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Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity

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ABSTRACT **The signaling mechanisms responsible for the induced expression of interferon (IFN) genes by viral infection or double-stranded RNA (dsRNA) are not well understood. Here we investigate the role of the interferon-induced dsRNA-dependent protein kinase PKR in the regulation of IFN induction. Biological activities attributed to PKR include regulating protein synthesis, mediating IFN actions, and functioning as a possible tumor suppressor. Since binding of dsRNA is required for its activation, PKR has been considered as a candidate signal transducer for regulating IFN expression. To examine this role of PKR, loss-of-function phenotypes in stable transformants of promonocytic U-937 cells were achieved by two different strategies, overexpression of an antisense PKR transcript or a dominant negative PKR mutant gene. Both types of PKR-deficient cells were more permissive for viral replication than the control U-937 cells. As the result of PKR loss, they also showed impaired induction of IFN-α and IFN-β genes in response to several inducers—specifically, encephalomyocarditis virus, lipopolysaccharide, and phorbol 12-myristate 13-acetate. Interestingly, while IFN-α induction by dsRNA was impaired in PKR-deficient cells, IFN-β induction remained intact. Loss of PKR function also resulted in decreased antiviral activity as elicited by IFN-α and, to a greater extent, by IFN-γ. These results implicate PKR in the regulation of several antiviral activities.**

Type I interferons (IFNs) regulate diverse biological processes including antiviral activities, cellular growth and differentiation, and modulation of immune functions (1, 2). The induced expression of type I IFN genes, which include the IFN-α and IFN-β gene families, is detected typically following viral infections. Previous studies have identified promoter elements and transcription factors involved in regulating the expression of type I IFNs (3–5). However, it remains unclear what are the particular biochemical cues that signify viral infections to the cell and the signaling mechanisms involved. Since many forms of double-stranded RNA (dsRNA) are capable of inducing type I IFNs, this led to suggestions that the common inducing molecule among different viruses was a viral replicative intermediate containing dsRNA (6). It seems reasonable, therefore, to hypothesize that the regulation of IFN genes and antiviral activities involves effector proteins responsive to dsRNA.

Of the many RNA-binding proteins, the few which are capable of binding dsRNA are distinguished by a conserved 65- to 68-amino acid "dsRNA-binding domain" (7). Among these, the IFN-induced dsRNA-dependent protein kinase PKR is the only one with kinase function. PKR is a serine/threonine kinase whose enzymatic activation requires dsRNA binding and consequent autophosphorylation (8, 9). The best characterized substrate of PKR is the α subunit of eukaryotic initiation factor 2, which once phosphorylated leads to inhibition of cellular and viral protein synthesis (10). This function of PKR has been suggested as one of the mechanisms responsible for mediating the antiviral and antiproliferative activities of IFNs. An additional putative function for PKR is its role as a signal transducer, since 2-aminopurine, a relatively specific inhibitor of PKR, can block the induction of IFN-α and IFN-β genes by viral infection or dsRNA (11, 12). In support of this, Kumar *et al.* (13) have demonstrated that PKR can phosphorylate IκBα, resulting in the release and activation of the transcription factor NF-κB (13). Given the well-characterized NF-κB site in the IFN-β promoter and that dsRNA alone can induce NF-κB activity (14), it has been postulated that PKR mediates the induction of IFN-β transcription by dsRNA.

To investigate the role of PKR in IFN gene regulation and cellular antiviral responses, we have utilized two different strategies to achieve a loss-of-PKR-function phenotype. This involved overexpression of a dominant negative PKR mutant gene, encoding [Arg^{296}]PKR, or an antisense PKR gene in stable transformants of a promonocytic cell line, U-937. Monocytes represent a primary source of type I IFNs *in vivo* and accordingly, we and others have found U-937 cells useful for studying IFN-α and IFN-β gene expression (15). The mutant [Arg^{296}]PKR contains a single amino acid substitution of arginine for the invariant lysine in catalytic domain II at position 296 and is a dominant negative protein which can specifically suppress the activity of endogenous wild-type PKR *in vivo* (14, 15). An alternative approach to specifically inhibit gene expression involves antisense strategies. Recently, Maran *et al.* (16) showed that 2′-5′-oligoadenylate-linked antisense oligonucleotides specific for PKR suppressed PKR activity and NF-κB activation by dsRNA. However, it is not known whether IFN production or IFN-mediated antiviral responses were affected as a result of suppressed PKR function in the above studies. Here we report that loss of PKR activity in U-937 cells results in multiple defects both in IFN production and in antiviral responsiveness to IFN-α and IFN-γ.

METHODS AND MATERIALS

Plasmids and Stable Transformants. The wild-type human PKR gene and the dominant negative [Arg^{296}]PKR mutant gene were released by *Hin*dIII digestion from the plasmids pBSKS and p6M (provided by B. R. G. Williams, Cleveland Clinic Research Institute, Cleveland), respectively. They were then subcloned into the eukaryotic expression vector pRC-CMV (Invitrogen) to generate the plasmids used in this study, namely, pPKR-AS (antisense) and p[Arg^{296}]PKR. Stable transformants were generated by electroporation of U-937 cells with 10 μg of each plasmid by use of a Gene Pulser apparatus (Bio-Rad). Clonal lines were obtained by selection with Geneticin (400 μg/ml; GIBCO/BRL) and limiting dilu-

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Abbreviations: IFN, interferon; dsRNA, double-stranded RNA; EMCV, encephalomyocarditis virus; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription–polymerase chain reaction; $TCID_{50}$, median tissue culture infective dose.

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tion cloning. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and Geneticin.

PKR Analysis. The PKR autophosphorylation assay was performed essentially as described by Maran *et al.* (16), with the following modifications. Cell extracts (100 μg) were incubated with poly(I)·poly(C)-cellulose for 1 hr on ice, washed three times, and incubated for 30 min at 30°C in 50 μl of reaction buffer [20 mM Hepes, pH 7.5/50 mM KCl/5 mM 2-mercaptoethanol/1.5 mM $Mg(OAc)_2$/1.5 mM $MnCl_2$] containing 1 μCi (37 kBq) of [γ-^{32}P]ATP. Samples were analyzed by SDS/10% PAGE and autoradiography. For immunoblot analysis of PKR, cell extract proteins (100 μg) were separated by SDS/10% PAGE and electrotransferred onto nitrocellulose membranes. Membranes were incubated with anti-PKR monoclonal antibody at 1:1000 in "Blotto" (5% nonfat dry milk/0.05% Tween 20 in Tris-buffered saline), with final detection provided by horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotech) and a chemiluminesence method (ECL; Amersham).

Encephalomyocarditis Virus (EMCV) Replication and IFN Assay. For determination of EMCV replication, U-937-derived transformants were cultured in complete medium alone or pretreated with recombinant human IFN-α2 (Schering) or IFN-γ (Amgen Biologicals) for 18 hr. Following two washes with phosphate-buffered saline, the cells were incubated with the indicated amounts of EMCV in serum-free medium for 2 hr. The cells were washed again and 10^6 cells per sample were suspended in 1 ml of medium containing 1% fetal bovine serum. Samples were collected at the required time points and lysed by three rounds of freeze–thaw. Fourfold serial dilutions of the samples were added onto L929 mouse fibroblast monolayers and incubated for 48 hr before the monolayers were stained with 0.05% crystal violet to determine cytopathic effects and median tissue culture infective dose ($TCID_{50}$). In assays of IFN production, U-937-derived transformants were similarly pretreated with IFNs as described above. Then, the cells were incubated with inducers [poly(I)·poly(C) (Pharmacia), EMCV, lipopolysaccharide (LPS; Sigma), or phorbol 12-myristate 13-acetate (PMA; Sigma)] for 2 hr. Cells were washed and cultured in medium containing 1% fetal bovine serum. Supernatants were collected after 24 hr and IFN activity was measured by a bioassay (17).

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). Priming and induction of U-937-derived transformants were performed as above. Total RNA was extracted from cell samples by an acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed with 2 μg of each RNA sample primed with random hexamer in a 25-μl reaction volume with 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). All PCRs were performed with 2 μl out of each cDNA mixture in a 50-μl reaction volume containing 50 pmol of each upstream and downstream primer, 2 units of *Taq* DNA polymerase (Promega), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 10× reaction buffer. IFN-α PCR used consensus primers, capable of amplifying all 14 known human IFN-α subtype genes, 5′-GGAAGCTTYCTC-CTGYYTGAWGGACAGA-3′ and 5′-GGGGATCCTCTGA-CAACCTCCCANGCACA-3′, which generate an expected product of 372 bp. IFN-β PCR used primers, 5′-GTGTCAG-AAGCTCCTGTGGC-3′ and 5′-CTTCAGTTTCGGAGGT-AACC-3′, which generate an expected product of 456 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR used primers, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CA-AAGTTGTCATGGATGACC-3′, which generate an expected product of 196 bp.

RESULTS

Characterization of PKR-Deficient Stable Transformants. Stable transformant cell lines were obtained by transfecting U-937 cells with the following expression plasmids. Five representative cell lines were selected for characterization: (*i*) U937-neo was the control cell line transfected with the parental vector, pRC/CMV; (*ii*) U937-AS1 and U937-AS3 were independent clones transfected with pPKR-AS; and (*iii*) U937-M13 and U937-M22 were independent clones transfected with p[Arg^{296}]PKR. PKR kinase activity was measured with an assay that uses poly(I)·poly(C)-cellulose for binding and activation of PKR enzyme. IFN-treated HeLa and mouse L929 cells were used as positive controls (Fig. 1*A*, lanes 1 and 8), since PKR activity in these cells had been described (9). Similar to the untransfected U-937 parents, U937-neo cells contained basal levels of PKR activity which increased following treatment with IFN-α (Fig. 1*A*, lanes 2 and 3). In contrast, PKR activity was not detected in any of the four cell lines transformed with pPKR-AS or p[Arg^{296}]PKR (results not shown). Furthermore, PKR activity was not restored in these cells by treatment with IFN-α (Fig. 1*A*, lanes 4–7) or IFN-γ (results not shown). To further confirm the inhibition of PKR expression in the pPKR-AS-transformed cells, Western blot analysis was performed with a monoclonal antibody specific for human PKR. Basal levels of PKR protein were detectable in U937-neo cells (Fig. 1*B*, lane 1), which increased following treatment with IFN-α or IFN-γ (lanes 2 and 3). In contrast, PKR expression was diminished in U937-AS1 and U937-AS3 cells (Fig. 1*B*, lanes 4 and 6) and did not increase with IFN-α treatment (lanes 5 and 7).

Enhanced EMCV Replication in PKR-Deficient Cells. We investigated whether loss of PKR function would affect the rate of EMCV replication. In control U937-neo cells following challenge with EMCV at 0.1 $TCID_{50}$ per cell, viral titers peaked at ≈10^4 $TCID_{50}$/ml after 48 hr (Fig. 2*A*). However, in U937-AS1 and U937-M22 cells, EMCV replication was substantially higher, reaching titers of 10^4–10^5 $TCID_{50}$/ml after only 24 hr and 10^8 $TCID_{50}$/ml by 48 hr, a 1000-fold increase over U937-neo cells. With a lower virus inoculum, 0.001

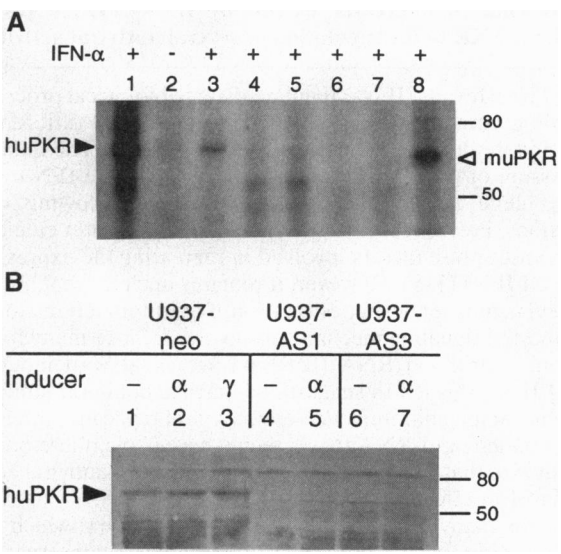

FIG. 1. PKR activity and protein levels in U-937-derived stable transformant cell lines. (*A*) PKR activity was determined by a poly(I)·poly(C)-cellulose assay for PKR autophosphorylation. Cell extracts were prepared from HeLa cells and the various U-937-derived cell lines following incubation with or without recombinant human IFN-α2 (200 units/ml) as indicated, while L929 cells were similarly treated with mouse IFN-α/β. Lane 1, HeLa; lanes 2 and 3, U937-neo; lane 4, U937-AS1; lane 5, U937-AS3; lane 6, U937-M13; lane 7, U937-M22; lane 8, L929. Positions of the human (68 kDa) and murine (65 kDa) PKR proteins and the molecular size standards (80 and 50 kDa) are indicated. (*B*) Cell extracts were prepared as above after induction with IFN-α or -γ, and PKR protein levels were determined by Western blot analysis.

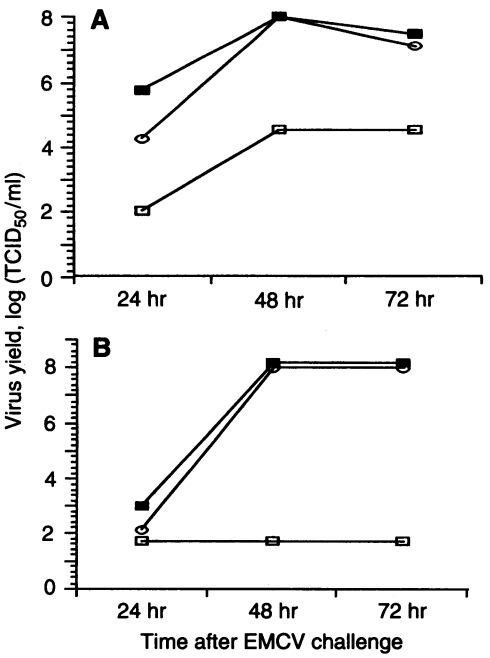

FIG. 2. Kinetics of EMCV replication are enhanced in PKR-deficient cells. The U937-neo (□), U937-M22 (○), and U937-AS1 (■) cell lines were challenged with EMCV at 0.1 (*A*) or 0.001 (*B*) $TCID_{50}$ per cell. Samples were harvested at the indicated times and viral yields were measured in terms of $TCID_{50}$.

$TCID_{50}$ per cell, more dramatic differences in EMCV susceptibility were observed. While EMCV replication in U937-neo cells did not exceed 10^2 $TCID_{50}$/ml, high viral titers of 10^8 $TCID_{50}$/ml were attained in both U937-AS1 and U937-M22 cells (Fig. 2*B*).

A Role for PKR in IFN Expression. The commonly invoked model for IFN action proposes that an essential function for the IFNs secreted from virus-infected cells is to protect neighboring cells against subsequent rounds of infection by progeny virus (1, 2, 5). Accordingly, the higher rates of EMCV replication in the PKR-deficient cells could have resulted from impaired IFN production or defective antiviral responses to the paracrine actions of induced IFNs. Here, we first showed that loss of PKR activity resulted in impaired IFN production in both U937-AS1 and U937-M22 cells compared with the control U937-neo cells. With U937-neo cells, induction by EMCV alone produced substantial amounts of secreted IFN protein (512 units/ml; Fig. 3*A*). In a phenomenon known as IFN priming, pretreatment of the IFN-producer cells with even small amounts of IFN enhances subsequent IFN production upon stimulation with inducers (18). Consistent with this, priming of U937-neo cells with either IFN-α or IFN-γ resulted in increased production of EMCV-induced IFN activity (Fig. 3*A*). The effects of priming were more significant for IFN induction by nonviral inducers. Stimulation of U937-neo cells with poly(I)·poly(C), LPS, or PMA alone did not induce any detectable levels of IFN unless the cells had been primed with IFN-α or IFN-γ (Fig. 3*A*). In contrast, IFN production was significantly impaired in both types of PKR-deficient cells under each of the above induction conditions (Fig. 3*A*). Compared with U937-neo cells, IFN levels from U937-AS1 and U937-M22 cells following EMCV induction were reduced nearly 50-fold (≤16 units/ml). Further, this impairment was not alleviated by IFN-α or IFN-γ priming. Also, IFN induction by poly(I)·poly(C), LPS, or PMA, following IFN priming, was impaired as well in both PKR-deficient cell lines (≤8 units/ml). The IFN activity produced by U-937 cells was composed

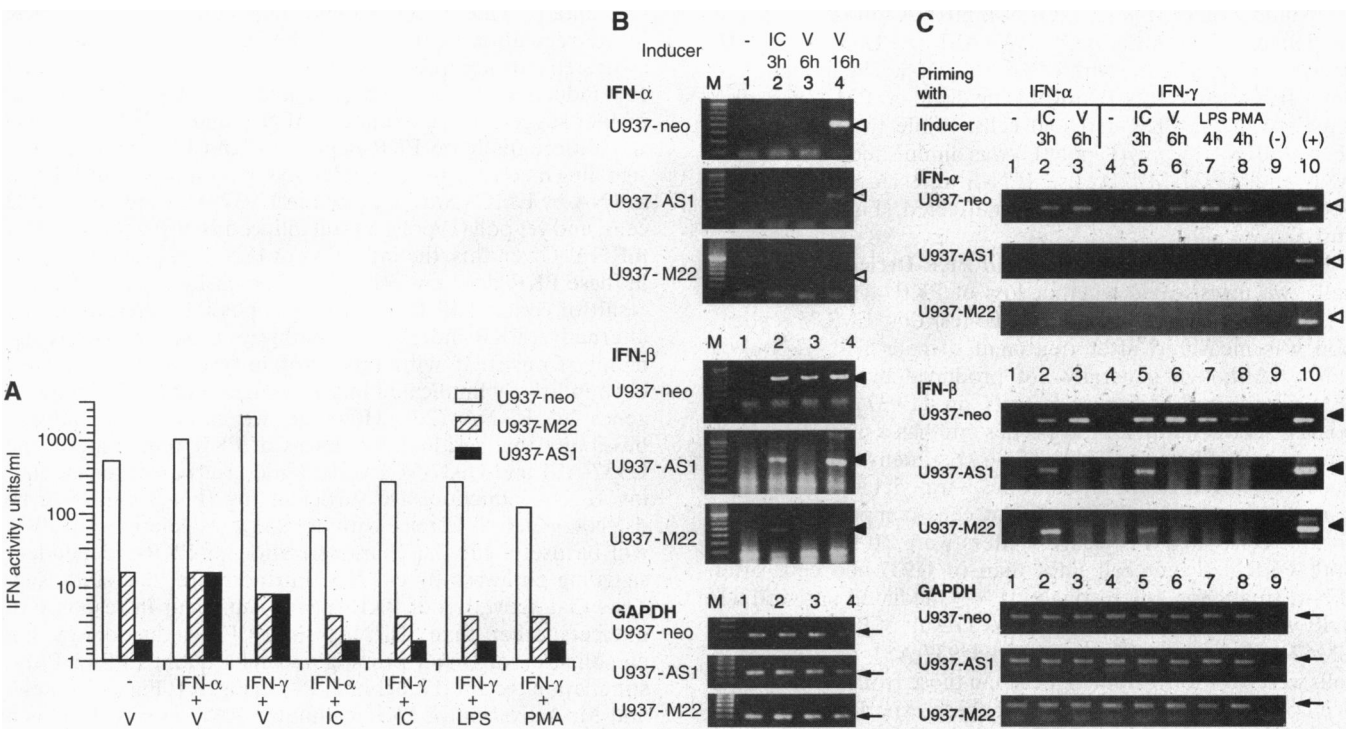

FIG. 3. IFN expression is impaired in PKR-deficient cells. (*A*) The different U937 cell lines were primed where specified with IFN-α or IFN-γ (200 units/ml). Cells were then incubated with the indicated inducers and IFN levels from each sample were determined. Following our induction conditions, U937 cells did not produce detectable IFN in response to poly(I)·poly(C) (IC, 100 μg/ml), LPS (50 ng/ml), or PMA (50 nM) alone, or after IFN priming in the absence of inducers. V, EMCV. (*B* and *C*) Cells were primed as described above and then incubated with inducers for the indicated times. IFN-α, IFN-β, and GAPDH mRNA were detected by RT–PCR. PCR products were visualized by ethidium bromide staining after 1.5% agarose gel electrophoresis. Negative controls (−) represent PCR performed on RT reagents without sample RNA. Positive controls (+) represent PCR amplification of 1.0 ng of human genomic DNA. DNA markers (M) represent a ladder of 100-bp increments.

of both IFN-α (>80%) and IFN-β proteins, as determined with neutralizing antibodies (results not shown).

To examine the role of PKR in regulating the differential expression of IFN-α and IFN-β genes, steady-state levels of the respective IFN mRNAs were determined by RT–PCR. Optimal induction of IFN-α mRNA in U937-neo cells by EMCV alone required stimulation for 16 hr (Fig. 3*B*, lane 4). Peak induction of IFN-β mRNA by poly(I)·poly(C) or EMCV alone was more rapid, occurring at 3 or 6 hr, respectively (Fig. 3*B*, lanes 2 and 3). In contrast, the induction of IFN-α and IFN-β mRNA following viral infection was impaired in both PKR-deficient cell lines. IFN-α mRNA induction by EMCV at 16 hr was diminished in U937-AS1 and U937-M22 cells (Fig. 3*B*, lane 4). Also, the early EMCV induction of IFN-β mRNA at 6 hr was absent (Fig. 3*B*, lane 3). However, IFN-β mRNA remained inducible in response to poly(I)·poly(C) (Fig. 3*B*, lane 2), and various levels were induced by EMCV only after 16 hr (lane 4) in the PKR-deficient cell lines.

Next, the effect of PKR loss on the induction of IFN mRNAs was examined in IFN-primed cells. We have determined that priming enhances IFN-α induction in U-937 cells. Stimulation of U937-neo cells with poly(I)·poly(C) alone resulted in weak IFN-α mRNA induction after 16 hr (results not shown). However, following priming with either IFN-α or IFN-γ, poly(I)·poly(C) stimulation resulted in a rapid induction of IFN-α mRNA, peaking after 3 hr (Fig. 3*C*, lanes 2 and 5, versus Fig. 3*B*, lane 2). Similarly, IFN priming also enabled a rapid induction of IFN-α mRNA in response to EMCV, peaking after 6 hr (Fig. 3*C*, lanes 3 and 6 versus Fig. 3*B*, lane 3). Further, the induction of IFN-α mRNA in U937-neo cells by LPS or PMA was dependent on priming with IFN-γ (Fig. 3*C*, lanes 7 and 8). Consistent with the patterns observed earlier for IFN protein production, IFN mRNA expression was impaired in both PKR-deficient cell lines despite priming. The early induction of IFN-α and IFN-β mRNA following EMCV challenge was absent in both U937-AS1 and U937-M22 cells, irrespective of priming with IFN-α or IFN-γ. The induction of both IFN-α and IFN-β mRNA by LPS or PMA was also impaired in the PKR-deficient cells. While the induction of IFN-α mRNA by poly(I)·poly(C) was diminished in the U937-AS1 and U937-M22 cells, IFN-β mRNA induction by poly(I)·poly(C) again appeared unaffected (Fig. 3*C*, lanes 2 and 5).

Impaired IFN Responsiveness in PKR-Deficient Cells. Finally, we investigated whether loss of PKR activity affected IFN-induced antiviral responses. To test this, EMCV replication was measured after treatment of cells with IFN-α or IFN-γ. Although generally not produced by macrophages, IFN-γ was studied for its effects on U-937 cells since it possesses direct antiviral properties and has a primary role in macrophage activation (19). While treatment with IFNs reduced EMCV titers in all cell lines, viral yields were consistently higher in the PKR-deficient cells compared with the control cells (Fig. 4*A*). EMCV titers were 10-fold higher in both PKR-deficient cell lines than in U937-neo cells after IFN-α treatment. Interestingly, IFN-γ-mediated antiviral activity was more severely impaired as a result of PKR loss, since EMCV titers from IFN-γ-primed U937-AS1 or U937-M22 cells were 10^2- to 10^3-fold higher than those from control cells. We considered the possibility that these experimental conditions involving a relatively low virus inoculum may have magnified the differences in IFN-mediated antiviral responses between these cell lines. However, similar results were observed when we applied more stringent conditions for comparing IFN responsiveness, by increasing the EMCV inoculum 100-fold and harvesting samples earlier, at 24 hr rather than 48 hr (Fig. 4*B*).

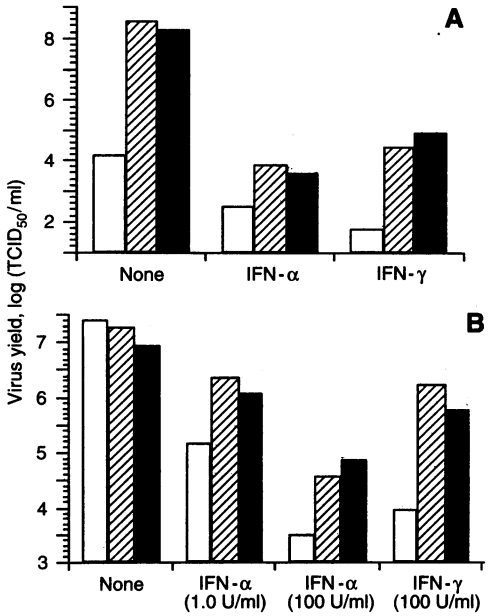

FIG. 4. Inhibition of EMCV replication by IFN-α or IFN-γ is impaired in PKR-deficient cells. (*A*) U937-neo (open bars), U937-AS1 (filled bars), and U937-M22 (hatched bars) cells were cultured in the absence or presence of the indicated concentrations of IFN-α or IFN-γ at 100 units/ml for 18 hr and challenged with EMCV at 0.1 $TCID_{50}$ per cell. Samples were harvested after 48 hr for determination of virus yield. (*B*) Cells (bars as in *A*) were similarly pretreated with or without IFNs [1 or 100 units (U)/ml], except they were challenged with EMCV at 10.0 $TCID_{50}$ per cell. Samples were then harvested after 24 hr for determination of virus yield.

DISCUSSION

Our data provide direct evidence implicating a role for PKR in the regulation of IFN-α and IFN-β genes. We have demonstrated that suppression of PKR function results in impaired IFN induction at both the protein and mRNA levels. Our data further suggest that induction of IFN-α and IFN-β genes may rely differentially on PKR-dependent and PKR-independent signaling mechanisms. The induction of both IFN-α and IFN-β mRNA by EMCV was impaired in U937-AS1 and U937-M22 cells, and yet poly(I)·poly(C) still induced IFN-β but not IFN-α mRNA. Given this, the induction of IFN-β by poly(I)·poly(C) in these PKR-deficient cells cannot be easily explained as the result of residual PKR activity. It is possible, therefore, that alternative, PKR-independent pathways exist for dsRNA signaling. Consistent with this, protein-tyrosine kinases have been indirectly implicated in the induction of IFN-stimulated genes by dsRNA (20). However, we cannot rule out the possibility that residual, low levels of PKR remaining in the U937-AS1 and U937-M22 cells, while insufficient for mediating IFN-α induction, are sufficient for IFN-β induction by dsRNA. Analysis of mice with homozygous deletions for PKR will be useful for the characterization of PKR-independent signaling pathways by dsRNA. Furthermore, this study suggests that activation of PKR *in vivo* can occur in response to inducers other than dsRNA, since IFN induction by the combination of IFN-γ priming and subsequent LPS or PMA stimulation required functional PKR (Fig. 3*A*; Fig. 3*C*, lanes 7 and 8). Activation of PKR without dsRNA *in vitro* has been described using heparin and other polyanionic molecules, and PKR activation *in vivo* was observed following interleukin 3 deprivation of an interleukin 3-dependent murine cell line (21, 22).

Our results also provide evidence for the participation of PKR in mediating the antiviral actions of IFN-α and IFN-γ. While PKR has not been commonly considered as a mediator

of IFN-γ actions, the presence of a consensus IFN-γ responsive element, GAS, within the PKR promoter suggests that PKR may be regulated by IFN-γ (23). Consistent with this, our results demonstrated the induction of PKR protein levels by IFN-γ (Fig. 1*B*). Previous studies have linked several proteins, including the Mx, 2-5-oligoadenylate synthetase, and 2-5-oligoadenylate-dependent RNase proteins, to IFN-α-induced antiviral activities (24–26). In particular, stable expression of the human PKR gene in mouse cells confers partial resistance to EMCV (27). Also, in embryonic fibroblasts from mice deleted for the gene encoding the IFN-responsive transcription factor IRF-1, anti-EMCV activity by IFN-γ was even more impaired than the reduced IFN-α-mediated activity, characteristics similar to the PKR-deficient cells in this report (28). It is likely that the concerted actions of several genes, including PKR, contribute to the antiviral activities of IFN-α and IFN-γ. Interestingly, a tumor-suppressor function for PKR has been suggested from studies showing that a malignant transformation phenotype correlates with overexpression of dominant negative PKR proteins (29, 30). Since IFNs have direct antitumor and antiproliferative activities (31), it is possible that the IFN-related deficiencies resulting from loss of PKR activity noted here may represent mechanisms which contribute to a transformation process.

PKR has been suggested to be important for controlling viral replication. However, many viruses, including adenovirus, influenza virus, vaccinia virus, and human immunodeficiency virus, possess mechanisms for inactivating PKR function as means to evade the antiviral actions of the IFN system (32). We have shown that specific suppression of PKR in U-937 cells resulted in a profound inability to restrict EMCV replication and that this was due to the impairment of at least two biological functions, type I IFN expression and IFN-mediated antiviral responses. While it remains unclear which cellular proteins mediate these activities in pathways downstream from PKR, transcription factors including IRF-1, ATF-2/c-Jun, and the STAT family, already implicated with regulation of type I IFNs and IFN-stimulated genes, are possible substrates for PKR (3, 4, 33).

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