The interferon-inducible double-stranded RNA (dsRNA)-activated protein kinase, PKR, plays an important role in messenger (m) RNA translation by phosphorylating the α subunit of eukaryotic initiation factor 2. Through this capacity PKR is thought to be a mediator of the antiviral and antiproliferative actions of interferon. In addition to translational function, PKR has been implicated in many signaling pathways to gene transcription by modulating the activities of a number of transcription factors, including NF-κB and STATs. However, experiments with two different PKR knockout (PKR−/−) mouse models have failed to verify many of the biological functions attributed to PKR. In addition, results with cells from the two PKR−/− mice have been contradictory and confusing. Here, we show that the first PKR−/− mouse with deletion of exons 2 and 3, corresponding to the N terminus domain of PKR (N-PKR−/−), expresses a truncated protein, resulting from the translation of the exon-skipped mouse PKR (ES-mPKR) mRNA. The ES-mPKR protein is defective in dsRNA binding but remains catalytically active both in vitro and in vivo. Furthermore, we show that the second PKR−/− mouse with a targeted deletion of exon 12, which corresponds to the C terminus of the molecule (C-PKR−/−), expresses a truncated mPKR produced by alternative splicing of exon 12. Although the spliced form of mPKR (SF-mPKR) is catalytically inactive, it retains the dsRNA-binding properties of the wild type mPKR. Reverse transcription-PCRs demonstrate that SF-mPKR mRNA is expressed in several normal mouse tissues, and appears to be under developmental control during embryogenesis. Our data demonstrate that both PKR−/− models are incomplete knockouts, and expression of the PKR variants may account, at least in part, for the significant signaling differences between cells from the two PKR−/− mice.

One of the best characterized interferon (IFN) inducible proteins is the double-stranded (ds) RNA-activated protein kinase PKR (1, 2). PKR is a 68-kDa protein in humans and a 65-kDa protein in mice that displays distinct activities: (i) autophosphorylation upon binding to dsRNA on multiple serine and threonine sites (1, 2), and (ii) phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) on serine 51, a modification that leads to the inhibition of protein synthesis (3). Through this capacity, PKR is believed to be a mediator of the biological functions of IFN (4). In addition to translational control, PKR has been implicated in various signaling pathways leading to gene transcription by modulating the activities of various transcription factors including NF-κB, signal transducer and activator of transcription (STATs), and the tumor suppressor p53 (1).

Work with cultured cells in many laboratories has assigned PKR antiviral, antiproliferative, and tumor suppressor functions in vitro (1, 2). These PKR functions were based on the observations that the overexpression of wild-type human PKR (hPKR) can inhibit growth and induce apoptosis, whereas the overexpression of hPKR mutants are capable of inducing malignant transformation when expressed in mouse NIH3T3 cells (1, 2, 5). However, the antiproliferative and tumor suppressor functions of PKR have not yet been verified in vivo. Specifically, two distinct PKR knockout mice, generated by either disruption of exons 2 and 3 of the pkr gene (herein referred as N-PKR−/− mice (6)) or elimination of exon 12 (herein referred as C-PKR−/− mice (7)), did not exhibit growth abnormalities nor were they tumorigenic. In addition, both types of PKR−/− mice have not displayed any significant susceptibility to infections with a variety of viruses (6, 7) with the exception of encephalomyocarditis virus infection of N-PKR−/− mice (6) or vesicular stomatitis virus infection of C-PKR−/− mice after intranasal inoculation (8–10). The contradictory results obtained with cells expressing dominant negative PKR mutants and PKR−/− cells have been explained by the possible expression of other PKR-like molecules, whose function should be possibly blocked by the PKR mutants (1, 2, 7). In fact, the cloning and characterization of PKR-related genes, such as the PKR-like endoplasmic reticulum kinase/pancreatic eIF-2α kinase, which functions as an eIF-2α kinase (11), and the mouse homologue of the yeast eIF-2α kinase, general control nonderepressible 2 (12), have supported this notion. Thus, PKR appears to be the prototype of a family of kinases with overlapping biochemical and biological functions (13).

Concerning cell signaling, studies with mouse embryonic fibroblasts (MEFs) from the N-PKR−/− mouse were found to be
defective in IκBα phosphorylation and NF-κB activation by dsRNA (14–16). Moreover, N-PKR−/− MEFs are susceptible to apoptosis induced by tumor necrosis factor α (TNF-α), dsRNA, or lipopolysaccharide treatment (17). Furthermore, activation of the STAT-1α protein by phosphorylation on serine 727 is impaired in N-PKR−/− MEFs treated with IFNs (18). In contrast to the N-PKR−/− cells, the C-PKR−/− cells respond normally to the above stimuli (7). For example, the susceptibility of C-PKR−/− MEFs to apoptotic death in response to dsRNA, virus infection, lipopolysaccharide, or TNF-α treatment is normal compared with genetically matched PKR+/+ MEFs (7), as is normal the activation of NF-κB by dsRNA (19).

These striking signaling differences between the two types of PKR−/− cells prompted us to investigate the possible expression of truncated PKRs resulting from incomplete disruption of the pkr gene. Specifically, Yang et al. (6) initially hypothesized that the N-PKR−/− mice might express a truncated PKR, which is the product of translation of a PKR mRNA devoid of exons 2 and 3. Translation of this exon-skipped mRNA could initiate from an AUG that corresponds to methionine 136 producing a 382-amino acid C-terminal fragment of mPKR (6). In fact, the 42–44-kDa exon-skipped mouse PKR (ES-mPKR) protein was detected in reticulocyte lysates programmed to translate the ES-mPKR mRNA from N-PKR−/− cells (6). However, Yang et al. (6) were not able to detect ES-mPKR expression in extracts from N-PKR−/− cells because of the unavailability of antibodies against the C terminus part of mPKR (6). Here, using antibodies specific to the C terminus of mPKR we demonstrate that ES-mPKR is indeed expressed in N-PKR−/− MEFs. We also show that the ES-mPKR is inducible by IFN-α/β and contains eIF2α kinase activity both in vitro and in vivo. Furthermore, we demonstrate that the C-PKR−/− mouse also contains a partial PKR product. Specifically, we show the expression of an alternatively spliced form of mPKR (SF-mPKR) in these cells, which is catalytically inactive but fully active for binding to dsRNA. Our data demonstrated that ES-mPKR expression in extracts from N-PKR−/− mice was not able to detect

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Materials and Methods

Cell Strains and Culture—The MEFs from the N-PKR−/− mice were in 129/SvEv x C57BL/6 genetic background (6), whereas the C-PKR−/− mice were in 129/terSv x BALB/c background (7). The strain-matched MEFs and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (100 units/ml), 0.1 mM minimal essential medium nonessential amino acids (Invitrogen).

Protein Extraction—Cells were washed twice with ice-cold phosphate-buffered saline and proteins were extracted in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KC1, 2 mM MgCl2, 1% Triton X-100, 3 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM dithiothreitol, 1 mM Na2VO4, and 1 mM phenylmethylsulfonyl fluoride. Extracts were kept on ice for 15 min, centrifuged at 10,000 × g for 15 min (4°C), and supernatants were stored at −80°C.

Western Blot Analysis—Protein extracts were subjected to SDS-PAGE as described (20). Proteins were then electroblotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore), which were incubated with any of the following antibodies: mouse monoclonal antibody to PKR (B-10; sc-6282, Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes an epitope within the C terminus half of the kinase; rabbit polyclonal antibody to PKR (n-20; sc-708, Santa Cruz), which recognizes an epitope at the very end of the C terminus of the kinase2; rabbit antiserum to phosphoserine 51 of eIF-2α (21); rabbit polyclonal anti-human eIF-2α (sc-11386, Santa Cruz); rabbit antiserum to residues 101–112 within the N terminus of mouse PKR (22); rabbit antiserum to human PKR (first 80 amino acids) (21); rabbit antiserum to Trp51-yeast eIF2α fusion protein (CM-217); rabbit antiserum to mammalian anticistatine antibodies (sc-803, Santa Cruz); mouse monoclonal antibody to actin (Clone C4; 69100; ICN Biomedicals); rabbit antiserum to phosphoserine 32 of IκBα (sc-9251, Santa Cruz Biotechnology, personal communication); rabbit antiserum to human PKR (first 80 amino acids) (21); rabbit antiserum to IFN-γ (American Biosciences). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences).

Phosphorylation in Vitro—Protein extracts were immunoprecipitated with rabbit polyclonal antibody to mouse PKR (D-20, SC-708, Santa Cruz) and protein A-agarose (American Biosciences). The immunoprecipitates were equilibrated in 1 × PKR kinase buffer containing 10 mM Tris-HCl, pH 7.7, 50 mM KCl, 2 mM MgCl2, 3 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Activator revirus dsRNA was then added to the final concentration of 0.1 μg/ml in the presence of 1 μg of bacteria purified histidine-tagged eIF-2α (23) and 1 μl ATP. After incubation at 30°C for 50 min, the reactions were subjected to SDS-PAGE and then transferred to nitrocellulose membrane to detect phosphoserine 51 of eIF-2α following immunoblotting with rabbit polyclonal histidine antibodies. Proteins were visualized by ECL according to the manufacturer’s specification (American Biosciences).

DNA-binding and PKR Autophosphorylation Assays—Protein extracts were used for a pulldown with poly(rI−c−R). dsRNA coupled to agarose beads (Type 6, American Biosciences). The dsRNA-bound proteins were subjected to SDS-PAGE followed by immunoblot analysis with the mouse monoclonal antibody to mouse PKR (B-10, SC-6282, Santa Cruz). Alternatively, dsRNA-bound proteins were equilibrated in PKR kinase buffer and incubated in the presence of 1 μCi of [γ-32P]ATP. The reactions were incubated at 30°C for 30 min and subjected to SDS-PAGE. The radioactive bands were visualized by autoradiography.

Recombinant Vaccinia/TT Virus Expression System—One day before transfection, 0.8 × 106 HeLa or HuH7 cells were seeded in 6-cm plates. Cells were infected with recombinant vaccinia virus containing the bacterial T7 RNA polymerase gene (24) for 1 h followed by transfection with LipofectAMINE Plus reagents (Invitrogen) and 2 μg of DNA containing the gene of interest in expression vector under the control of T7 promoter. Cells were incubated in serum-free medium at 37°C for 6 h followed by the addition of complete medium and incubation for an additional 18 h before protein extraction.

RNA Isolation, reverse transcriptase-PCR, and Cloning—Exon-skipped mPKR cDNA from N-PKR−/− cells was cloned as described (6) and subcloned into BamHI/XbaI sites of pYES2 expression vector (Invitrogen). Table I summarizes all the primers used for reverse transcription-PCR analysis of RNA from C-PKR−/− cells. The primers were designed according to the mouse PKR sequence (accession number: M65629; locus: MUSSTKINA (25)). Total RNA isolation was established using TRIzol (Invitrogen) according to the protocol provided by the manufacturer. One μg of RNA was used for reverse transcription with the Moloney murine leukemia virus reverse transcriptase (Invitrogen) using the oligo(dT)18 primer (reverse transcription was performed as described by the manufacturer). The single-stranded cDNA was amplified by PCR using the T502/T302 or T503/T303 set of primers and Taq DNA polymerase (catalog number 1146 165; Roche Molecular Biochemicals) with 35 PCR cycles to generate the final product: 1 min at 94°C for the denaturing step, 1.5 min at 58°C for the annealing step, and 2 min at 72°C for the elongation step. PCR products were visualized by ethidium bromide staining under a UV light source. The PCR products generated by the T503/T303 primer set were subcloned into the pCRII.2 vector provided by the TA cloning kit (K2000-01, Invitrogen) and subjected to DNA sequencing.

Yeast Transformations—The yeast strain used was J110 (Mata ura3-52 leu2-3, 2-112 trpl-1363 gen2A::LEU2::Ade2 (21)). Wild type mPKR and hPKR were inserted in pYES3/CT vector (Invitrogen), respectively, using the HI/His/Enzymes IHI sites of pYES2 expression vector (Invitrogen). J110 yeast cells were transformed with DNA using the LiAc method (26). Transformed strains were grown in SGL liquid medium overnight at 30°C. Yeast protein extracts were prepared as described (26). Immunoblot analysis was performed as described below.

Expression of mPKR Isolforms in Normal Mouse Tissue—Normalized cDNAs from 12 different mouse tissues (mouse MTC Panel I; K1425-1;
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RESULTS

Detection of the ES-mPKR Product in N-PKR−/− MEFs—To test whether N-PKR−/− cells express the PKR fragment produced from the translation of exon-skipped mouse PKR mRNA (Fig. 1A) (6), we performed Western blot analysis with protein extracts from strain-matched PKR+/+ and N-PKR−/− MEFs (Fig. 1B). To induce PKR protein expression, MEFs were treated with mouse IFN-α/β. Immunoblot analysis with an anti-PKR monoclonal antibody (mAb) specific for an epitope within the C terminus end of the kinase2 (clone B-10, top panel) detected the expression of full-length PKR in PKR+/+ cells (lanes 1 and 2) and the presence of a 45-kDa protein in isogenic N-PKR−/− MEFs (lane 3), which was highly induced by IFN treatment (lane 4). To further substantiate this finding, we performed a Western blot analysis with protein extracts from isogenic PKR+/+ and N-PKR−/− MEFs using a rabbit polyclonal anti-PKR Ab (clone D-20) specific for an epitope at the very C terminus end of the kinase3 (Fig. 1B, bottom panel). Similarly, we observed the presence of the 45-kDa protein in N-PKR−/− cells (lane 3), which was significantly induced after IFN treatment (lane 4). Note that this protein was undetectable in protein extracts from isogenic PKR+/+ cells (lanes 1 and 2). To confirm that the 45-kDa protein is specifically expressed in N-PKR−/− cells, we performed an immunoprecipitation with the rabbit polyclonal anti-mouse PKR Ab (clone D-20) followed by immunoblotting with the monoclonal anti-mouse PKR Ab (clone B-10; Fig. 1C). This assay demonstrated the expression of the 45-kDa ES-mPKR protein in the N-PKR−/− cells.

ES-mPKR Contains eIF-2α Kinase Activity in Vitro and in Vivo—Next, we examined whether ES-mPKR retains the catalytic activity of PKR. To this end, protein extracts from isogenic PKR+/+ and N-PKR−/− cells were subjected to immunoprecipitation with the rabbit polyclonal anti-mouse PKR antibody (clone D-20; Fig. 2A). Phosphorylation of eIF-2α was then detected by immunoblot analysis using a phosphoserine 51-specific anti-eIF-2α antibody (top panel). The levels of recombinant eIF-2α in the kinase reactions were measured by immunoblot analysis with anti-histidine antibodies (bottom panel). These assays showed that eIF-2α is phosphorylated on serine 51 in N-PKR+/+ cells (lane 1), and this phosphorylation was more highly induced after IFN treatment (lane 2). On the other hand, eIF-2α phosphorylation in the PKR immunoprecipitates from N-PKR−/− MEFs (lane 3) was lower than in PKR+/+ MEFs (lane 1) and detectable only after IFN treatment (lane 4). These differences most likely reflected the different levels of expression of full-length mPKR and ES-mPKR in PKR+/+ and N-PKR−/− cells, respectively (Fig. 1B).

We also examined whether ES-mPKR retains its capacity to bind dsRNA and autophosphorylate. To this end, we used HeLa cells to express transiently wild type (WT) mPKR or ES-mPKR using the recombinant vaccinia/T7 virus method. In this method, transfected genes under the control of the bacteriophage T7 promoter are efficiently transcribed in the cytoplasm by the T7 RNA polymerase delivered into the cells by infection with recombinant vaccinia viruses (24). This method is suitable for expressing PKR and studying the translational functions of the kinase (21). First, we tested the dsRNA binding capacity of ES-mPKR. Protein extracts from HeLa cells expressing WT-mPKR and ES-mPKR were incubated with poly(rI-rC)-agarose beads, and proteins bound to dsRNA were detected by immunoblotting (Fig. 2B). We found that WT-mPKR was capable of binding to dsRNA (lane 5) unlike ES-mPKR (lane 6). However, ES-mPKR was expressed in HeLa cells at higher levels than WT-mPKR (compare lanes 2 and 3). When the dsRNA-bound proteins were subjected to in vitro autophosphorylation assay in the presence of [γ-32P]ATP (Fig. 2C), we found that WT-mPKR was highly autophosphorylated (lane 2) whereas no kinase activity was detected for ES-mPKR (lane 3). The high molecular mass band (~110 kDa) phosphoprotein was nonspecific. These data suggested that ES-mPKR lacks the ability to

FIG. 1. Immunodetection of ES-mPKR in N-PKR−/− MEFs. A, schematic representation of ES-mPKR. ES-mPKR is translated from an alternative start site at position 553 within exon 5 of the PKR mRNA (Met-136). The primary initiator AUG within exon 2 was disrupted by the neomycin resistance gene (neo) to generate the N-PKR−/− mouse. ES-mPKR is a 382-amino acid, 45-kDa protein, which contains part of the dsRNA-binding motif 2 (dsRBM2) and an intact kinase domain (KD). B, ES-mPKR is recognized by two different PKR antibodies. Protein extracts (50 μg) from isogenic (i.e. genetic background 129Sv/ev x C57BL/6J) PKR+/+ and N-PKR−/− MEFs before (lanes 1 and 3) or after stimulation with mIFNα/β (1000 IU/ml for 18 h; lanes 2 and 4) were subjected to SDS-10% PAGE, transferred onto Immobilon P membrane (Millipore), and Western blotted (WB) with an anti-mPKR Ab (B-10, Santa Cruz; top panel) or with a rabbit polyclonal anti-PKR antibody (D-20, Santa Cruz; bottom panel), both specific for the C terminus domain of mPKR. The positions of mPKR and ES-mPKR protein are indicated. C, immunoprecipitation of ES-mPKR from N-PKR−/− MEFs. Protein extracts (500 μg) of PKR+/+ and N-PKR+/+ MEFs before (lanes 1 and 3) and after stimulation with mIFNα/β (1000 IU/ml; lanes 2 and 4) were subjected to SDS-10% PAGE and Western blotted (WB) with the anti-mPKR mAb (B-10, Santa Cruz). The positions of mPKR and ES-mPKR proteins are indicated.
bind dsRNA and conceivably the capacity to autophosphorylate in the presence of dsRNA.

Next, we tested the ability of ES-mPKR to induce the phosphorylation of eIF-2α in vivo. For this, we used the human hepatocarcinoma Huh7 cells, which exhibit 80–90% transfection efficiency as judged by the expression of the green fluorescence protein (data not shown). That is, WT-mPKR and ES-mPKR were transiently expressed in Huh7 cells using the vaccinia/T7 virus system (Fig. 2D). Protein extracts were subjected to immunoblot analysis for mPKR (top panel), eIF-2α serine 51 phosphorylation (middle panel), or total eIF-2α levels (bottom panel). We found that expression of both WT-mPKR and ES-mPKR was able to induce the phosphorylation of endogenous eIF-2α (lanes 2 and 3) demonstrating that ES-mPKR indeed acts as an eIF2α kinase in vivo. In these experiments, we noticed that ES-mPKR migrated higher than the 45-kDa protein marker (Fig. 2B, lanes 4–6) or kinase assay in the presence of [γ-32P]ATP (C, lanes 1–3). E, ES-mPKR exhibits eIF-2α kinase activity in vivo. Huh7 cells (D) were infected with vaccinia virus/T7 and transfected with pYES2 vector DNA (mock; lane 1), WT-mPKR in pYES3/CT vector (lane 2), or ES-mPKR in pYES2 vector (lane 3). Whole cell extracts (WCE) containing 10 μg of protein used to detect the expression of PKR proteins by immunoblotting with the rabbit polyclonal anti-mPKR Ab (D-20) (B, lanes 1–3). For the dsRNA pull-down assays, 200 μg of protein extracts were incubated with poly(rI-rC)-agarose beads, and the dsRNA-bound proteins were subjected to immunoblotting with rabbit polyclonal anti-mPKR Ab (D-20; lane 4), or WT-mPKR in pYES3/CT vector (lane 1), or ES-mPKR was able to induce the phosphorylation of endogenous eIF-2α (lanes 2 and 3) demonstrating that ES-mPKR indeed acts as an eIF2α kinase in vivo. In these experiments, we noticed that ES-mPKR migrated higher than the 45-kDa protein marker (Fig. 2B, lane 3; Fig. 2D, lane 3) probably because of its phosphorylation caused by overexpression.

It has been demonstrated that hPKR substitutes the function for general control nondepressible 2 (28), the only eIF-2α kinase in Saccharomyces cerevisiae (29). Because expression of PKR in yeast induces endogenous eIF-2α phosphorylation (28), we examined whether expression of ES-mPKR in the yeast strain J110, which lacks general control nondepressible 2 (gen2Δ) (30), was also capable of inducing eIF-2α phosphorylation in vivo (Fig. 2E). To do so, ES-mPKR cDNA from
FIG. 3. Immunodetection of a 40-kDa PKR-like protein in C-PKR−/− MEFs. A, a PKR-like protein (mPKR*) is recognized by two different PKR antibodies. Protein extracts (50 μg) from isogenic PKR+/+ and C-PKR−/− MEFs (i.e. genetic background 129/terSv x BALB/c) before (lanes 1 and 3) or after stimulation with mIFNαβ (1000 IU/ml) for 18 h (lanes 2 and 4) were subjected to SDS-12% PAGE, transferred onto Immobilon P membrane (Millipore), and immunoblotted with anti-mPKR mAb (B-10; top panel), rabbit polyclonal anti-mPKR Ab specific for the N terminus domain of the kinase (middle panel), or anti-actin antibody mAb (bottom panel). The PKR-like protein is indicated as mPKR* (lanes 3 and 4). B, immunoprecipitation of mPKR*. Protein extracts (500 μg) of PKR+/+ and C-PKR−/− MEFs before (lanes 5 and 7) and after stimulation with mIFNαβ (1000 IU/ml, 18 h; lanes 6 and 8) were subjected to immunoprecipitation with 1 μg of anti-mPKR B-10 mAb. Immunoprecipitates were then subjected to SDS-12% PAGE and immunoblotted with anti-mPKR B-10 mAb. Lanes 1–4 are 50 μg of whole protein extracts (WPE) from PKR+/+ and C-PKR−/− MEFs before (lanes 1 and 3) or after mIFNαβ stimulation (lanes 2 and 4). C, mPKR* is a 40-kDa protein. Fifty μg of protein extracts from C-PKR−/− MEFs after stimulation with mIFNαβ (1000 IU/ml, 18 h; lanes 1) was immunoblotted with anti-mPKR B-10 mAb. The same blot was stripped and re-probed with rabbit polyclonal anti-phosphoserine 51 eIF-2α Ab (lanes 2), which recognizes the 38-kDa eIF-2α phosphorylase.

N-PKR−/− MEFs was subcloned into the pYES2 expression vector that allows expression under the galactose-inducible promoter (21). J110 cells were transformed with pYES2 vector DNA (mock, lane 1), pYES2/CT mPKR DNA (lane 2), pYES2 ES-mPKR DNA (lane 3), or pYES3/CT hPKR DNA (lane 4). The expression of the PKR forms was induced after growth of yeast cells in galactose-rich medium. Yeast protein extracts were subjected to immunoblot analysis with antibodies against mPKR (first and second from the top panels), hPKR (third from the top panel), phosphoserine 51 of eIF-2α (fourth from the top panel), and endogenous eIF-2α (bottom panel). We found that the two different anti-mPKR antibodies (B-10, top panel; D-20, second from the top panel) recognized mPKR and ES-mPKR in lanes 2 and 3, respectively, but not hPKR (lane 4, third panel). We noticed that the D-20 Ab recognized full-length mPKR better than the B-10 mAb (lane 2) for reasons that are not immediately clear. We also noticed a couple of slower migrating band(s) with the anti-mPKR antibodies in lane 3, which may represent phosphorylated forms of ES-mPKR. More importantly, however, we observed that phosphorylation of endogenous eIF-2α was induced in cells expressing ES-mPKR (fourth from the top panel, lane 3) and that eIF-2α phosphorylation levels were proportional to the amounts of mPKR and ES-mPKR expressed in yeast cells. These findings clearly demonstrate that ES-mPKR is catalytically active in yeast cells in vivo.

MEFs from the C-PKR−/− Mice Express a 40-kDa PKR-like Protein—The above findings prompted us to perform similar experiments with MEFs from the C-PKR−/− mouse (7). We performed immunoblot analysis of protein extracts from untreated and IFN-α/β-treated cells with either the anti-PKR B-10 mAb (Fig. 4A, top panel) or a rabbit polyclonal anti-PKR
Ab specific for the dsRBM1 within the N terminus domain of mouse PKR (22) (Fig. 3A, middle panel). Both antibodies detected the expression of a ~40-kDa protein in C-PKR*−/− MEFs, which was more highly induced after IFN treatment (lane 4). This PKR-like protein, herein designated as mPKR*, was also detected by immunoprecipitation and immunoblotting with the anti-N terminus mPKR Ab (data not shown), we conclude that mPKR* in C-PKR* and ES-mPKR proteins are different (see also below).

To do so, protein extracts from isogenic PKR*−/− and C-PKR*−/− MEFs were incubated with poly(rI·rC) dsRNA-agarose beads (Fig. 4B), and phosphorylation of dsRNA-bound proteins on the pulled down beads was tested in the presence of [γ-32P]ATP. The 65-kDa dsRNA-bound phosphorylated protein in PKR*−/− extracts (lane 1), whose phosphorylation was more highly induced after treatment with IFN (lanes 2 and 3), was mPKR. Contrary to PKR*−/− extracts, C-PKR*−/− protein extracts were free of kinase activity (lanes 3 and 4) demonstrating that mPKR* is catalytically inactive. Note that the 65-kDa protein recognized by the anti-PKR B-10 mAb (see also below).

FIG. 5. Cloning and sequencing of two alternative SF of mPKR in C-PKR*−/− MEFs. A, reverse transcription-PCR analysis of C-PKR*−/− MEFs. Exponentially grown PKR *−/− and C-PKR*−/− MEFs both with genetic background 129/terSv x BALB/c were treated with 1000 IU/ml mIFNα/β for 18 h. Reverse transcription (RT) was performed using 1 μg of total RNA, oligo(dT)18 primer, and Moloney murine leukemia virus-reverse transcriptase followed by PCR with either the T503/T303 set of primers (top panel) or with the T502/T302 set of primers (bottom panel). Amplified DNA was subjected to 1.5% agarose electrophoresis and bands were visualized by EtBr staining.

Lane 1, marker DNA (4X174 DNA digested with HaeIII). Lane 4, negative control (dH2O). Lane 5, mPKR gene in pcDNA3/neo. B, DNA sequence and schematic representation of SF-mPKR. The DNA sequence around the junctions between exon 10/exon 11, exon 11/exon 12, exon 12/exon 13 of wild type (WT) mPKR, exon 10/exon 11 and exon 11/exon 13 of SF1-mPKR, and exon 10/exon 13 of SF2-mPKR are shown (top panels). The predicted structures and molecular sizes of SF1-mPKR and SF2-mPKR (bottom panels) are shown and compared with full-length mPKR (top panel).
therefore, it is unlikely to be a PKR-related protein.

mPKR* Is an Alternative Spliced Form of mPKR—Next, we sought to explain the presence of mPKR* in C-PKR/−/− cells. We speculated that mPKR* might be a product of alternative splicing based on our previous findings that spliced forms of PKR are expressed in human cells (21). To test this hypothesis, RNA from PKR+/− and C-PKR/−/− MEFs was subjected to reverse transcription-PCR analysis using two sets of primers, one that amplifies the region of mPKR cDNA between exons 5 and 14 (Fig. 5A, top panel), and the other the region between exons 7 and 13 (Fig. 5A, bottom panel). We found that full-length PKR was not amplified in RNA samples from C-PKR−/− cells providing further evidence that the 65-kDa protein recognized by the anti-PKR B-10 mAb in C-PKR−/− cells (Fig. 4A) was not a contamination with PKR+/− cells. Instead, we observed the presence of two smaller amplified bands (herein designated as SF1-mPKR and SF2-mPKR), which were subsequently identified as SF of the pkr gene. That is, sequencing of the cloned PCR products using the primers indicated in Table 1 demonstrated that SF1-mPKR is a product of alternative splicing that occurs between exons 11 and 13 (Fig. 5B, middle panel), whereas SF2-mPKR is made by alternative splicing between exons 10 and 13 (Fig. 5B, bottom panel). Thus, both SF-mPKR products bypass exon 12, which contains the neomycin resistance gene used for the generation of the C-PKR−/− mouse (7).

Analysis of the sequencing data also showed that the alternative splicing mechanisms generated nonsense mutations for both alternatively spliced products with a stop codon a few nucleotides downstream of the splicing site. Thus, SF1-mPKR consists of 323 residues with a predicted molecular size of 40 kDa, whereas SF2-mPKR generates a protein of 283 residues with a molecular size of 35 kDa (Fig. 5B). However, the immunoblot analysis in Fig. 3C clearly identified mPKR* as a 40-kDa protein suggesting that mPKR* is identical to the SF1-mPKR product. Detection of the SF2-mPKR protein in C-PKR−/− cells was not possible.

Tissue Distribution of SF1-mPKR mRNA—The lack of any detectable levels of SF-mPKR proteins in PKR−/− cells prompted us to examine whether these PKR forms are expressed in normal mouse tissues at low levels and whether their expression is tissuespecific. To this end, we performed PCR analysis of normalized single-stranded cDNAs prepared from 12 different normal mouse tissues using the T502/T302 set of primers. To increase the sensitivity of the screening, the PCR products were detected by Southern blotting (Fig. 6). These experiments showed that expression of full-length mPKR did not significantly vary between the mouse tissues, whereas SF1-mPKR was expressed at very low levels in brain, spleen, lung, kidney, and testis (Fig. 6). Interestingly, SF1-mPKR was also detected in tissues from 7-, 11-, and 17-day-old mouse embryos but not in tissue from 15-day-old mouse embryos indicating that its expression could be under developmental control. Detection of SF2-mPKR mRNA in these assays was not possible suggesting that its expression is probably unique for C-PKR−/− MEFs.

Control of IκBα and eIF-2α Phosphorylation in C-PKR−/− MEFs—The partial inactivation of PKR in both PKR−/− mice may account, at least in part, for the signaling differences between the two PKR−/− cell types. For example, previous work with N-PKR−/− cells showed an impaired IκBα phosphorylation and NF-κB activation in response to dsRNA treatment (6, 15, 16). Contrary to this, NF-κB activation by dsRNA is normal in C-PKR−/− cells compared with isogenic PKR+/−/− cells (19). We further confirmed this finding by assessing IκBα phosphorylation levels in PKR−/−/− and C-PKR−/−/− cells in response to dsRNA (Fig. 7A). We found that IκBα phosphorylation in vivo was induced at equal levels in both PKR−/−/− and C-PKR−/−/− cells (Fig. 7A, top panel). This finding demonstrates that the catalytic activity of mPKR plays no role in dsRNA-mediated signaling to NF-κB-dependent transcription. This is in agreement with recent findings from us and others showing that the catalytic activity of hPKR is dispensable for NF-κB-mediated gene transcription (31, 32).

We also examined the eIF-2α phosphorylation levels in virus-infected C-PKR−/− MEFs. To this end, cells derived from isogenic PKR+/−/− and C-PKR−/− mice bred onto 129SvEv background (10) were infected with Sendai virus (Fig. 7B). Immunoblot analysis with anti-eIF-2α phosphoserine 51 antibodies (Fig. 7B, top panel) followed by immunoblotting with anti-mouse eIF-2α mAb (bottom panels) showed no significant differences in eIF-2α phosphorylation between PKR+/−/− and C-PKR−/−/− cells. These data show the redundancy of PKR in eIF-2α phosphorylation in response to Sendai virus infection.

**DISCUSSION**

PKR was initially characterized as a protein kinase with an important role in the control of mRNA translation through its capacity to induce eIF-2α phosphorylation (1, 2). The transcriptional induction of the pkr gene by type I IFNs provided further evidence that PKR is a mediator of the antiviral and antiproliferative effects of IFN (4). The cloning of the human and mouse PKR cDNAs (25, 33–35) assisted in the identification of novel biological properties of the kinase in cultured cells. To date, numerous reports have assigned PKR anti-proliferative and tumor suppressor functions in vitro and have implicated it in many signaling pathways that control gene expression at both translational and transcriptional levels (1, 2). However, the generation and characterization of two different PKR−/−...
mice did not yield the anticipated results, as many of the important in vitro functions of PKR have not been verified in vivo. In addition, experiments with cells from the two PKR−/− mice have led to contradictory and confusing results concerning the PKR functions in vivo. Here, we demonstrate that neither of the two PKR−/− mice is completely devoid of PKR. The N-PKR−/− mouse (6) expresses a dsRNA-binding defective but catalytically active PKR protein, whereas the C-PKR−/− mouse (7) expresses an alternatively spliced form of the kinase with intact dsRNA-binding and impaired catalytic properties.

In regard to cell signaling, it was previously shown that N-PKR−/− MEFs display an impaired NF-κB activation in response to dsRNA and this defect was restored after priming with IFN-α/β or IFN-γ possibly by the expression of a molecule that substitutes PKR function (6). Subsequent work with the N-PKR−/− MEFs concluded that PKR functions as an important signal transducer for the induction of IFN-stimulated genes through pathways that implicate interferon regulatory factor-1 and NF-κB (14). Moreover, N-PKR−/− MEFs were found to be resistant to apoptotic death in response to dsRNA, TNF-α, or lipopolysaccharide providing further evidence for the role of PKR in stress-induced apoptosis (17). Furthermore, work with the N-PKR−/− MEFs established a requirement of the kinase for p38 MAPK activation (36) and for serine phosphorylation of STAT1α in response to IFNs (18). More recent work with the N-PKR−/− MEFs showed that the defective NF-κB activation by dsRNA is because of the impaired IKK activation and phosphorylation of IkBα (15, 16).

The above signaling defects of N-PKR−/− cells were not found in C-PKR−/− cells (7). Specifically, IFN signaling and transcriptional induction of IFN-inducible genes is normal in C-PKR−/− cells (7), indicating that the lack of the catalytic activity of PKR does not interfere with the Jak-STAT pathway. In accord with this, we found that phosphorylation of STAT1 on serine 727 is not impaired in IFN-treated C-PKR−/− MEFs (data not shown) as opposed to N-PKR−/− MEFs (18). In regard to NF-κB activation, it has been documented that C-PKR−/− MEFs respond normally to dsRNA (19) or TNF-α (7), suggesting that the catalytic activity of PKR is dispensable for NF-κB-mediated gene transcription. This is further supported by our data showing that induction of IkBα phosphorylation by dsRNA is normal in C-PKR−/− MEFs compared with isogenic PKR+/+ MEFs (Fig. 7A).

The above striking differences between the two PKR−/− mice may be attributed to the expression of the PKR forms. It is possible that the N-terminal dsRNA-binding domain of PKR is essential for mediating some signaling properties of PKR, and therefore the lack of this domain in ES-mPKR may account for the signaling defects reported for N-PKR−/− MEFs. Specifically, PKR through its N terminus domain may participate in cell signaling as a scaffold protein by mediating protein-protein interactions and/or subcellular localization and compartmentalization of the molecule. This would explain the lack of the signaling defects that were reported for N-PKR−/− cells in C-PKR−/− MEFs, because the latter cell type expresses SF-PKR with an intact dsRNA-binding domain. This would also imply that PKR may have the capacity to modulate cell signaling independently of its enzymatic activity. Interestingly, a kinase-independent role of PKR has been reported for signaling pathways leading to the transcriptional activation of NF-κB and STAT1 (31, 32, 37).

Concerning viral infection, both PKR−/− mice exhibit a very modest susceptibility to viruses. Specifically, N-PKR−/− mice are susceptible to infection with encephalomyocarditis virus (6), whereas C-PKR−/− mice are susceptible to vesicular stomatitis virus infection only after intranasal inoculation (8, 9).

Interestingly, a recent report demonstrates that the increased susceptibility of C-PKR−/− mice to vesicular stomatitis virus is mouse strain-dependent because animals with BALB/c genetic background are 5 orders of magnitude more sensitive to infection than those with 129SvEv background (10). Nevertheless, PKR−/− mice bred onto resistant 129SvEv background still exhibited a 10-fold increased sensitivity compared with the strain-matched PKR+/+ mice (10). These data raise the possi-
bility that the signaling properties attributed to PKR may also be affected by mouse strain differences. We also found that eIF-2α phosphorylation in response to Sendai virus infection is not impaired in C-PKR/−/− MEFs compared with isogenic PKR+/+/− MEFs (Fig. 7B). This may indicate that the loss of the catalytic activity of PKR in C-PKR/−/− cells could be compensated by another eIF-2α kinase activated by virus infection. This is consistent with our previous observations that expression of the vaccinia virus K3L protein, which functions as a pseudosubstrate inhibitor of PKR (38), was still capable of enhancing translation from reporter gene constructs in transiently transfected C-PKR/−/− MEFs (21). The newly identified eIF-2α kinases, PKR-like endoplasmic reticulum kinase and general control nonderepressible 2, might be implicated in this process although it is not yet known how these enzymes are regulated in virus-infected cells. In regard to an antiviral role of PKR independently of eIF2α, the JNK and p38 MAPK pathways have been recently implicated in virus replication (15, 39). Although activation of p38 MAPK was partially impaired in N-PKR/−/− MEFs (36), our experiments with C-PKR/−/− MEFs showed normal patterns of activation of both JNK and p38 MAPK pathways in response to vesicular stomatitis virus or Sendai virus infection (data not shown).

The functional characteristics of the ES-mPKR and SF1-mPKR proteins may provide some clues to molecular mechanisms of PKR action. For example, current models for PKR activation propose that dsRNA binding to the dsRBMs leads to structural rearrangement of PKR relieving an autoinhibitory mechanism (32). However, our results indicate that SF1-mPKR, its expression in certain mouse tissues might be indicative of a specific function of this protein. Because induction of PKR activity proceeds through dsRNA binding, dimerization, and interphosphorylation, a predicted function of the SF1-mPKR protein is the inhibition of mPKR activity in a dominant negative fashion, as previously demonstrated for a similar catalytically inactive PKR form expressed in a mouse pre-B leukemia cell line (22). However, expression of SF1-mPKR in normal tissues is very little compared with the full-length PKR suggesting that a dominant negative effect of this protein could possibly be local and/or RNA specific. Alternatively, SF1-mPKR could function as a RNA-binding protein independent of PKR by modulating, for example, RNA editing (42), RNA trafficking (43), or RNA processing (44).

In conclusion, our data clearly demonstrate that both PKR/−/− animal models are incomplete knockouts, and this may account for the differential responses of the two PKR/−/− cell types to various intracellular and extracellular signals. Considering the crucial biological functions that have been attributed to PKR in tissue culture systems in vitro, it is apparent that a complete disruption of the pkr gene may be necessary to verify the biological functions of the kinase in vivo.

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Functional Characterization of *pkr* Gene Products Expressed in Cells from Mice with a Targeted Deletion of the N terminus or C terminus Domain of PKR
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