The EMBO Journal Vol.16 No.6 pp.1291–1304, 1997

Physical association between STAT1 and the interferon-inducible protein kinase PKR and implications for interferon and double-stranded RNA signaling pathways

Andrew Hoi-Tao Wong, Nancy Wai Ning Tam, Yi-Li Yang[1], Andrew R.Cuddihy, Suiyang Li, Sabine Kirchhoff[2], Hansjörg Hauser[2], Thomas Decker[3] and Antonis E.Koromilas[4]

Departments of Oncology and Medicine, McGill University, Montreal, Quebec H3T 1E2, Canada, [1]Institüt für Molekularbiologie I, Universität Zürich, Hönggerberg, CH 8093, Zürich, Switzerland, [2]Gesellschaft für Biotechnologische Forschung mbH, Genetik von Eukaryonten, D-38124 Braunschweig, Germany and [3]Vienna Biocenter, Institute for Microbiology and Genetics, 1030 Vienna, Austria

[4]Corresponding author at: Lady Davis Institute for Medical Research, Sir Mortimer B.Davis-Jewish General Hospital, 3755 Cote-Ste-Catherine Road, Room 528.1, Montreal, Quebec H3T 1E2, Canada

A.H.-T.Wong and N.W.N.Tam should be considered joint first authors

The interferon-inducible double-stranded RNA protein kinase PKR controls protein synthesis through the phosphorylation of eukaryotic translation initiation factor (eIF)-2. In addition to its demonstrated role in translational control, several reports have suggested a transcriptional role for PKR. Here we report that PKR is involved in IFN- and dsRNA-signaling pathways by modulating the function of the signal transducer and activator of transcription STAT1. We also show that PKR associates with STAT1 in mouse and human cells. The association is not a kinase–substrate interaction since STAT1 phosphorylation is not modified by PKR *in vitro* or *in vivo*. In addition, the formation of the PKR–STAT1 complex is not dependent upon the enzymatic activity of PKR but does require the dsRNA-binding domain of PKR. Moreover, there is a concomitant decrease in PKR–STAT1 interaction and increase in STAT1 DNA binding in response to IFNs or dsRNA. These findings suggest that PKR plays an important role in IFN and dsRNA-signaling pathways by modulating the transcriptional function of STAT1.

Keywords: DNA binding/double-stranded RNA/interferon/protein phosphorylation/signal transduction

Introduction

The signal transducers and activators of transcription (STAT) proteins function by transducing signals from ligand-activated receptor kinase complexes and then localizing to the nucleus, whereupon they bind DNA and activate transcription (reviewed in Darnell *et al.*, 1994; Gilmour and Reich, 1995; Schindler and Darnell, 1995). STAT1α and 1β were originally identified as components of the interferon (IFN) signaling system. They are both derived from alternatively spliced transcripts of the same gene and differ in their carboxy termini (Schindler *et al.*, 1992). IFN-α/β (type I IFN) induces the formation of the multimeric IFN-stimulated gene factor-3 (ISGF-3), which consists of the STAT1α/β (p91/p84) heterodimer, STAT2 (p113) (Fu *et al.*, 1992) and ISGF-3γ (p48) (Veals *et al.*, 1992). Once ISGF-3 has translocated to the nucleus (Kessler *et al.*, 1992), it binds to the IFN-stimulated response element (ISRE) found upstream of many IFN-α/β-stimulated genes and then transactivates expression of these genes.

Similar to IFN-α/β, IFN-γ (type II IFN) utilizes STAT1 by inducing the dimerization of STAT1 to form the transactivator protein complex termed γ-IFN activated factor (GAF) (Decker *et al.*, 1991; Shuai *et al.*, 1992, 1993, 1994; Heim *et al.*, 1995). GAF then binds to the γ-IFN activating sequence (GAS), an element specific for IFN-γ-stimulated genes. Recently, GAF has also been shown to bind and transactivate ISRE-containing genes, thus demonstrating the overlapping nature of IFNs (Bluyssen *et al.*, 1995). In addition, targeted disruption of the *stat1* gene in mice results in unresponsiveness to either type of IFN (Durbin *et al.*, 1996; Meraz *et al.*, 1996).

In addition to IFNs, STAT1 has been shown to be activated by growth hormone (Han *et al.*, 1996), IL-6 (Zhang *et al.*, 1995), IL-10 (Larner *et al.*, 1993), epidermal growth factor (EGF) (Ruff-Jamison *et al.*, 1993), platelet-derived growth factor (PDGF) (Vignais *et al.*, 1996) and colony-stimulating factor 1 (CSF-1) (Larner *et al.*, 1993). Furthermore, double-stranded (ds)RNA, which is often produced in a cell during viral replication, can induce transcription of type I IFN-inducible genes (Pine *et al.*, 1990; Decker, 1992) through STAT1-dependent (Bandyopadhyay *et al.*, 1995) and STAT1-independent pathways (Daly and Reich, 1993, 1995).

In response to IFNs, a large number of genes are induced. One of the best characterized IFN-induced proteins is the dsRNA-activated protein kinase, PKR (reviewed in Proud, 1995). PKR is a serine/threonine protein kinase which is activated by autophosphorylation upon binding to dsRNA and then phosphorylates the α subunit of the eukaryotic initiation factor eIF-2, a modification that results in the inhibition of protein synthesis (Hershey, 1989). PKR exhibits antiviral (Meurs *et al.*, 1992; reviewed in Katze, 1995; Yang *et al.*, 1995), antiproliferative (Chong *et al.*, 1992; Koromilas *et al.*, 1992) and tumor suppressor functions (Koromilas *et al.*, 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). In addition to its demonstrated role in translational control, PKR is able to regulate gene expression at the transcriptional level (Kumar *et al.*, 1994; Maran *et al.*, 1994; Koromilas *et al.*, 1995; Mundschau and Faller, 1995; Yang *et al.*, 1995).

At the same time that IFNs are capable of eliciting gene expression by activating components of the JAK–STAT signaling pathway, they may also have the capacity to induce the expression of proteins that are able to bind to and impair the activity of sequence-specific transcription

factors (Min *et al.*, 1996). To date, molecules that are able to modify the functions of STATs without interfering with ligand-induced phosphorylation cascades remain to be identified. Here we report that PKR, an IFN-inducible protein, associates with STAT1. Interestingly, this is not a kinase–substrate interaction since STAT1 is not phosphorylated by PKR *in vitro* or *in vivo*. Rather, in response to IFNs or dsRNA, conditions under which STAT1 becomes activated, the interaction between PKR and STAT1 diminishes. We demonstrate that the interaction is not dependent upon the enzymatic activity of PKR but requires the dsRNA-binding domain of PKR. Furthermore, in cells expressing increased levels of catalytically inactive PKR relative to wild type endogenous PKR, there is a decrease in STAT1 DNA binding and transactivation in response to IFNs or dsRNA. In contrast, STAT1 DNA binding is induced in cells expressing a dsRNA-binding-defective PKR protein or in cells deficient in the PKR gene ($PKR^{-/-}$). As such, there appears to be an inverse correlation between the formation of the PKR–STAT1 complex and the ability of STAT1 to bind DNA. Taken together, these data suggest a novel function of PKR in the transcriptional regulation of IFN-inducible gene expression: the ability to modulate STAT1 function.

Results

Inhibition of STAT1 DNA binding and transactivation by the catalytically inactive dominant-negative PKRΔ6

The aggregation of activated STATs and the subsequent binding to DNA is a prerequisite for transactivation of IFN-inducible genes. In addition to tyrosine phosphorylation, several reports have implicated serine/threonine phosphorylation in the activation of STATs (Eilers *et al.*, 1995; Wen *et al.*, 1995; Zhang *et al.*, 1995). The role of PKR in STAT activation was examined in NIH 3T3 cells expressing catalytically inactive dominant-negative mutants of PKR. Cells were stimulated with IFN-α/β and the ability of STAT1 to bind DNA was measured by gel mobility shift assays, employing a dsDNA oligonucleotide which encompassed the ISRE of the ISG-15 gene (Reich and Darnell, 1989). In control NIH 3T3 cells (neomycin resistant), DNA binding of ISGF-3 was first observed at 30 min after stimulation and peaked at 120 min (Figure 1A, lanes 2–4) whereas in NIH 3T3 cells expressing the dominant-negative catalytically inactive PKRΔ6 (Koromilas *et al.*, 1992), ISGF-3 binding to ISRE was reduced 8-fold (lanes 7–9). In the same experiment, DNA binding of IRF-1 and IRF-2 to the ISG-15 ISRE was not affected by PKRΔ6 (data not shown). The identity of the induced band was ascertained by supershifting the ISGF-3–ISRE complex with a monoclonal antibody to STAT1α (lanes 12 and 15). Similar results were obtained with the use of an ISRE dsDNA oligonucleotide from the promoter of the 561 IFN-inducible gene (Bandyopadhyay *et al.*, 1995) (data not shown).

The overlapping nature of IFN signaling warranted an examination of STAT1 DNA binding in the presence of PKRΔ6 in response to IFN-γ. The binding of GAF to the GAS DNA sequence was tested by gel mobility shift assays, using an IFP-53 GAS dsDNA oligonucleotide (Strehlow *et al.*, 1993; Figure 1B). We noted a 3-fold reduction of GAF DNA binding in NIH 3T3 cells expressing PKRΔ6 (compare lanes 2–4 with 7–9). Supershift analyses with an antibody to STAT1α verified the identity of GAF (lanes 12 and 15). Similar results were obtained with the Ly6E/A GAS dsDNA oligonucleotide (Khan *et al.*, 1990) (data not shown).

IFN-γ also induces binding of GAF to ISRE-containing genes through the association with ISGF-3γ (p48) and in the absence of activated STAT2 (Bluyssen *et al.*, 1995). We then tested whether GAF–ISGF-3γ complex formation was affected in cells expressing PKRΔ6. Gel mobility shift assays were performed with the ISG-15 ISRE dsDNA oligonucleotide and the GAF–ISGF-3γ complex was identified based on its mobility and binding site specificity (Bluyssen *et al.*, 1995). Although the GAF–ISGF-3γ complex was induced in control NIH 3T3 cells (Figure 1C, lanes 2–4), the complex was not formed in cells expressing PKRΔ6 (lanes 7–9). Supershift analyses with an anti-STAT1α antibody indicated the presence of STAT1 in the GAF–ISGF-3γ complex (lanes 12 and 15).

Similarly, dsRNA can elicit transcriptional induction of IFN-inducible genes through the activation of STAT1 (Bandyopadhyay *et al.*, 1995). We then examined the levels of STAT1 DNA binding in NIH 3T3 cells expressing PKRΔ6 after dsRNA treatment. Although it has been previously shown that IFN production is impaired by PKRΔ6 (Kirchhoff *et al.*, 1995), any residual autocrine effect of IFNs produced in response to dsRNA was minimized by the addition of neutralizing antibodies against mouse type I IFNs to the cell media. DNA binding was analyzed with the use of the ISG-15 ISRE dsDNA oligonucleotide. We observed that dsRNA treatment induced an ISGF-3-like protein–DNA complex formation at 4 and 6 h after transfection of poly(rI)–poly(rC) in control cells (Figure 1D, lanes 3 and 4). However, in cells expressing PKRΔ6, no induction of the ISGF-3-like protein–DNA complex was observed (lanes 7–9). Supershift analyses demonstrated the presence of STAT1 in the induced DNA–protein complex (lanes 12 and 15). Similar results were obtained with the 561 ISRE dsDNA oligonucleotide (data not shown). It is noteworthy that dsRNA can induce the binding of unique transcription factors known as dsRNA-activated factors (DRAFs) to the ISG-15 ISRE, which do not contain STAT1 (Daly and Reich, 1993, 1995). It is likely, then, that induction of IFN-inducible genes by dsRNA proceeds through distinct pathways which are cell type dependent.

Northern blot analyses showed that expression of PKRΔ6 inhibited gene transactivation by IFNs or dsRNA (Figure 2). For example, expression of ISG-15 RNA was not observed in NIH 3T3 cells expressing PKRΔ6 (Figure 2A, lanes 8–14) by IFN-α/β (compare lanes 5–7 with 12–14) or dsRNA (compare lanes 2–4 with 9–11). Moreover, expression of IFP-53 RNA after treatment with IFN-γ was reduced by ~50% in NIH 3T3 cells expressing PKRΔ6 (Figure 2B, compare lanes 2–4 with 6–8).

Similar to PKRΔ6, STAT1 DNA binding and transactivation capacity were inhibited in NIH 3T3 cells expressing the dominant-negative catalytically inactive mutant PKRK296R (Lys296 to Arg; Katze *et al.*, 1991) (data not shown). Together, these data indicate that STAT1 DNA-binding activity and transactivation are impaired by the expression of dominant-negative catalytic mutants of PKR.

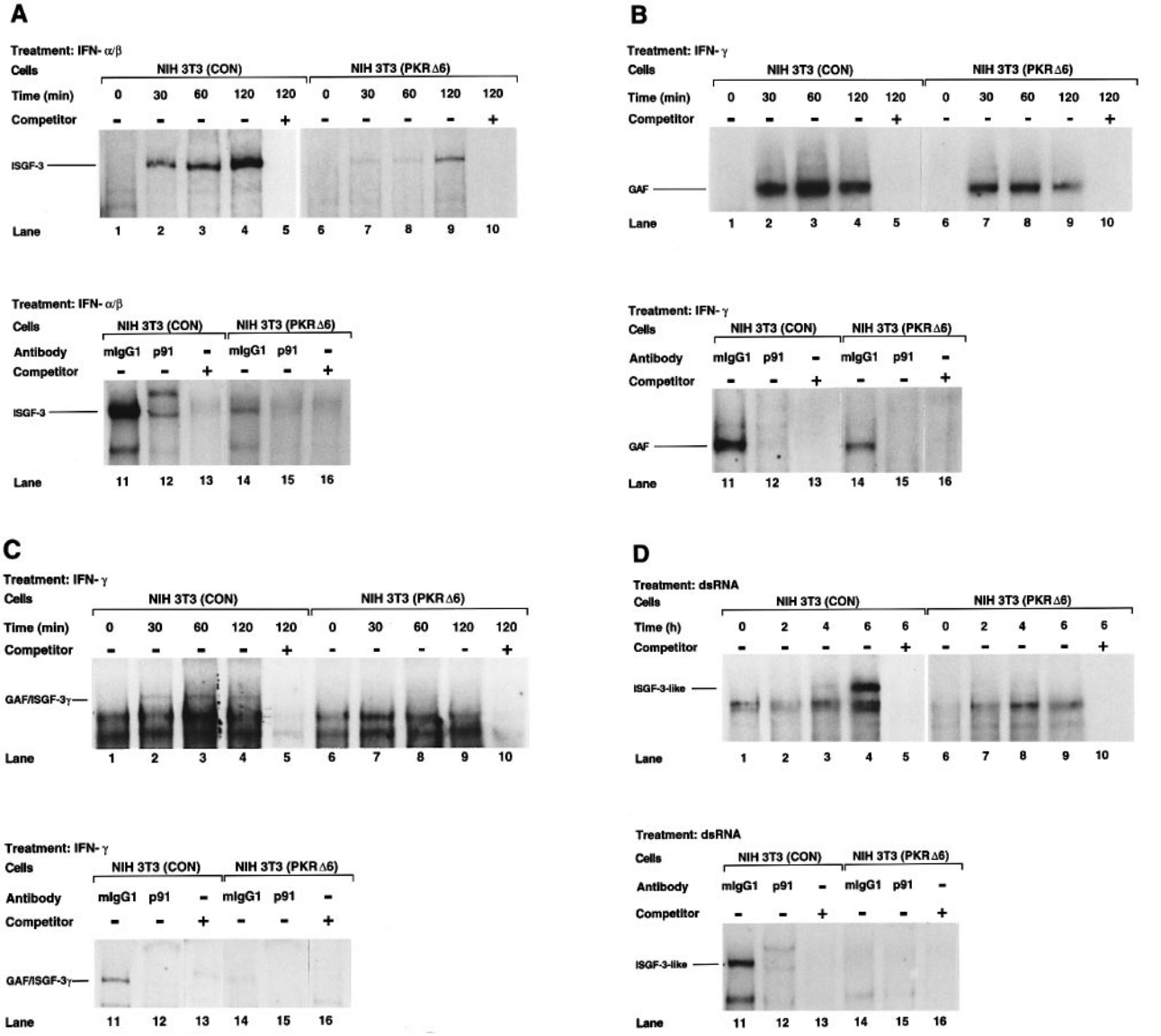

Fig. 1. Inhibition of STAT1 DNA binding by the dominant-negative mutant PKRΔ6. NIH 3T3 control (lanes 1–5 and 11–13) and PKRΔ6-expressing NIH 3T3 cells (lanes 6–10 and 14–16) were treated with 1000 IU/ml IFN-α/β (**A**), 100 IU/ml IFN-γ (**B** and **C**) for 30, 60 and 120 min or 100 μg/ml poly(rI)–poly(rC) for 2, 4 and 6 h (**D**). Protein extracts were used for ISGF-3 DNA binding assays either with the ISG-15-ISRE (A, C and D) or the IFP-53-GAS (B) probe. A 200-fold excess of unlabeled dsDNA oligonucleotide was added in cold competition reactions (lanes 5, 10, 13 and 16). For supershift assays, protein extracts from time point 120 min for IFN-stimulation (A, B and C) and 6 h for dsRNA treatment (D) were pre-incubated with 3 μg of either mouse IgG1 (lanes 11 and 14) or anti-STAT1α antibody (lanes 12 and 15).

Expression of a dsRNA-binding-defective mutant of PKR enhances STAT1 DNA binding

The inhibition of ISGF-3 and GAF DNA binding by catalytically inactive PKR mutants prompted us to examine whether PKR defective in RNA binding mediates a similar effect. To this end, we examined STAT1 DNA binding in cells expressing a mutant of PKR which is defective in RNA binding but which possesses an intact catalytic domain, PKRLS4 (substitutions of Arg58Ser59Lys60 to Gly58Ala59Leu60 in RNA-binding domain I which abolish PKR binding to dsRNA; Green and Mathews, 1992). In this regard, mutants of PKR defective in dsRNA binding have been shown to function in a dominant-negative manner in the phosphorylation of eIF-2α (Barber *et al.*, 1995).

Stimulation with IFN-α/β resulted in a 5-fold increase in the induction of ISGF-3 binding to the ISG-15 ISRE in cells expressing PKRLS4 compared with control NIH 3T3 cells (Figure 3A, compare lanes 2–4 with 7–9) whereas DNA binding of IRF-1 and IRF-2 to the ISRE was not affected by PKRLS4 (data not shown). Similar levels of induction were observed in IFN-γ (Figure 3C) and dsRNA-treated cells (Figure 3D) where the ISG-15 ISRE probe was also used. However, GAF binding to the IFP-53 GAS probe was only moderately enhanced (~2-fold) by PKRLS4 relative to control NIH 3T3 cells (Figure 3B, compare lanes 2–4 with 7–9). Supershift analyses indicated that STAT1 was present in all induced protein–DNA complexes (Figure 3A, B, C and D, lanes 12 and 15). These data show a stimulatory effect by PKRLS4 on STAT1 DNA binding in response to IFNs or dsRNA.

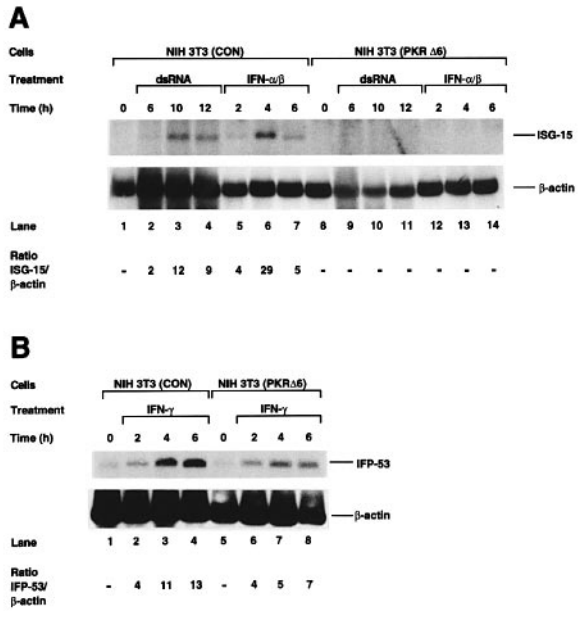

Fig. 2. Inhibition of STAT1 transactivation capacity by PKRΔ6. Control NIH 3T3 cells (**A**, lanes 1–7; **B**, lanes 1–4) and NIH 3T3 cells expressing PKRΔ6 (A, lanes 8–14; B, lanes 5–8) were stimulated with either IFN-α/β (A; 1000 IU/ml) or IFN-γ (B; 100 IU/ml) for 2 h (A, lanes 5 and 12; B, lanes 2 and 6), 4 h (A, lanes 6 and 13; B, lanes 3 and 7) and 6 h (A, lanes 7 and 14; B, lanes 4 and 8) or transfected with poly(rI)–poly(rC) (100 μg/ml) for 6 h (A, lanes 2 and 9), 10 h (A, lanes 3 and 10) and 12 h (A, lanes 4 and 11). Total RNA (10 μg) was subjected to Northern blot analysis using ^{32}P-labeled ISG-15 (A, upper panel) or IFP-53 cDNA (B, upper panel) as a probe. The same blots were stripped and reprobed with [^{32}P]β-actin cDNA (A and B, lower panels). Quantification of radiolabeled bands was performed by scanning autoradiograms in the linear range of exposure with an enhanced laser densitometer Ultroscan XL (LKB).

Phosphorylation of STAT1 is not mediated by PKR

The inhibition of ISGF-3, GAF and GAF–ISGF-3γ DNA binding by the catalytically inactive dominant-negative mutants of PKR suggested a modification of STAT1 activity by phosphorylation. To investigate whether PKR could phosphorylate STAT1, an *in vitro* kinase assay was performed, using HeLa S3 cell extracts in which PKR was activated by autophosphorylation in the presence of reovirus dsRNA and [γ-^{32}P]ATP (Figure 4A). After incubation, one-third of the reaction was subjected to PKR immunoprecipitation, one-third to STAT1α immunoprecipitation and the rest to eIF-2α immunoprecipitation. Immunoprecipitiation with an anti-PKR antibody indicated that PKR was autophosphorylated and thus catalytically active (lanes 1–4). Phosphorylation of PKR was induced by dsRNA (lanes 2 and 4) and was dependent upon the amount of PKR protein (compare lanes 2 and 4; IFN treatment upregulated PKR protein in lanes 3 and 4). Immunoprecipitiation with an anti-STAT1α antibody, though, did not precipitate a phosphorylated protein the size of STAT1α (~90 kDa) (lanes 5–8). However, we noted that STAT1α could co-precipitate with a phosphoprotein the size of PKR (lanes 6 and 8). In contrast, immunoprecipitation with anti-eIF-2α antiserum indicated the phosphorylation of eIF-2α by activated PKR (lanes 10 and 12).

Although STAT1 did not prove to be a direct substrate of PKR *in vitro*, it was possible that PKR mediated STAT1 phosphorylation *in vivo* by functioning as an intermediate kinase in a phosphorylation cascade induced by IFNs or dsRNA. To explore this possibility, we examined the *in vivo* phosphorylation of STAT1α in NIH 3T3 cells expressing the mutants of PKR. Cells labeled with [^{32}P]-orthophosphate *in vivo* were treated with IFNs, followed by immunoprecipitation with an anti-STAT1α antibody (Figure 4B). In this and several other experiments we observed that the phosphorylation of STAT1α did not vary significantly between control NIH 3T3 cells (lanes 2 and 3) and NIH 3T3 cells expressing PKRLS4 (lanes 5 and 6) or PKRΔ6 (lanes 8 and 9). Similar results were obtained when cells expressing the mutants of PKR were treated with dsRNA for 4 h (data not shown).

Since tyrosine phosphorylation of STAT1 has been shown to represent a small fraction of the phosphorylated protein (Eilers *et al.*, 1995), we then tested the possibility that tyrosine phosphorylation of STAT1α may be altered in the presence of the PKR mutants. To do so, STAT1α was immunoprecipitated from IFN-stimulated cells and subjected to immunoblotting analysis, first with anti-phosphotyrosine antibodies and then with an anti-STAT1α antibody. As shown in Figure 4C and D, no significant differences in STAT1α tyrosine phosphorylation were observed between control cells and cells expressing PKRΔ6 after treatment with either IFN-α/β (Figure 4C) or IFN-γ (Figure 4D). Similar results were obtained when extracts from PKRLS4- or PKRK296R-expressing cells were used (data not shown). These data suggest that PKR does not mediate STAT1 phosphorylation *in vitro* or *in vivo*.

PKR associates with STAT1

The immunoprecipitation of autophosphorylated PKR by the anti-STAT1α antibody (Figure 4A, lanes 6 and 8) implied an association between PKR and STAT1. We further examined the nature of this association in response to IFNs or dsRNA. HeLa S3 cells were treated with IFN-α/β, IFN-γ or dsRNA. STAT1 DNA binding and PKR–STAT1 association were then examined in parallel. Gel shift analyses demonstrated that treatment with IFN-α/β (Figure 5A, lanes 1–3), IFN-γ (lanes 5–7) or dsRNA (lanes 9–12) resulted in an induction of STAT1 DNA binding. Interestingly, corresponding immunoblotting analysis indicated the association of STAT1 with PKR before and after IFN stimulation at 30 min (Figure 5B, lanes 1, 2 and 4), whereas the association decreased at 60 min post-stimulation (lanes 3 and 5). Similarly, dsRNA reduced the association of STAT1 with PKR at 7 h after stimulation (lane 9). Notably, the point at which PKR and STAT1 dissociated coincided with the maximal DNA binding of STAT1 (Figure 5A, lanes 3, 7 and 12).

We then examined whether PKR–STAT1 complex formation was dependent upon PKR binding to RNA. As shown in Figure 5C, equal amounts of STAT1α (p91) and STAT1β (p84) were co-precipitated with PKR from HeLa cell extracts before (lane 1) and after treatment with RNase A (lane 2), indicating that the PKR–STAT1 interaction is not mediated by RNA. We next tested whether the interaction between PKR and STAT1 was direct. Mixing experiments with ^{35}S-labeled PKR and ^{35}S-labeled STAT1 proteins synthesized *in vitro* resulted in a significant amount of STAT1 (~10%) co-precipitating with PKR (Figure 5D, lane 5), showing a direct interaction between

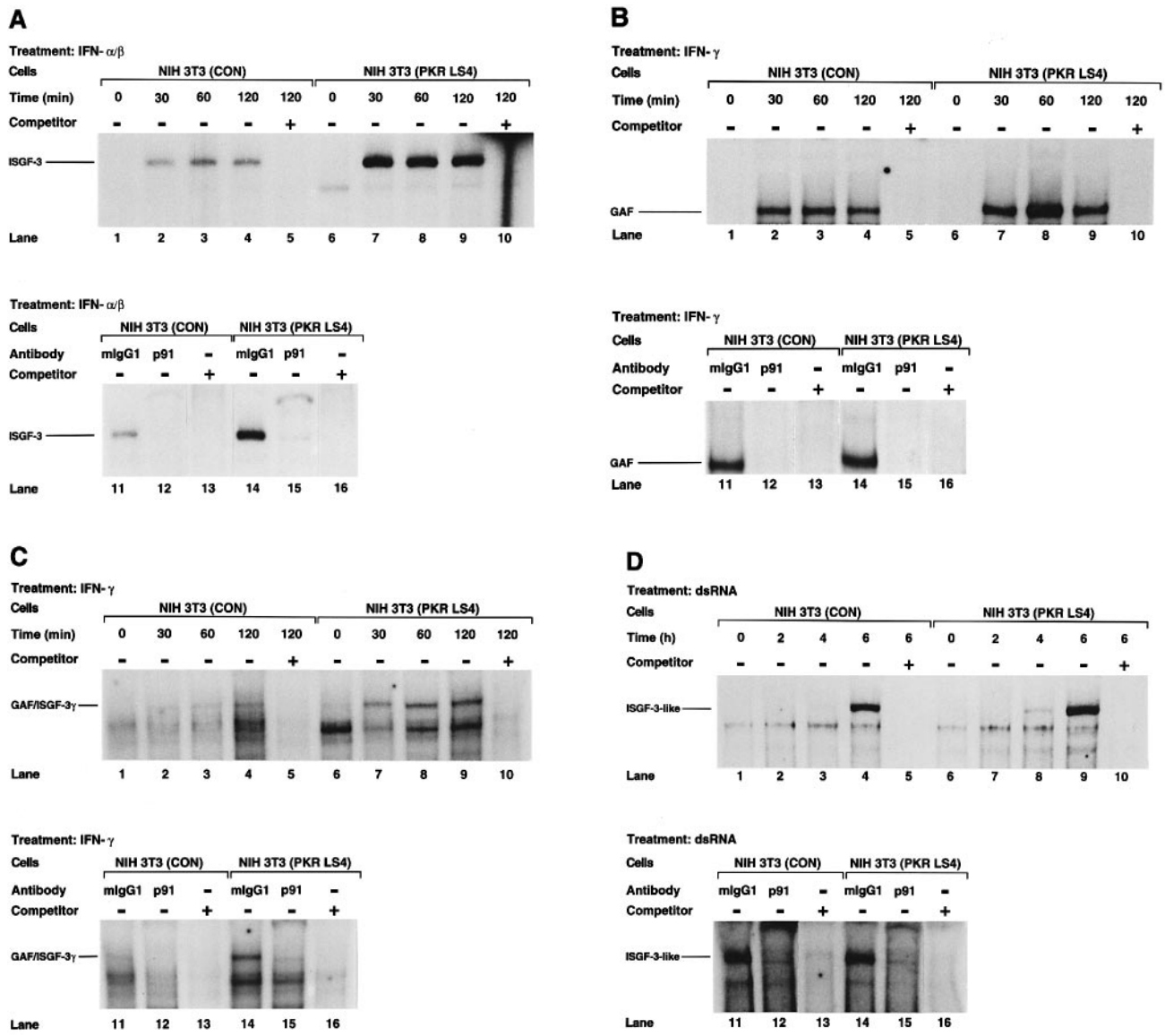

Fig. 3. Enhanced STAT1 DNA binding in cells expressing a dsRNA-binding-defective mutant of PKR. Control NIH 3T3 cells (lanes 1–5 and 11–13) and PKRLS4-expressing NIH 3T3 cells (lanes 6–10 and 14–16) were treated with 1000 IU/ml IFN-α/β (**A**), 100 IU/ml IFN-γ (**B** and **C**) for 30, 60 and 120 min or 100 μg/ml poly(rI)–poly(rC) for 2, 4 and 6 h (**D**). Protein extracts were used for ISGF-3 DNA-binding assays either with the ISG-15-ISRE (A, C and D) or the IFP-53-GAS (B) probe. A 200-fold excess of unlabeled dsDNA oligonucleotide was added in cold competition reactions (lanes 5, 10, 13 and 16). For supershift assays, protein extracts from time point 120 min for IFN stimulation (A, B and C) and 6 h for dsRNA treatment (D) were pre-incubated with 3 μg of either mouse IgG1 (lanes 11 and 14) or anti-STAT1α antibody (lanes 12 and 15).

the two proteins. Note the association of PKR with the full length of STAT1α only (compare lanes 3 and 5).

The interaction between PKR and STAT1 was also observed in NIH 3T3 cells. Control NIH 3T3 cells and NIH 3T3 cells expressing the dominant-negative PKRK296R were stimulated with either IFN-α/β or IFN-γ and cell extracts were immunoprecipitated with an anti-STAT1α antibody (Figure 6A). Co-immunoprecipitated PKR was detected by immunoblotting analysis, using a rabbit anti-mouse PKR antiserum which recognizes both the murine (65 kDa) and human forms (68 kDa) of PKR. Similar to HeLa S3 cells, the interaction between STAT1 and PKR occurred before stimulation with IFNs (lanes 1 and 3). In addition, the level of murine PKR or PKRK296R associated with STAT1 decreased after IFN-α/β treatment (lanes 2 and 4). These data suggest that the catalytic activity of PKR is not required for the association with STAT1.

Next we examined whether the interaction between PKR and STAT1 was dependent upon the ability of PKR to be activated (autophosphorylated). To this end, we measured the levels of free and STAT1-bound ^{32}P-labeled PKR in control NIH 3T3 cells and in NIH 3T3 cells expressing PKRK296R in response to IFNs (Figure 6B). We reasoned that if PKR phosphorylation was due to its activation by autophosphorylation, then the ^{32}P-labeled PKR levels would be lower in cells expressing PKRK296R. Extracts from [^{32}P]orthophosphate-labeled cells were first immunoprecipitated with an anti-STAT1α antibody (lanes 1–4) followed by a second immunoprecipitation with antiserum to mouse PKR (lanes 5–8).

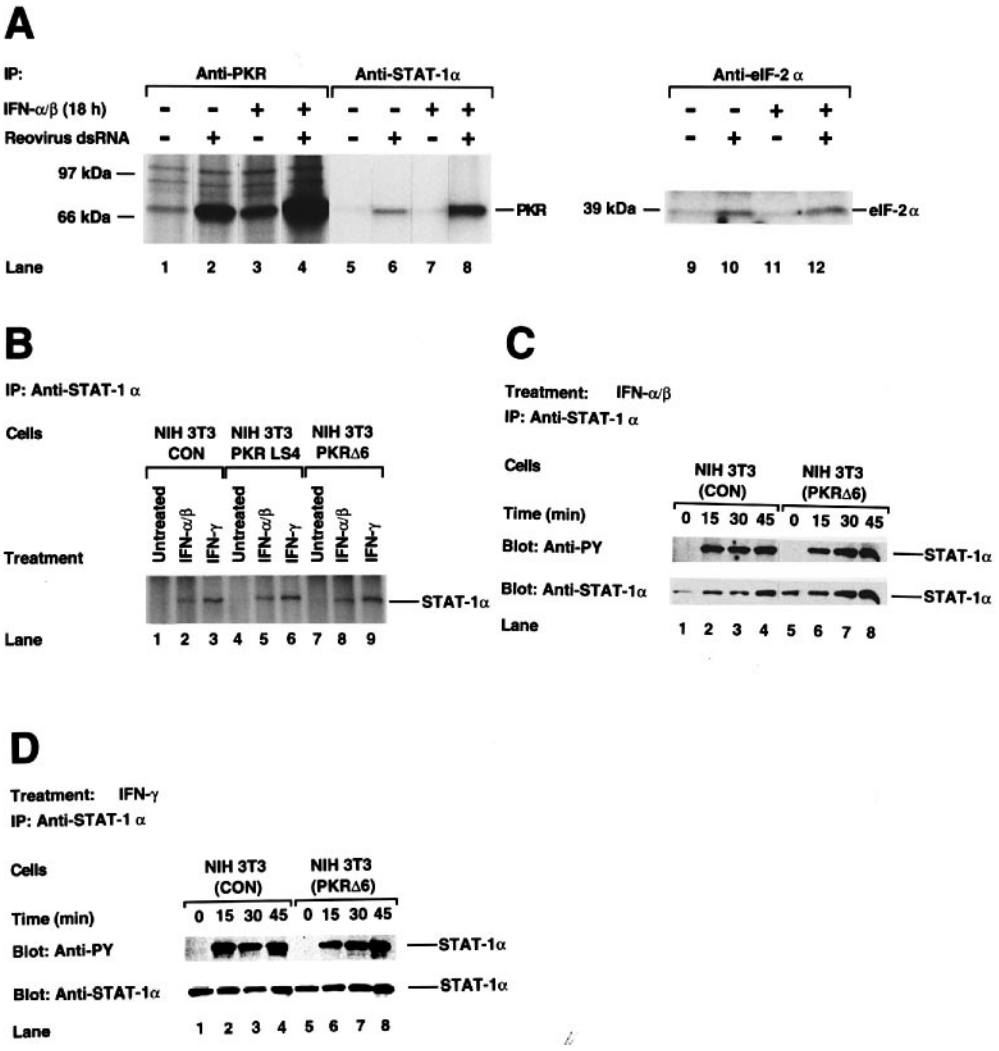

Fig. 4. STAT1 phosphorylation is not mediated by PKR. (**A**) Phosphorylation of STAT1 *in vitro*. Untreated (lanes 1, 2, 5, 6, 9 and 10) or IFN-α/β-treated (1000 IU/ml, 18 h; lanes 3, 4, 7, 8, 11 and 12) HeLa S3 extracts were incubated with [γ-^{32}P]ATP in absence (lanes 1, 3, 5, 7, 9 and 11) or presence (lanes 2, 4, 6, 8, 10 and 12) of activator reovirus dsRNA (0.1 μg/ml). Reactions were immunoprecipitated against human PKR (lanes 1–4), STAT1α (lanes 5–8) or eIF-2α (lanes 9–12) and subjected to SDS–PAGE. (**B**) STAT1 phosphorylation *in vivo*. Control NIH 3T3 cells (lanes 1–3) and NIH 3T3 cells expressing PKRLS4 (lanes 4–6) or PKRΔ6 (lanes 7–9) were labeled with [^{32}P]orthophosphate (200 μCi/ml) and subsequently left untreated (lanes 1, 4 and 7) or treated with IFN-α/β (lanes 2, 5 and 8) or IFN-γ (lanes 3, 6 and 9) for 30 min. Immunoprecipitates were subjected to SDS–PAGE. (**C** and **D**) Tyrosine phosphorylation of STAT1. NIH 3T3 control cells (lanes 1–4) or PKRΔ6-expressing NIH 3T3 cells (lanes 5–8) were treated with IFN-α/β (C) or IFN-γ (D) for 15 (lanes 2 and 6), 30 (lanes 3 and 7) and 45 min (lanes 4 and 8). Lysates were immunoprecipitated with anti-STAT1α antibody followed by immunoblotting with either anti-phosphotyrosine antibodies (top panels) or anti-STAT1α antibody (bottom panels).

We observed that the relative amounts of ^{32}P-labeled PKR associated with STAT1α did not vary significantly in control (lanes 1 and 2) or in PKRK296R-expressing cells (lanes 3 and 4) either before (lanes 1 and 3) or after stimulation with IFN-α/β (lanes 2 and 4). We also noted that the total amounts of ^{32}P-labeled PKR did not differ between control (lanes 5 and 6) and PKRK296R-expressing cells (lanes 7 and 8) before (lanes 5 and 7) and after stimulation with IFN-α/β (lanes 6 and 8), indicating that PKR activation by autophosphorylation is not induced by IFN-α/β. In addition, we observed that STAT1 phosphorylation levels were induced after IFN treatment, indicating that STAT1 activation is not dependent upon PKR activation. Lastly, we noted that the levels of ^{32}P-labeled PKR were equal in unstimulated control cells (lane 5) and in cells expressing the dominant-negative PKRK296R (lane 7), indicating that this basal phosphorylation of PKR was not due to its activation by autophosphorylation. Thus, PKR may serve as a substrate for another kinase(s) whose activity is not regulated by IFNs. The two closely migrating ~66 kDa phosphoproteins recognized by antiserum to murine PKR in NIH 3T3 cells (lanes 1–8) but not in PKR$^{-/-}$ MEFs (lane 9) most probably represent phosphorylated isoforms of PKR, whereas the 68 kDa phosphoprotein (lanes 3, 4, 7 and 8) phosphorylated PKRK296R.

Since NIH 3T3 cells expressing PKRLS4 exhibited an increased induction of STAT1 DNA binding in response to IFNs or dsRNA, we then examined the ability of STAT1 to interact with PKRLS4 (Figure 6C). The amount of PKRLS4 associated with STAT1 was compared with PKRΔ6 since these two PKR mutants were expressed in

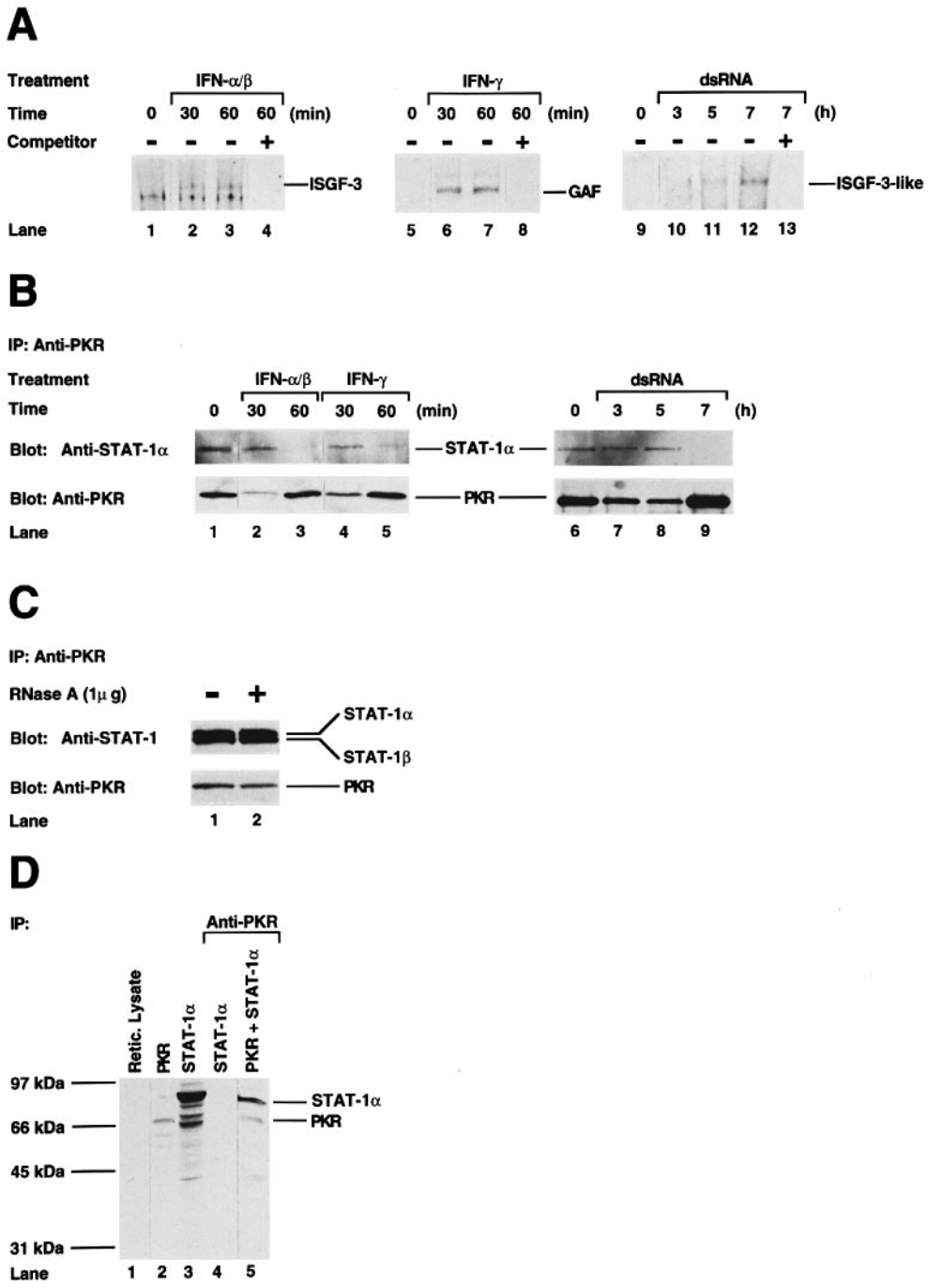

Fig. 5. Interaction between PKR and STAT1. (**A**) HeLa S3 cells were treated with IFN-α/β (1000 IU/ml), IFN-γ (100 IU/ml) for 30 and 60 min or poly(rI)-poly(rC) (100 μg/ml) for 3, 5 and 7 h. Gel mobility shift assays were performed using the ISG-15 ISRE (lanes 1–4 and 9–13) or the IFP-53 GAS (lanes 5–8). A 200-fold excess of the appropriate unlabeled oligonucleotide was used in cold competition reactions (lanes 4, 8 and 13). (**B**) The same HeLa S3 cell extracts were also used to monitor the association between PKR and STAT1. Equal amounts of protein extract were immunoprecipitated against human PKR followed by immunoblotting against STAT1α (top panels) or against PKR (bottom panels). (**C**) HeLa S3 cell extracts untreated (lane 1) or treated with RNase A (lane 2) were subjected to immunoprecipitation with anti-PKR antibodies followed by immunoblotting analysis with either rabbit antisera against STAT1 (p84/91) (top panel) or anti-PKR antibody (bottom panel). (**D**) PKR binding to STAT1α *in vitro*. ^{35}S-labeled human PKR and human STAT1α proteins were synthesized *in vitro* (lanes 2 and 3 respectively). ^{35}S-labeled STAT1 (equal amount to lane 3) was either immunoprecipitated with anti-PKR antibody only (lane 4) or incubated with ^{35}S-labeled PKR (equal to lane 2) followed by immunoprecipitation with anti-PKR antibody (lane 5), SDS–PAGE and fluorography. Lane 1, a reticulocyte lysate reaction not programmed with RNA is shown.

NIH 3T3 cells at comparable levels (compare lanes 3 and 4 with 5 and 6). Equal amounts of cell extracts from PKRΔ6 or PKRLS4-expressing cells were immunoprecipitated with an anti-STAT1α antibody and the levels of PKR mutants which co-precipitated with STAT1α were detected by immunoblotting analysis, using a monoclonal anti-human PKR antibody which does not cross-react with murine PKR (lanes 1 and 2). STAT1 was able to co-precipitate with PKRΔ6 (lanes 11 and 12) but not PKRLS4 (lanes 9 and 10), indicating that the dsRNA-binding

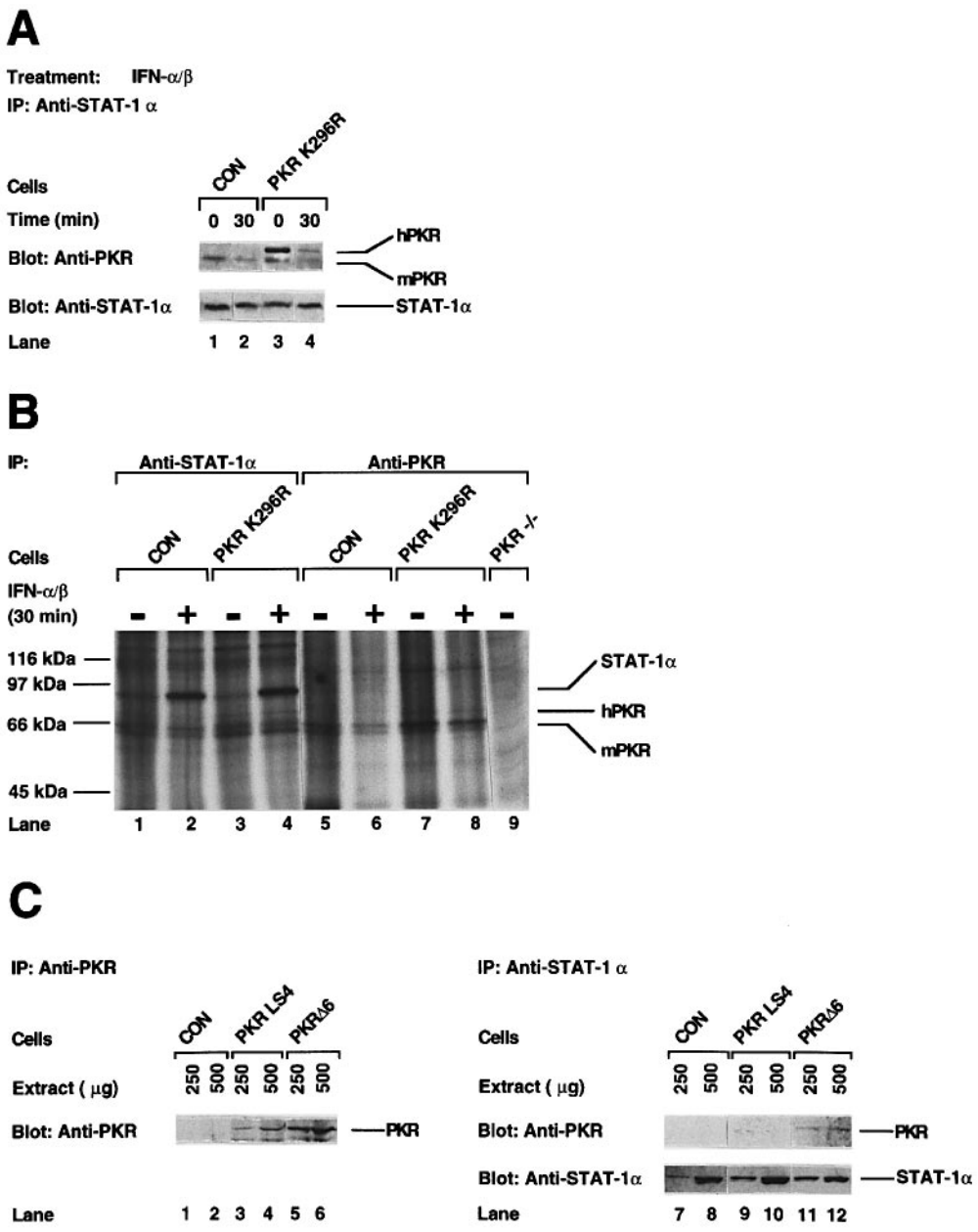

Fig 6. Interaction between mutants of PKR and STAT1. (**A**) Protein extracts from NIH 3T3 control (lanes 1 and 2) and PKR K296R-expressing NIH 3T3 cells (lanes 3 and 4) treated with IFN-α/β for 30 min were immunoprecipitated against STAT1α and immunoblotted with a human PKR cross-reactive, polyclonal antibody against mouse PKR (top panel). The immunoblot was re-probed with antibodies against STAT1α (bottom panel). (**B**) Control NIH 3T3 cells (lanes 1, 2, 5 and 6) and NIH 3T3 cells expressing PKRK296R (lanes 3, 4, 7 and 8) were labeled with [^{32}P]orthophosphate and stimulated with IFN-α/β for 30 min (lanes 2, 4, 6 and 8). Whole cell extracts normalized to TCA counts were first immunoprecipitated with anti-STAT1α antibody (lanes 1–4) followed by a second immunoprecipitation with rabbit antiserum to mouse PKR (lanes 5–8). Immunoprecipitates were analyzed on SDS–8% polyacrylamide gels. As a negative control [^{32}P]orthophosphate-labeled whole cell extracts from PKR$^{-/-}$ MEFs immunoprecipitated with antiserum to PKR were used (lane 9). The migration of ^{32}P-labeled mouse (m)PKR, human (h)PKR (i.e. PKRK296R) and STAT1α are indicated. (**C**) Two hundred and fifty and 500 μg of extracts from NIH 3T3 control (lanes 1, 2, 7 and 8), PKRLS4-expressing NIH 3T3 (lanes 3, 4, 9 and 10) and PKRΔ6-expressing NIH 3T3 cells (lanes 5, 6, 11 and 12) were immunoprecipitated against human PKR (lanes 1–6) or STAT1α (lanes 7–12) and immunoblotted against human PKR (lanes 1–6 and 7–12, top panel) or STAT1α (lanes 7–12, bottom panel).

domain of PKR is required for the interaction with STAT1.

Induction of STAT1 DNA binding in PKR$^{-/-}$ MEFs

The above data implied that dissociation of the PKR–STAT1 complex had to occur in order for STAT1 to exhibit its maximal DNA binding capacity. We pursued this hypothesis further by using cells which lack PKR (PKR$^{-/-}$) (Yang *et al.*, 1995). Mouse embryonic fibroblasts (MEFs) with a PKR$^{+/+}$ or PKR$^{-/-}$ genotype were treated with IFN-α/β, IFN-γ or dsRNA and the DNA binding capacity of STAT1 was tested by gel shift analysis (Figure 7).

In MEFs treated with IFN-α/β (Figure 7A), ISGF-3–DNA complex formation was induced 3- to 5-fold in PKR$^{-/-}$ MEFs (lanes 7–9) relative to PKR$^{+/+}$ MEFs (lanes

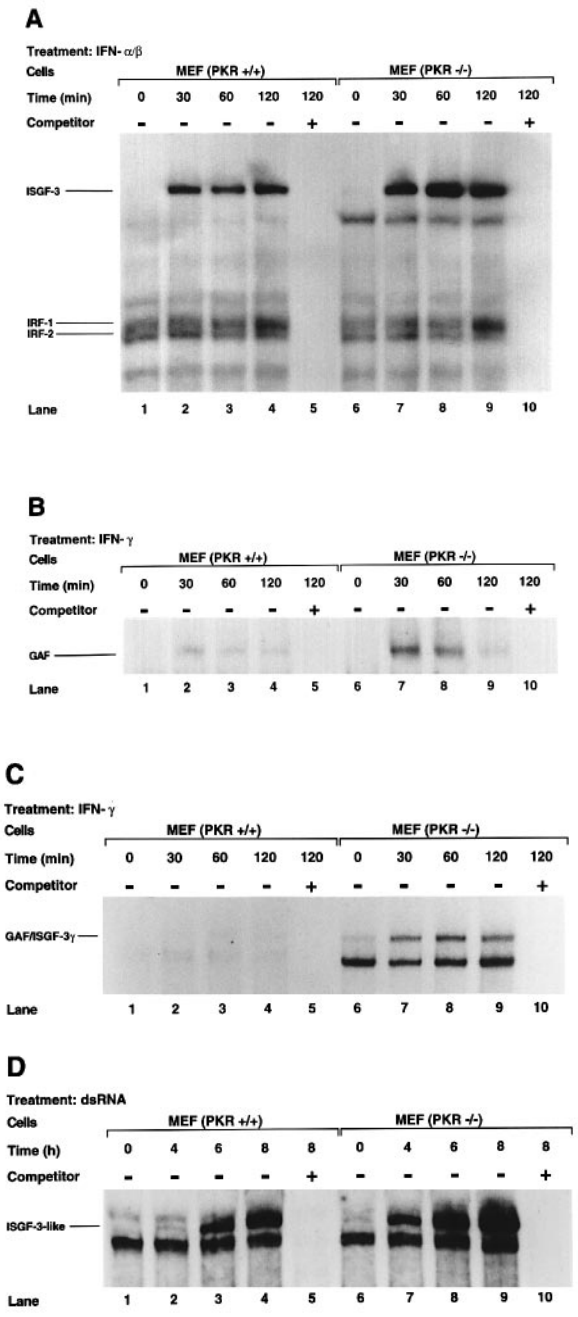

Fig. 7. Induction of STAT1 DNA binding in $PKR^{-/-}$ MEFs. $PKR^{+/+}$ (lanes 1–5) and $PKR^{-/-}$ (lanes 6–10) MEFs were treated with 1000 IU/ml IFN-α/β (**A**), 100 IU/ml IFN-γ (**B** and **C**) for 30, 60 and 120 min or 100 μg/ml poly(rI)–poly(rC) for 2, 4 and 6 h (**D**). Protein extracts were used for ISGF-3 DNA-binding assays either with the ISG-15 ISRE (A, C and D) or the IFP-53 GAS (B) probe. A 200-fold excess of unlabeled dsDNA oligonucleotide was added in cold competition reactions (lanes 5 and 10).

2–4). The DNA binding of IRF-1 and IRF-2 to ISRE was not altered in $PKR^{-/-}$ MEFs compared with $PKR^{+/+}$ MEFs (compare lanes 2–4 with 7–9). Similar results were obtained when the assays were performed with the 561 ISRE dsDNA oligonucleotide (data not shown). In the case of MEFs treated with IFN-γ (Figure 7B), we noted a 3- to 5-fold increase in GAF DNA binding in $PKR^{-/-}$ MEFs (lanes 7–9) compared with $PKR^{+/+}$ MEFs (lanes 2–4). We also detected an increase in GAF–ISGF-3γ DNA binding in $PKR^{-/-}$ MEFs (Figure 7C, lanes 7–9) relative to $PKR^{+/+}$ MEFs (lanes 2–4). Notably, the degree of induction was greater in the case of ISRE binding than in that of GAS binding. For MEFs that had been treated with dsRNA (Figure 7D), an induction of ISGF-3 DNA binding was also observed in $PKR^{-/-}$ MEFs relative to $PKR^{+/+}$ MEFs (compare lanes 2–4 with 7–9). Supershift analyses with an anti-STAT1 antibody indicated that STAT1 was present in the above protein–DNA complexes (data not shown). Interestingly, we noted the presence of a constitutively induced factor in $PKR^{-/-}$ MEFs (Figure 7A and C, lanes 6–9) migrating below the level of ISGF-3 (A) and GAF–ISGF-3γ (C) complexes, which was present at a significantly lower level in $PKR^{+/+}$ MEFs (Figure 7A and 7C, lanes 1–4). It is possible that loss of PKR may also induce the binding of one or more yet identified transcription factors to the ISRE.

Discussion

In this report, we have shown that PKR plays an important role in IFN and dsRNA signaling pathways by modulating the function of the transcription factor STAT1. Specifically, expression of dominant-negative catalytically inactive forms of PKR (PKRK296R and PKRΔ6) results in inhibition of STAT1 DNA binding and gene transactivation in response to IFNs or dsRNA (Figure 1). In contrast, expression of an RNA-binding-defective mutant (PKRLS4) or depletion of PKR ($PKR^{-/-}$) results in enhanced STAT1 DNA binding induced by IFNs or dsRNA (Figures 3 and 7).

Upon further analysis, we have demonstrated an interaction between PKR and STAT1 *in vitro* and *in vivo*. The interaction is specific since neither STAT2 nor ISGF-3γ co-precipitates with PKR and accounts for 10% of each protein present in mouse and human cell extracts prior to IFN or dsRNA treatment (data not shown). Additionally, the association between PKR and STAT1 does not represent a kinase–substrate interaction since phosphorylation of STAT1 is not modified by PKR *in vitro* or *in vivo* (Figure 4). Instead, stimulation with IFNs or dsRNA, conditions under which STAT1 becomes phosphorylated and activated, causes the dissociation of the PKR–STAT1 complex (Figures 5B and 6A). Notably, the minimal point of PKR–STAT1 association correlates with the maximal induction of STAT1 DNA binding (Figure 5A and B).

Although there is a clear inverse correlation between dissolution of the PKR–STAT1 complex and STAT1 DNA-binding capacity, the molecular mechanism(s) by which PKR exerts its effect on the ability of STAT1 to bind DNA is not immediately clear. The observation that induction of STAT1 phosphorylation in response to IFNs remains unchanged in the presence of dominant-negative PKR mutants indicates that early events required for the onset of IFN signaling may not be affected by PKR. For example, since STAT1 tyrosine phosphorylation is not altered by PKR (Figure 4C and D), we reason that events prior to STAT1 phosphorylation, such as JAK activation and STAT1 docking to the IFN receptor (IFNR) (Yan *et al.*, 1996), may not be modulated by PKR. In addition, since PKR does not associate with tyrosine phosphorylated STAT1 (data not shown), PKR is probably not part of the IFNR–JAK–STAT1 complex. Furthermore, it can be

inferred from the lack of PKR phosphorylation (activation) concomitant with STAT1 phosphorylation (activation) in response to IFNs (Figure 6B) that PKR does not modify by phosphorylation any of the phosphatase activities that have been implicated in the regulation of STAT1 (David *et al.*, 1993, 1995; Igarashi *et al.*, 1993a,b; Haque *et al.*, 1995; Shuai *et al.*, 1996). In contrast to STAT1, PKR-mediated effects on STAT2 and ISGF-3γ activation have not been examined due to a lack of antibodies specific for the murine forms of these proteins. However, the inhibition of ISGF-3 DNA binding in response to IFN-α/β by the catalytic inactive mutants of PKR is unlikely to be due to an inhibition of PKR-mediated phosphorylation of STAT2 and/or ISGF-3γ for the following reasons: (i) STAT2 phosphorylation precedes the phosphorylation of STAT1 (Qureshi *et al.*, 1996) and therefore any inhibition of STAT2 phosphorylation by the catalytic mutants of PKR should also affect STAT1 phosphorylation and (ii) phosphorylation of STAT2 and/or ISGF-3γ should also be inhibited in $PKR^{-/-}$ cells or in cells expressing the RNA-binding-defective mutant PKRLS4, which functions in a dominant-negative manner in PKR activation. However, an inhibition of phosphorylation of STAT2 and/or ISGF-3γ would be inconsistent with the induction of ISGF-3 DNA binding observed in these cell types (Figures 3A and 7A).

Instead, a possible mechanism by which PKR modulates STAT1 function may become evident by a recent report demonstrating that a single phosphotyrosyl–SH2 interaction is sufficient to mediate association between STATs *in vitro* (Gupta *et al.*, 1996). Critically, we do not detect interaction between PKR and tyrosine phosphorylated STAT1. However, non-phosphorylated STAT1 has been shown to associate with tyrosine-phosphorylated STAT1 and STAT2, thereby forming transcriptionally active STAT1:STAT1 homodimers and STAT1:STAT2 heterodimers *in vitro* (Gupta *et al.*, 1996). In the same study, it was also suggested that non-phosphorylated STAT1 can be detected in the ISGF-3 complex formed in response to IFN α/β (Gupta *et al.*, 1996). In addition, non-phosphorylated STAT1 has been found in transcriptionally active STAT1:STAT3 heterodimers formed in response to IL-6 (Zhang *et al.*, 1995). As such, the inhibitory effect of PKR may lie in the ability of PKR to bind and sequester STAT1, thereby preventing STAT1 addition into ISGF-3, GAF–ISGF-3γ and GAF complexes. Since the ISGF-3 and GAF–ISGF-3γ complexes are more transient and less stable than the GAF homodimer (Bluyssen *et al.*, 1996; Gupta *et al.*, 1996), any inhibition of STAT1 incorporation into these complexes by PKR would have a more pronounced effect on ISGF-3 and GAF–ISGF-3γ than GAF. Consistent with this notion, a much higher degree of inhibition of ISGF-3 and GAF–ISGF-3γ DNA binding relative to GAF DNA binding is observed in cells expressing the catalytically inactive PKRΔ6 (Figure 1).

Alternatively, it is possible that PKR either facilitates or inhibits the interaction between STAT1 and another protein which might bear a negative or a positive regulatory effect on STAT1 respectively. We postulate that this factor(s) would not mediate its effect through either kinase or phosphatase activity as STAT1 phosphorylation levels remain unchanged in the presence of PKR mutants. Rather, a protein–protein interaction modulated by PKR may be responsible for modification of STAT1 DNA-binding ability.

In either capacity, PKR would assume a structural role similar to the one suggested for the recently cloned STAT-interacting protein (STIP-1). Comparatively, the interaction of both proteins with non-phosphorylated STATs is thought to alter the ability of these STATs to be functional. It has been proposed that STIP-1 provides a positive effect on signaling by interacting with non-phosphorylated STAT3 and facilitating phosphorylation of STAT3 by JAKs in response to IL-6 (Collum and Schindler, 1996); PKR, as demonstrated, exerts a negative effect on signaling by mediating STAT1 DNA binding and transactivation capacities. In further support of a structural role for PKR, there have been other reports indicating that conformational and not catalytic requirements of kinases are important for the progression of signaling pathways. For instance, 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). An analogy could also be drawn with the tyrosine kinase $p56^{lck}$ involved in T cell activation. In this case, it has been shown that the kinase activity of $p56^{lck}$ is dispensable for CD4 co-receptor activity (Collins and Burakoff, 1993; Xu and Littman, 1993). Instead, $p56^{lck}$ may function as an adaptor protein, recruiting downstream signaling components to CD4 via its SH2 domain (reviewed in Ravichandran *et al.*, 1996).

The notion that PKR functions in IFN and dsRNA signaling pathways in a kinase-independent manner is strengthened by the observation that STAT1 is able to associate with both wild type and dominant-negative catalytic mutants of PKR (Figure 6A and C). Additionally, formation of the PKR–STAT1 complex and STAT1 DNA binding are not affected by the PKR inhibitor 2-aminopurine (data not shown). Furthermore, the lack of induced PKR autophosphorylation and activation within the period of IFN treatment (Figure 6B) is in agreement with previous reports showing that modulation of protein synthesis in 3T3 cells, as measured by the appearance of autophosphorylated (activated) PKR, does not occur until at least 3 h after IFN treatment (Petryshyn *et al.*, 1988, 1996). It is noteworthy that the increased expression of either of the PKR catalytic mutants relative to endogenous wild type PKR correlates with a greater inhibition of STAT1 DNA binding (data not shown), a finding which would be consistent with a model in which PKR is able to sequester STAT1 from incorporation into transactivation complexes independently of catalytic activity. Thus, it is conceivable that upregulation of PKR at the protein level by IFNs may represent a feedback mechanism to control the duration and strength of IFN signaling through the regulation of STAT1 function. For example, genes whose expression is regulated by the GAF–ISGF-3γ complex might be a specific target for PKR since this complex is formed by prolonged IFN treatment (Bluyssen *et al.* 1996), a condition under which PKR protein synthesis is induced.

In the case of the dominant-negative dsRNA-binding-defective mutant PKRLS4, we find that the PKR–STAT1 interaction is not dependent upon RNA (Figure 5C) but does require the dsRNA-binding domain of PKR since PKRLS4 fails to co-precipitate with STAT1 (Figure 6C).

If PKR–STAT1 interaction is responsible for modification of STAT1 DNA binding, then loss of this complex would account for the induction of STAT1 DNA binding observed in PKRLS4-expressing cells (Figure 3). Likewise, the induction of STAT1 DNA binding observed in PKR$^{-/-}$ MEFs (Figure 7) could be attributed to the lack of PKR in these cells and thus the absence of PKR–STAT1. Furthermore, although complex formation is not dependent on STAT1 dimerization as implied by the lack of association between PKR and tyrosine phosphorylated STAT1, whether it requires PKR dimerization remains to be clarified. It has been shown that all these dominant-negative PKR mutants are capable of dimerization (Cosentino *et al.*, 1995; Patel *et al.*, 1995). Since dimerization does not necessarily require the dsRNA-binding properties of PKR (Patel *et al.*, 1995), dimerization of the dominant-negative PKRLS4 with the endogenous mouse PKR may be responsible for inhibition of PKR–STAT1 complex formation in cells expressing PKRLS4 and may account for the induction of STAT1 DNA binding in these cells (Figure 3).

Presently, it is not known what processes are involved in the dissociation of PKR and STAT1. It is unlikely that it requires PKR activation since (i) a large amount of autophosphorylated PKR co-precipitates with STAT1 from HeLa S3 cells (Figure 4A, lanes 6 and 8); (ii) dissociation also occurs in the presence of the dominant-negative PKRK296R (Figure 6A); (iii) PKR autophosphorylation and subsequent activation is not induced by IFNs (Figure 6B) although disruption of the PKR–STAT1 complex is (Figure 5B); and (iv) treatment with the PKR inhibitor 2-aminopurine does not affect PKR–STAT1 dissociation (data not shown). Rather, the dissociation of PKR and STAT1 may be effected by another protein(s) whose activity is induced by IFNs or dsRNA. Although the interaction of PKR and STAT1 is direct *in vitro* (Figure 5D), it remains possible that complex dissociation is dependent upon the presence and/or activity of another protein(s). Alternatively, structural changes in signaling components induced by IFN or dsRNA might facilitate dissociation of PKR and STAT1.

It should also be noted that PKR may be able to negatively regulate ISRE DNA binding by modifying the activity of one or more transcription factors other than STAT1. For example, a DNA–protein complex bound to ISRE migrating below the ISGF-3 and GAF–ISGF-3γ complexes was present in PKR$^{-/-}$ MEFs (Figure 7A and C) as well as in NIH 3T3 cells expressing PKRLS4 (Figure 3A and C). The constitutive activation of this DNA-binding factor(s) may indicate that its activity is not regulated by the cascade of tyrosine phosphorylation induced by IFNs. Thus, loss of PKR and/or the PKR–STAT1 complex may induce the binding of one or more yet identified transcription factors to the ISRE.

An interesting question which arises is whether the PKR–STAT1 complex bears any effect on the function of PKR in the regulation of protein synthesis. To date, the role of PKR in translational control via phosphorylation of eIF-2α is well documented (reviewed in Proud, 1995). As a consequence of its ability to inhibit protein synthesis, PKR assumes a critical role in the antiviral response (reviewed in Katze, 1994; Yang *et al.*, 1995). In addition to PKR, the importance of STAT1 in the response to viral infection is highlighted by recent reports which demonstrate that *stat1*-deficient mice are more susceptible to viral infection compared with normal mice (Durbin *et al.*, 1996; Meraz *et al.*, 1996). Such an increase in viral susceptibility of *stat1*$^{-/-}$ mice has been attributed mostly to the failure of genes encoding proteins with antiviral effects such as PKR to be upregulated by IFNs at the transcriptional level. An intriguing possibility that remains to be determined, however, is that STAT1 might interact with other non-STAT proteins that mediate antiviral effects, like PKR, and modify their functions. That is, STAT1 may serve as a mediator of signaling events induced either by IFNs and/or viral infection which regulate protein synthesis by modulating PKR activity. In this regard, crosstalk between components of membrane-to-transcriptional and translational pathways has already been documented for a growth factor-regulated pathway (reviewed in Brown and Schreiber, 1996).

In conclusion, the data presented here suggest that PKR is able to modify the pattern of gene expression that takes place in response to IFN, dsRNA and possibly other stimuli. PKR appears to do so by modulating the activity of STAT1, apparently without directly binding to specific DNA sequences. In this manner, PKR can be compared with p202, another IFN-inducible protein which functions as a modulator of NF-κB, c-Fos and c-Jun activities (Min *et al.*, 1996). As such, the regulation of cell growth by PKR may be the consequence of the ability of PKR to alter the expression patterns of cell cycle regulatory proteins. For instance, if PKR is able to regulate the activity of requisite transcription factors, then this may be, at least in part, the mechanism for the control of cell proliferation by PKR (Koromilas *et al.*, 1992; Meurs *et al.*, 1993; Barber *et al.*, 1995). In pursuit of this, the recent finding that STAT1 plays a role in p21 transcription (Chin *et al.*, 1996) may have established a tentative yet important link between the abilities of PKR to modulate transcription and to regulate cell growth: the regulation of cell cycle progression.

Materials and methods

Cell culture and transfections

NIH 3T3 cells (ATCC CRL-1658) and MEFs were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc.). HeLa S3 cells (ATCC CCL-2.2) were grown in RPMI 1640 (Life Technologies Inc.). Both media were supplemented with 10% fetal calf serum (FCS; Life Technologies Inc.), 2 mM L-glutamine (Life Technologies Inc.) and penicillin–streptomycin (100 units/ml; Life Technologies Inc.).

For IFN treatment, NIH 3T3 cells and MEFs were incubated with 1000 IU/ml of recombinant murine IFN-α/β (Lee Biomolecules, CA) or 100 IU/ml of recombinant murine IFN-γ (Cedarlane, Canada). HeLa S3 cells were stimulated with IFN-α_2/α_1 (1000 IU/ml, provided by C.Weissmann) or with IFN-γ (100 IU/ml, Collaborative Res.).

NIH 3T3 clones expressing mutants of PKR were generated by stable transfection of PKR K296R, PKRΔ6 or PKRLS4 cDNA, cloned in the *Hin*dIII–*Bam*HI sites of the pcDNA3/neo vector (Invitrogen) and selected in G418 (400 μg/ml; Life Technologies Inc.) as described elsewhere (Koromilas *et al.*, 1992). Transfections with dsRNA were conducted in a DEAE-dextran-dependent manner (Yang *et al.*, 1995) with 100 μg/ml of poly(rI)–poly(rC) (Pharmacia Biotech Inc.) in the presence of an anti-mouse Type I IFN monoclonal antibody (Kirchhoff *et al.*, 1993).

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays, the following double-stranded oligonucleotides were used (Sheldon Biotechnology Centre, Montreal, Canada): the ISRE of the IFN-α/β-inducible ISG-15 gene (5′-GATCGG-

GAAAGGGAAACCGAAA CTGAAGCC-3′) (Reich and Darnell, 1989) and the GAS of the IFN-γ-inducible IFP-53 gene (5′-G ATCCAGATTCT-CAGAAA-3′) (Strehlow *et al.*, 1993).

Gel mobility shift experiments were performed with whole-cell extracts as previously described (Eilers *et al.*, 1995). 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 ~2×10^5 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, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml pepstatin. 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× TBE at 400 V at 4°C. To identify the components contained within the protein–DNA complexes, antiserum to STAT1α (Santa Cruz Biotechnology Inc.) was added to the binding reactions. To ensure the specificity of interactions, a 100- to 200-fold excess of unlabeled dsDNA oligonucleotide was used in cold competition reactions. Induced DNA–protein complexes were visualized by autoradiography and quantified by scanning autoradiograms in the linear range of exposure with an enhanced laser densitometer Ultroscan XL (LKB).

Northern blot analysis

Total RNA was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA (10 μg) was denatured with glyoxal and dimethylsulfoxide and subjected to electrophoresis on a 1% agarose gel in 10 mM sodium phosphate buffer pH 7.0 (Sambrook *et al.*, 1989). RNA was transferred onto a nylon membrane (BioTans, ICN). Hybridization was performed at 65°C for 16 h with [α-^{32}P]dATP-labeled random primed cDNA probes (Feinberg and Vogelstein, 1983) (5×10^6 c.p.m./ml) consisting of either the 0.95 kbp *Eco*RI–*Hin*dIII fragment of the human ISG-15 cDNA, 1.5 kbp *Eco*RI fragment of the human IFP-53 cDNA or the entire sequence of mouse β-actin cDNA. After hybridization, the filters were washed with 0.1× SSC (150 mM NaCl and 15 mM sodium citrate pH 7.0) plus 0.1% SDS for 1 h at 50°C. The filters were exposed to X-ray film for 16 h.

In vitro phosphorylation assay of PKR

One hundred μg of untreated HeLa S3 cells or HeLa S3 cells treated with human IFN-α/β for 18 h (1000 IU/ml; Lee Biomolecules) were suspended in 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 10 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 equally into three fractions. Immunoprecipitations were performed with antibodies to PKR (13B8-F9), STAT1 (anti-91T; C.Schindler) or eIF-2α, using protein G–Sepharose as a carrier (Pharmacia). Immunoprecipitates were fractionated on SDS–7% polyacrylamide gels and visualized by autoradiography.

[^{32}P]orthophosphate cell labeling and STAT1 phosphorylation

Cells were incubated in phosphate-free DMEM media (Life Technologies Inc.), supplemented first with dialyzed FCS (Life Technologies Inc.) for 3 h and then with [^{32}P]orthophosphate (200 μCi/ml; Dupont) for an additional 3 h. Following stimulation with IFNs, cells were washed twice with ice-cold 1× phosphate buffered saline (PBS) containing 100 mM NaF, 20 mM β-glycerophosphate and 20 mM Na_2MdO_4 and lysed in 1× RIPA lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% SDS) containing 1 mM DTT, 0.2 mM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The lysate was centrifuged at 10 000 *g* for 10 min and equal counts of ^{32}P-labeled proteins (10% trichloroacetic acid precipitates) from the supernatants were pre-cleared with rabbit pre-immune serum (Sigma, MO) and then incubated with 5 μg of anti-STAT1α monoclonal antibody (Santa Cruz Biotechnology, CA) overnight at 4°C under rotation. Immunoprecipitates were captured with protein G–Sepharose and washed three times with cold 1× RIPA supplemented with 1 M NaCl, 2 mM DTT, 0.2 mM PMSF and 4 μg/ml aprotinin and then three times with cold 1× RIPA containing 2 mM DTT, 0.2 mM PMSF and 4 μg/ml aprotinin. Proteins were fractionated by SDS–7% polyacrylamide gels and visualized by autoradiography.

Immunoprecipitation and immunoblotting

Whole cell extracts were prepared either as previously described (Eilers *et al.*, 1995) or by extraction with 1× RIPA buffer supplemented with 1 mM DTT, 0.2 mM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 mM NaF and 0.1 mM Na_3VO_4. Immunoprecipitations were performed using 100, 250 or 500 μg of whole-cell extracts, which were pre-cleared with rabbit pre-immune serum or mouse IgG1 (Sigma, MO). Five μg of antisera to human PKR (13B8-F9), mouse PKR (TIK), or STAT1 (anti-91T) were used. Reactions were rotated at 4°C for 2 h and antibody–antigen complexes were captured by protein G–Sepharose. The beads were washed three times with ice cold 1× extraction buffer. Immunoprecipitates were electrophoresed on SDS–7% polyacrylamide gels and proteins were transferred onto nitrocellulose filters. Immunoblotting analyses with anti-human PKR (1 μg/ml), anti-mouse PKR (rabbit serum, 1:500), anti-STAT1α (1 μg/ml; Santa Cruz Biotechnology, CA) or anti-phosphotyrosine [4G10 (1 μg/ml); Upstate Biotechnology Inc. and PY20 (1 μg/ml); Transduction Laboratories] antibodies were performed as previously described (Eilers *et al.*, 1995; Koromilas *et al.*, 1995). After incubation with anti-mouse or anti-rabbit peroxidase-conjugated antibody (1:1000; Amersham Corp.). Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Corp.).

In vitro transcription and translation

Human PKR or human STAT1α protein was synthesized from human PKR or STAT1α cDNA under the control of 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. Immunoprecipitation of ^{35}S-labeled PKR–STAT1α complex was performed as described above. Protein preparations and immunoprecipitates were electrophoresed on SDS–8% polyacrylamide gels which were treated with EN^{3}HANCE (Dupont) according to the manufacturer's specifications before visualization by fluorography.

Acknowledgements

We are indebted to Dr C.Weissmann for stimulating discussions and suggestions during the course of the work and to Drs C.Schindler and R.Pine for critical comments. We thank Dr C.Schindler for rabbit anti-STAT1α polyclonal antibody (anti-91T) and STAT1α cDNA; Drs G.N.Barber, A.Darveau and M.G.Katze for anti-human PKR monoclonal antibodies; Dr J.Bell for rabbit antiserum to mouse PKR (TIK); Dr B.Safer for sheep antiserum to eIF-2α; Dr M.G.Katze for PKRK296R cDNA; Dr M.Mathews for PKRLS4 cDNA; Luc Chandonnet for technical assistance. Many thanks to the members of the Terry Fox Molecular Oncology Group for helpful comments. This work has been supported by grants from the National Cancer Institute of Canada and Health Welfare Canada to A.E.K. N.W.N.T. is a recipient of the Gertrude and Charles Clark Cancer Research Fellowship from the Sir Mortimer B.Davis-Jewish General Hospital. A.H.-T.W. and A.R.C. are recipients of the Defi Corparatif Canderel Studentship from the McGill Cancer Center. A.E.K. is a member of the Terry Fox Molecular Oncology Group and an awardee from the American Foundation for AIDS Research (AmFAR).

References

Bandyopadhyay,S.K., Leonard,G.T.,Jr, Bandyopadhyay,T., Stark,G.R. and Sen,G.C. (1995) Transcriptional induction by double-stranded RNA is mediated by interferon-stimulated response elements without activation of interferon-stimulated gene factor 3. *J. Biol. Chem.*, **270**, 19624–19629.

Barber,G.N., Wambach,M., Thompson,S., Jagus,R. and Katze,M.G. (1995) Mutants of the RNA-dependent protein kinase (PKR) lacking double-stranded RNA domain I can act as transdominant inhibitors and induce malignant transformation. *Mol. Cell. Biol.*, **15**, 3138–3146.

Bluyssen,H.A.R., Muzaffar,R., Vlieststra,R.J., Van der Made,A.C.J., Leung,S., Stark,G.R., Kerr,I.M., Trapman,J. and Levy,D.E. (1995) Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. *Proc. Natl Acad. Sci. USA*, **92**, 5645–5649.

Bluyssen,H.A.R., Durbin,J.E. and Levy,D.E. (1996) ISGF3γ p48, a specificity switch for interferon activated transcription factors. *Cytokine Growth Factor Rev.*, **7**, 11–17.

Briscoe,J., Rogers,N.C., Witthuhn,B.A., Watling,D., Harpur,A.G., Wilks,A.F., Stark,G.R., Ihle,J.N. and Kerr,I.M. (1996) Kinase-negative mutants of JAK1 can sustain interferon-γ-inducible gene expression but not an antiviral state. *EMBO J.*, **15**, 799–809.

Brown,E.J. and Schreiber,S.L. (1996) A signaling pathway to translational control. *Cell*, **86**, 517–520.

Chin,Y.E., Kitagawa,M., Su,W.-C.S., You,Z.-H., Iwamoto,Y. and Fu,X.-Y. (1996) Cell growth arrest and induction of cyclin-dependent kinase inhibitor $p21^{WAF/CIP1}$ mediated by STAT1. *Science*, **272**, 719–722.

Chomczynski,P. and Sacchi,N. (1987) Single-step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Chong,K.L., Feng,L., Schappert,K., Meurs,E., Donahue,T.F., Friesen,J.D., Hovanessian,A.G. and Williams,B.R.G. (1992) Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator GCN2. *EMBO J.*, **11**, 1553–1562.

Collins,T.L. and Burakoff,S.J. (1993) Tyrosine kinase activity of CD4-associated $p56^{lck}$ may not be required for CD4-dependent T-cell activation. *Proc. Natl Acad. Sci. USA*, **90**, 11885–11889.

Collum,R.G. and Schindler,C. (1996) Identification of a novel factor in the JAK-STAT signaling pathway. *Eur. Cytokine Network*, **7**, 518.

Cosentino,G.P., Venkatesan,S., Serluca,F.C., Green,S.R., Mathews, M.B. and Sonenberg,N. (1995) Double-stranded-RNA-dependent protein kinase and TAR RNA-binding protein form homo- and heterodimers *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 9445–9449.

Daly,C. and Reich,N. (1993) Double-stranded RNA activates novel factors that bind to the interferon-stimulated response element. *Mol. Cell. Biol.*, **13**, 3756–3764.

Daly,C. and Reich,N.C. (1995) Characterization of specific DNA-binding factors activated by double-stranded RNA as positive regulators of interferon alpha/beta-stimulated genes. *J. Biol. Chem.*, **270**, 23739–23746.

Darnell,J.E.,Jr, Kerr,I.M. and Stark,G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415–1421.

David,M., Romero,G., Zhang,Z.-Y., Dixon,J.E. and Larner,A.C. (1993) *In vitro* activation of the transcription factor ISGF3 by interferon α involves a membrane-associated tyrosine phosphatase and tyrosine kinase. *J. Biol. Chem.*, **268**, 6593–6599.

David,M., Chen,H.E., Goelz,S., Larner,A.C. and Neel,B.G. (1995) Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol. Cell. Biol.*, **15**, 7050–7058.

Decker,T. (1992) Double-stranded RNA and interferon-α induce transcription through different molecular mechanisms. *J. Interferon Res.*, **12**, 445–448.

Decker,T., Lew,D.J., Mirkovitch,J. and Darnell,J.E.,Jr (1991) Cytoplasmic activation of GAF, an IFN-γ-regulated DNA-binding factor. *EMBO J.*, **10**, 927–932.

Durbin,J.E., Hackenmiller,R., Simon,M.C. and Levy,D.E. (1996) Targeted disruption of the mouse *Stat1* gene results in compromised innate immunity to viral disease. *Cell*, **84**, 443–450.

Eilers,A., Georgellis,D., Klose,B., Schindler,C., Ziemiecki,A., Harpur,A.G., Wilks,A.F. and Decker,T. (1995) Differentiation-regulated serine phosphorylation of STAT1 promotes GAF activation in macrophages. *Mol. Cell. Biol.*, **15**, 3579–3586.

Feinberg,A.P. and Vogelstein,B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132**, 6–13.

Fu,X.-Y., Schindler,C., Improta,T., Aebersold,R. and Darnell,J.E.,Jr (1992) The proteins of ISGF-3, the interferon α-induced transcriptional activator, define a gene family involved in signal transduction. *Proc. Natl Acad. Sci. USA*, **89**, 7840–7843.

Gilmour,K.C. and Reich,N.C. (1995) Signal transduction and activation of gene transcription by interferons. *Gene Expression*, **5**, 1–18.

Green,S.R. and Mathews,M.B. (1992) Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI. *Genes Dev.*, **6**, 2478–2490.

Gupta,S., Yan,H., Wong,L.-H., Ralph,S., Krolewski,J. and Schindler,C. (1996) The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-α signals. *EMBO J.*, **15**, 1075–1084.

Han,Y., Leaman,D.W., Watling,D., Rogers,N.C., Groner,B., Kerr,I.M., Wood,W.I. and Stark,G.R. (1996) Participation of JAK and STAT proteins in growth hormone-induced signaling. *J. Biol. Chem.*, **271**, 5947–5952.

Haque,S.J., Flati,V., Deb,A. and Williams,B.R.G. (1995) Roles of protein-tyrosine phosphatases in Stat1α-mediated cell signaling. *J. Biol. Chem.*, **270**, 25709–25714.

Heim,M.H., Kerr,I.M., Stark,G.R. and Darnell,J.E.,Jr (1995) Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. *Science*, 267, 1347–1349.

Hershey,J.W.B. (1989) Protein phosphorylation controls translation rates. *J. Biol. Chem.*, **264**, 20823–20826.

Igarashi,K.-I., David,M., Finbloom,D.S. and Larner,A.C. (1993a) In vitro activation of the transcription factor gamma interferon activation factor by gamma interferon: evidence for a tyrosine phosphatase/kinase signaling cascade. *Mol. Cell. Biol.*, **13**, 1634–1640.

Igarashi,K.-I., David,M., Larner,A.C. and Finbloom,D.S. (1993b) In vitro activation of a transcription factor by gamma interferon requires a membrane-associated tyrosine kinase and is mimicked by vanadate. *Mol. Cell. Biol.*, **13**, 3984–3989.

Katze,M.G. (1995) Regulation of the interferon-induced PKR: can viruses cope? *Trends Microbiol.*, **3**, 75–78.

Katze,M.G., Wambach,M., Wong,M.-L., Garfinkel,M., Meurs,E., Chong,K., Williams,B.R.G., Hovanessian,A.G. and Barber,G.N. (1991) Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-M_r protein kinase in a cell-free system. *Mol. Cell. Biol.*, **11**, 5497–5505.

Kessler,D.S., Veals,S.A., Fu,X.-Y. and Levy,D.E. (1990) Interferon-α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. *Genes Dev.*, **4**, 1753–1765.

Khan,D.K., Lindwall,G., Maher,S.E. and Bothwell,A.L.M. (1990) Characterization of promoter elements of an interferon-inducible Ly-6E/A differentiation antigen, which is expressed on activated T cells and hematopoietic stem cells. *Mol. Cell. Biol.*, **10**, 5150–5159.

Kirchhoff,S., Schaper,F. and Hauser,H. (1993) Interferon regulatory factor 1 (IRF-1) mediates cell growth inhibition by transactivation of downstream target genes. *Nucleic Acids Res.*, **21**, 2881–2889.

Kirchhoff,S., Koromilas,A.E., Schaper,F., Grashoff,M., Sonenberg,N. and Hauser,H. (1995) IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. *Oncogene*, **11**, 439–445.

Koromilas,A.E., Roy,S., Barber,G.N., Katze,M.G. and Sonenberg,N. (1992) Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. *Science*, **257**, 1685–1689.

Koromilas,A.E., Cantin,C., Craig,A.W.B., Jagus,R., Hiscott,J. and Sonenberg,N. (1995) The interferon-inducible protein kinase PKR modulates transcriptional activation of the immunoglobulin κ gene. *J. Biol. Chem.*, **270**, 25426–25434.

Kumar,A., Haque,J., Lacoste,J., Hiscott,J. and Williams,B.R.G. (1994) Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating IκB. *Proc. Natl Acad. Sci. USA*, **91**, 6288–6292.

Larner,A.C., David,M., Feldman,G.M., Igarashi,K.-I., Hackett,R.H., Webb,D.S.A., Sweitzer,S.M., Petricoin,E.F.,III and Finbloom,D.S. (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science*, **261**, 1730–1733.

Maran,A., Maitra,R.K., Kumar,A., Dong,B., Xiao,W., Li,G., Williams,B.R.G., Torrence,P.F. and Silverman,R.H. (1994) Blockage of NF-κB signaling by selective ablation of an mRNA target by 2–5A antisense chimeras. *Science*, **265**, 789–792.

Meraz,M.A. *et al.* (1996) Targeted disruption of the *Stat* 1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*, **84**, 431–442.

Meurs,E.F., Watanabe,Y., Kadereit,S., Barber,G.N., Katze,M.G., Chong,K., Williams,B.R.G. and Hovanessian,A.G. (1992) Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth. *J. Virol.*, **66**, 5805–5814.

Meurs,E.F., Galabru,J., Barber,G.N., Katze,M.G. and Hovanessian,A.G. (1993) Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc. Natl Acad. Sci. USA*, **90**, 232–236.

Min,W., Ghosh,S. and Lengyel,P. (1996) The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF-κB, c-fos, and c-jun activities. *Mol. Cell. Biol.*, **16**, 359–368.

Mundschau,L.J. and Faller,D.V. (1995) Platelet-derived growth factor signal transduction through the interferon-inducible kinase PKR. *J. Biol. Chem.*, **270**, 3100–3106.

Patel,R.C., Stanton,P., McMillan,N.M.J., Williams,B.R.G. and Sen,G.C. (1995) The interferon-inducible double-stranded RNA-activated protein kinase self-associates *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 8283–8287.

Petryshyn,R., Chen,J.-J. and London,I.M. (1988) Detection of activated double-stranded RNA-dependent protein kinase in 3T3-F442A cells. *Proc. Natl. Acad. Sci. USA*, **85**, 1427–1431.

Petryshyn,R., Chen,J.-J., Danley,L. and Matts,R.L. (1996) Effect of interferon on protein translation during growth stages of 3T3 cells. *Arch. Biochem. Biophys*., **328**, 290–297.

Pine,R., Decker,T., Kessler,D.S., Levy,D.E. and Darnell,J.E.,Jr (1990) Purification and cloning of interferon-stimulated gene factor 2 (ISGF2):ISGF2(IRF-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either. *Mol. Cell. Biol.*, **10**, 2448–2457.

Proud,C.G. (1995) PKR: a new name and new roles. *Trends Biochem. Sci*., **20**, 241–246.

Qureshi,S.A., Leung,S., Kerr,I.M., Stark,G.R. and Darnell,J.E.,Jr (1996) Function of Stat2 protein in transcriptional activation by alpha interferon. *Mol. Cell. Biol*., **16**, 288–293.

Ravichandran,K.S., Collins,T.L. and Burakoff,S.J. (1996) CD4 and signal transduction. In Littman,D.R. (ed.), *The CD4 Molecule.* Springer-Verlag, Berlin, Germany, pp. 47–62.

Reich,N.C. and Darnell,J.E.,Jr (1989) Differential binding of interferon-induced factors to an oligonucleotide that mediates transcriptional activation. *Nucleic Acids Res*., **17**, 3415–3424.

Ruff-Jamison,S., Chen,K. and Cohen,S. (1993) Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. *Science*, **261**, 1733–1736.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schindler,C. and Darnell,J.E.,Jr (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem*., **64**, 621–651.

Schindler,C., Fu,X.-Y., Improta,T., Aebersold,R. and Darnell,J.E.,Jr (1992) Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84- kDa ISGF-3 proteins that are activated by interferon α. *Proc. Natl Acad. Sci. USA*, **89**, 7836–7839.

Shuai,K., Schindler,C., Prezioso,V.R. and Darnell,J.E.,Jr (1992) Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91-kD DNA binding protein. *Science*, **258**, 1808–1812.

Shuai,K., Stark,G.R., Kerr,I.M. and Darnell,J.E.,Jr (1993) A single phosphotyrosine residue of Stat91 required for gene activation by interferon-γ. *Science*, **261**, 1744–1746.

Shuai,K., Horvath,C.M., Huang,L.H.T., Qureshi,S.A., Cowburn,D. and Darnell,J.E.,Jr (1994) Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions.*Cell*, **76**, 821–828.

Shuai,K., Liao,J. and Song,M.M. (1996) Enhancement of anti-proliferative activity of gamma interferon by the specific inhibition of tyrosine dephosphorylation of Stat1. *Mol. Cell. Biol*., **16**, 4932–4941.

Strehlow,I., Seegert,D., Frick,C., Bange,F.-C., Schindler,C., Böttger,E.C. and Decker,T. (1993) The gene encoding IFP 53/tryptophanyl-tRNA synthetase is regulated by the γ-interferon activation factor. *J. Biol. Chem*., **268**, 16590–16595.

Veals,S.A., Schindler,C., Leonard,D., Fu,X.-Y., Aebersold,R., Darnell, J.E.,Jr and Levy,D.E. (1992) Subunit of an alpha-interferon responsive transcription factor is related to interferon regulatory factor and myb families of DNA-binding proteins. *Mol. Cell. Biol.*, **12**, 3315–3324.

Vignais,M.-L., Sadowski,H.B., Watling,D., Rogers,N.C. and Gilman,M. (1996) Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins. *Mol. Cell. Biol*., **16**, 1759–1769.

Wen,Z., Zhong,Z. and Darnell,J.E.,Jr (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*, **82**, 241–250.

Xu,H. and Littman,D.R. (1993) A kinase-independent function of Lck in potentiating antigen-specific T cell activation. *Cell*, **74**, 633–643.

Yan,H., Krishnan,K., Greenlund,A.C., Gupta,S., Lim,J.T.E., Schreiber, R.D., Schindler,C.W. and Krolewski,J.J. (1996) Phosphorylated interferon-α receptor 1 subunit (IFNαR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. *EMBO J*., **15**, 1064–1074.

Yang,Y.-L., Reis,L.F.L., Pavlovic,J., Aguzzi,A., Schäfer,R., Kumar,A., Williams,B.R.G., Aguet,M. and Weissmann,C. (1995) Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J*., **14**, 6095–6106.

Zhang,X., Blenis,J., Li,H.-C., Schindler,C. and Chen-Kiang,S. (1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science*, **267**, 1990–1994.

Received on July 1, 1996; revised on August 29, 1996