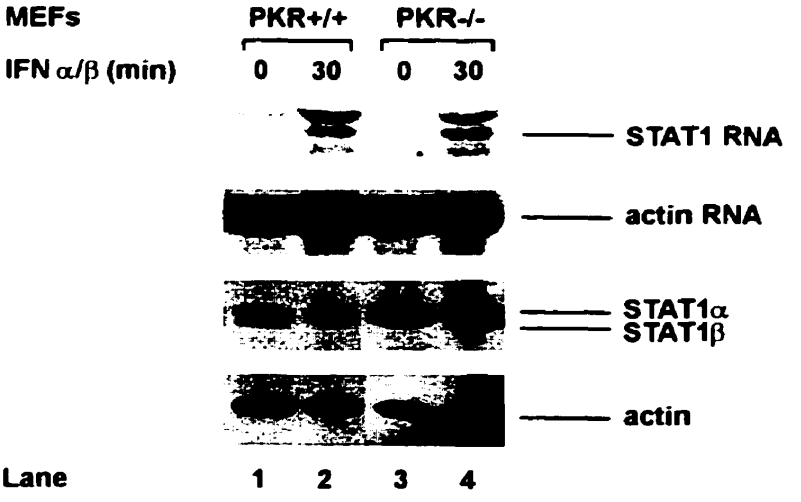

MEFs
PKR+/+
PKR-/-
(μg)
10
20
50
10
20
50
STAT1α
actin
Lane
1
2
3
4
5
6

Figure 10

(A) STAT1 mRNA and protein expression in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. STAT1 (first row) and actin (second row) RNA levels in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were examined by Northern blot analysis of 10 μg of RNA with an [α-^{32}P]-labeled STAT1 and β-actin cDNA probe respectively. STAT1 (third row) and actin (fourth row) protein expression in $PKR^{+/+}$ (lanes 1 and 2) and $PKR^{-/-}$ (lanes 3 and 4) MEFs were analyzed by Western blot analysis of 40 μg of whole cell extracts with an anti-STAT1α/β and anti-actin antibody respectively. Lane 1 and 3, untreated; 2 and 4, IFN-α/β-treated. (B) STAT1 mRNA stability of $PKR^{+/+}$ and $PKR^{-/-}$ 3T3-like cells. $PKR^{+/+}$ (lanes 1-4) and $PKR^{-/-}$ (lanes 5-8) 3T3-like cells were treated with 10 μg/ml actinomycin D for times indicated, and STAT1 (first row) and actin (second row) RNA levels were examined by Northern blot analysis of 20 μg RNA with an [α-^{32}P]-labeled STAT1 and β-actin cDNA probe respectively. Lanes 1 and 5, untreated; 2 and 6, treated for 45 min; 3 and 7, treated for 90 min; 4 and 8, treated for 120 min.

A


MEFs
PKR+/+
PKR-/-
IFN α/β (min)
0 30 0 30
STAT1 RNA
actin RNA
STAT1α
STAT1β
actin
Lane
1 2 3 4

B

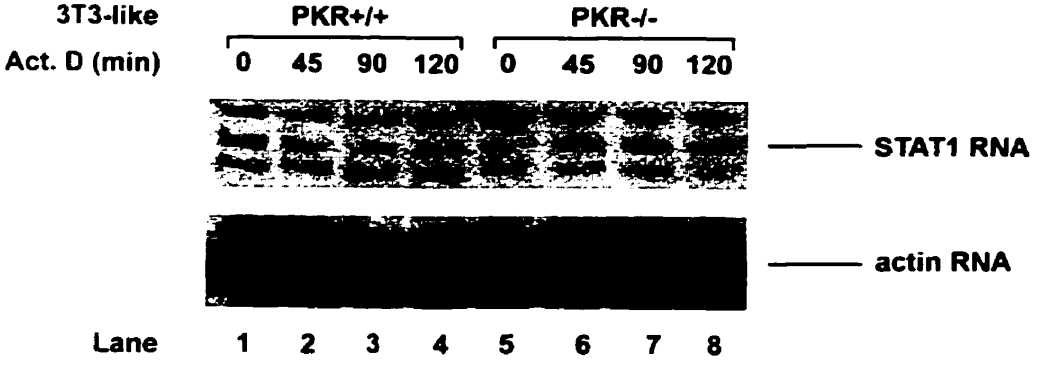

3T3-like
PKR+/+
PKR-/-
Act. D (min)
0 45 90 120 0 45 90 120
STAT1 RNA
actin RNA
Lane
1 2 3 4 5 6 7 8

Figure 11

(A, upper panel) Pulse chase analysis of STAT1 protein in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. $PKR^{+/+}$ (lanes 1-4) and $PKR^{-/-}$ (lanes 5-8) MEFs were labeled with [^{35}S]-methionine for 2 h and chased with cold methionine for 0 (lanes 1 and 5), 30 (lanes 2 and 6), 60 (lanes 4 and 7), and 90 (lanes 5 and 8) min. An equal amount of total [^{35}S]-labeled proteins from cell lysates was immunoprecipitated with an anti-STAT1α antibody. Immunoprecipitates were then analyzed by SDS PAGE and fluorography. (B, upper panel) Cycloheximide treatment of $PKR^{+/+}$ and $PKR^{-/-}$ 3T3-like cells. $PKR^{+/+}$ (lanes 1-4) and $PKR^{-/-}$ (lanes 5-8) 3T3-like cells were treated with 50 μg/ml cycloheximide (CHX) for times indicated, and STAT1 and actin protein levels were analyzed by Western blot analysis of 25 μg of protein with an anti-STAT1α and anti-actin antibody respectively. Lanes 1 and 5, untreated; 2 and 6, treated for 30 min; 3 and 7, treated for 60 min; 4 and 8, treated for 90 min. (A and B, lower panels) Quantification of bands was performed by scanning autoradiograms within the linear range of exposure with a Chemiimager 4000 Imaging system and analyzing with spot densitometry software (Alpha Innotech Corporation).

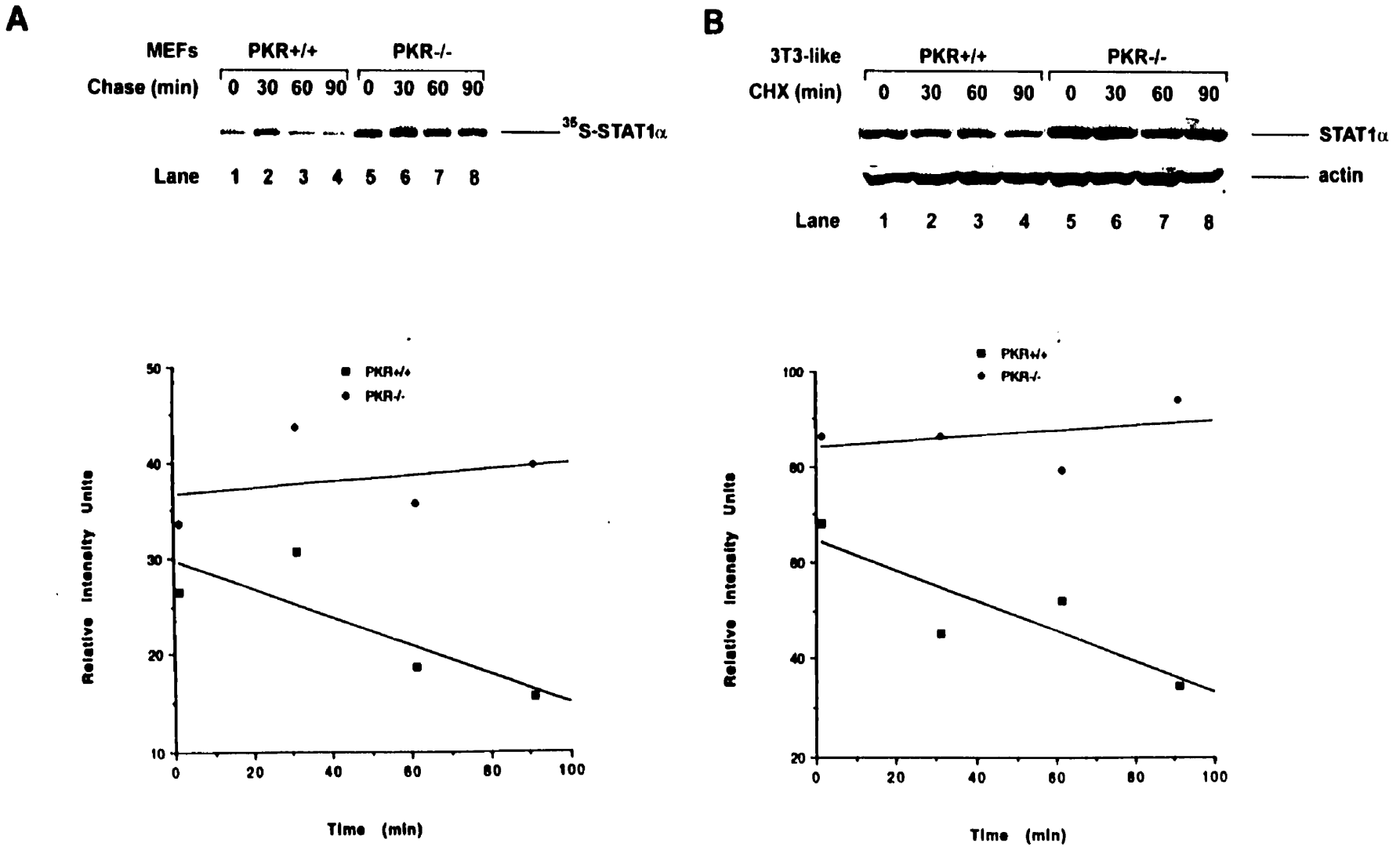

A
MEFs
PKR+/+
PKR-/-
Chase (min)
0 30 60 90 0 30 60 90
35S-STAT1α
Lane
1 2 3 4 5 6 7 8
B
3T3-like
PKR+/+
PKR-/-
CHX (min)
0 30 60 90 0 30 60 90
STAT1α
actin
Lane
1 2 3 4 5 6 7 8
PKR+/+
PKR-/-
Relative Intensity Units
Time (min)
PKR+/+
PKR-/-
Relative Intensity Units
Time (min)

Figure 12

STAT1 protein expression in cells expressing a PKR catalytic (PKRΔ6) or RNA-binding (PKR LS4) mutant. Fifty μg of whole cell extracts from NIH 3T3 control (lane 1) and NIH 3T3 cells stably expressing the PKRΔ6 (lane 2) or PKR LS4 (lane 3) were tested for STAT1 (top row) and actin (bottom row) protein levels by Western blot analysis with an anti-STAT1α and anti-actin antibody respectively.

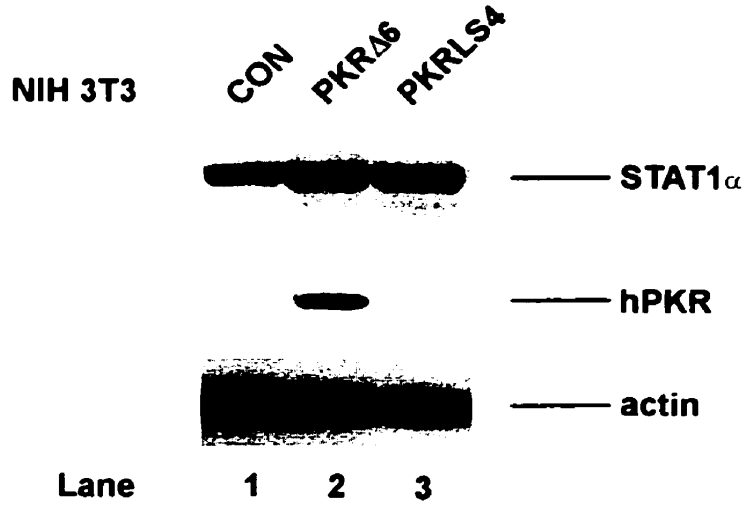
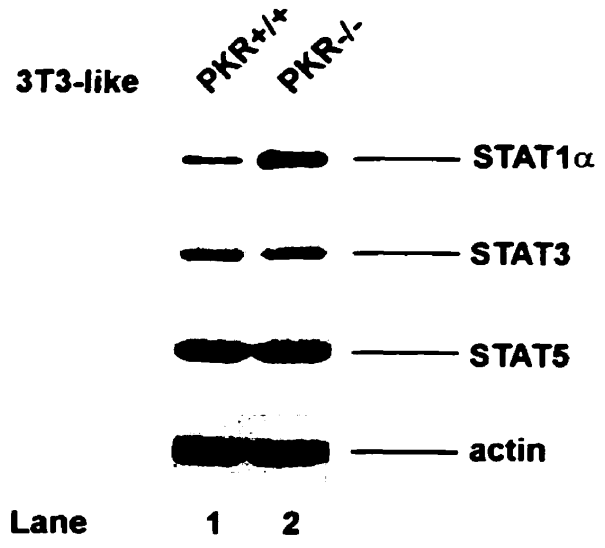

NIH 3T3
CON
PKRΔ6
PKRLS4
STAT1α
hPKR
actin
Lane
1
2
3

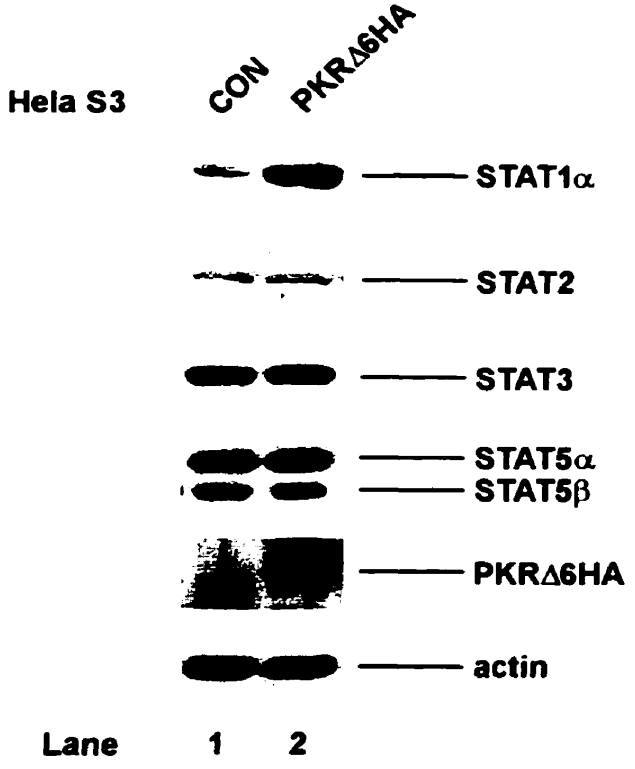
Figure 13

(A) Expression of STATs in $PKR^{+/+}$ and $PKR^{-/-}$ 3T3-like cells. Twenty-five μg of cytoplasmic cell extracts from $PKR^{+/+}$ (lane 1) and $PKR^{-/-}$ (lane 2) 3T3-like cells were tested for STAT1 (first row), STAT3 (second row), STAT5 (third row), and actin (fourth row) protein levels by Western blot analysis with an anti-STAT1α, anti-STAT3, anti-STAT5, and anti-actin antibody respectively. (B) Expression of STATs in HeLa S3 cells expressing the PKR catalytic mutant PKRΔ6. Fifty μg of cytoplasmic cell extracts from HeLa S3 control (lane 1) and Hela S3 cells stably expressing the HA epitope tagged PKRΔ6 catalytic mutant (lane 2) were analyzed for STAT1 (first row), STAT2 (second row), STAT3 (third row), STAT5 (fourth row), PKRΔ6HA (fifth row), and actin (sixth row) protein levels by Western blot analysis with an anti-STAT1α, anti-STAT2, anti-STAT3, anti-STAT5, anti-HA, and anti-actin antibody respectively.

A


3T3-like
PKR+/+
PKR-/-
STAT1α
STAT3
STAT5
actin
Lane
1
2

B


Hela S3
CON
PKRΔ6HA
STAT1α
STAT2
STAT3
STAT5α
STAT5β
PKRΔ6HA
actin
Lane
1
2

DISCUSSION

1. Summary of results

The action of IFNs is an important feature of the host immune response that develops during viral infection and is mediated primarily through the activity of proteins that are upregulated at the transcriptional level. Of these IFN-inducible proteins, the dsRNA-dependent protein kinase PKR has been well characterized for its ability to regulate protein synthesis and to inhibit viral replication. In addition to its role in translational regulation, several reports have indicated that PKR also functions in the regulation of transcription.

1.1 Regulation of STAT1 function by PKR

The work presented in this thesis has extended these earlier reports. The objective was to determine whether PKR is involved in the signal transduction pathways that mediate IFN-inducible patterns of gene transcription. Using embryonic fibroblasts derived from mice with a targeted deletion in the *pkr* gene as a model system (Yang *et al.*, 1995), a requirement for PKR has been demonstrated in the signaling pathways induced by IFN-α/β, IFN-γ, and dsRNA. In cells lacking PKR, there is an increase in the transcriptional response to all these extracellular stimuli.

It has been further shown that the signaling component regulated by PKR is the STAT1 transcription factor, which participates in all of these signal transduction pathways. In trying to elucidate the mechanism by which PKR modulates STAT1 transcriptional function, an interaction between PKR and STAT1 has been identified *in vitro*. This association does not represent a kinase-substrate interaction as data have been provided to indicate that PKR does not phosphorylate STAT1 *in vitro*. Instead, the interaction is not dependent upon the catalytic activity of PKR as wildtype and mutant PKR can associate with STAT1 with approximately the same efficiency.

These findings are corroborated by parallel studies conducted in cells overexpressing dominant negative mutants of PKR that indicate that STAT1 DNA-binding activity induced by IFN-α/β, IFN-γ, and dsRNA is differentially regulated by PKR mutants (Wong/Tam *et al.*, 1997). That is, it is downregulated in cells overexpressing PKR catalytic mutants and upregulated in cells expressing a dsRNA-binding defective mutant of PKR. Importantly, STAT1 can interact with the former but not with the latter. In addition, it has been shown that the interaction between PKR and STAT1 is disrupted in response to IFNs and dsRNA. Therefore, it is concluded that the interaction between PKR and STAT1 serves to inhibit the transcriptional activity of STAT1 induced by IFNs or dsRNA and that loss of this interaction in cells lacking PKR results in an increase in STAT1 transcriptional function.

1.2 Regulation of STAT1 expression by PKR

In addition, data has been provided that indicates a regulation of STAT1 expression by PKR. It has been shown that in cells lacking PKR, there is an increase in STAT1 protein expression. This PKR-mediated effect is specific for STAT1 as the expression of a number of other STAT family members is not affected by PKR. The regulation of STAT1 expression does not occur at the either the transcriptional or post-transcriptional level as STAT1 mRNA transcription and stability do not differ between cells expressing or lacking PKR.

Instead, preliminary evidence suggests a possible regulation of STAT1 protein stability by PKR. The STAT1 protein appears to be more stable in $PKR^{-/-}$ cells than in $PKR^{+/+}$ cells, suggesting that the interaction between PKR and STAT1 might contribute to STAT1 protein stability. However, the significance of this effect has been shown to vary across multiple experiments. At the same time, STAT1 expression appears to be regulated by mutants of PKR that function in a dominant negative manner in PKR-mediated translational regulation, suggesting that PKR may regulate translation of the STAT1 protein. Therefore,

the mechanism by which PKR regulates STAT1 expression remains unresolved and requires further experimental clarification. An experimental approach that would distinguish between these two possibilities is polysome profile analysis. This technique measures the ability of a particular mRNA species to be translated based on its relative association with monosomes and polysomes.

2. *Analysis of PKR-mediated regulation of STAT1 function*

Although the results presented here indicate that PKR is able to modulate STAT1 function, the molecular mechanism(s) by which PKR is able to do so is not fully clear. Since it has been shown PKR itself does not induce serine phosphorylation or regulate tyrosine phosphorylation of STAT1(Tam/Wong *et al.*, 1997), it is reasonable to eliminate PKR-mediated regulation of the early events required for the onset of signaling. For instance, PKR most likely does not affect the activation of JAKs and by extension STAT2, the recruitment of STAT1 to the proximity of the activated receptor-kinase, or the activity of the one or more phosphatases that have been implicated in STAT1 regulation complex (Igarishi *et al.*, 1993; David *et al.*, 1993; Haque *et al.*, 1995; Haspel *et al.*, 1996; Shuai *et al.*, 1996; Williams and Haque, 1997; Haque and Williams, 1998).

Instead, a possible mechanism by which PKR may be able to regulate STAT1 activity would involve direct sequestration of the transcription factor. In this scenario, the inhibitory effect of PKR may lie in the ability of PKR to bind and sequester STAT1, thereby preventing STAT1 addition into the ISGF-3, GAF/ISGF-3γ, and GAF transcriptional complexes. Alternatively, it could be postulated that a ternary protein interaction mediated by PKR is responsible for regulation of STAT1 activity. That is, it may be possible that PKR may affect the interaction between STAT1 and another protein that might bear a negative or a positive regulatory on STAT1.

In either capacity, PKR would assume a structural role similar to a number of proteins that have been shown to interact with components of the JAK/STAT signal transduction pathway and to regulate their activities. For instance, a STAT-inhibitory family of proteins termed protein inhibitors of activated STAT (PIAS) has been identified (Chung *et al.*, 1997; Liu *et al.*, 1998). PIAS1 has been demonstrated to interact with STAT1 and to inhibit its transcriptional function (Liu *et al.*, 1998), whereas PIAS3 has been shown to interact with interleukin-6-activated STAT3 and to block the DNA-binding activity of this transcription factor (Chung *et al.*, 1997). Another STAT-interacting protein (STIP-1) that has been cloned is proposed to provide a positive effect on signaling by interacting with STAT3 and facilitating its phosphorylation by JAKs in response to interleukin-6 (Collum and Schindler, 1996). As well, the nuclear import of IFN-γ-activated STAT1 has been reported to require the small G protein Ran that catalyzes energy-dependent nuclear translocation (Sekimoto *et al.*, 1996a) and NPI-1 which is a member of the nuclear pore-targeting complex (Sekimoto *et al.*, 1996b). In addition, a family of SH2-containing proteins (SOCS, JAB, SSI), identified by several independent groups, is reported to inhibit the activity of STATs upstream of tyrosine phosphorylation and phosphorylation of cytokine receptors (Starr *et al.*, 1997; Naka *et al.*, 1997; Endo *et al.*, 1997). Although these proteins have been shown to interact with JAKs and cytokine receptors, the regulatory mechanisms employed by these proteins to regulate signaling remains unclear. Interestingly, all members of this family of inhibitors are transcriptionally induced by cytokines and represent a potential negative feedback in the regulation of cytokine signaling. This example provides a particularly apt description of a potential feedback mechanism of PKR on IFN and dsRNA signal transduction pathways; it could be postulated that one of the downstream effects of IFN-mediated induction of PKR is the establishment of a negative regulatory loop that can attentuate IFN signaling. In further support of a structural role for PKR, there have been reports indicating that conformational and not catalytic requirements of kinases are important for the progression of signaling pathways. In this regard, structural properties of JAK-1 have been suggested to be

important for the propagation of IFN-γ signaling; that is, kinase-negative mutants of JAK-1 are able to support IFN-γ-inducible gene expression (Briscoe *et al.*, 1996).

3. Analysis of PKR-mediated regulation of STAT1 expression

The findings presented here also indicate that PKR plays a role in the regulation of STAT1 expression. Presently, the mechanism by which PKR is able to modulate STAT1 expression remains unclear. With the use of PKR dominant negative mutants, it is apparent that both the catalytic and RNA-binding properties of PKR are required for regulation of STAT1 expression. It is reasonable to conclude then that the interaction between PKR and STAT1 probably does not play a role in the ability of PKR to modulate STAT1 expression since it has been demonstrated that the PKRΔ6 catalytic mutant but not the PKR LS4 RNA-binding mutant can interact with STAT1 (Tam/Wong *et al.*, 1997).

A possible mechanism by which PKR regulates STAT1 expression that is consistent with the function of these dominant negative PKR mutants in PKR-mediated translation is the phosphorylation of a factor that can regulate STAT1 protein expression. Another plausible mechanism might involve the ability of PKR to regulate the specific translation of a factor involved in STAT1 protein stability since STAT1 appears to be more stable in cells lacking PKR than in cells expressing PKR. In this case, the data provided would imply that this factor should be a fairly stable species since STAT1 turnover was still observed in $PKR^{+/+}$ cells after CHX treatment. However, in the absence of direct evidence indicating that the rates of STAT1 translation do not differ between cells expressing or lacking PKR, the possibility that STAT1 translation may be regulated by PKR directly and that the observed differences in STAT1 protein stability may be a secondary effect cannot be precluded.

4. Biological implications of STAT1 regulation by PKR

The finding that PKR, an IFN-inducible protein, can regulate the function and expression of the STAT1 transcription factor provides insight into the mechanisms underlying the biological effects of the kinase. Although the ability of PKR to negatively regulate protein synthesis is considered an integral component of the anti-viral state induced by IFNs, PKR-mediated regulation of STAT1 suggests that the kinase may confer immunity to viral infection in other ways as well. Preceded by reports concerning the mode of action of the IFN-inducible p202 protein (Min *et al.* 1996; Choubrey *et al.*, 1996; Datta *et al.*, 1996), the ability of PKR to interact with STAT1 and to inhibit the function of the transcription factor is the second example of an IFN-inducible protein regulating transcription through protein-protein interactions. Furthermore, the data provided also suggest that PKR might assume independent roles in regulating the expression and function of STAT1. It has been argued that PKR, by interacting with STAT1, is able to impair the activity of this transcription factor and thereby to negatively regulate signaling pathways involved in IFN action. At the same time, it could be suggested that the ability of PKR to negatively regulate STAT1 expression may account for the observed effects on STAT1 function. However, two main observations argue against the latter conclusion. First, in cells lacking PKR, an increase in basal STAT1 protein expression is observed prior to the onset of any signaling event. Second, in cells expressing either the catalytic or the RNA-binding defective dominant negative mutant of PKR, there is an increase in STAT1 protein expression. However, these same PKR mutants regulate STAT1 function differently. That is, STAT1 transcriptional activity is upregulated in cells expressing an RNA-binding mutant of PKR but actually downregulated in cells expressing PKR catalytic mutants (Tam/Wong *et al.*, 1997). As such, it is concluded that the abilities of PKR to regulate STAT1 function and expression do not overlap and may affect different cellular processes.

In trying to understand the significance of PKR-mediated regulation of STAT1, the anti-viral effects of PKR should be noted (Der and Lau, 1995; Yang *et al.*, 1995). It has been previously suggested that regulation of STAT1 transcriptional function by PKR may serve as a negative feedback mechanism in signal transduction pathways induced by IFNs and dsRNA, i.e. viral infection. Appropriate cessation of STAT transcriptional activity appears to be important in homeostasis of the cell since chronic STAT activation has been documented in the development of certain cancers, some of which are virally-induced (Pelligrini and Dusanter-Fourt, 1997; Liu *et al.*, 1998; Schindler, 1998). Although it is not entirely clear how constitutive activation of STATs contributes to virally-induced malignant transformation, it is suggested that the function of these transcription factors is usurped by viruses for their own propagation. Until now, the anti-viral effects of PKR have been largely attributed to the ability of the kinase to inhibit protein synthesis. However, it could be postulated that the ability of PKR to inhibit the adverse effects of viral infection, including the development of associated malignancies, may be due in part to downregulation of STAT1 transcriptional function.

Within a similar context, PKR-mediated regulation of STAT1 expression may also have implications for host protection during viral infection. To prevent the inhibition of protein synthesis and thus to enhance replicative capacity, several viruses have evolved mechanisms to downregulate PKR (Gale and Katze, 1998). It is possible to envisage that virus-mediated downregulation of PKR may result in a concomitant increase in STAT1 expression. The increase in STAT1 expression might then represent an alternative pathway against certain viral infections in the event that virus-mediated activation of PKR is subverted. In this regard, it is noteworthy that mice lacking the *pkr* gene display varying degrees of susceptibility to infection by different viruses (Yang *et al.*, 1995).

These findings relating to PKR-mediated regulation of STAT may also shed insight on the role of PKR in the regulation of normal cell growth (Koromilas

et al., 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). In this area, PKR and STAT1 share some functional overlap as they have both been implicated in transcriptional regulation of Fas expression which is important in apoptosis (Der *et al.*, 1997; Xu *et al.*, 1998). Whether PKR regulates this particular aspect of STAT1 transcriptional function remains an unexplored possibility. In addition, both have been reported to be required for TNF-α-induced apoptosis (Yeung *et al.*, 1996; Der *et al.*, 1997; Kumar *et al.*, 1997; Srivastava *et al.*, 1998; Yeung and Lau, 1998). Interestingly, STAT1 has been implicated in this process in a manner that is independent of its transcriptional function (Kumar *et al.*, 1997). As such, regulation of STAT1 expression may in part account for the role of PKR in TNF-α-induced apoptosis.

5. Potential significance for PKR function

Until now, attention has been focused chiefly on the significance of the regulatory relationship between PKR and STAT1 for STAT1 biology. However, the interaction identified between the two proteins provides impetus to examine potential effects on the function of PKR, namely, the ability of the kinase to inhibit protein synthesis. To date, the role of PKR in translational control via phosphorylation of eIF-2α has been well documented, and because of this function PKR is thought to play critical role in the anti-viral response. In addition to PKR, the importance of STAT1 in the response to viral infection is highlighted by the demonstration that *stat1*-deficient mice are more susceptible to viral infection compared to normal mice (Meraz *et al.*, 1996; Durbin *et al.*, 1996). Such an increase in viral susceptibility of *stat1*$^{-/-}$ mice has been attributed mostly to the failure of genes encoding proteins with anti-viral effects such as PKR to be upregulated by IFNs at the transcriptional level. An alternative possibility that remains to be explored, however, is that STAT1 might interact with other non-STAT proteins that mediate anti-viral effects, like PKR, and modify their functions. That is, STAT1 may mediate signaling events induced by IFN and/or viral infection that regulate not only transcription but possibly translation by regulating

PKR activity. In this perspective, then, it is significant that crosstalk between components of transcriptional and translational pathways has already been documented for a growth factor-regulated pathway (Brown and Schreiber, 1996).

6. Conclusions

The results presented in this study indicate that the interferon-inducible dsRNA-dependent protein kinase PKR regulates both the activity and expression of the transcription factor STAT1. Considering the increasingly prominent roles of PKR and STAT1 in various cellular processes, they suggest a number of potentially important implications in the regulation of gene expression, immunity to viral infection, and normal cell growth.

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