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Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase

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Double-stranded RNA-dependent protein kinase (PKR) has been implicated in interferon (IFN) induction, antiviral response and tumor suppression. We have generated mice devoid of functional PKR (*Pkr*$^{o/o}$). Although the mice are physically normal and the induction of type I IFN genes by poly(I)·poly(C) (pIC) and virus is unimpaired, the antiviral response induced by IFN-γ and pIC was diminished. However, in embryo fibroblasts from *Pkr* knockout mice, the induction of type I IFN as well as the activation of NF-κB by pIC, were strongly impaired but restored by priming with IFN. Thus, PKR is not directly essential for responses to pIC, and a pIC-responsive system independent of PKR is induced by IFN. No evidence of the tumor suppressor activity of PKR was demonstrated.

Keywords: antiviral response/double-stranded RNA-dependent protein kinase/interferon induction/tumor suppression

Introduction

Double-stranded RNA-dependent protein kinase (PKR), a serine/threonine protein kinase, is one of the best characterized of the many proteins induced by type I interferon (IFN; for reviews see Hovanessian, 1989, 1993; Katze, 1992; Lengyel, 1993; Samuel, 1993). Upon activation by double-stranded RNA (dsRNA; Galabru *et al.*, 1989) or by stem–loop structures of certain single-stranded RNAs (SenGupta *et al.*, 1990; Roy *et al.*, 1991), the murine 65 kDa (p65^{muPKR}) or human 68 kDa (p68^{huPKR}) protein autophosphorylates and phosphorylates the eukaryotic initiation factor eIF-2, thereby throttling protein synthesis (Galabru and Hovanessian, 1987; Hovanessian and Galabru, 1987; Hershey, 1989; Chong *et al.*, 1992). It was proposed early on that PKR plays an important role in the cellular antiviral response, and it has been shown recently that the overexpression of p68^{huPKR} in cultured cells inhibits the replication of vaccinia virus (Lee and Esteban, 1993) and encephalomyocarditis virus (EMCV), but not vesicular stomatitis virus (VSV; Meurs *et al.*, 1992). The postulated biological significance of PKR in antiviral responses is supported by the finding that several viruses have developed different strategies for abrogating PKR activity (for a review see Katze, 1992). In addition, on the basis of indirect evidence, it has been suggested that PKR may be essential for the induction of IFN-β gene expression by both virus and double-stranded RNA (Marcus and Sekellick, 1988; Zinn *et al.*, 1988). A role for PKR has also been proposed in the activation of at least some IFN-inducible genes in HeLa cell lines (Tiwari *et al.*, 1988).

More recently, evidence has been proffered that PKR is also involved in growth control. The expression of p68^{huPKR} in yeast leads to the phosphorylation of eIF-2 and slows down the proliferation of the transformed cells (Chong *et al.*, 1992; Dever *et al.*, 1993). Interestingly, the introduction of expression plasmids encoding enzymatically inactive human PKR into NIH 3T3 cells led to transformed cell lines capable of generating tumors in nude mice. This result has been ascribed to a transdominant inhibitory effect of the mutant enzyme on the resident wild-type PKR resulting in the derepression of growth, and led to the designation of PKR as a tumor suppressor gene (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993).

To determine whether PKR plays an essential role in the activation of IFN genes and IFN-stimulated genes and in growth control, we generated mice devoid of p65^{muPKR} by disrupting the *Pkr* gene by homologous recombination. Homozygous *Pkr* knockout (*Pkr*$^{o/o}$) mice developed normally and were fertile. The induction of IFN-α and IFN-β gene transcription by virus or poly(I)·poly(C) (pIC) was the same in spleen, lung and liver from *Pkr*$^{o/o}$ and wild-type mice. The IFN-α-induced antiviral response against EMCV was the same in *Pkr*$^{o/o}$ and wild-type mice; however, IFN-γ- and pIC-induced responses were impaired. In mouse embryo fibroblasts (MEFs), the induction of type I IFN genes by pIC was diminished, as was the activation of NF-κB by pIC [but not by tumor necrosis factor (TNF)-α], but this defect was corrected if cells were first primed with IFN-α. Thus, PKR is not directly essential for responses to pIC, and a pIC-responsive system independent of PKR is induced by IFN. So far, no spontaneous tumors in *Pkr*$^{o/o}$ animals have been observed. In addition, inoculation into nude mice of embryonic fibroblasts, embryonic fibroblasts transformed by ras or established 3T3-like cell lines derived from *Pkr*$^{o/o}$ mice has not resulted in tumor formation, arguing against the role of PKR as a direct tumor suppressor.

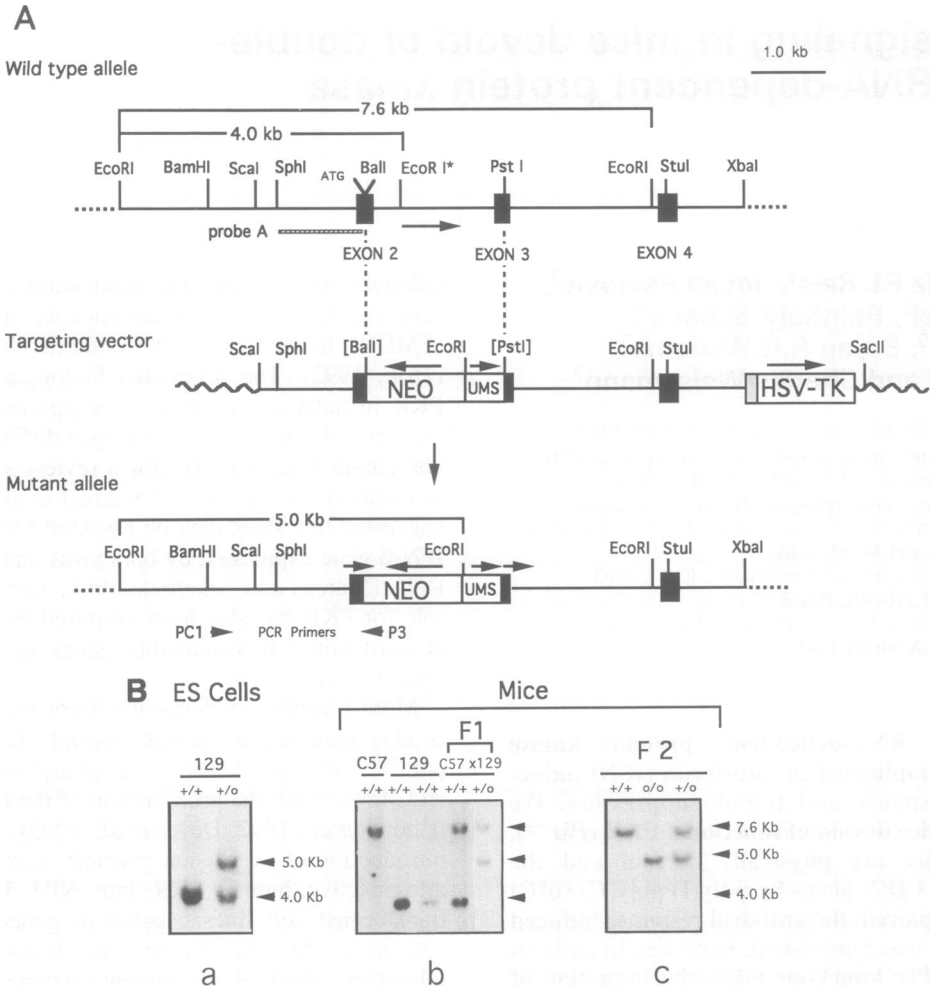

Fig. 1. Targeted disruption of the PKR gene by homologous recombination. (**A**) (Top) The wild-type allele of 129/Sv(ev) mice has an *Eco*RI site (marked with *) which is absent in C57BL/6J mice; this gives rise to a polymorphic Southern blot pattern. (Middle) The targeting vector is the 7.6 kb genomic *Bam*HI–*Xba*I segment in which the *Bal*I–*Pst*I segment was replaced by a PGK-NEO-UMS cassette and to which the HSV-TK cassette was added. (Bottom) The allele generated by homologous recombination. The lengths of diagnostic restriction fragments and the probe used for Southern blot analysis are shown. Black boxes indicate exons. Exon 2 contains the initiation codon indicated by ATG. (~) Vector sequences; (→) orientation of the DNA segments. (**B**) Southern blot analysis of *Eco*RI-cleaved genomic DNA using the ^{32}P-labeled probe A indicated in (A), top. (Panel a) Wild-type and $Pkr^{+/o}$ GS-1 ES cell lines [129/Sv(ev)]. Genomic DNA of (panel b) 129/Sv(ev), C57BL/6J and the F1 progeny of 129/Sv(ev)×C57BL/6J mice, demonstrating polymorphism and (panel c) F2 of a $Pkr^{+/o}$ [129/Sv(ev)×C57BL/6J] self-cross.

Results

Generation and characterization of $Pkr^{o/o}$ mice

Using a probe derived from $p65^{muPKR}$ cDNA (Feng *et al.*, 1992), we isolated a 7.6 kb genomic mouse DNA fragment that contained exons 2–4 (Tanaka and Samuel, 1994), encoding amino acids 1–124 (Figure 1A, top). A targeting plasmid was constructed (Figure 1A, middle) in which a 2.0 kb DNA segment, comprising parts of exons 2 and 3 and the intron in between, was replaced by a neomycin resistance (NEO) gene in an antisense orientation and the upstream mouse sequence (UMS) element which purportedly mediates transcription termination (Heard *et al.*, 1987). The disrupted *Pkr* gene was introduced into embryonic stem (ES) cells [strain 129/SV(ev)]. Homologous recombinants (Figure 1A, bottom) were identified by PCR or Southern blot analysis and injected into blastocysts from C57BL/6J mice, following the strategy described previously (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987; Capecchi, 1989; McMahon and Bradley, 1990). The resulting chimeric mice were bred to C57BL/6J mice and the progeny homozygous for the disrupted *Pkr* allele ($Pkr^{o/o}$) were identified by PCR and Southern blot analysis (Figure 1B). The ratio of wild-type, heterozygous and homozygous mice was 1:2:0.8 (n = 100).

$Pkr^{o/o}$ mice developed normally and showed no visible differences compared with their littermates with regards appearance and behavior. Homozygous $Pkr^{o/o}$ mice were fertile and produced litters of normal size. No gross anatomical changes were noted, and a histological examination of the internal organs revealed no consistent abnormalities.

Figure 1B shows a Southern blot of DNA from wild-type mice and animals heterozygous ($Pkr^{o/+}$) and homozygous ($Pkr^{o/o}$) for the disrupted *Pkr* gene. The 7.6 and 4.0 kb *Eco*RI fragments are characteristic of the wild-type *Pkr* alleles of strains C57BL/6J and SV129, respectively, while the 5.0 kb *Eco*RI fragment is characteristic of the disrupted SV129 *Pkr* allele. A Northern blot analysis of primary fibroblast RNA using a probe for the deleted *Pkr* segment

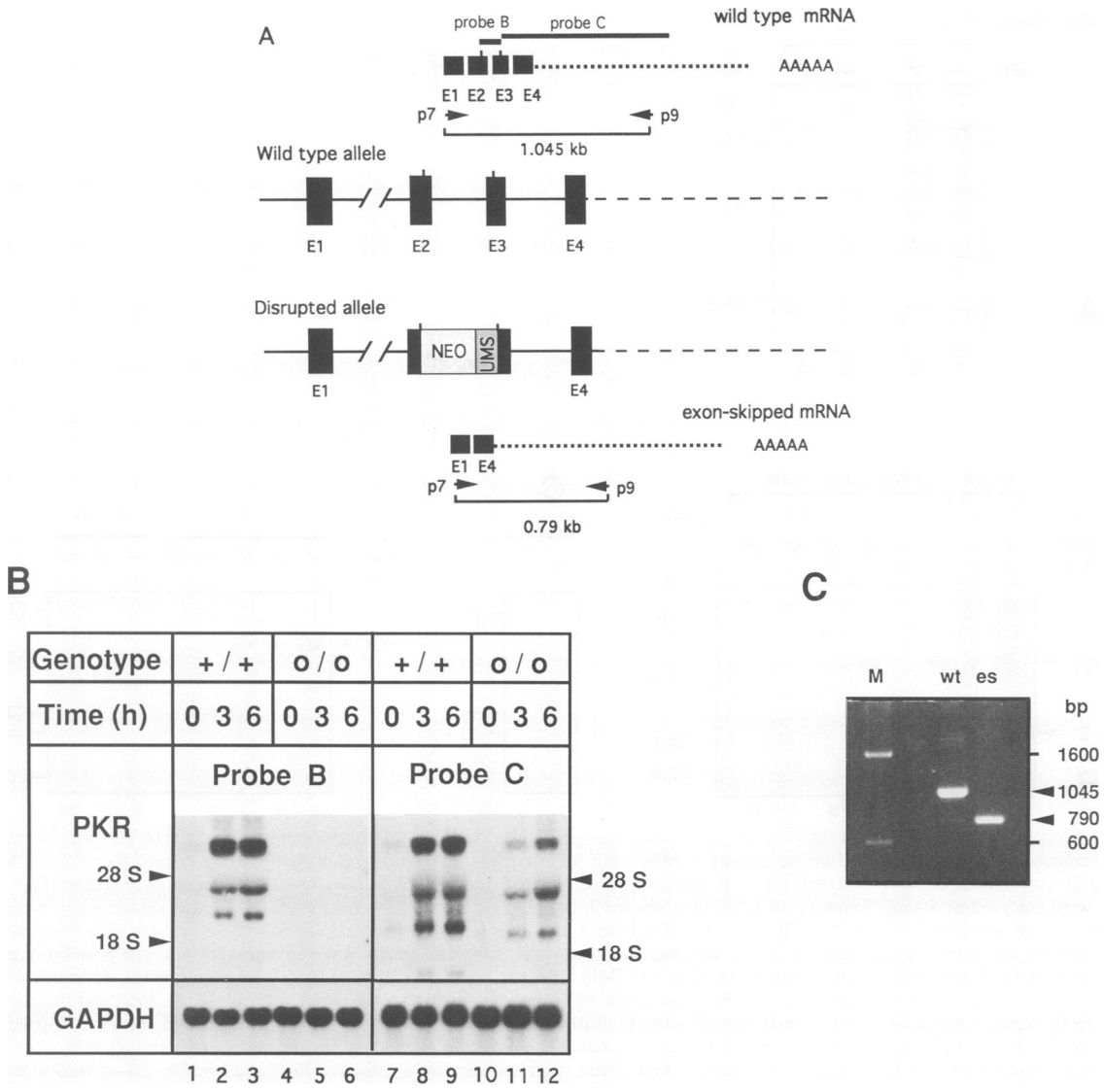

Fig. 2. Analysis of PKR transcripts in $Pkr^{o/o}$ mice. (**A**) Scheme of the wild-type and disrupted genome sequences and the resulting transcripts. The wild-type PKR allele and the normally spliced product are shown in the top two lines; the disrupted allele and the RNA product resulting from exon skipping are given in the lower two lines. (**B**) For Northern blot analysis, total RNA was isolated from primary MEFs derived from wild-type and mutant embryos mock-induced or -induced with rhuIFN-α2/α1 (500 U/ml) for 3 or 6 h. Blots (10 μg RNA per lane) were hybridized with the probes indicated in (A). (**C**) RT-PCR was performed as described in Materials and methods, using the primers P7 and P9 as indicated in (A).

shows the IFN-inducible 2.5, 4.0 and 6.0 kb *Pkr* transcripts described previously in wild-type mice (Icely *et al.*, 1991; Samuel, 1993) and the complete absence of a signal in the $Pkr^{o/o}$ sample (Figure 2B, lanes 1–6). This finding strongly suggests that the three bands are derived from the same gene from which parts of exons 2 and 3 had been deleted. However, when a probe from downstream of the deletion (probe C, Figure 2A) was used, the $Pkr^{o/o}$ RNA also showed three bands, albeit of lower intensity and, at least in the case of the two lower bands, with distinctly higher mobility than in the case of wild-type (Figure 2B, lanes 8, 9, 11 and 12). A NEO probe gave rise to a weak signal for a 2.5 kb IFN-induced transcript and a strong signal for a constitutive low molecular weight transcript that decreased after IFN induction, presumably representing the NEO sequence transcribed from its PGK promoter (data not shown). We conclude that the UMS sequence did not efficiently terminate the transcription in fibroblasts and that the artifactual exon, comprising the NEO/UMS insert, had been spliced out ('exon skipping') in the majority of the transcripts. This conclusion was supported by a PCR analysis (Figure 2C). Reverse transcripts of $Pkr^{+/+}$ and $Pkr^{o/o}$ RNA were amplified using two primers flanking the disrupted exons. $Pkr^{+/+}$ RNA gave a product of about the expected size (1045 bp), while $Pkr^{o/o}$ RNA gave a product of about the size expected if exons 2 and 3 had been spliced out, namely 790 bp. A sequence analysis of the $Pkr^{o/o}$ RT-PCR product confirmed that the DNA was precisely lacking the segment corresponding to exons 2 and 3.

mRNA containing the NEO/UMS substitution would have an interrupted reading frame (Figure 2A). 'Exon-skipped' mRNA, devoid of exons 2 and 3, lacks 17 nucleotides of the 5′ non-coding region and the coding sequence up to amino acid 80, including of course the authentic initiation codon AUG. The most upstream met

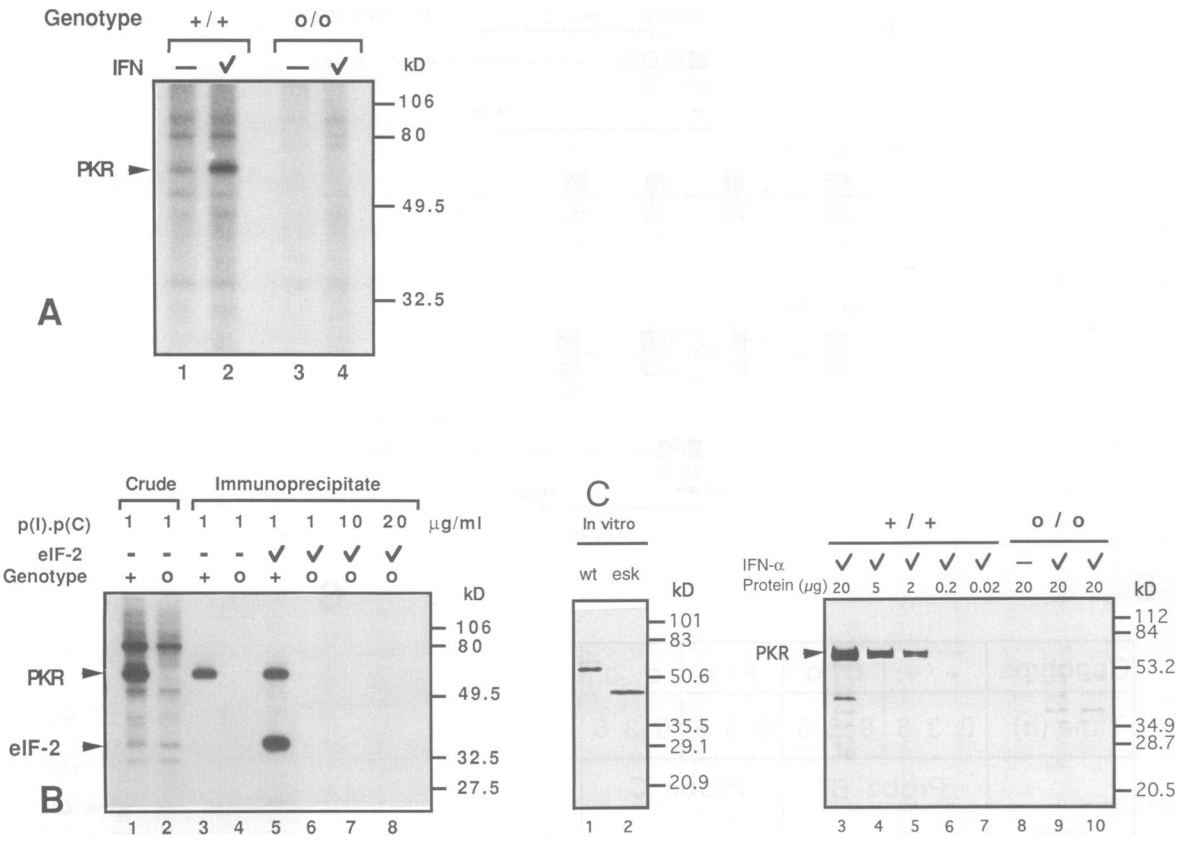

Fig. 3. Determination of PKR protein and kinase activity in MEFs and MEF extracts. (**A**) Wild-type or *Pkr*$^{o/o}$ MEFs, treated (lanes 2 and 4) or not (lanes 1 and 3) with rhuIFN-α2/α1 (500 U/ml) for 17 h, were incubated with pIC and [^{32}P]phosphate, lysed and immunoprecipitated. The precipitates were analyzed by PAGE. Exposure to X-ray film was for 7 days. (+/+) Wild type; (o/o) *Pkr*$^{o/o}$. (**B**) Protein kinase assays were performed with crude whole-cell extracts from IFN-treated cells (lanes 1 and 2) or with immunoprecipitates of these extracts with PKR antiserum (lanes 3–6), with or without 0.5 μg purified eIF-2. pIC was present as indicated. Autoradiography was performed for 4 h. The position of eIF-2α is indicated. The PKR band represents autophosphorylated PKR. (+) Wild type; (o) *Pkr*$^{o/o}$. (**C**) Western blots were performed as described in Materials and methods. Lanes 1 and 2 show that the polyclonal PKR antiserum BC1 gives an equally strong signal with full-length PKR and that the 42–44 kDa PKR fragment synthesized *in vitro* using equal amounts (4 μl incubation mix, 40 ng mRNA/μl) of full-length and exon-skipped mRNAs, respectively, as described in Materials and methods. Exposure, 15 s. Lanes 3–10, lysates containing the amounts of protein indicated, from wild-type or *Pkr*$^{o/o}$ MEFs induced with 500 U IFN-α2/α1 or mock-induced. There is no evidence for a truncated PKR at a level ≥~2% of wild-type PKR, as judged by comparing the *Pkr*$^{o/o}$ sample (20 μg) with the dilution series of the wild-type sample. Exposure, 15 min; in vitro, *in vitro* translation reaction mixture; wt, wild-type sample; esk, exon-skipped sample.

codon in the correct reading frame is in position 136 and has an acceptable Kozak sequence, so that in principle a 379 amino acid C-terminal PKR fragment could be synthesized. To determine whether the exon-skipped mRNA was potentially able to give rise to a translation product, wild-type and exon-skipped *Pkr* cDNAs were constructed. The cognate mRNAs were prepared and translated in a reticulocyte system. Major ^{35}S-labeled products of ~60 kDa resulted from the reaction directed by wild-type *Pkr* mRNA and of ~42–44 kDa resulted from that directed by exon-skipped mRNA. Both products were immunoprecipitated to the same extent by an anti-mouse PKR serum (Petryshyn *et al.*, 1988; data not shown). We concluded that, at least in a cell-free system, a C-proximal fragment of PKR could be translated from the exon-skipped RNA and that it could be precipitated by the antiserum. Therefore we examined cell extracts from *Pkr*$^{o/o}$ MEFs for the presence of a kinase-active PKR fragment.

Cell extracts were subjected to the classic PKR autophosphorylation assay as follows. pIC agarose was added to the extracts. The agarose was washed and incubated with [γ-^{32}P]ATP, and the radioactive products were eluted and analyzed by SDS–PAGE. No autophosphorylated PKR could be detected in the case of the *Pkr*$^{o/o}$ MEFs, while the wild-type control gave a strong signal (data not shown). This was as expected for a putative fragment lacking 135 N-proximal amino acids, because the deletion of 34 N-terminal residues abolished the RNA binding capacity of PKR (Green and Mathews, 1992; Patel and Sen, 1992).

It was reported recently that N-terminal deletion mutants of PKR which no longer bind dsRNA are not constitutively active, but they may be phosphorylated *in trans* and lead to the inhibition of translation (Lee *et al.*, 1994). Therefore we searched for a putative phosphorylated PKR fragment and for eIF-2-phosphorylating activity. MEFs were incubated with or without IFN-α [rhIFNα2/α1; Weber *et al.*, 1987; shown to be as active as muIFN-β in the induction of IFN-inducible genes in the mouse (Müller *et al.*, 1994)], treated with pIC and labeled with [^{32}P]phosphate. Cell lysates were treated with PKR antiserum and the precipitates analyzed by PAGE and autoradiography. As shown in Figure 3A, samples from IFN-α-treated (lane 2) but not from untreated (lane 1) cells gave a strong radioactive

PKR band at ~65 kDa, while $Pkr^{o/o}$ samples showed neither a band at that position nor a new band of lower molecular weight (lanes 3 and 4). Similarly, when extracts from IFN-α-treated cells were incubated with pIC and [γ-^{32}P]ATP and analyzed by PAGE, a labeled PKR band was found in the case of the $Pkr^{+/+}$ but not in the $Pkr^{o/o}$ samples (Figure 3B, lanes 1 and 2). In a further experiment, extracts from IFN-α-treated cells were immunoprecipitated with PKR antiserum and the washed precipitate incubated with various amounts of pIC, [γ-^{32}P]ATP and purified eIF-2, a specific substrate of PKR (Farrell *et al.*, 1978). $Pkr^{+/+}$ immunoprecipitates gave strong radioactive PKR and eIF-2α bands, whereas no corresponding signals were detected in the case of the $Pkr^{o/o}$ immunoprecipitates (Figure 3B, lanes 3–8). Thus, $Pkr^{o/o}$ cells did not contain a PKR fragment with detectable kinase activity. Finally, total proteins from IFN-α-induced $Pkr^{+/+}$ and $Pkr^{o/o}$ fibroblasts were analyzed by Western blotting, using an antibody (BC1) raised against the N-terminal 170 residues of human PKR which cross-reacts with murine PKR. As shown in Figure 3C, this antibody revealed a strong 65 kDa band in the wild-type (lane 3) but not in the $Pkr^{o/o}$ sample (lanes 9 and 10). Moreover, although the antibody reacts with the 42–44 kDa PKR fragment synthesized *in vitro* (lane 2), it failed to reveal any band in the $Pkr^{o/o}$ preparation which was not also present in the $Pkr^{+/+}$ sample; from the dilution series of the $Pkr^{+/+}$ sample (lanes 3–7), the detection limit was estimated to be 2% of the wild-type PKR level. Similar results were obtained with a murine PKR antiserum (data not shown).

Activation of NF-κB in $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs treated with pIC or TNF-α: effect of priming with IFN

The transcription factor NF-κB is found as an inactive cytoplasmic complex with IκB. Virus infection or the treatment of cells with a variety of agents, such as phorbol myristate acetate (Sen and Baltimore, 1986), TNF-α, interleukin-1 (Osborn *et al.*, 1989) or dsRNA (Visvanathan and Goodbourn, 1989), leads to the release of NF-κB and its translocation to the nucleus. At least one mechanism of release involves the phosphorylation of IκB (Ghosh and Baltimore, 1990; Diaz *et al.*, 1993; Li and Sedivy, 1993) which can be mediated by protein kinase C (Ghosh and Baltimore, 1990), Raf-1 protein kinase (Li and Sedivy, 1993) or PKR in a dsRNA-dependent reaction (Kumar *et al.*, 1994; Maran *et al.*, 1994).

To assess whether NF-κB activation was impaired in $Pkr^{o/o}$ cells, MEFs were treated with pIC or TNF-α and nuclear extracts were subjected to a bandshift analysis using the γ-^{32}P-labeled NF-κB binding sequence PRDII (position –55 to –66 of the human IFN-β promoter; Goodbourn and Maniatis, 1988). As shown in Figure 4A, NF-κB was detected in pIC-treated $Pkr^{+/+}$ and $Pkr^{+/o}$ (lanes 5 and 6), but not in $Pkr^{o/o}$ nuclear extracts (lane 4). The incubation of the binding complex with antiserum to p50 (lane 7), but not antisera to p65 or rel (lanes 8 and 9), caused a decrease in mobility, indicating the presence of at least p50 in the complex. In cells stimulated with TNF-α, NF-κB was activated to the same extent in wild-type, heterozygous and knockout cells (lanes 10–12). Interestingly, when the MEFs were pretreated (primed; Stewart *et al.*, 1971; Raj and Pitha, 1981; Enoch *et al.*, 1986; Dron *et al.*, 1990) with type I or type II IFN, the activation of NF-κB in $Pkr^{o/o}$ was restored to about half the wild-type level (Figure 4B).

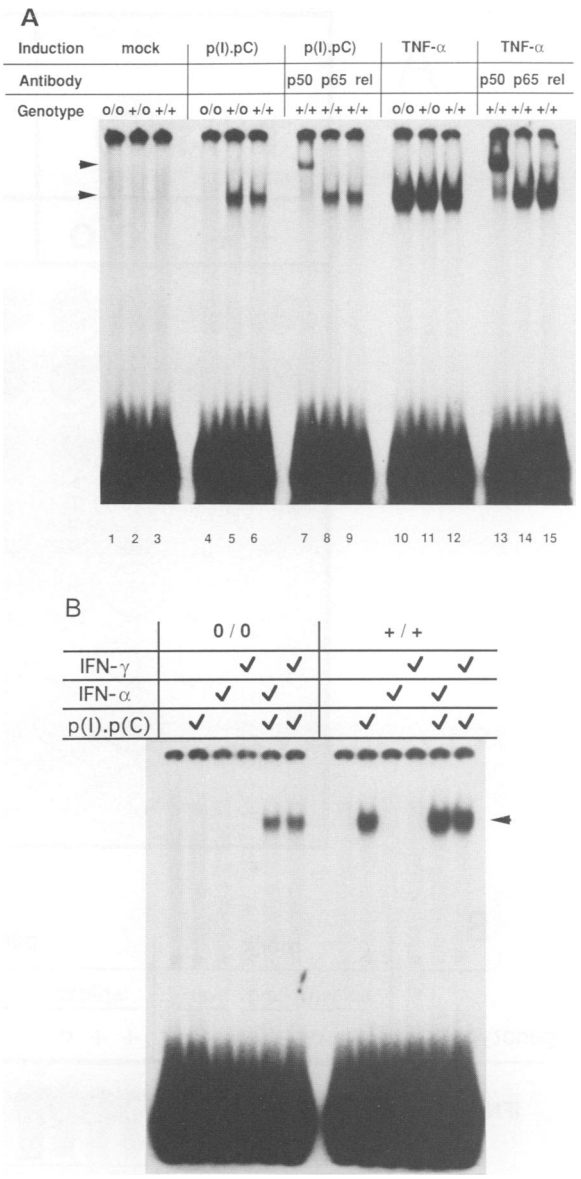

Fig. 4. Activation of NF-κB by pIC or TNF-α in MEFs. (**A**) Nuclear extracts were prepared from wild-type (+/+) or $Pkr^{o/o}$ (O/O) MEFs induced by pIC or TNF-α and subjected to an electrophoretic mobility shift assay using the ^{32}P-labeled NF-κB binding oligonucleotide PRDII as a ligand. Where indicated, extracts were preincubated with antibody to p50, p65 or rel prior to the addition of the oligonucleotide. (**B**) MEFs were primed with IFN-α or IFN-γ as indicated, induced with pIC or mock-induced. The analysis of nuclear extracts was as in (A).

This experiment shows that NF-κB activation by dsRNA and TNF-α proceeds via different pathways and that activation by dsRNA can occur by a PKR-independent pathway, at least in MEFs primed with IFN. The experiment also confirms that a functional knockout has been achieved.

Induction of type I IFN genes by virus and pIC in mice and MEFs

Two mice were each injected intravenously with Newcastle disease virus (NDV) or intraperitoneally with pIC. No

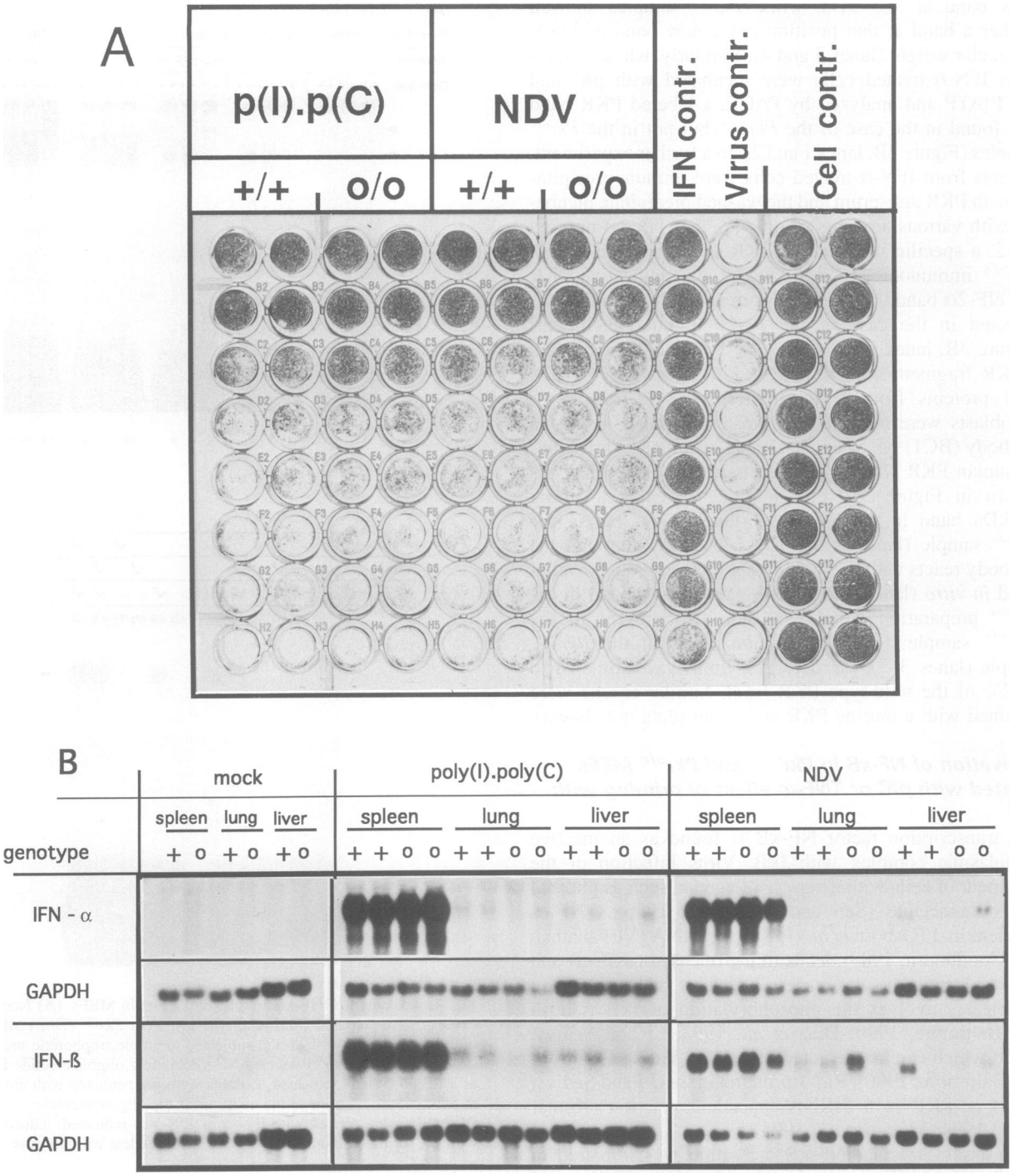

Fig. 5. Induction of type I IFN and IFN mRNA by pIC or NDV in wild-type and *Pkr*$^{o/o}$ mice. Mice were not injected or injected either intraperitoneally with pIC (200 μg/mouse) 6 h prior to killing or intravenously with NDV (10^7 p.f.u./mouse) 4 h prior to killing. (**A**) Two mice of each genotype were bled after 4 h and sera were assayed for antiviral activity as described in Materials and methods. The first dilution was 1:20 followed by a 2-fold serial dilution. (**B**) Total RNA (10 μg/lane) of the indicated organs was subjected to a Northern blot analysis using a ^{32}P-labeled IFN-α (upper panel) or IFN-β (lower panel) cDNA probe. After stripping, the filters were hybridized with a GAPDH cDNA probe. (+) Wild-type; (○) *Pkr*$^{o/o}$.

significant difference in antiviral activity levels in the sera of wild-type or *Pkr*$^{o/o}$ mice could be observed (Figure 5A) with either inducer. IFN-α and IFN-β mRNA levels were determined in organs of *Pkr*$^{o/o}$ and wild-type controls. Substantial differences in the mRNA levels of individual animals can occur (Reis *et al.*, 1994). After NDV challenge, notable levels of IFN-β mRNA were detectable in spleen, and less so in lung and liver, in both wild-type and PKR-deficient mice (Figure 5B). In the case of IFN-α mRNA, spleen samples gave a strong and liver a weak signal (Figure 5B); no bands were detectable in lung. In repeated experiments there was no significant difference between wild-type and *Pkr*$^{o/o}$ mice. Challenge with pIC led to high levels of mRNA for both IFN-β and IFN-α in spleen,

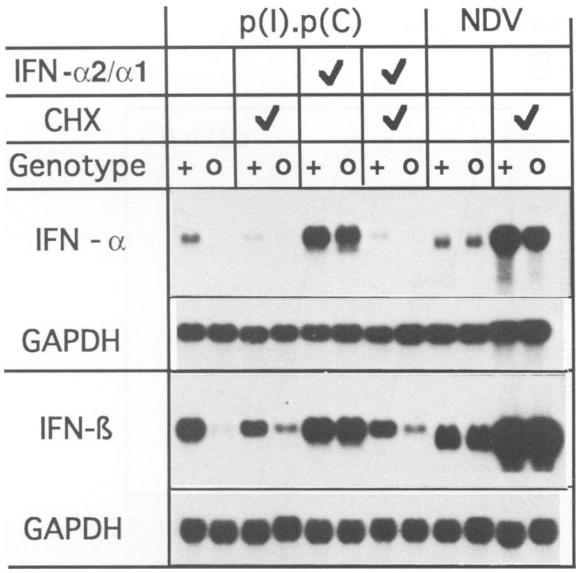

Fig. 6. Induction of type I IFN transcripts in wild-type and $Pkr^{o/o}$ MEFs with pIC or NDV. Wild-type (+) or $Pkr^{o/o}$ (○) MEFs were mock-induced or induced with either pIC for 4 h or NDV for 8 h. Where indicated, rhuIFN-α2/α1 (500 U/ml) or cycloheximide (CHX, 75 μg/ml) was added prior to induction. Total RNA was loaded at 10 μg/lane. Northern blots were hybridized with a ^{32}P-labeled IFN-α or IFN-β probe. After stripping, the filters were hybridized with a GAPDH cDNA probe.

whereas in lung there was the weak expression of IFN-β but not of IFN-α (Figure 5B). There was no significant difference between wild-type and mutant mice.

MEFs showed a distinctly different behavior. After induction with an optimized dose of NDV, RNA was collected after 8 h and subjected to a Northern blot analysis with an IFN-β and an IFN-α probe. As shown in Figure 6, the same levels of both IFN-β and IFN-α mRNA were found in wild-type and $Pkr^{o/o}$ MEFs. Therefore, as in organs, PKR is not essential for virus induction of type I IFN genes in MEFs. In contrast, the treatment of MEFs with pIC resulted in a much lower level of type I IFN mRNA in $Pkr^{o/o}$ than in $Pkr^{+/+}$ cells. However, when the MEFs were primed with type I IFN, both wild-type and $Pkr^{o/o}$ cells showed about the same levels of type I IFN mRNA, which were two to five times higher than the levels in unprimed wild-type cells. This effect was abolished by cycloheximide, suggesting that the priming effect involves protein synthesis. The experiment shows that in $Pkr^{o/o}$ MEFs, as in the case of NF-κB, induction with pIC is impaired but the deficiency can be overcome by IFN-inducible protein(s); thus, PKR is not essential for dsRNA-elicited IFN induction. NF-κB is involved in the activation of the IFN-β gene (Fujita *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Xanthoudakis *et al.*, 1989; Thanos and Maniatis, 1992), but its role in the induction of murine IFN-α genes is questionable (MacDonald *et al.*, 1990); it cannot be excluded that the deficit in induction of the IFN genes is caused by the deficit in the activation of NF-κB.

Induction of IFN-stimulated genes (ISGs) with IFN or pIC in mice and MEFs

Mice were injected intraperitoneally with rhuIFN-α2/α1 (which is fully active on murine cells; Weber *et al.*, 1987; 5×10^5 U/mouse) or pIC (200 μg/mouse). Spleen, lung, liver and thymus samples were collected for RNA extraction after 7.5 h. A Northern blot analysis revealed no difference in the type I IFN-induced levels of the mRNA of major histocompatibility complex (MHC) class I, 2′,5′-oligoadenylate synthetase (OAS), 1-8 and 202 in $Pkr^{+/+}$ and $Pkr^{o/o}$ organs. However, in $Pkr^{o/o}$ MEFs treated for 3 or 6 h with rhuIFN-α2/α1 or murine IFN-γ, expression levels of MHC class I mRNA were 2- to 3-fold lower than those in $Pkr^{+/+}$ cells. Induction by pIC was low and late in both wild-type and mutant MEFs (data not shown).

Antiviral response in mice and MEFs

Wild-type and $Pkr^{o/o}$ mice were injected intravenously with EMCV and blood samples were taken at 0, 2, 4 and 6 and 24 h post-infection. IFN concentrations were maximal between 4 and 6 h; there was no significant difference between mutant and wild-type mice (data not shown).

The average survival time was ~3 days for both groups (Figure 7A). However, when the animals were pretreated with pIC, the average survival time of wild-type mice was extended to >6 days, while that of the $Pkr^{o/o}$ mice remained unchanged at 3 days (Figure 7B). The same results were obtained when EMCV was administered by the intracerebral or intraperitoneal route (data not shown). Surprisingly, similar results were obtained when the mice were pretreated with IFN-γ. The minimal survival time for wild-type mice was 6 days and two out of five wild-type mice survived EMCV infection for >21 days. In contrast, IFN-γ-treated $Pkr^{o/o}$ mice survived EMCV infection for only 4 days (Figure 7C). Pretreatment with IFN-α extended the survival time of both wild-type and $Pkr^{o/o}$ mice to the same extent (Figure 7D), implying that the IFN-α-induced antiviral state directed against EMCV does not depend on PKR. Increased survival times were reflected in lower EMCV levels in brain and serum from animals 72 h after infection (Figure 7E and F). These experiments indicate that there may be two pathways leading to an antiviral state, one dependent on PKR (elicited by pIC or IFN-γ) and another independent of PKR (elicited by IFN-α).

The intravenous injection of up to 10^6 p.f.u. VSV did not lead to the death of either wild-type or $Pkr^{o/o}$ mice (data not shown).

The diminished antiviral response to IFNs and pIC was reflected in the virus yields of MEFs challenged with EMCV. The titers were about the same in untreated $Pkr^{o/o}$ and $Pkr^{+/+}$ cells, but whereas they were reduced by 4.0 and 4.7 logs after pretreatment with pIC and IFN-γ, respectively, in the case of $Pkr^{+/+}$ MEFs, the corresponding reductions were only 2.7 and 1.5 for $Pkr^{o/o}$ cells. These results confirm at the level of MEFs the dependence on PKR of the antiviral response induced by pIC and IFN-γ.

Cellular transformation in vitro and tumorigenicity

It has been reported that NIH 3T3 cells expressing a transdominant mutant *PKR* were tumorigenic, indicating that PKR is a tumor suppressor gene (Koromilas *et al.*, 1992; Meurs *et al.*, 1993). Therefore, we analyzed $Pkr^{o/o}$ fibroblasts for the expression of transformed phenotypes *in vitro* and *in vivo*. MEFs from two wild-type and

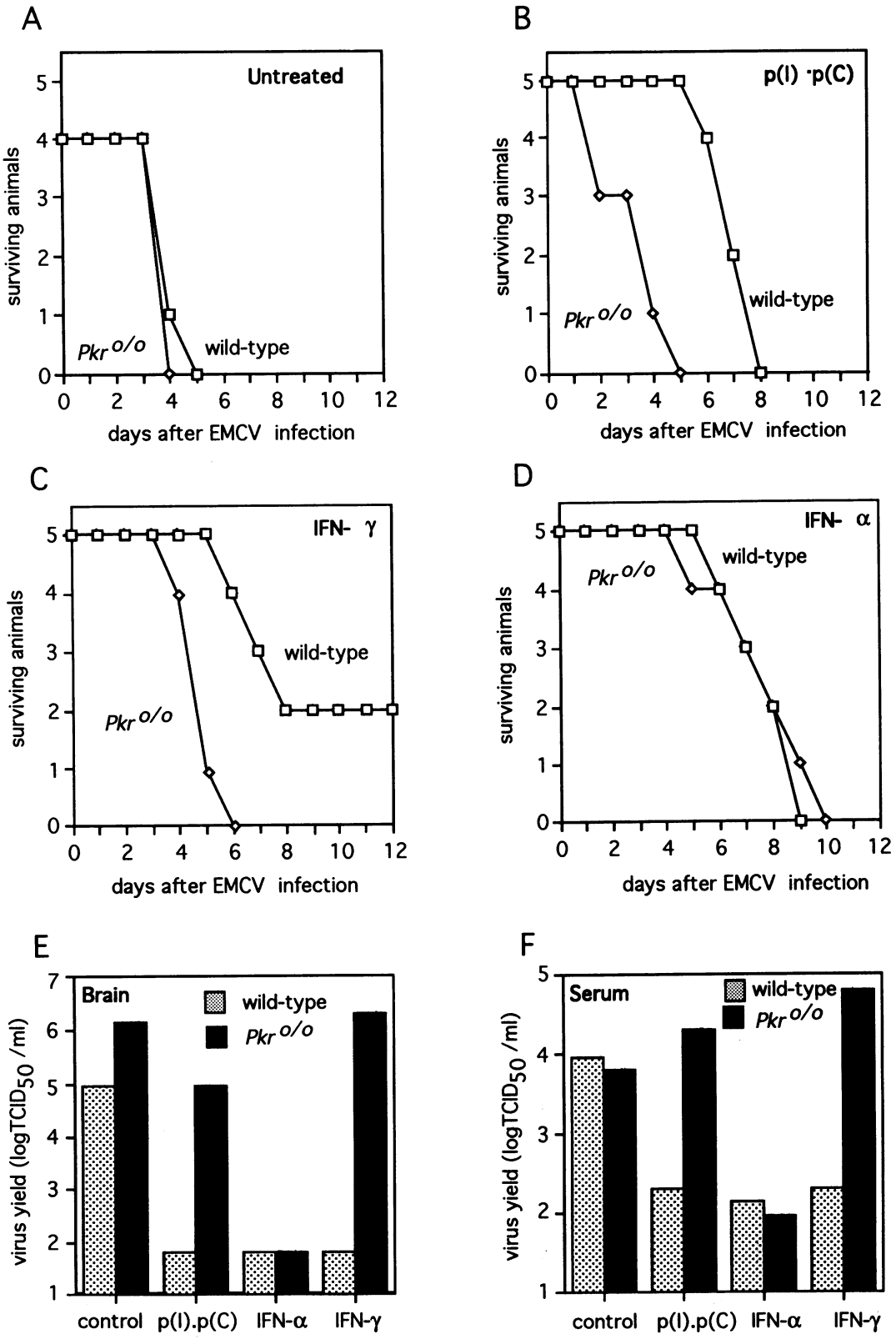

Fig. 7. Susceptibility of mice to EMCV infection after pretreatment with pIC, IFN-α or IFN-γ. Groups of four to five wild-type and $Pkr^{o/o}$ mice were inoculated with EMCV intravenously. The mice were (**A**) not pretreated, (**B**) injected intraperitoneally with pIC 18 h prior to infection or (**C**) injected intravenously with rmuIFN-γ or (**D**) rhuIFN-α2/α1 simultaneously with the virus. The data for (A), (B) and (D) were compiled from two independent experiments. EMCV titers in brain (**E**) and serum (**F**) of treated and untreated mice were determined 72 h after infection. $TCID_{50}$, tissue culture 50% infectious dose.

six $Pkr^{o/o}$ cultures were injected subcutaneously into nude mice ($2–8\times10^6$ cells per site; two to four sites injected per sample). No tumors were noted in either case after 50–78 days.

Because MEFs have a limited life-span, we generated cell lines from the same embryos according to the 3T3 protocol (Todaro and Green, 1963). 3T3-like cells are immortal and likely to be predisposed to neoplastic transformation by a mutation such as the inactivation of a tumor suppressor gene (Koi and Barrett, 1986; Iten *et al.*, 1989). Cells were passaged every 3–4 days, replating at 1.8×10^4 cells/cm^2. After 10 passages, cells went into crisis. Five of six MEF $Pkr^{o/o}$ cultures and both wild-type MEF cultures were recovered as permanent lines. $Pkr^{+/+}$ and $Pkr^{o/o}$ 3T3-like cell lines exhibited indistinguishable cloning efficiencies and saturation densities. All cell lines were unable to form colonies in soft agar medium and to initiate tumor growth in nude mice ($2–9\times10^6$ cells injected per site; one to four sites per line; observation period 40–130 days). *Ras* oncogene-transformed NIH 3T3 control cells formed colonies >1 mm in diameter after 2 weeks in soft agar, and produced tumors in nude mice after 16 days.

In a further experiment, NIH 3T3 cells and MEFs derived from two individual embryos of each genotype ($Pkr^{o/o}$ and $Pkr^{+/+}$) were infected with a retrovirus containing the *c-Ha-ras*^{val12} gene (pGDV12ras) or control virus (pGD) at a multiplicity of infection (MOI) of 10. After seeding into soft agar, 26–31% of NIH 3T3 cells infected with pGDV12ras formed colonies after 2 weeks, whereas none of the MEFs formed colonies up to 12 weeks after seeding at 2×10^5 cells/bottle. In methylcellulose, 20% of NIH 3T3 cells infected with PGDV12ras, but <0.5% of the MEFs, be they $Pkr^{o/o}$ or $Pkr^{+/+}$, formed colonies. The injection of nude mice with 10^6 pGDV12ras-infected NIH 3T3 cells gave rise to tumors of >5 mm in diameter after 2 weeks. However injection with 10^6 pGDV12ras-infected MEFs of either genotype did not give rise to tumors after 4 weeks. Thus, we have so far been unable to demonstrate a tumor suppressor effect of PKR.

Discussion

Various roles have been assigned to PKR, namely involvement in the induction of type I IFN genes, in the induction of IFN-stimulated genes by type I IFN and in growth control.

We have generated mice with *Pkr* genes in which a segment spanning parts of two exons and the intervening intron were replaced by foreign DNA sequences, including a sequence purportedly causing transcriptional termination. Nevertheless, substantial levels of mRNA were produced, from which the two disrupted exons were deleted. Despite a potential in-frame initiator codon at amino acid position 136, no PKR fragment was observed by either Western blotting or an autophosphorylation assay, and no kinase activity with eIF-2 as substrate was detected.

Virus-induced IFN-α and IFN-β mRNA levels were not affected by the absence of PKR in either MEFs or organs of $Pkr^{o/o}$ mice. While the induction of type I IFN mRNA by pIC was normal in $Pkr^{o/o}$ mouse organs, it was impaired in $Pkr^{o/o}$ MEFs; however, priming with type I IFN restored the full response. We conclude that in MEFs, induction by virus and by dsRNA is mediated by pathways that differ partly or entirely, that PKR is not essential in either pathway but that in the absence of PKR dsRNA-induced signal transmission depends on a type I IFN-inducible component. Interestingly, analogous findings were made with regards to IRF-1 (Matsuyama *et al.*, 1993; Reis *et al.*, 1994), and analogous conclusions were drawn (Reis *et al.*, 1994).

Double-stranded RNA can lead to the activation of NF-κB through the phosphorylation of IκB (Kumar *et al.*, 1994). In $Pkr^{o/o}$ MEFs, treatment with pIC does not activate NF-κB, in agreement with the findings of Maran *et al.* (1994) who proposed that the selective ablation of *Pkr* mRNA abolishes NF-κB activation but, again, priming with IFN (type I or type II) redresses this defect. Therefore there must be a second, PKR-independent activation pathway which is induced by IFN. Because NF-κB plays an essential role in the induction of the IFN-β gene (Fujita *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Xanthoudakis *et al.*, 1989; Thanos and Maniatis, 1992), it is tempting to consider that the failure of MEFs to accumulate IFN mRNA after pIC induction is because of the absence of activated NF-κB. However, the induction of IFN-α genes by pIC is also impaired in $Pkr^{o/o}$ MEFs, although the induction of the IFN-α genes is thought not to involve NF-κB (MacDonald *et al.*, 1990). For this reason, and because a knockout of the p50 subunit of NF-κB does not abolish IFN-β induction (Sha *et al.*, 1995), it is possible that the impairment of IFN induction in $Pkr^{o/o}$ MEFs is not a direct consequence of the lack of active NF-κB. The discrepancy between the results with MEFs and the findings *in vivo*, where we did not detect a difference in the inducibility of type I IFN genes by pIC, could be explained if the mice, which are kept under conventional conditions, were in a permanently 'primed' state due to the constant presence of low concentrations of IFN (Belardelli *et al.*, 1984; Galabru *et al.*, 1985), or if the treatment with pIC generated low concentrations of IFN which then led to priming. To resolve this question, we are generating double knockout mice lacking both PKR and the type I IFN-α receptor chain (Müller *et al.*, 1994).

Although the constitutive expression of PKR in cultured cells increases their resistance to EMCV (Meurs *et al.*, 1992), there was no difference in the susceptibility of untreated wild-type and $Pkr^{o/o}$ mice to infection by EMCV. Pretreatment with IFN-α increased the survival time of both mouse strains to an equal extent, showing that the establishment of an IFN-α-dependent antiviral state does not depend on a PKR-mediated process. Indeed, the induction in MEFs of a number of ISGs by IFN-α was not dependent on PKR (data not shown). The pretreatment of wild-type mice with pIC or IFN-γ extended the survival time after EMCV infection to the same extent as type I IFN. However, surprisingly, this process was dependent on PKR, showing that there exist both PKR-dependent and PKR-independent pathways for the establishment of an antiviral state. In $Pkr^{o/o}$ MEFs, there was a 2- to 3-fold lower induction of MHC class I mRNA in response to IFN-γ or IFN-α.

It is possible that PKR-independent pathways are not present in all cell types, and in particular not in all cell lines, which might explain the results of Der and Lau

(1995). They reported recently that the inhibition of PKR activity in the promonocytic cell line U937, by PKR antisense RNA or dominant negative PKR mutants, led to the impaired induction of IFN-α genes but not the IFN-β gene in response to dsRNA, despite priming with IFN.

The fact that mice developed normally in the absence of PKR shows that this enzyme does not play an essential role in growth control, either because this is not its function or because it is redundant in this regard. In addition, we have not found evidence for a tumor suppressor function in the experiments carried out so far. In particular, the injection of primary or immortalized $Pkr^{o/o}$ MEFs, or $Pkr^{o/o}$ MEFs transformed with ras into nude mice, did not give rise to tumors. This raises interesting questions as to why 3T3 cells overexpressing an inactive mutant form of PKR efficiently give rise to tumors in nude mice (Koromilas *et al.*, 1992; Meurs *et al.*, 1993). Perhaps PKR is not a tumor suppressor and the transdominant mutant PKR causes tumorigenicity by interfering with some other 'suppressor pathway' which is unaffected by the complete ablation of PKR. Alternatively, if PKR is a tumor suppressor, one would have to assume that some other system can replace it functionally and that this system is absent in 3T3 cells.

Materials and methods

Generation of $Pkr^{o/+}$ ES cells

A PKR cDNA probe extending from codon 2 to codon 66 (Feng *et al.*, 1992) was generated by RT–PCR on mouse spleen RNA and used to isolate a PKR genomic clone from an isogenic λGem11 genomic library constructed from the AB-1 [129/Sv(ev)] ES cell DNA (Müller *et al.*, 1994). A 7.6 kb genomic *Bam*HI–*Xba*I segment was subcloned in Bluescript KS–. A 2.0 kb *Bal*I–*Pst*I fragment containing parts of exons 2 and 3 (including the coding sequence from the first ATG up to codon 66) and the intron in between was replaced by the 2.0 kb pPGK-NEO-UMS cassette described previously (Ruffner *et al.*, 1993; Müller *et al.*, 1994). A herpes simplex virus thymidine kinase (HSV-TK) gene was inserted into the vector *Not*I site downstream of this segment to allow for counter-selection (Thomas and Capecchi, 1987). The targeting vector was linearized at the *Sac*II site proximal to the long arm of the PKR genomic sequence.

GS-1 ES cells were derived from 129/Sv(ev) blastocysts, as described previously (Bradley, 1987). Cells were maintained on a feeder layer of irradiated SNL cells (McMahon and Bradley, 1990), transfected with leukocyte inhibitory factor expression plasmid and a neomycin resistance gene, in DMEM, 20% fetal calf serum (FCS) and 0.14 mM 2-mercaptoethanol (D20). *Sac*II-linearized targeting vector DNA was electroporated into ES cells, plated on SNL cell feeder layers and selected in D20 containing 400 μg/ml G418 and 0.2 μM 1-(2-deoxy,2-fluoro-β-D-arabinofuranosyl)-5-iodouracil. After 10–12 days, individual colonies were picked and analyzed for homologous recombination by PCR using a primer complementary to the 3′ end of the neomycin cassette (P3; Büeler *et al.*, 1992) and a primer (PC1) corresponding to a genomic sequence beyond the 5′ end of the targeting vector (5′-GTTTGGCTATTTCTCTGTGTTCATTGGA; Figure 1A, bottom). PCR-positive colonies were expanded and confirmed to be heterozygous by a Southern blot analysis (Figure 1B).

ES cells carrying the PKR mutation were injected into 3.5 day old blastocysts of C57BL/6J mice and implanted into ICR foster mice (Bradley, 1987). The resulting male chimeras gave germline transmission of the mutated PKR allele when mated with C57BL/6J females, and heterozygous mice ($Pkr^{o/+}$) were intercrossed to generate homozygous offspring ($Pkr^{o/o}$). The genetic background of these animals was a mixture of the strains 129/Sv(ev)×C57BL/6J, and all animals were typed by a Southern blot analysis of tail DNA.

Analysis of ES cell and mouse tail DNA

ES cells depleted of feeder cells or pieces of mouse tail were lysed in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 1% SDS and 0.1 mg/ml proteinase K, and incubated for 16 h at 37°C for ES cells and at 55°C for tail tissue. DNA was precipitated with one volume of isopropanol, washed with 70% ethanol and dissolved in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). In all, 10 μg DNA were digested with *Eco*RI, fractionated on a 0.8% agarose gel and transferred to a nitrocellulose membrane (Amersham). Hybridizations were performed (Church and Gilbert, 1984) with random-primed α-^{32}P-labeled probes (PrimeIt, Stratagene).

RNA analysis

For Northern blot analysis, total RNA (Chomczynski and Sacchi, 1987; 10 μg per lane) was fractionated on a 1% denaturing agarose gel. Northern blots were hybridized with random-primed α-^{32}P-labeled probes. The following probes were used: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA, the rat 490 bp GAPDH *Xho*II fragment subcloned in pSP64 (Fort *et al.*, 1985); for IFN-α RNA, the 690 bp *Hin*dIII–*Eco*RI fragment of murine IFN-α1 cDNA (Shaw *et al.*, 1983); for IFN-β RNA, the 430 bp *Bam*HI–*Kpn*I fragment of murine IFN-β cDNA (Higashi *et al.*, 1983); for OAS RNA, the 2.2 kb *Eco*RI murine OAS cDNA fragment (Rutherford *et al.*, 1991); for PKR RNA, the 194 bp *Bal*I–*Pst*I murine PKR cDNA fragment corresponding to the deleted segment of the PKR gene (Feng *et al.*, 1992) and the 3′ proximal 1.4 kb *Pst*I–*Pst*I murine PKR cDNA fragment corresponding to the region downstream of the deleted segment; for 1-8 RNA, a 0.2 kb *Eco*RI–*Xba*I fragment of the 1-8 cDNA (Flenniken *et al.*, 1988); for MHC-1 RNA, a 1.8 kb genomic *Xba*I fragment of the H-2D^b MHC class I gene (Weiss *et al.*, 1984); and for IRF-1 RNA, a 1.1 kb *Nco*I fragment of murine IRF-1 cDNA (Ruffner *et al.*, 1993). The radioactive bands were quantified using a PhosphorImager (Molecular Dynamics), and all values were normalized relative to the GAPDH value in the cognate lane. Autoradiograms were prepared for illustrations.

RT–PCR was performed according to the Clontech manual using 5 μg total RNA per 20 μl reaction. First-strand cDNA was synthesized using the antisense primer p9 (Figure 2A; position 1135–1112) and 200 U Mo-MLV reverse transcriptase (BRL) at 23°C for 10 min, at 42°C for 60 min and at 95°C for 10 min. For 25 μl PCRs, 2 μl RT-reaction mix were adjusted to 6 mM Tris–HCl (pH 8.4), 40 mM KCl, 1.5 mM $MgCl_2$, 40 μg gelatin/ml, 0.18% Tween-20, 0.18% NP-40 and 200 μM dNTPs. To this, 16 pmol each of sense primer (p7) located 5′ of the deletion (position 91–114), antisense primer p9 and 0.625 U *Taq* polymerase were added. PCR was started with an initial denaturation at 94°C for 4 min, followed by 35 cycles consisting of 30 s at 94°C, 45 s at 50°C and 2 min at 72°C. Products were analyzed on 1% Metaphor (FMC Bioproducts) agarose gels.

In vitro translation and immunoprecipitation of wild-type and exon-skipped RNA

A *Bam*HI–*Pst*I PKR cDNA fragment containing the murine PKR coding sequence was cloned into pGEM-3Z (Promega) to yield pGEM-3Z–p65. To obtain an expression plasmid for the exon-skipped PKR, the RT–PCR product described above (see also Figure 2C) was cleaved with *Bam*HI and *Hae*II, and the 630 bp product inserted into *Bam*HI–*Hae*II-cleaved pGEM-3Z–p65. Linearized expression plasmids were transcribed with T7 RNA polymerase in the presence of 25 mM m^{7}GpppG (Promega). For *in vitro* translation, 2 μg mRNA (PKR, exon-skipped PKR or luciferase as control) were incubated for 90 min at 30°C with 35 μl nuclease-treated rabbit reticulocyte lysate (Promega), 40 U RNasin, 1 μl 1 mM amino acid mixture (without methionine) and 40 μCi [^{35}S]methionine (final volume 50 μl). For immunoprecipitation experiments, 35 μl product were diluted 1:10 in immunoprecipitation buffer [50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin 100 U/ml] and 3 μl mouse PKR antiserum (a gift from J.J.Chen) were added (Petryshyn *et al.*, 1988). After 1 h on ice, protein G–agarose (50% v/v) in 50 μl wash buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.05% Triton X-100, 150 mM NaCl, 50 mM NaF) was added and the mixture was agitated for 30 min at 4°C. The immunoprecipitates were washed 12 times with wash buffer and eluted by heating at 95°C in 40 μl SDS–PAGE sample buffer. SDS–PAGE was performed on a 20 μl sample.

Western blot analysis for PKR

Preparation of cell extracts. About 10^7 MEFs, untreated or induced with rhuIFN-α2/α1 [which shows full activity in mice (Weber *et al.*, 1987); 500 U/ml] for 17 h, were washed twice with cold PBS and 10 mM EDTA, and scraped off the plates. Cell extracts were prepared as described previously (Petryshyn *et al.*, 1984).

Western blotting. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes in 25 mM Tris–HCl (pH 8.3), 0.192 M glycine, 20% methanol and 0.1% SDS. After blocking for 1 h in PBS–5% non-fat dry milk, the blot was washed twice in PBS and then incubated for 1 h in a 1/500 dilution of BC-1 (a polyclonal rabbit antiserum raised against the N-terminal 170 residues of human PKR) in PBS–10% FCS. After treatment with the primary antibody, the blot was washed three times in PBS–0.5% NP-40, reblocked for 30 min, washed with PBS, incubated for 1 h with 1:3000 swine anti-rabbit antiserum coupled to peroxidase (Dako) in PBS–10% FCS, and washed three times in PBS–0.5% NP-40. Detection was by enhanced chemiluminescence (Amersham) following the protocol of the manufacturer.

Analysis of PKR activity

dsRNA binding and autophosphorylation assay. Cell extract (10 μg protein) was incubated with 15 μl packed pIC cellulose (Silverman and Krause, 1987) for 1 h on ice. The cellulose–PKR complex or crude cell extract (10 μg protein) was subjected to the kinase assay, as described previously (Feng *et al.*, 1992).

Phosphorylation of eIF-2 by immunoprecipitated PKR. Cell extract (40 μg protein) from MEFs treated for 17 h with rhuIFN-α2/α1 (500 U/ml) was diluted to 250 μl with immunoprecipitation buffer. In all, 3 μl anti-PKR serum were added and the extract was kept for 1 h on ice. Protein G–agarose (50 μl of a 50% v/v suspension in wash buffer) was added and the mixtures were agitated for 30 min at 4°C. The immunoprecipitates were washed 12 times with wash buffer and twice with kinase buffer (10 mM Tris–HCl, pH 7.6, 50 mM KCl, 2 mM Mg-acetate, 2.5 mM $MnCl_2$, 7 mM 2-mercaptoethanol, 20% glycerol). The precipitates were incubated with or without 0.5 μg purified eIF-2 (a gift from W.Merrick), pIC as indicated and 1.25 μM [γ-^{32}P]ATP for 15 min at 30°C, heated for 2 min at 95°C in 2× SDS–PAGE sample buffer and analyzed by SDS–PAGE.

In vivo autophosphorylation of the endogenous PKR. 10^7 MEFs, uninduced or induced with rhuIFN-α2/α1 (500 U/ml) in DMEM, 10% FCS (D10) for 17 h, were serum-starved in DMEM lacking phosphate (Sigma) for 3 h. Labeling was in the same medium containing 200 μCi/ml [^{32}P]orthophosphate (Amersham) and pIC (100 μg/ml). After 3 h, the cells were washed twice with cold PBS and 10 mM EDTA, and scraped off the plates (Katze *et al.*, 1988; Lee *et al.*, 1994). The cells were lysed for 10 min on ice in 100 μl lysis buffer (Petryshyn *et al.*, 1984). The extracts were diluted to 300 μl with immunoprecipitation buffer and pretreated with protein G–agarose (100 μl of a 50% v/v suspension in wash buffer) for 1 h on ice. The agarose was removed and 3 μl PKR antiserum were added. After 1 h on ice, protein G–agarose (100 μl) was added and the suspensions were agitated for 30 min at 4°C. The beads were washed 12 times with wash buffer, heated for 2 min at 95°C in 2× SDS–PAGE sample buffer, analyzed by 10% SDS–PAGE and autoradiographed for 7 days.

Induction of IFN genes and ISGs in mice and MEFs

For the induction of IFN genes *in vivo*, 4–6 week old mice were injected either intraperitoneally with pIC (Sigma; 200 μg/mouse) 6 h or intravenously with NDV (10^7 p.f.u./mouse) 4 h prior to killing. Sera were collected for the determination of antiviral activity. Organs were frozen at –80°C until RNA preparation. For the induction of ISGs *in vivo*, mice were injected intraperitoneally with rhuIFN-α2/α1 (5×10^5 U/mouse) or pIC (200 μg/mouse) and organs were collected for RNA extraction after 7.5 or 6.0 h, respectively. For induction *in vitro*, MEFs were treated with an optimized amount of NDV for 8 h or with pIC (100 μg/ml) and DEAE dextran (500 μg/ml) for 1 h in the presence or absence of cycloheximide (75 μg/ml). Medium was removed and the cells were washed with PBS before adding 10% FCS–DMEM. After 3 h at 37°C, RNA was prepared. For priming, cells were treated with rhuIFN-α2/α1 (500 U/ml) 3 h prior to pIC induction. For the induction of ISGs, MEFs were treated with rhuIFN-α2/α1 (500 U/ml) or muIFN-γ (50 U/ml) for 3 and 6 h, or with pIC (100 μg/ml) and DEAE dextran (500 μg/ml) for different time periods.

Cytopathic effect assay for type I IFNs

Titration was performed as described previously (Stewart, 1981), using an IFN-γR$^{o/o}$ cell line (Huang *et al.*, 1993) to exclude IFN-γ activity. In the case of NDV-induced mice, sera were diluted 1:20 in pH 2.0 buffer and stored at 4°C for 48 h to inactivate the circulating virus. After neutralization, samples were diluted 1:20 and titrated in a 2-fold serial dilution. VSV was used as the challenging virus.

Activation of NF-κB

MEFs (2×10^6 cells/10 cm dish) were serum-starved for 4 h. Where indicated, cells were then primed for 2 h with 500 U rhuIFN-α2/α1 or rmuIFN-γ/ml and induced for 2 h with 500 μg DEAE dextran/ml (mock-induced), 100 μg/ml pIC and 500 μg/ml DEAE dextran or 20 ng/ml murine rTNF-α (Boehringer Mannheim), all in serum-free medium. 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, 1 mM PMSF, 0.15% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After 15 min on ice, the nuclei were pelleted from the cleared suspension by microfugation at 14 000 r.p.m. for 10 min at 4°C. The pellet was resuspended in an equal volume of nuclear extract buffer (20 mM Tris–HCl, pH 8.0, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) and NaCl was adjusted to 400 mM. After 10 min at 4°C, the suspension was vortexed and cleared by microfugation at 14 000 r.p.m. for 5 min at 4°C. Nuclear extract (2 μg protein) was subjected to an electrophoretic mobility shift assay in 16 μl 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)·poly(dC) and 200 000 c.p.m. γ-^{32}P-labeled PRDII (Goodbourn and Maniatis, 1988) 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 preincubated with antibody for 10 min at room temperature prior to the addition of the PRDII probe. p50, p65 and rel antibodies were obtained from Santa Cruz Biotechnology, Inc., and all were used at a final concentration of 0.063 μg/ml.

Susceptibility of mice and MEFs to viral infection

EMCV stock (3×10^8 p.f.u./ml) was prepared from supernatants of virus-infected Swiss 3T3 cells (Pavlovic *et al.*, 1990). Mice were injected intravenously with 300 μl 3×10^3 p.f.u./ml EMCV. Where indicated, the mice (7–8 weeks old) were primed by the intraperitoneal injection of 100 μg pIC 18 h prior to infection, or by adding 5×10^5 U rhuIFN-α2/α1 or 10^5 U rmuIFN-γ to the EMCV solution. To determine EMCV yields, mice were anaesthetized 72 h after infection, exsanguinated and the organs removed. Homogenates (10% w/v) of brain were prepared (Gresser *et al.*, 1976), and virus yields of the cleared homogenates and sera were determined by the 50% tissue culture infectious dose method (Pavlovic *et al.*, 1990).

MEFs were seeded at 3.5×10^5 cells/well into 24-well plates in D10. After 48 h, the cells were treated with rhuIFN-α2/α1 (500 U/ml), rmuIFN-γ (50 U/ml) or pIC (100 μg/ml) and DEAE dextran (500 μg/ml) for 18 h, washed twice with PBS and incubated for 1 h with serum-free DMEM containing EMCV at an MOI of 1. The medium was replaced by D10 and incubation continued for 24 h. Virus yields were determined on NIH 3T3 cell monolayers (Pavlovic *et al.*, 1990).

Preparation of 3T3-like cell lines

Tertiary cultures of MEFs from 15 day old wild-type and *Pkr$^{o/o}$* embryos were replated in two flasks at 1.35×10^6 cells per 75 cm^2 flask. Cells were repassaged after 3–4 days. At each passage, the cells from the two flasks were mixed and replated at the same cell density as above. When it was no longer possible to replate two flasks at the required density, only one 75 cm^2 flask or one or two 25 cm^2 flasks were used.

Soft agar, methyl cellulose and in vivo tumorigenicity assay

MEFs from 12–15 day old wild-type and *Pkr$^{o/o}$* mouse embryos as well as NIH 3T3 cells were infected with recombinant retrovirus containing the 2c-*Ha-ras^{Val12}* gene (pGDV12 ras) or control virus pGD (Tanaka *et al.*, 1994) at an MOI of 10 and assayed after 48 h. For colony formation in soft agar, 2×10^3 or 2×10^5 uninfected or infected cells were incubated for 12 weeks in 65 ml Falcon tissue culture flasks (Schäfer *et al.*, 1983) or 5×10^5 cells were incubated per 6 cm dish with 1.3% methyl cellulose (Tanaka *et al.*, 1994). For the determination of tumorigenicity, $1–9\times10^6$ cells in 0.2 ml PBS were injected subcutaneously into the flanks of ICR *nu/nu* mice. Mice were inspected for palpable tumors at daily intervals.

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