Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-κB

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The interferon (IFN)-induced double-stranded RNA (dsRNA)-activated Ser/Thr protein kinase (PKR) plays a role in the antiviral and antiproliferative effects of IFN. PKR phosphorylates initiation factor eIF2α, thereby inhibiting protein synthesis, and also activates the transcription factor, nuclear factor-κB (NF-κB), by phosphorylating the inhibitor of NF-κB, IκB. Mice devoid of functional PKR (*Pkr*$^{o/o}$) derived by targeted gene disruption exhibit a diminished response to IFN-γ and poly(rI:rC) (pIC). In embryo fib oblasts derived from *Pkr*$^{o/o}$ mice, interferon regulatory factor 1 (IRF-1) or guanylate binding protein (*Gbp*) promoter–reporter constructs were unresponsive to IFN-γ or pIC but response could be restored by co-transfection with PKR. The lack of responsiveness could be attributed to a diminished activation of IRF-1 and/or NF-κB in response to IFN-γ or pIC. Thus, PKR acts as a signal transducer for IFN-stimulated genes dependent on the transcription factors IRF-1 and NF-κB.

Keywords: cytokine signaling/interferon/IRF-1/NF-κB/ PKR gene

Introduction

Interferons (IFNs) are a family of proteins with distinct biological properties, the most prominent of which is their ability to impair viral replication (Samuel, 1991; Hovanesian, 1994). Double-stranded RNA (dsRNA) which accumulates during the replication of many viruses activates the dsRNA-dependent protein kinase (PKR; Meurs *et al*., 1990; Garfinkel and Katze, 1993) which in turn phosphorylates different substrates including eukaryotic protein synthesis initiation factor 2 (eIF2) and IκB (Chong *et al*., 1992; Meurs *et al*., 1992; Kumar *et al*., 1994; Williams, 1995). The phosphorylation and inactivation of eIF2 results in a decrease in total cellular protein synthesis (Hovanessian, 1994) and, in the context of a virus-infected cell, leads to cell death, possibly by apoptotic pathways (Lee and Esteban, 1994). In many unstimulated cells, transcription factor NF-κB is found localized to the cytoplasm as a latent heterodimeric complex bound to its subunit-specific inhibitor IκB (Haskill *et al*., 1991). NF-κB is a multisubunit transcription factor comprising p50, p65 and *rel* proto-oncogene products (Hill and Treisman, 1995). In response to dsRNA, PKR phosphorylates IκB releasing an active form of NF-κB (Kumar *et al*., 1994; Maran *et al*., 1994). Active NF-κB translocates to the nucleus where it regulates IFN-β (Hiscott *et al*., 1989; Lenardo *et al*., 1989; Visvanathan and Goodbourn, 1989; Xanthoudakis *et al*., 1989; Leblanc *et al*., 1990; Du *et al*., 1993) and a number of genes involved in mediating the antiproliferative and antiviral effects of IFN, including class I major histocompatibility complex (MHC) (Weiss *et al*., 1984; Ten *et al*., 1993) and interferon regulatory factor 1 (IRF-1) (Reis *et al*., 1992, 1994; Ruffner *et al*., 1993). Transcription factor IRF-1 is required for the induction of the inducible nitric oxide synthase (iNOS) gene by IFN-γ and lipopolysaccharide (LPS) (Kamijo *et al*., 1994), plays a role in the regulation of the IFN-β (Reis *et al*., 1992) and guanylate binding protein (*Gbp*) genes (Briken *et al*., 1995) and is involved in cellular apoptotic responses (Tanaka *et al*., 1994; Tamura *et al*., 1995).

We have produced mice devoid of p65 Mu*Pkr* (Feng *et al*., 1992) by homologous recombination (Yang *et al*., 1995). *Pkr*$^{o/o}$ mouse embryo fibroblasts (MEFs) derived from these mice are deficient in dsRNA-dependent NF-κB activation. Since the mice also exhibited a diminished antiviral response to IFN-γ, we have analyzed signal transduction pathways in *Pkr*$^{o/o}$ MEFs using reporter constructs responsive to IFN-γ as well as to dsRNA, IFN-α and tumor necrosis factor (TNF)-α. IFN-γ and poly(rI:rC) (pIC) induction of IRF-1 or *Gbp* promoter luciferase reporters was deficient in *Pkr*$^{o/o}$ MEFs but could be rescued by co-expression of wild-type human PKR. The deficiency in signaling could be attributed to an inability of IFN-γ or pIC to activate IRF-1 or NF-κB. Thus, PKR acts as a signal-transducing kinase for IRF-1- and NF-κB-dependent gene induction.

Results

Deficient signaling to the IRF-1 promoter in Pkr$^{o/o}$ *MEFs*

Pkr$^{o/o}$ mice exhibit a diminished antiviral response to IFN-γ and pIC (Yang *et al*., 1995). To determine whether this impaired response was reflected in a promoter normally responsive to either IFN or pIC, we cloned a 1308 bp fragment of the IRF-1 promoter (IRF1-WT, Sims *et al*., 1993; Haque and Williams, 1994) upstream of the luciferase gene and used this reporter in transcriptional assays. *Pkr*$^{+/+}$ MEFs transiently transfected with IRF1-WT showed responsiveness to IFN-γ, IFN-α, dsRNA and

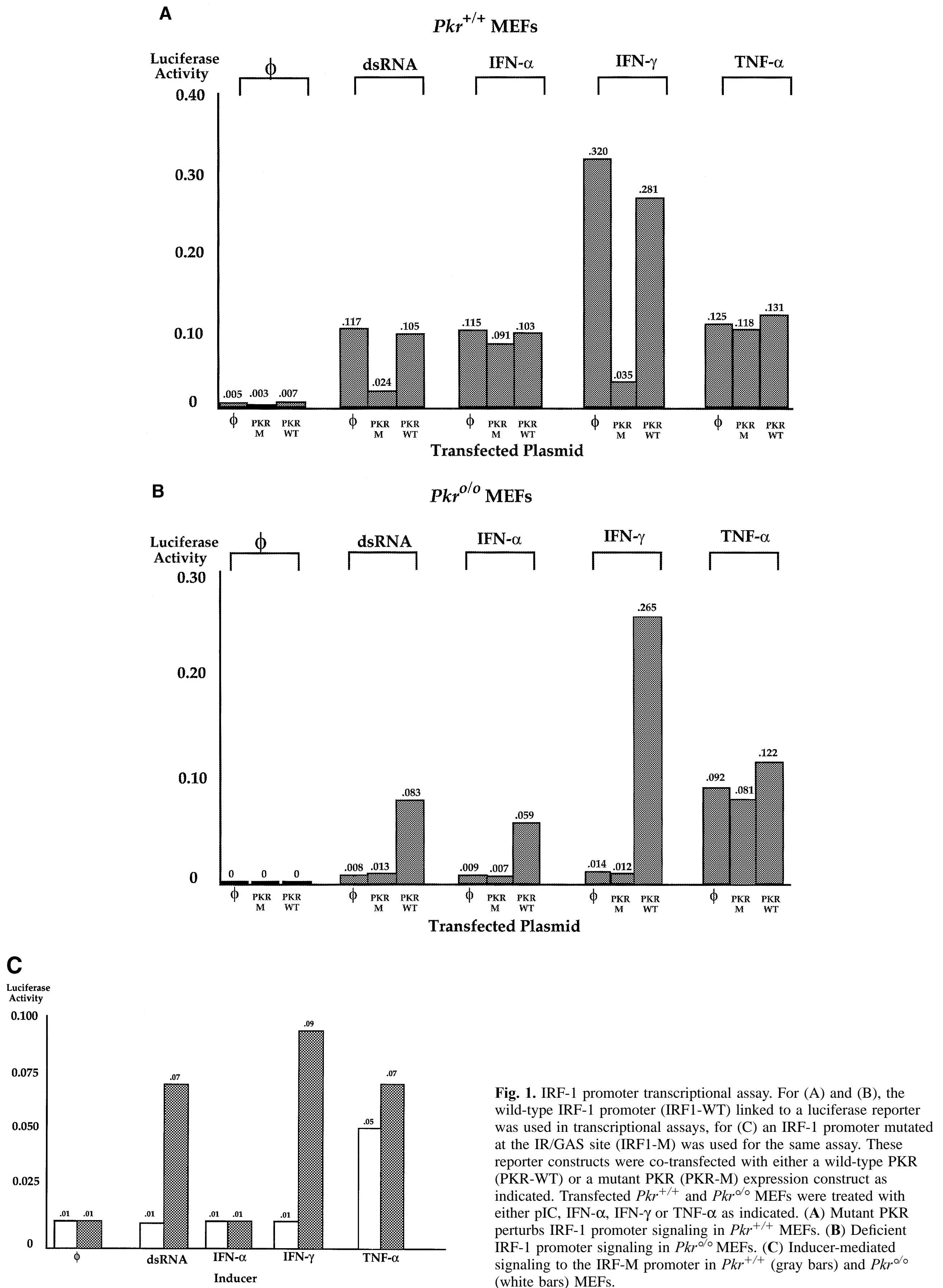

Fig. 1. IRF-1 promoter transcriptional assay. For (A) and (B), the wild-type IRF-1 promoter (IRF1-WT) linked to a luciferase reporter was used in transcriptional assays, for (C) an IRF-1 promoter mutated at the IR/GAS site (IRF1-M) was used for the same assay. These reporter constructs were co-transfected with either a wild-type PKR (PKR-WT) or a mutant PKR (PKR-M) expression construct as indicated. Transfected *Pkr*$^{+/+}$ and *Pkr*$^{o/o}$ MEFs were treated with either pIC, IFN-α, IFN-γ or TNF-α as indicated. (**A**) Mutant PKR perturbs IRF-1 promoter signaling in *Pkr*$^{+/+}$ MEFs. (**B**) Deficient IRF-1 promoter signaling in *Pkr*$^{o/o}$ MEFs. (**C**) Inducer-mediated signaling to the IRF-M promoter in *Pkr*$^{+/+}$ (gray bars) and *Pkr*$^{o/o}$ (white bars) MEFs.

TNF-α (Figure 1A). In contrast, $Pkr^{o/o}$ MEFs transiently transfected with IRF1-WT exhibited a 23-fold, 13-fold and 14-fold decrease in luciferase activity in response to IFN-γ, IFN-α or pIC respectively (Figure 1B). Importantly, TNF-α induction of the IRF1-WT construct was normal in $Pkr^{o/o}$ MEFs (Figure 1B).

To determine whether this signaling defect could be rescued by restoring PKR function, co-transfection of constructs which expressed either wild-type or mutant PKR was performed on cells treated with the different inducers. Co-transfection of a construct which expressed wild-type PKR (PKR-WT) rescued IFN-γ-, IFN-α- and pIC-dependent signaling in $Pkr^{o/o}$ MEFs (Figure 1B), whereas a catalytically inactive PKR (PKR-M) did not restore responsiveness to these inducers (Figure 1B). These results demonstrate that PKR is essential for IFN-γ-, IFN-α- and pIC-dependent signaling to the IRF-1 promoter in MEFs. In accord with this, co-transfection of PKR-M into $Pkr^{+/+}$ MEFs disrupted dsRNA and IFN-γ signaling (Figure 1A). TNF-α (or IFN-α, see Discussion) signaling was unaffected by PKR-M co-expression. We previously have demonstrated a transdominant effect of PKR-M on pIC signaling (Kumar *et al.*, 1994; McMillan *et al.*, 1995) in a murine macrophage cell line.

It has been shown previously that STAT1α (also known as p91) binding to the inverted repeat element/gamma activated sequence (IR/GAS) of the IRF-1 promoter is sufficient to confer IFN-γ (and IFN-α) inducibility (Sims *et al.*, 1993; Haque and Williams, 1994). To determine whether this site could be implicated in PKR-mediated signaling, the IR/GAS element in the IRF-1 promoter was mutated (as described in Materials and methods) to abrogate the binding of STAT1α. Transfection of this construct into $Pkr^{+/+}$ MEFs showed that, as expected, pIC and TNF-α, which activate NF-κB, induced the IRF1-M reporter, whereas IFN-α which activates STAT1α did not (Figure 1C). Surprisingly, IFN-γ was able to induce the IRF1-M construct (albeit at a reduced level), suggesting that IFN-γ is able to activate the IRF-1 promoter in the absence of STAT1α binding (Figure 1C). TNF-α signaled to IRF1-M in both $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs (Figure 1C).

Deficiencies in activation of NF-κB and IRF-1 in $Pkr^{o/o}$ MEFs

In order to obtain mechanistic insights into the signaling defects noted in the absence of PKR, we investigated the activation in $Pkr^{o/o}$ MEFs of different transcription factors known to be regulated by pIC, IFN-α, IFN-γ and TNF-α. To determine whether NF-κB and IR/GAS element binding factors were misregulated in $Pkr^{o/o}$ MEFs, electrophoretic mobility shift assays (EMSAs) were performed using either a κB (position –37 to –48) binding element or the IR/GAS (position –110 to –128) derived from the IRF-1 promoter as radiolabeled probes. pIC treatment of $Pkr^{+/+}$ MEFs resulted in an increased level of five complexes with the IR/GAS element (Figure 2A, lane 4); however, no increase of these complexes occurred in $Pkr^{o/o}$ MEFs (Figure 2A, lane 2). Antibody supershift analysis indicated that these complexes did not contain the IRF family members (IRF-1, IRF-2 or p48), subunits of NF-κB (p50, p65 or rel) or STAT1α (data not shown). Since IFN-γ treatment activated STAT1α binding to the IR/GAS sequence in both $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs (Figure 2B, lanes 3 and 6), we conclude that PKR does not play a role in the STAT activation pathway that leads to DNA binding.

pIC treatment of $Pkr^{+/+}$ MEFs activated factor binding to the putative κB regulatory element from the IRF-1 promoter (position –37 to –48) (Sims *et al.*, 1993) (Figure 3A, lane 7). In contrast, treatment of $Pkr^{o/o}$ MEFs failed to activate this factor (Figure 3A, lane 2). TNF-α elicited complex formation with this κB regulatory element in both $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs (Figure 3A, lanes 5 and 10). The pIC- and TNF-α-activated factors were identified as NF-κB containing the p50 and p65 subunits since p50 antibody supershifted and p65 antibody abolished the complex (Figure 3B, lanes 1, 2, 4, 5, 7 and 8). Although in these EMSAs IFN-γ treatment did not result in measurable NF-κB activation, in cells with lower basal NF-κB activity this is clearly observable (A.Deb, J.Haque and B.R.G.Williams, unpublished observations). NF-κB is also known to positively regulate the IFN-β promoter through the PRDII element (position –55 to –66, Lenardo *et al.*, 1989; Xanthoudakis *et al.*, 1989), and we have shown previously that PKR plays a crucial role in this process (Kumar *et al.*, 1994; Maran *et al.* 1994; Yang *et al.*, 1995). As expected, pIC treatment activates NF-κB which binds the PRDII element in extracts from $Pkr^{+/+}$ MEFs but not in extracts from $Pkr^{o/o}$ MEFs (Figure 3C, lanes 3 and 4). As is the case with the κB binding element from the IRF-1 promoter, TNF-α-dependent NF-κB signaling to the PRDII element was normal in both $Pkr^{o/o}$ and $Pkr^{+/+}$ MEFs (data not shown). This signaling defect is in accord with Northern blot analysis of IFN-β RNA which showed a several-fold reduction in pIC induction in $Pkr^{o/o}$ MEFs (Yang *et al.*, 1995).

There is indirect evidence that PKR may regulate the activity of transcription factor IRF-1 (Watanabe *et al.*, 1991; Kirchhoff *et al.*, 1995). Although the activity of IRF-1 is usually measured by transient transfection assays on reporter constructs, we used EMSA to determine the DNA binding status of IRF-1 and IRF-2 proteins in response to pIC and IFN-γ. Treatment of $Pkr^{+/+}$ MEFs with pIC or IFN-γ resulted in the activation of a factor to a multimerized hexamer element (sequence derived from position –49 to –54, *Gbp-2* promoter) (Miyamoto *et al.*, 1988; Briken *et al.*, 1995) (Figure 4, lanes 3 and 5). These pIC- and IFN-γ-activated factors in $Pkr^{+/+}$ MEFs were identified as IRF-1 since IRF-1-specific antibody abolished these complexes in the EMSA (Figure 4, lane 7 for IFN-γ treatment; data not shown for pIC). In contrast to $Pkr^{+/+}$ MEFs, pIC and IFN-γ activation of IRF-1 was reduced in $Pkr^{o/o}$ MEFs (Figure 4, lanes 4 and 6). IRF-2 was not modulated in either $Pkr^{+/+}$ or $Pkr^{o/o}$ MEFs in response to pIC or IFN-γ (Figure 4, lanes 3–6 and 8). We also noted a low mobility complex that was activated in $Pkr^{+/+}$ but not $Pkr^{o/o}$ MEFs in response to pIC (Figure 4, lane 5). We currently are attempting to identify this PKR-dependent factor.

The data presented above lead to the conclusion that in cells lacking PKR there is a defect in activation of NF-κB and IRF-1 by pIC and IFN-γ. The activation of STATs on the other hand appears to be normal. Consequently, we would predict that genes that are induced largely or exclusively via NF-κB and/or IRF-1 would be activated inefficiently in $Pkr^{o/o}$ MEFs. Northern blot analyses of

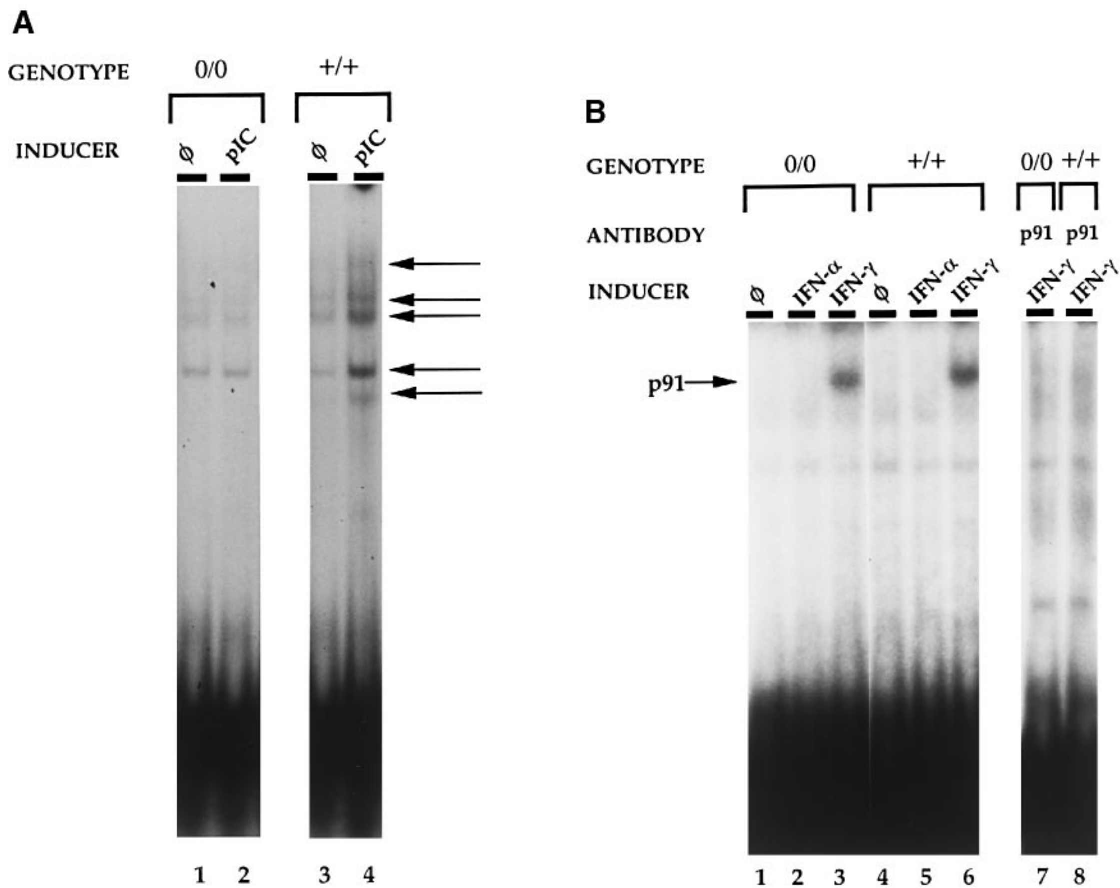

Fig. 2. EMSA showing deficient activation of transcription factors in $Pkr^{o/o}$ MEFs in response to pIC or IFN-γ. $Pkr^{o/o}$ and $Pkr^{+/+}$ MEFs were treated with inducers (pIC at 100 μg/ml with 500 μg/ml DEAE-dextran, IFN-α at 1000 U/ml, IFN-γ at 1000 U/ml or TNF-α at 20 ng/ml for 2 h in serum-free media) and EMSA was performed using 2 μg of nuclear extract and the –110 to –128 IR/GAS sequence from the IRF-1 promoter. (**A**) $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs treated with pIC. (**B**) $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs treated with IFN-α or IFN-γ. Where indicated, nuclear cell extract was pre-incubated with STAT1α/p91 antiserum.

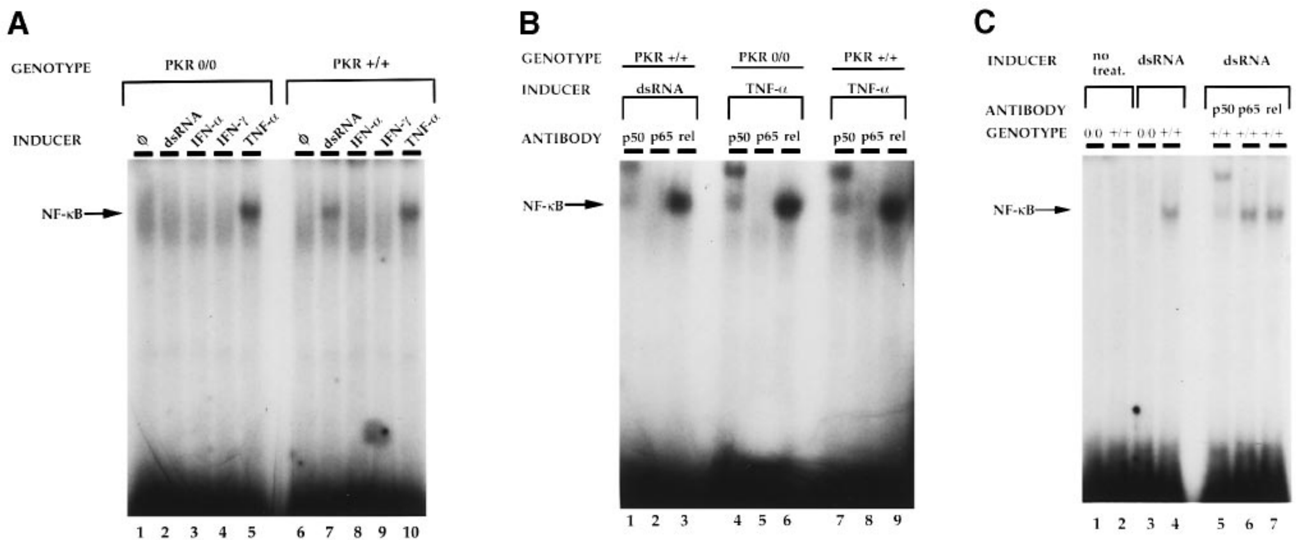

Fig. 3. EMSA showing deficient activation of NF-κB in $Pkr^{o/o}$ MEFs. $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated with pIC, IFN-α, IFN-γ or TNF-α and EMSA was performed using 2 μg of nuclear extract (as described in Figure 2). (**A**) $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated as indicated and EMSA was performed using the –37 to –48 κB sequence from the IRF-1 promoter. (**B**) $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated as indicated and EMSA was performed as in (A). Where indicated, nuclear extract was pre-incubated with p50, p65 or rel antisera. (**C**) $Pkr^{+/+}$ or $Pkr^{o/o}$ MEFs were treated with pIC and EMSA was performed using the –55 to –66 PRDII sequence from the IFN-β promoter; where indicated, nuclear extract was pre-incubated with either p50, p65 or rel antisera.

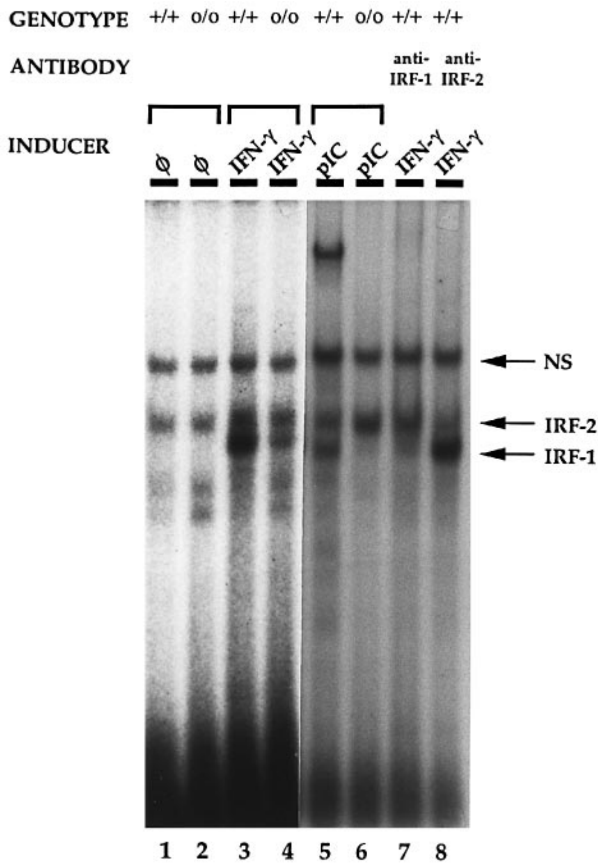

Fig. 4. EMSA showing deficient activation of IRF-1 in $Pkr^{o/o}$ MEFs. $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated with pIC, IFN-α or IFN-γ and EMSA was performed as described in Figure 2, using 10 μg of whole cell extract. The radiolabeled probe is the –49 to –54 hexamer sequence from the *Gbp-2* promoter and, where indicated, whole cell extract was pre-incubated with either IRF-1 or IRF-2 antisera.

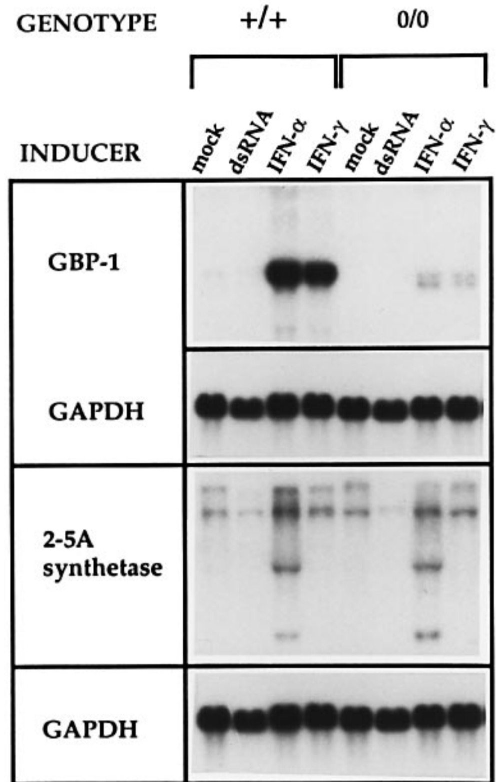

Fig. 5. Northern analysis showing induction of GBP-1 and 2–5A synthetase mRNA in $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs. $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated with pIC, IFN-α or IFN-γ for 4 h and total RNA was harvested. Total RNA (10 μg/lane) was subjected to Northern analysis using either GBP-1 or 2–5A synthetase cDNAs as the radiolabeled probe. After stripping, the filters were hybridized with a GAPDH cDNA probe.

different IFN-regulated genes in $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs support this prediction. 2–5A synthetase gene expression, which is dependent on the transcription factor complex ISGF3, shows no deficiency in induction in $Pkr^{o/o}$ MEFs (Figure 5). In contrast, the murine *Gbp* or class I MHC genes which are dependent on IRF-1 (Briken *et al.*, 1995; Drew *et al.*, 1995) for transcriptional activation by IFN-γ show defects in induction by IFN-α or IFN-γ in $Pkr^{o/o}$ MEFs (Figure 5, and data not shown for MHCI). This was confirmed to occur at the transcription level in the case of the *Gbp*-2 gene by transient transfection analyses. A *Gbp* promoter–reporter construct (GBP2-WT) was responsive to pIC, IFN-α, IFN-γ and TNF-α in $Pkr^{+/+}$ MEFs (Figure 6A). Co-transfection of GBP2-WT with PKR-M reduced pIC, IFN-γ and IFN-α signaling, consistent with a role for endogenous PKR in signal transduction by these inducers. TNF-α signaling was also slightly decreased. In contrast, only TNF-α signaled to GBP2-WT in $Pkr^{o/o}$ MEFs (Figure 6B). However, the pIC, IFN-α and IFN-γ signaling defects were rescued in $Pkr^{o/o}$ MEFs by co-transfection with PKR-WT (Figure 6B). These results are consistent with the Northern blot experiments and define PKR as a critical signal-transducing kinase for genes dependent on IRF-1 and/or NF-κB for transcriptional activation.

Changes in the phosphorylation of PKR induced by IFN-γ

The observed IFN-γ signaling deficiencies in $Pkr^{o/o}$ MEFs beg the question of whether IFN-γ treatment of mammalian cells induces the phosphorylation (and, by implication, activation) of PKR in the absence of added dsRNA. Accordingly, we treated HeLa S3 cells with IFN-γ for different times, immunoprecipitated cell lysates and analyzed the immunoprecipitates by polyacrylamide gel electrophoresis and Western blot. PKR was present constitutively at all time points (Figure 7A, lanes 1–7). However, after 30 min of IFN-γ treatment, a discernible decrease in mobility of PKR can be observed which increases at 4 h (Figure 7A, lane 7). This shift is consistent with an IFN-γ-induced change in the phosphorylation of PKR. To confirm this, two-dimensional gel analysis was performed following IFN-γ treatment and immunoprecipitation of PKR. The results (Figure 7B) show a shift in PKR protein to both the acidic and basic pH range as early as 30 min following IFN-γ treatment and is most pronounced at 1 h. An IFN-γ-induced shift in PKR mobility is also observed when immunopreciptates from $Pkr^{+/+}$ MEFs are analyzed by one-dimensional SDS–PAGE (Figure 7C).

Discussion

We have investigated the molecular basis of a signaling defect in mice devoid of PKR. At physiological levels,

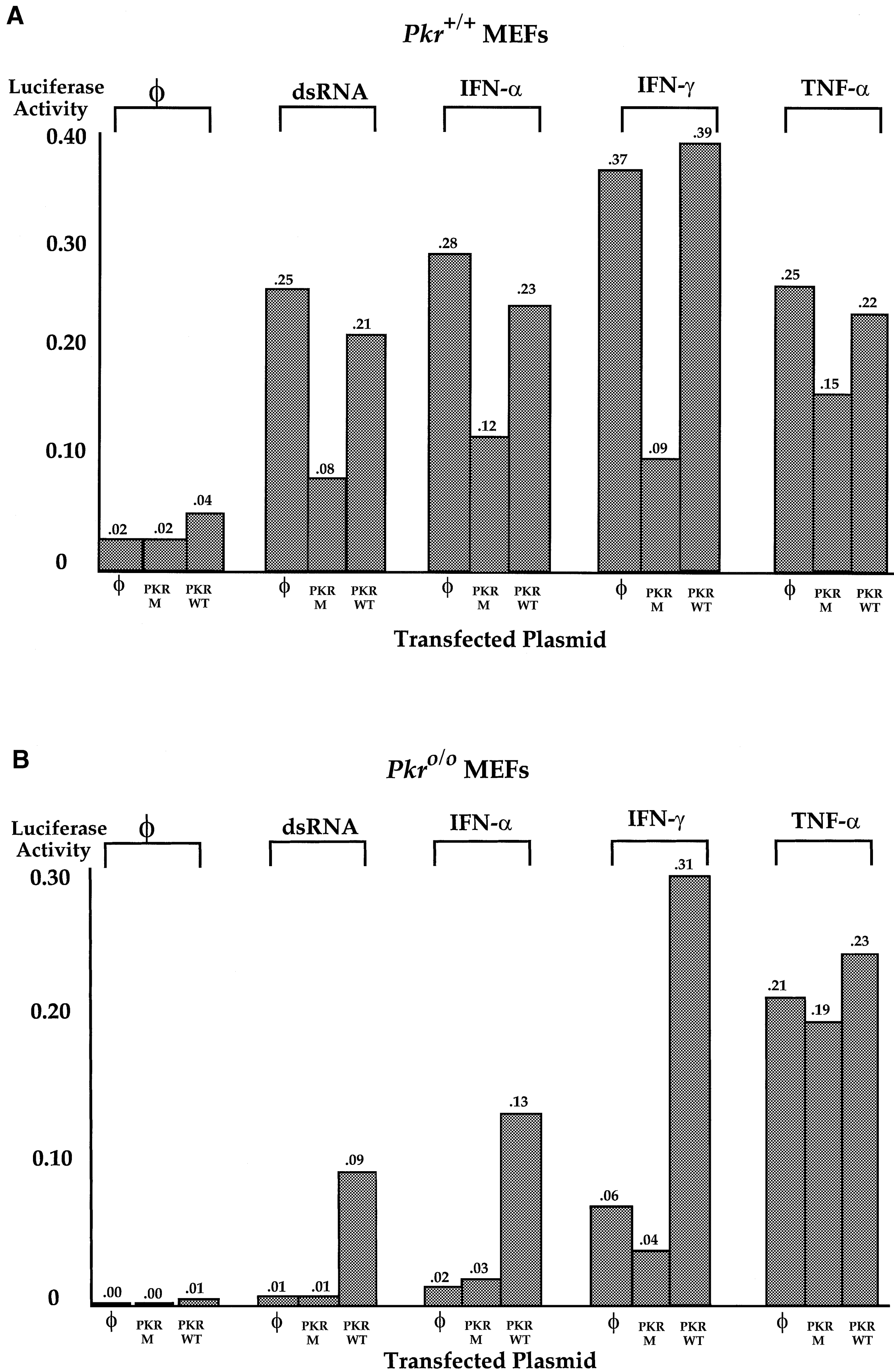

Fig. 6. GBP-2 promoter transcriptional assay. For (A) and (B) the wild-type *Gbp-2* promoter (GBP2-WT) linked to a luciferase reporter was used in transcriptional assays. This reporter construct was co-transfected with either a wild-type PKR (PKR-WT) or a mutant PKR (PKR-M) expression construct as indicated. Transfected $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were treated with either pIC, IFN-α, IFN-γ or TNF-α as indicated. (**A**) Mutant PKR perturbs *Gbp-2* promoter signaling in $Pkr^{+/+}$MEFs. (**B**) Deficient *Gbp-2* promoter signaling in $Pkr^{o/o}$ MEFs.

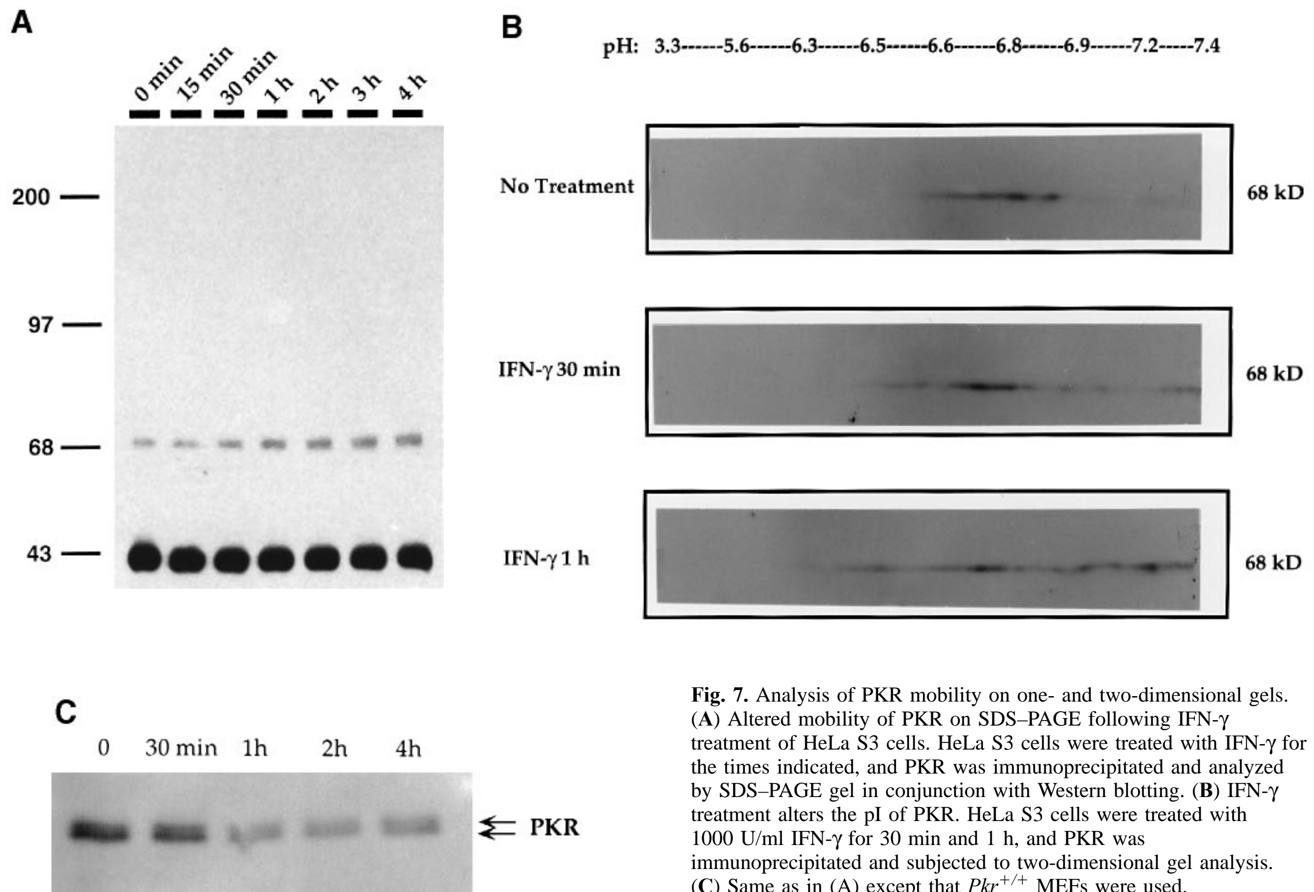

Fig. 7. Analysis of PKR mobility on one- and two-dimensional gels. (**A**) Altered mobility of PKR on SDS–PAGE following IFN-γ treatment of HeLa S3 cells. HeLa S3 cells were treated with IFN-γ for the times indicated, and PKR was immunoprecipitated and analyzed by SDS–PAGE gel in conjunction with Western blotting. (**B**) IFN-γ treatment alters the pI of PKR. HeLa S3 cells were treated with 1000 U/ml IFN-γ for 30 min and 1 h, and PKR was immunoprecipitated and subjected to two-dimensional gel analysis. (**C**) Same as in (A) except that $Pkr^{+/+}$ MEFs were used.

these mice fail to show enhanced protection against encephalomyocarditis virus infection by pIC or IFN-γ, while IFN-α did provide protection analogous to that observed in wild-type animals (Yang *et al.*, 1995). In transcriptional assays in MEFs using the IRF-1 promoter driving the luciferase gene as a reporter, we observed IFN-α, IFN-γ and dsRNA signaling deficiencies in $Pkr^{o/o}$ MEFs, thereby implicating PKR in the regulation of this promoter (Figure 1B). TNF-α signaled to the IRF-1 promoter in both $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs, indicating that this cytokine utilizes a largely non-PKR-dependent signal transduction pathway (Figure 1A and B). However, we did notice a small but consistent decrease in transcriptional activity of different reporter constructs induced by TNF-α when the transdominant PKR construct was co-expressed (Figures 1A and B, and 6A and B). This suggests that a minor component of TNF signaling (probably NF-κB activation) may be contributed through PKR.

When $Pkr^{+/+}$ MEFs were co-transfected with the dominant-negative mutant PKR expression plasmid PKR-M, both IFN-γ and pIC signaling to the IRF-1 promoter–reporter was reduced markedly (Figure 1A). We have shown previously that this mutant is able to reduce pIC signaling to a NF-κB-dependent reporter construct (Kumar *et al.*, 1994; McMillan *et al.*, 1995) and have suggested that the mechanism probably involves the formation of inactive heterodimers between the transfected mutant and endogenous wild-type PKR. The alternative mechanism involving the sequestration of dsRNA was deemed less likely as mutant PKR devoid of dsRNA binding activity were still partially transdominant in the reporter assay (McMillan *et al.*, 1995). This is in accord with the results presented here, where IFN-γ signaling through PKR, which is unlikely to involve dsRNA intermediates, is also inhibited by PKR-M. Interestingly, co-transfection of PKR-M did not affect IFN-α signaling in $Pkr^{+/+}$ MEFs even though IFN-α signaling is defective in $Pkr^{o/o}$ MEFs (Figure 1A and B). Since IFN-α is a more potent inducer of the PKR gene than either IFN-γ or dsRNA (Thomis *et al.*, 1992; Tanaka *et al.*, 1994), it seems likely that IFN-α treatment of the transfectants resulted in higher levels of endogenous PKR overcoming the transdominant effect of PKR-M.

Initially, the obvious target for PKR-mediated signaling appeared to be STAT binding to the IR/GAS site in the IRF-1 promoter. This site was characterized as a target for both IFN-γ and IFN-α signaling (Sims *et al.*, 1993; Haque and Williams, 1994), and previous studies have shown that treatment of cells with IFN-α, IFN-β or IFN-γ activates the binding of STAT1α-containing complexes to the IR/GAS element (Shuai *et al.*, 1993; Darnell *et al.*, 1994; Pine *et al.*, 1994). This site also cooperates with the –43 κB site in synergistic induction of the IRF-1 gene by IFN-γ and TNF-α (Pine, 1995). However, when this site was mutated such that STAT1α binding was abolished, the mutant IRF-1 reporter construct retained pIC, IFN-γ and TNF-α responsiveness (Figure 1C). This mutant promoter was not responsive to IFN-α, indicating that the IR/GAS regulatory element is essential for signaling by IFN-α. Since dsRNA-, TNF-α- and IFN-γ-mediated signaling to IRF1-M was retained (although at a reduced level compared with IRF1-WT), these inducers are most

likely utilizing an alternative regulatory element in the IRF-1 promoter (discussed below). When the same experiment was performed in *Pkr*$^{o/o}$ MEFs, signaling was deficient in response to dsRNA, IFN-α and IFN-γ (Figure 1C). However, TNF-α signaling remained normal, indicating that TNF-α signaling to IRF1-M is not dependent on PKR. These results point to a role for NF-κB in IFN-γ signaling, and a more detailed analysis of the IRF-1 promoter reveals that NF-κB activation contributes to ~30% of the IFN-γ response in HeLa cells (A.Deb, J.Haque and B.R.G.Williams, unpublished observations). TNF-α activates the binding of a p50/p65 NF-κB complex to both the IR/GAS and the putative –43 κB regulatory elements (Pine, 1995). It has also been shown that virus infection of cells activates an NF-κB complex (presumably through dsRNA) to the putative –43 κB site in this promoter (Harada *et al.*, 1994).

The dsRNA signaling deficiency to the IRF-1 promoter in *Pkr*$^{o/o}$ MEFs correlates with NF-κB misregulation, since dsRNA is unable to signal to the –43 κB site in this promoter (Figure 3A, lane 2). Antibody supershift analysis of pIC-treated *Pkr*$^{+/+}$ MEFs indicated that this NF-κB complex consisted of the p50/p65 NF-κB heterodimer (Figure 3B, lanes 1 and 2). As expected, the same signaling deficiency was observed when using the κB site from the IFN-β promoter (Figure 3C, lanes 3 and 4). Interestingly, dsRNA treatment of *Pkr*$^{+/+}$ MEFs enhances the formation of five complexes with the IR/GAS element, and this enhancement is not found with *Pkr*$^{o/o}$ MEFs (Figure 2A). Antibody supershift analysis indicates that these factors do not contain IRF-1, IRF-2, p48, p50, p65, rel or STAT1α (Figure 2A; data not shown for antibody analysis) and, therefore, may represent a novel class of PKR-dependent dsRNA-activated factors. Novel dsRNA-activated transcription factors have been reported and termed dsRNA-activated transcription factors (DRAF) (Daly and Reich, 1993, 1995). The dsRNA-activated factors that we have observed may be related to the DRAF family members or to vesicular stomatitis virus-induced binding proteins (VIBP) (Bovolenta *et al.*, 1995), both of which bind to the ISRE of ISG15.

IFN-γ treatment of both *Pkr*$^{+/+}$ and *Pkr*$^{o/o}$ MEFs resulted in the normal activation of STAT1α binding to the IR/GAS element (Figure 2B, lanes 3 and 6). However, it has been shown that serine phosphorylation of STAT1α at amino acid 727 is required for optimal activity of this factor in the transcriptional response to IFN-γ (Wen *et al.*, 1995). Although binding of STAT1α to DNA in response to IFN-γ treatment of *Pkr*$^{o/o}$ MEFs appeared normal, we cannot exclude a role for PKR in phosphorylation of STAT proteins *in vivo* (Kessler and Levy, 1991). However, *in vitro*, STAT1α does not appear to be a substrate for PKR (V.Flati and B.R.G.Williams, unpublished observations).

Although IFN-α treatment of either *Pkr*$^{+/+}$ or *Pkr*$^{o/o}$ MEFs did not activate the binding of factors to the IRF-1 IR/GAS element (Figure 2B), an IFN-α signaling defect was observed in the *Pkr*$^{o/o}$ MEFs using the more sensitive IRF-1 reporter construct assays. As we have reported previously (Haque and Williams, 1994), IFN-α is able to activate the binding of STAT1 to the IR/GAS element and induce transcription of the IRF-1 gene, but levels of induction vary with different cell types. In the case of either *Pkr*$^{+/+}$ or *Pkr*$^{o/o}$ MEFs, STAT1 activation by IFN-α is not detected by EMSA although activation of ISGF3 binding to an ISRE is normal (data not shown), consistent with the induction of ISRE-dependent genes (Figure 5).

The defect in IFN-γ signaling to the IRF-1 promoter can be correlated with a failure to activate NF-κB. This is apparent from the transfection experiments using the IRF1-M construct (Figure 1C). When this mutation is combined with a mutation in the –43 κB site, the IFN-γ response is blunted further (A.Deb and B.R.G.Williams, unpublished observations). However, Northern blot analysis of RNA extracted from spleens of IFN-treated *Pkr*$^{o/o}$ mice did not reveal a defect in IRF-1 mRNA induction (data not shown). Moreover, there was no apparent defect in IFN-γ induced transcription in *Pkr*$^{o/o}$ MEFs as measured by nuclear run-on assays (our unpublished observations). We assume that in *Pkr*$^{o/o}$ mice STAT1α levels are elevated sufficiently to activate the IRF-1 promoter in the absence of activation of NF-κB. In MEFs transfected with reporter constructs, NF-κB activation is necessary for full activation of the IRF-1 promoter or perhaps NF-κB is activated via the IFN-α-primed alternative pathway due to constitutive IFN-α expression (Yang *et al.*, 1995).

Recently, it has been demonstrated that IRF-1 plays an essential role in the induction of the *Gbp* gene. The *Gbp-2* promoter is regulated by STAT1 binding an IR/GAS site at –536 and IRF-1 acting on a hexamer element at –49 (Briken *et al.*, 1995). However, the –536 IR/GAS site is not required to confer IFN-γ or IFN-α inducibility on this promoter, while the –49 hexamer IRF-1 binding regulatory element is essential. In *IRF-1*$^{o/o}$ ES cells, the *Gbp-2* gene is not induced with either IFN-γ or IFN-α treatment (Kimura *et al.*, 1994; Briken *et al.*, 1995). Northern blot analyses of *Pkr*$^{o/o}$ MEFs treated with pIC, IFN-α or IFN-γ revealed a deficiency in *Gbp* gene induction (Figure 5), consistent with a requirement for PKR activation of IRF-1 (the pIC induction in *Pkr*$^{+/+}$ MEFs was apparent only after 6 h treatments, data not shown). This was confirmed by transfection assays using a *Gbp-2* luciferase construct where pIC, IFN-γ and IFN-α failed to signal to the *Gbp-2* promoter in *Pkr*$^{o/o}$ MEFs (Figure 6B) but could be rescued by co-transfection with PKR-WT (Figure 6B). Taken together with the experiments which demonstrate PKR-M perturbation of signaling to the *Gbp* promoter (Figure 6A) and EMSA showing a lack of IRF-1 activation (Figure 4, lanes 4 and 6), these results demonstrate conclusively that the pIC and IFN-γ signaling deficiencies to the *Gbp*-2 promoter in *Pkr*$^{o/o}$ MEFs can be attributed to defective IRF-1 activation. Although there has been some controversy as to the role which phosphorylation plays in the activation of IRF-1 (Pine *et al.*, 1990), it has been reported that mouse L929 cells, treated with dsRNA and the Ser/Thr kinase inhibitor staurosporin, fail to induce a *tk*–CAT gene construct regulated by the IRF-1 binding site hexamer (Watanabe *et al.*, 1991). Moreover, PKR has been implicated in the IRF-1-dependent induction by LPS of the Ig κ gene (Koromilas *et al.*, 1995).

The class I MHC gene is known to be regulated synergistically by IRF-1 and NF-κB transcription factors in response to Newcastle disease virus, IFN-γ and IFN-α (Ten *et al.*, 1993; Drew *et al.*, 1995). Northern analysis of the MHCI gene in response to IFN-γ and IFN-α in *Pkr*$^{o/o}$ MEFs indicates that the induction of this gene is

reduced as compared with *Pkr*$^{+/+}$ MEFs (Yang *et al.*, 1995), providing further evidence that PKR is utilized as a signal transducer for NF-κB- and IRF-1-dependent genes, and we expect that other genes which depend predominantly on NF-κB and/or IRF-1 activation for induction by IFN-γ or pIC, such as *ICAM*, *VCAM*, *E selectin* or *INOS*, will be shown to utilize PKR as a signal transducer (Williams, 1995). Indirect evidence for a role for PKR in VCAM signaling by pIC in vascular endothelial cells has already been presented (Offerman *et al.*, 1995).

PKR has been implicated directly in dsRNA signaling of NF-κB via IκB phosphorylation (Kumar *et al.*, 1994; Maran *et al.*, 1994; McMillan *et al.*, 1995). However, the mechanism that results in the activation of PKR by IFN-γ is not clear. One- and two-dimensional gel analyses of extracts from IFN-γ-treated cells indicate a rapid modification of PKR in response to IFN-γ treatment consistent with a phosphorylation event (Figure 7A–C). Since this is not the result of tyrosine phosphorylation (V.Flati and B.R.G.Williams, unpublished observation), the linkage of this to IFN-γ-activated Jak kinase activity remains to be defined. PKR may be activated by IFN-γ via the mobilization of intracellular calcium, an early event in IFN-γ signaling (Celada and Schreiber, 1986). Calcium-mediated activation of PKR has been reported recently (Prostko *et al.*, 1995; Srivastava *et al.*, 1995).

PKR has been implicated as a growth factor and cytokine signal transducer in other systems. For example, some evidence has emerged suggesting a role for PKR in platelet-derived growth factor (PDGF) and interleukin-3 (IL-3) signaling (Ito *et al.*, 1994; Mundschau and Faller, 1995). In NFS/N1.H7 mouse cells, IL-3 activates a 97 kDa phosphatase-like protein that transiently associates with PKR resulting in PKR dephosphorylation and inactivation (Ito *et al.*, 1994). Antisense ablation of PKR message or use of the PKR inhibitor 2-aminopurine markedly reduces PDGF induction of the c-*myc*, c-*fos* and *JE* genes in Balb/c/3T3 mouse cells (Mundschau and Faller, 1995), implicating PKR in this pathway.

Here we have shown that PKR acts as an essential molecule in at least some signal transduction pathways initiated by IFN-α, IFN-γ and dsRNA. We have shown previously that PKR acts as a dsRNA signal transducer; here we have shown that PKR plays a selective role as an IFN signal transducer. PKR is essential in regulating genes that are dependent on IRF-1 and NF-κB, which include the *Gbp* and MHCI genes. There is some evidence that the role of IRF-1 may be in maintaining, rather than initiating, the transcriptional activity of ISGs (Iman *et al.*, 1990) and perhaps PKR is involved in this mechanism. The availability of cell lines with a targeted deletion in PKR will allow for a precise description of the role of PKR as a general cytokine signal transducer.

Materials and methods

Promoter transcriptional assays

The IRF-1 promoter (–1308/+1) was cloned upstream of the luciferase reporter gene of the pGL2 vector (Promega) and the construct termed IRF1-WT. This IRF-1 promoter (–1308/+1) was mutated in the inverted repeat regulatory element (IR/GAS) and termed IRF1-M (wild-type IRF-1 IR/GAS sequence; GATTTCCCCGAAATGACGGC: IRF-1 M; GATTTCCCCGAC̲ATGACGGC). The *Gbp*-2 promoter (–550/+1, kindly provided by P.Staeheli) was cloned upstream of the pGL2 vector and termed GBP2-WT. These reporter constructs were used for transcriptional assays. Wild-type PKR (PKR-WT) and a catalytically inactive mutant Lys296→Arg (PKR-M) were cloned into the *Hin*dIII site of pRcCMV vector (Invitrogen) and constitutively expressed under the cytomegalovirus promoter. The Rous sarcoma virus vector RSVβ-gal was used to express β-galactosidase. The plasmids were transfected using the Lipofectin transfection reagent (Gibco, BRL). Briefly, *Pkr*$^{o/o}$ MEFs or *Pkr*$^{+/+}$ MEFs (4×10^6 cells per 100 mM plate) were serum starved for 4 h in αMEM and transfected with the different plasmid cocktails. A typical plasmid transfection cocktail contained 5 μg of IRF1-WT, 5 μg of PKR-WT and 5 μg of RSVβ-gal plasmids and 20 μl of Lipofectin reagent in 600 μl of serum-free media. The plasmid transfection cocktail was added dropwise to 3 ml of serum-free αMEM containing the MEFs. After 6 h, the cells were washed three times with phosphate-buffered saline (PBS) and various inducers were added to the MEFs for 4 h in serum-free αMEM. Murine IFN-γ (Boehringer Mannheim) was added at 1000 U/ml, murine IFN-α (Boehringer Mannheim) at 1000 U/ml, murine rTNF-α at 20 ng/ml (Boehringer Mannheim) and pIC at 100 μg/ml (Pharmacia) with a final concentration of DEAE-dextran of 500 μg/ml (Sigma). The cells were washed three times with 4°C PBS, scraped in PBS at 4°C and transferred to 1.5 ml microfuge tubes. The MEFs were centrifuged in a microfuge for 15 s at 4°C, luciferase assays (Technical bulletin, Promega, Part #TB101 and #TB161) were performed and transfection efficiency was standardized using β-galactosidase assays.

Electrophoretic mobility shift assays

MEFs (2×10^6/10 cm dish) were serum starved for 4 h and treated in serum-free media with 500 μg of DEAE-dextran/ml (mock-induced), 100 μg of pIC and 500 μg DEAE-dextran/ml, 20 ng of murine rTNF-α/ml, 1000 U/ml IFN-γ or 1000 U/ml IFN-α for 2 h. After washing in PBS, cells were resuspended in lysis buffer [10 mM Tris–HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.15% NP-40, 10 μg leupeptin/ml]. After 15 min on ice, the suspension was cleared and nuclei were pelleted by centrifugation in a microfuge for 10 min at 4°C. The pellet was resuspended in an equal volume of nuclear extract buffer [20 mM Tris–HCl (pH 8), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol] and NaCl was adjusted to 400 mM. After 10 min at 4°C, the suspension was vortexed and cleared by centrifugation in a microfuge for 5 min at 4°C. Nuclear extract (2 μg of protein) was subjected to EMSA in 16 μl of 8 mM HEPES (pH 7.0), 8% glycerol, 20 mM KCl, 4 mM $MgCl_2$, 1 mM sodium phosphate, 0.2 mM EDTA containing 0.5 μg poly(dI)·(dC) (Boehringer Mannheim) and 200 000 c.p.m. of [γ-^{32}P]ATP-labeled PRDII regulatory element from the IFN-β promoter (position –55 to –66), κB regulatory element from the IRF-1 promoter (position –37 to –48), IR/GAS element from the IRF-1 promoter (position –110 to –128) or four tandem copies of the hexamer element $(AAGTGA)_4$ from the *Gbp*-2 promoter (position –49 to –54) for 20 min at room temperature. Products were analyzed by electrophoresis through a 4% polyacrylamide gel in 0.5× TBE running buffer. The dried gel was exposed to X-ray film. Where indicated, nuclear extracts were pre-incubated with antibody for 10 min at room temperature prior to addition of the radiolabeled probe. p50, p65, rel, IRF-1 and IRF-2 antibodies (Santa Cruz Biotechnology, Inc.) and p48 polyclonal antibody (Signal Transduction Laboratory Inc.) were used at a final concentration of 0.063 μg/ml. p91 polyclonal antiserum was developed in this laboratory and was used at a dilution of 1:20.

RNA analysis

For Northern blot analysis, 10 μg of total RNA per lane were fractionated on a 1% denaturing agarose gel (Chomczynski and Sacchi, 1987). Northern blots were hybridized with random-primed α-^{32}P-labeled probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fort *et al.*, 1985) and for *Gbp-1* (Briken *et al.*, 1995). The radioactive bands were quantified using a Phosphorimager (Molecular Dynamics) and all values were normalized relative to the GAPDH value in the cognate lane.

Analysis of PKR mobility shift by SDS–PAGE

Approximately 4×10^6 HeLa S3 cells in 100 mm dishes were treated with IFN-γ at 1000 U/ml for the times indicated. The cells were washed three times with 0°C PBS, frozen on a dry-ice–ethanol bath, scraped in 1 ml of lysis buffer [50 mM Tris–HCl (pH 7.8), 1% Triton X-100, 0.1% SDS, 250 mM NaCl, 5 mM EDTA, 2 mM NaPi, 2 mM Na_3VO_4 and 1 mM PMSF] and incubated on ice for 20 min. Cellular debris was removed by centrifugation in a microfuge for 15 min at 4°C. Stock monoclonal PKR antibody was diluted 1:20 in lysis buffer, added to

200 μg of cell extract and incubated on ice for 30 min. To each sample, two volumes of lysis buffer and 20 μl of protein G–Sepharose beads were added and incubated for 3 h at 4°C with rotation. The samples were centrifuged in a microfuge for 10 s at 4°C and the protein G–Sepharose beads were washed twice with 500 μl of lysis buffer at 4°C. Then 30 μl of 2× loading buffer was mixed with each sample and run on a 7.5% SDS–PAGE gel. The proteins were electrotransferred to an Immobilon P membrane (Millipore) which was blocked with 5% Carnation skimmed milk in 1× TBST for 1 h at room temperature, washed for 5 min in 1× TBST and incubated with polyclonal PKR antibody diluted 1:5000 in 1% Carnation skimmed milk in 1× TBST for 2 h at room temperature. The blot was washed extensively with 1× TBST and incubated in goat anti-rabbit IgG secondary antibody (Gibco, BRL) diluted 1:1000 in 1× TBST for 45 min. The blot was washed in 1× TBST, subjected to ECL detection reagent (Amersham) and exposed to X-ray film. For MEFs (Figure 7C), a polyclonal PKR antibody raised in the $Pkr^{o/o}$ mice was used.

Two-dimensional gel analysis of PKR

Total cell extracts from HeLa S3 cells were immunoprecipitated with monoclonal PKR antibodies (as described in Figure 7A) and separated in the first dimension by isoelectric focusing using a pH gradient between pH 7.4 and 3.3. The pH gradient was obtained by mixing equal parts of ampholine ranging from pH 7.9 to 9.0 and pH 8 to 10.5. For the second dimension, a 10% SDS–PAGE was used to separate the protein on the basis of molecular weight. Western analysis was performed using polyclonal PKR antibodies in conjunction with ECL (Amersham). For the detailed protocol, refer to Meurs *et al.* (1992).

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