Dominant Negative Function by an Alternatively Spliced Form of the Interferon-inducible Protein Kinase PKR

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The double-stranded RNA (dsRNA)-activated protein kinase PKR (protein kinase dsRNA-dependent) plays an important role in the regulation of protein synthesis by phosphorylating the α-subunit of eukaryotic initiation factor 2 (eIF-2α) (reviewed in Ref. 2). Phosphorylation of eIF-2α by PKR on serine 51 leads to an increased affinity of the initiation factor for eukaryotic translation initiation factor 2B, also known as guanine exchange factor, and thus increases the proportion of the latter that is trapped as an inactive complex with eIF-2 and GDP (reviewed in Ref. 3). The reduction in free eukaryotic translation initiation factor 2B results in a fall of the overall rate of guanine nucleotide exchange on the remaining unphosphorylated eIF-2, eventually leading to an inhibition of translation initiation (3). In addition to translational control, PKR has been implicated in signaling pathways leading to transcriptional activation by dsRNA, virus infection, various cytokines, or genotoxic stress (reviewed in Ref. 4).

Several reports have assigned to PKR a tumor suppressor function in vitro (2). Specifically, expression of wild type (WT) human PKR in yeast (5) or in mouse cells (6) results in cell growth inhibition and in some cases in the induction of cell death by apoptosis (7). On the other hand, expression of PKR mutants in NIH3T3 cells that are catalytically inactive or dsRNA binding-defective causes malignant transformation and induction of tumorigenesis after injection of the transformed cells in nude mice (6, 8–10). Contrary to these in vitro functions, deletion of the pkr gene by homologous recombination is not tumorigenic (11, 12). In addition, PKR knockout (PKR−/−) mice are not susceptible to virus infection (11, 12) with the exemption of encephalomyocarditis virus after priming with IFN-γ (12) or vesicular stomatitis virus after intranasal infection (13, 14). Therefore, it has been suggested that the lack of PKR may be compensated by the expression of other PKR-like molecules whose function is possibly blocked by the expression of the PKR mutants in vitro (2, 11). This is supported by the cloning and characterization of PKR-related genes, such as PKR-like endoplasmic reticulum kinase/pancreatic eIF-2α kinase, which functions as an eIF-2α kinase (15), and the mouse homologue of the yeast eIF-2α kinase GCN2 (16). Thus, PKR may be the prototype of a family of kinases with overlapping biochemical and biological functions (17).

Work in many laboratories using in vitro mutagenesis of human and mouse PKR has led to an extensive characterization of the structure-function relationship of the molecule (2). Briefly, in both species the amino-terminal half of PKR contains two RNA-binding motifs (dsRBMs) (2), which are conserved among most of the RNA-binding proteins (18). On the other hand, the carboxyl-terminal half of PKR is divided into 11 subdomains, which are required for catalytic activity (2) and are conserved among many serine/threonine protein kinases (19). At the genomic level, the human PKR gene contains 17 exons that vary in size from 18 nucleotides in exon 1 to 840 nucleotides in exon 17 (20), whereas the mouse gene contains...
16 exons varying from 35 nucleotides in exon 8 to 750 nucleotides in exon 16 (21).

Despite the tumor suppressor function in vitro, naturally occurring mutants of PKR in tumor cells have not as yet been identified with the exception of a mutant form of mouse PKR in a pro-B leukemia cell line (22). Here, we report the cloning of a point mutant of human PKR (Y176H) from Jurkat leukemia cells encoding for a protein that retains the RNA-binding and catalytic properties of wild type PKR in vitro. We also describe the cloning and characterization of an alternatively spliced form of PKR (PKRΔE7) from Jurkat cells. PKRΔE7 is a splicing product of exon 7 of the human PKR gene, which contains the two dsRBMs and exhibits a dominant negative function in vitro (23).

**MATERIALS AND METHODS**

**RNA Isolation and RT-PCRs**—The primers used in RT-PCRs are summarized in Table I. For sequencing of the protein-coding sequence of PKR, 1 μg of RNA isolated by the guanidium thiocyanate method (23) was reverse transcribed using the P303 primer. The single-stranded cDNA was then amplified by PCR using the P505 and PCR amplification using the P505/P305 set of primers with denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min for a total of 30 cycles. The PCR products, which spanned the entire protein-coding sequence of PKR, were subcloned into pCR™II (Invitrogen) and sequenced with T7 DNA Polymerase (U.S. Biological Corp.) or subjected to direct sequencing using the DeazaT7Sequencing™ Kit according to the supplier's instructions (Amersham Pharmacia Biotech). Quantification of radioactive bands was visualized by autoradiography. Alternatively, the in vitro kinase assay of PKR was performed with S10 protein extracts in 1× PKR kinase buffer in the conditions described above. The autophosphorylated PKR was then immunoprecipitated with mouse monoclonal anti-human PKR antibodies, subjected to SDS-PAGE, and radioactive bands were visualized by autoradiography.

**Generation of an Anti-Phosphoserine 51-specific eIF-2α Antibody**—Rabbit antiserum was produced against a chemically synthesized phosphopeptide ILLSELpSRRRIRS (where pS represents phosphoserine) that contains serine 51 of human eIF-2α. The antibody was purified from rabbit serum by sequence-specific chromatography and was negatively charged using protein-A-agarose beads. PKR immunoprecipitates were equilibrated in 1× PKR kinase buffer consisting of 10 mM Tris-HCl, pH 7.7, 50 mM KCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml peptatin. PKR autophosphorylation was performed in the presence of 0.1 μg/ml activator recombinant vaccinia virus containing the bacterial T7 RNA polymerase gene (25). After incubation at 30 °C for 20 min, the reactions were subjected to SDS-PAGE, and radioactive bands were visualized by autoradiography.

**Western Blotting**—Protein extracts or PKR immunoprecipitates were subjected to SDS-PAGE and proteins were transferred onto nylon membranes (Millipore Corp.). Immunoblots were performed with mouse monoclonal anti-human PKR antibodies, rabbit polyclonal anti-phosphoserine 51 eIF-2α antibodies (a gift from Dr. H. W. O'Shea), anti-human eIF-2α antibodies, rabbit polyclonal anti-phosphoserine 51 eIF-2α antibodies (homemade from or BIO-SOURCE, catalog no. 44-728), and rabbit antiserum to Trp-eIF-2α fusion protein (CM-217) at a concentration of 1 μg/ml using the standard protocol (27). After incubation with horseradish peroxidase-labeled rabbit anti-mouse IgG or anti-rabbit IgG antibodies (1:1000 dilution; Eastman Kodak Co.), or subjected to direct sequencing using the DeazaT7Sequencing™ Kit according to the supplier's instructions (Amersham Pharmacia Biotech). Quantification of the bands in the linear range of exposure was performed by densitometry using the NIH Image 1.54 software.

**TABLE I**

| Name       | Position | Sequence                     |
|------------|----------|------------------------------|
| P501       | 928      | 5'-GCTGAGAAGTCACCTTCAGA-3'   |
| P502       | 1249     | 5'-ACTAGTGTGTCGTTCATTTTTC-3' |
| P503       | 1567     | 5'-TGCCAAACCTCTTGTCCACA-3'   |
| P504       | 1899     | 5'-ACTAGTGTGTCGTTCATTTTTC-3' |
| P505       | 167      | 5'-GACCTTCCTGACATGAAAGA-3'   |
| P506       | 979      | 5'-TGCCAAACCTCTTGTCCACA-3'   |
| P507       | 979      | 5'-GACCTTCCTGACATGAAAGA-3'   |
| P508       | 654      | 5'-GCGGCTGAAACTGCTATAC-3'    |
| P509       | 815      | 5'-GCTGAGAAGTCACCTTCAGA-3'   |

* According to gbM35663.
2% raffinose, and the required amino acid supplement (SGAL). The cultures were incubated at 30 °C for various time periods, and a 0.5-ml liquid culture from each point was used to measure A$_{660}$.

RESULTS

Cloning of a Point Mutant and an Alternatively Spliced Form of PKR from the Human Leukemia Jurkat T Cells—We have reported a diminished PKR activation in various human leukemia cell lines including Jurkat T lymphocytes (26). We speculated that PKR inactivation in these cells might be caused by mutations in the PKR gene. To test this possibility, we amplified and sequenced the PKR cDNA from Jurkat cells and normal PBMCs after RT-PCR as described under “Materials and Methods.” Direct sequencing of the PCR products verified the presence of a T to C point mutation in Jurkat PKR cDNA at nucleotide 526 downstream from the initiator ATG (Fig. 1A), which results in a single substitution of tyrosine 176 to histidine. When the PCR products were subcloned into pCRRII vector and sequenced (see “Materials and Methods”), nine out of 10 clones contained the T to C mutation. One clone, however, harbored a 77-bp deletion, which corresponds to the entire exon 7 of the human PKR gene (Fig. 1B). Deletion of exon 7 leads to the conjunction of exons 6 and 8 with a frameshift that introduces a stop codon (TGA) within exon 8 and produces an RNA encoding for a 174 amino acid protein. This truncated protein contains the two dsRBMs of PKR and herein is named PKR$E_7$ (Fig. 2).

We confirmed the expression of the PKR$E_7$ RNA in Jurkat cells by an RT-PCR assay (see “Materials and Methods”). The PKR$E_7$ PCR product contains a 77-bp deletion and therefore migrates faster than the WT PKR PCR product on agarose gel electrophoresis (Fig. 3A, compare lanes 3 and 4). The ratio of the band intensities of the two PCR products is proportional to the amount of each PKR transcript within the cells. To examine whether PKR$E_7$ expression is unique for Jurkat cells, we performed an RT-PCR assay with RNA from normal PBMCs. We found that PBMCs contain very low levels of PKR$E_7$ RNA (<1% of full-length PKR RNA; see also Fig. 8), whereas PKR$E_7$ RNA levels in Jurkat cells is about ~10% of WT PKR transcript (Fig. 3A). We also verified the PKR$E_7$ protein expression by immunoblot analysis (Fig. 3B). To facilitate the detection of PKR$E_7$, Jurkat cells were treated with IFN-α/β to induce PKR RNA expression. The protein extracts before (lane 2) and after IFN treatment (lane 3) were subjected to immunoprecipitation and immunoblotting with antibodies specific to the N terminus domain of PKR to detect both full-length PKR (top panel) and PKR$E_7$ (bottom panel). These experiments showed that PKR$E_7$ protein is expressed in Jurkat cells at low levels (lane 2), and its expression is induced after IFN treatment (Fig. 3B, lane 3).

Biochemical Characterization of PKR$E_7$—Characterization of Y176H mutation showed that PKRY176H retains both the dsRNA binding and catalytic activities of WT PKR in vitro (data not shown). As a result of it, we concentrated our efforts on characterizing the function of PKR$E_7$. The N terminus domain of PKR is involved in dsRNA-binding (29). PKR$E_7$ is similar to an artificially made N terminus-truncated form of PKR, known as p20, which contains the two contiguous dsRBMs. p20 can bind to dsRNA (30–33) and heterodimerize with WT PKR in yeast two-hybrid assays (32). Based on this, we wished to examine the ability of PKR$E_7$ to self-associate and associate with WT PKR in the presence and absence of dsRNA. To do so, we constructed a fusion protein of PKR$E_7$ bearing the FLAG epitope in the N terminus. When FLAG-PKR$E_7$ and PKR$E_7$ were transiently co-expressed into HeLa cells, an equal amount of the two proteins was co-immunoprecipitated with anti-FLAG antibodies (Fig. 4A, lane 8), confirming their ability to self-associate. In similar assays, an equal amount of endogenous WT PKR (Fig. 4B, top panel, lane 1) and PKR$E_7$ (bottom panel, lane 1) was found to associate with FLAG-PKR$E_7$ (middle panel, lane 1). However, treatment with micrococcal nuclease (MN) diminished the association of FLAG-PKR$E_7$ with WT PKR (top panel, lane 2) without affecting its association with PKR$E_7$ (bottom panel, lane 2). These data suggested that self-association of PKR$E_7$ may take place in the absence of dsRNA, whereas its association with full-length PKR is dsRNA-dependent. To further investigate this possibility, we used a FLAG-PKR$E_7$ construct bearing the LS9 mutation (substitutions of alanine 66 and alanine 68 to glycine 66 and proline 68), which completely abolishes dsRNA binding (34). Co-expression of FLAG-PKR$E_7$ and PKR$E_7$ in HeLa cells and immunoprecipitation with anti-FLAG antibody revealed the lack of association of FLAG-PKR$E_7$ with either PKR$E_7$ (lanes 3 and 4, bottom row) or the endogenous PKR (lanes 3 and 4, top row). Since the LS9 mutation may affect the conformation of the dsRNA-binding domain of PKR (34), these data suggested that the integrity of dsRNA-binding domain is essential for PKR$E_7$ self-association and association with full-length PKR. In these experiments, we noticed that a higher amount of FLAG-PKR$E_7$ was immunoprecipitated with anti-FLAG antibodies after MN treatment. One plausible explanation is that binding of RNA to FLAG-PKR$E_7$ impedes the accessibility of the antibody to FLAG epitope, and this inhibition may be alleviated by MN treatment.

PKR$E_7$ Exhibits a Dominant Negative Function—The ability of PKR$E_7$ to associate with WT PKR prompted us to examine for a possible dominant negative function in PKR activation. To this end, first we assessed the ability of FLAG-PKR$E_7$ to inhibit the autophosphorylation of endogenous PKR when transiently expressed in HeLa cells using the vaccinia/T7 virus system (25). Because the vaccinia/T7 virus system is a two-step procedure utilizing transfection with LipofectAMINE and infection with recombinant virus (see “Materials and Methods”), we measured PKR activation in cells treated with LipofectAMINE and vector DNA (Fig 5A, mock, lane 1). LipofectAMINE plus vector DNA plus virus (mock, lane 2), or LipofectAMINE plus FLAG-PKR$E_7$ cDNA plus virus (lane 3). The activation of endogenous PKR was measured first by autophosphorylation in the protein extracts in vitro followed by immunoprecipitation with an anti-human PKR antibody (Fig 5A). We observed that PKR autophosphorylation, after normalization to protein levels, was more highly induced by the virus (Fig 5A, compare lane 2 with lane 1) presumably by the production of activator dsRNA during infection. On the other
hand, FLAG-PKRΔE7 expression resulted in the inhibition of PKR autophosphorylation (Fig. 5A, lane 3) compared with mock-transfected cells in the presence (lane 2) or absence (lane 1) of vaccinia/T7 virus. The levels of endogenous PKR or FLAG-PKRΔE7 were detected by immunoblot analysis with the anti-human PKR (middle panel) or anti-FLAG antibody (bottom panel), respectively. These data argued for a dominant negative effect of PKRΔE7 on PKR activation in vitro.

Similar observations were made when the dominant negative function of PKRΔE7 was tested in fibroblasts derived from a mouse with targeted disruption of the catalytic domain of PKR (C-PKR−/− cells; Ref. 11) (Fig. 5B). Transient expression of PKRΔE7 with human WT PKR into C-PKR−/− cells resulted in inhibition of PKR autophosphorylation (top panel, lane 4) compared with expression of WT PKR alone (top panel, lane 2). Immunoblot analysis with an anti-human PKR antibody veri-
were visualized after 1 min. As a positive control (lane 2) eIF-2α made rabbit polyclonal antibody specific to phosphoserine 51 of alanine; Ref. 35) cDNA (Fig. 5A and B). Phosphorylation of eIF-2α in vivo was then detected by immunoblot analysis using a homemade rabbit polyclonal antibody specific to phosphoserine 51 of eIF-2α (see "Materials and Methods"). Transient expression of WT eIF-2α resulted in induction of phosphorylation on serine 51 caused by the activation of endogenous HeLa PKR (36) (top panel, lane 2). Co-expression of WT eIF-2α with PKRAR7E (lanes 4) resulted in inhibition of eIF-2α serine 51 phosphorylation (top panel, compare lane 4 with lane 2), whereas the expression levels of eIF-2α remained stable (middle panel, lanes 2 and 6). Note the low (undetectable) levels of endogenous eIF-2α phosphorylation with this antibody (lanes 1) and the lack of its cross-reactivity with the serine 51 to alanine mutant of eIF-2α (lane 3). The phosphorylation levels of endogenous HeLa eIF-2α were detected, however, when a commercially available phosphoserine 51-specific antibody was used (Fig. 5D). We measured eIF-2α phosphorylation in cells treated with LipofectAMINE plus vector DNA alone (mock, lane 1), LipofectAMINE plus virus plus vector DNA (mock, lane 2), or LipofectAMINE plus vaccinia virus plus PKRAR7E cDNA (lane 3). 

Infection with vaccinia virus (lane 2) induced the phosphorylation of eIF-2α (compare lanes 1 and 2), which was diminished by PKRAR7E expression (lane 3) through the inhibition of endogenous PKR. Taken together, the above data demonstrate the dominant negative function of PKRAR7E in both PKR activation and eIF-2α phosphorylation.

Functional Characterization of the Dominant Negative Function of PKRAR7E in Yeast—It has been shown that high level of PKR expression in Saccharomyces cerevisiae is toxic due to inhibition of general translation (5). However, at a lower level of expression, PKR can substitute the function of GCN2, the only eIF-2α kinase known to exist in yeast (37), by phosphorylating eIF-2α on serine 51 and stimulating GCN4 translation, a transcription factor involved in amino acid biosynthesis (38). To verify the dominant negative function of PKRAR7E in vivo, we used a yeast strain that lacks endogenous GCN2 (J110) (39) and two strains that contain one (H2544) and two (H2543) alleles of WT human PKR, respectively, under the control of the galactose-inducible promoter (40). Induction of PKR expression in H2544 strain partially inhibits growth, whereas PKR induction in H2543 completely abolishes growth. Strains J110, H2544, and H2543 were transformed with vector alone, FLAG-PKR, or FLAG-PKR7E7. As positive control, we used the PKR inhibitor vaccinia virus K3L (40). The transformants were streaked onto minimal medium plates containing either glucose or galactose, and the effect of each of these proteins on PKR-mediated growth inhibition was monitored. All transformants of the isogenic J110 strain grew well in either glucose or galactose, indicating that expression of these exogenous proteins did not perturb normal yeast growth characteristics (Fig. 6A). In agreement with previous studies (40), H2544 transformants containing vector DNA without an insert demonstrated a slow growth phenotype after PKR induction (Fig. 6A, bottom plate). However, expression of K3L reversed this growth-inhibitory phenotype (bottom plate). Likewise, expression of FLAG-PKR7E7 also rescued yeast growth consistent with previous findings that the N-terminus domain of PKR from amino acid 1 to 262 rescues yeast growth inhibition by WT PKR (5). In contrast to this, FLAG-PKR7E7

FIG. 3. Detection of PKRAR7E7 RNA and protein expression in Jurkat cells. A, RNA isolated from normal PBMCs (lane 5) and Jurkat (lane 6) was subjected to RT-PCR. PCR products were fractionated in 2% agarose gel and stained with ethidium bromide. A DNA molecular weight (M.W.) marker was loaded to indicate the size of the PCR products (lane 1). As a negative control, a PCR containing no DNA template was used (lane 2). As positive controls, PCR products from wild type PKR (lane 3) and PKRAR7E7 (lane 4) cDNAs were 162 and 85 bp, respectively. B, 200 μg of Jurkat S10 protein extracts before (lane 2) or after IFN-α, treatment (1000 IU/ml, 18 h) (lane 3) were immunoprecipitated with a rabbit polyclonal anti-human PKR antibody specific for the N-terminal domain of PKR. Immunoprecipitates were then subjected to Western blotting with the mouse monoclonal anti-human PKR (F9) antibody. The expression levels of full-length PKR (top panel) and PKRAE7 (bottom panel) are shown. Note that PKR levels were visualized by ECL after film exposure for 10 s, whereas PKRAR7E7 levels were visualized after 1 min. As a positive control (Ctl) to PKRAR7E7, 10 μg of S10 protein extracts from HeLa cells transfected with PKRAR7E7 were run in lane 1. Detection of endogenous HeLa PKR (top panel, lane 1) was possible after long film exposure (data not shown).

FIG. 4. Self-association and association of PKRAR7E7 with wild type PKR. A, PKRAR7E7 and FLAG-PKR7E7 were expressed in HeLa cells with the vaccinia virus-E7 system. 100 μg of S10 protein extracts were immunoprecipitated (IP) with mouse monoclonal anti-FLAG antibody (lanes 5–8). Proteins were separated in SDS-14% PAGE and subjected to Western blotting (WB) using mouse monoclonal anti-human PKR (F9) antibody. In parallel, 20 μg of S10 protein extracts from each transfection was used to show the PKRAR7E7 expression levels (whole cell extracts (WCE), lanes 1–4). B, PKRAR7E7 and FLAG-PKR7E7 or PKRAR7E7 and FLAG-PKR7E7LS9 were co-expressed in HeLa cells as above. Protein extracts were left untreated (lanes 1 and 3) or treated (lanes 2 and 4) with MN and immunoprecipitated with mouse monoclonal anti-FLAG antibody. Proteins were separated in SDS-14% PAGE and subjected to Western blotting using mouse monoclonal anti-PKR (F9) antibody (top and bottom panels) followed by immunoblotting with mouse monoclonal anti-FLAG antibody (middle panel).
Dominant Negative Function by a Form of PKR

Fig. 5. Dominant negative function of PKRΔE7 in vitro and in vivo. A, HeLa cells were transfected with pFLAG-CMV-2 vector and LipofectAMINE in the absence (lane 1) or presence (lane 2) of recombinant vaccinia/T7 virus (mock transfections). FLAG-PKRΔE7 cDNA was overexpressed using LipofectAMINE and vaccinia/T7 virus (lane 3). 100 µg of S10 protein extracts was subjected to in vitro PKR kinase assay in the presence of reovirus activator dsRNA (0.1 µg/ml) and [γ-32P]ATP (1 µCi). PKR was then immunoprecipitated with a rabbit polyclonal anti-PKR antibody, and 32P-labeled proteins were separated in SDS-10% PAGE and visualized by autoradiography (top panel). In parallel, 20 µg of S10 protein extracts used in the in vitro kinase assay were separated in SDS-10% PAGE (middle panel) or SDS-14% PAGE (bottom panel) and subjected to Western blotting with mouse monoclonal anti-PKR (F9) antibody or mouse anti-FLAG antibody, respectively. B and C, PKRΔE7 fibroblasts were transfected with vector DNA (lane 1), WT human PKR cDNA (lane 2), PKRΔE7 cDNA (lane 3), or WT human PKR and PKRΔE7 cDNAs (lane 4) using the vaccinia/T7 virus system. 100 µg of S10 protein extracts was immunoprecipitated with a rabbit polyclonal anti-human PKR antibody and subjected to in vitro PKR kinase assay as described above. Proteins were separated in SDS-10% PAGE, and the PKR autophosphorylation was visualized by autoradiography (top panel). In parallel, 50 µg of S10 protein extracts from transfected cells was used to show the expression levels of PKR (middle panel) or PKRΔE7 (lower panel) by Western blotting using mouse monoclonal anti-PKR (F9) antibody. D, HeLa cells were transfected with WT elf-2α cDNA (lane 2), the serine 51 to alanine mutant of elf-2α cDNA (lane 3), or wild type elf-2α cDNA together with PKRΔE7 cDNA (lane 4) using the vaccinia/T7 virus system. 20 µg of S10 protein extracts was subjected to Western blotting first with a homemade rabbit polyclonal anti-phosphoserine 51 elf-2α specific antibody (top panel). An equal amount of the same extracts was used for immunoblotting with a mouse monoclonal anti-elf-2α antibody (middle panel) or mouse monoclonal anti-PKR (bottom panel). D, HeLa cells were transfected with vector DNA and LipofectAMINE in the absence (lane 1) or presence (lane 2) of recombinant vaccinia/T7 virus (mock transfections) or with PKRΔE7 cDNA, LipofectAMINE, and vaccinia/T7 virus (lane 3). Protein extracts (50 µg) were subjected to SDS-10% PAGE and immunoblot analysis with a phosphospecific anti-elf-2α serine 51 antibody (BIOSOURCE) (top panel). An equal amount of protein was used for Western blotting with a mouse monoclonal anti-elf-2α antibody (middle panel) or the mouse monoclonal anti-human PKR (F9) antibody (bottom panel). A–D, the intensity of the bands was quantified with the NIH Image 1.54 software, and the ratios of autophosphorylated PKR to PKR protein levels (A and B) or elf-2α serine 51 phosphorylation to the amount of elf-2α protein (C and D) are indicated. vv/T7, vaccinia/T7 virus system.

was unable to counteract the growth-inhibitory effects of PKR (bottom plate). Growth showed that the ability of FLAG-PKRΔE7 transformants to rescue growth was equally potent to K3L (Fig. 6B, middle and bottom graphs). The expression of PKR, FLAG-PKRΔE7, and FLAG-PKRLS9ΔE7 in yeast was then examined by Western blotting (Fig. 6C). PKR expression was detected in H2544 (top panel, lanes 3 and 4) and H2543 (top panels, lanes 5 and 6) but not in the control J110 strain (lanes 1 and 2). However, expression of WT PKR in H2544 and H2543 strains was more highly induced in the presence of FLAG-PKRΔE7 than FLAG-PKRLS9ΔE7 (top panel, compare lanes 3 with lane 4 and lane 5 with lane 6). This higher PKR induction could be translational in nature and caused by the dominant negative function of FLAG-PKRΔE7 but not FLAG-PKRLS9ΔE7. The migration of PKR in polyacrylamide gels as a doublet could have been a result of partial degradation or could represent the phosphorylated (upper band) and nonphosphorylated forms of PKR (lower band) as previously reported for human PKR expressed in mouse cells (41). On the other hand, both FLAG-PKRΔE7 and FLAG-PKRLS9ΔE7 were equally expressed in strain J110 (bottom panel, lanes 1 and 2). However, expression of FLAG-PKRLS9ΔE7 in strains H2544 and H2543 was very low under growth conditions in which PKR expression was induced (bottom panel, lanes 4 and 6). Since FLAG-PKRLS9ΔE7 does not exhibit a dominant negative function, we speculated that its low expression was due to translation inhibition by PKR. To further investigate this possibility, WT human PKR and FLAG-PKRΔE7 or FLAG-PKRLS9ΔE7 were co-expressed into yeast strains J80 and J82, which lack GCN2 but contain wild type elf-2α and the serine 51 to alanine mutant elf-2α, respectively (38). As shown in Fig. 6D, FLAG-PKRΔE7 was equally expressed in both strains in the absence (lanes 2 and 5) or presence of WT PKR (lanes 3 and 6). On the other hand, expression of FLAG-PKRLS9ΔE7 was significantly reduced in both strains when WT PKR was induced (lanes 9 and 12). These data indicated that the inhibition of FLAG-PKRLS9ΔE7 expression by WT PKR may not be translational in nature, since PKR-mediated inhibition of protein synthesis cannot take place in the elf-2α mutant-containing strain (38). The mecha-
nism of down-regulation of FLAG-PKRLS9ΔE7 by PKR is not presently known.

Next, we examined the dominant negative effect of PKRΔE7 on PKR-mediated eIF-2α phosphorylation in H2544 and H2543 strains (Fig. 6E). To do so, H2544 and H2543 strains were transformed with vector DNA alone (lanes 1 and 5), the vaccinia virus inhibitor K3L (lanes 2 and 6), PKRΔE7 (lanes 3 and 7), or PKRΔL9ΔE7 (lanes 4 and 8) followed by the induction of WT PKR in the presence of galactose. WT PKR expression was detected by immunoblot analysis using an anti-human PKR
specific antibody (top panel). Phosphorylation of eIF-2α was detected by immunoblotting using the homemade phosphospecific antibody (middle panel) and normalized to eIF-2α protein levels using a rabbit polyclonal antibody to yeast eIF-2α (bottom panel). These experiments proved that expression of K3L or PKRΔE7 inhibit the eIF-2α phosphorylation in both yeast strains (middle panel, compare lane 1 with lane 2 and 3 and compare lane 5 with lane 6 or 7). On the other hand, PKRΔS9ΔE7 expression did not affect eIF-2α phosphorylation by PKR (compare lane 1 with lane 4, and compare lane 5 with lane 8). Due to the dominant negative functions of K3L and PKRΔE7, expression of WT PKR was more highly induced in the presence of these inhibitors compared with PKRΔS9ΔE7 (top panel, compare lane 4 with lane 2 or 3, and compare lane 8 with lane 6 or 7). These data clearly demonstrate the dominant negative function of PKRΔS9ΔE7 in PKR activation and eIF-2α phosphorylation in yeast.

Activation of Reporter Gene Expression by PKRΔE7—The dominant negative function of PKRΔE7 was further verified in reporter assays in HeLa cells or in mouse fibroblasts derived from two different PKR knockout (PKR−/−) mice (11, 12) (Fig. 7). The first PKR−/− mouse was generated by the disruption of the N terminus domain of the kinase (deletion of exons 2 and 3; N-PKR−/−; Ref. 12), whereas the second was generated by the disruption of the catalytic domain of the molecule (deletion of exon 12; C-PKR−/−; Ref. 11). Cells were co-transfected with the β-galactosidase reporter gene and K3L or PKRΔE7 cDNA in the absence or presence of WT human PKR cDNA. Expression of K3L or PKRΔE7 alone induced β-galactosidase activity in HeLa cells (Fig. 7A), most likely due to the relief of translational inhibition caused by the activation of the endogenous PKR during transfection (36). As expected, expression of WT PKR in HeLa cells resulted in the inhibition of β-galactosidase activity compared with control, which was relieved by the co-expression of either K3L or PKRΔE7. Note that β-galactosidase activity was more highly inhibited when HeLa cells were transfected with a higher amount of WT PKR cDNA (data not shown). On the other hand, in N-PKR−/− (Fig. 7B) and C-PKR−/− cells (Fig. 7C) K3L but not PKRΔE7 expression resulted in an induction of β-galactosidase activity. This effect of K3L may indicate the presence of other eIF-2α kinase(s) that can be activated during transfection. Whether this is PKR-like endoplasmic reticulum kinase (15), GCN2 (16), or another PKR-like kinase is not presently known. Expression of WT human PKR in both knockout cells led to the inhibition of β-galactosidase activity (Fig. 7, B and C), which was relieved by the co-expression of either K3L or PKRΔE7. Taken together, these data demonstrate the dominant negative function of PKRΔE7 in PKR-mediated inhibition of reporter gene expression. Consistent with our data, Tian and Mathews (42) have recently reported that induction of reporter gene expression by p20 in transient transfection assays in human 293 cells.

Tissue Distribution of PKRΔE7 RNA—To investigate the physiological relevance of PKRΔE7 expression, we examined the expression levels of PKRΔE7 relative to WT PKR RNA in various types of normal human tissue by a RT-PCR assay. As shown in Fig. 8, PKRΔE7 RNA was expressed in a broad range of human tissues but at variable levels. In most tissues, expression was below 5% of WT PKR RNA, whereas expression in heart, placenta, liver, and skeletal muscle was as high as 5.3, 5.7, 5.3, and 8.4%, respectively. Expression of PKRΔE7 RNA in spleen was undetectable.

DISCUSSION

In this paper, we have characterized the function of an alternatively spliced form of PKR produced by a deletion of exon 7 (PKRΔE7). Although alternative splicing has been previously described for the 5′-untranslated region of PKR mRNA (43), to our knowledge this is the first study to describe the expression of an alternatively spliced product of human PKR with a dominant negative function. PKRΔE7 is composed of the two copies of the dsRBMs of PKR, a sequence motif found in many dsRNA-binding proteins (18).

Analysis of the biochemical characteristics of PKRΔE7 has shown that it binds to dsRNA and is capable of both self-associating and associating with full-length WT PKR. Both properties of PKRΔE7 appear to require the presence of dsRNA, since treatment with MN abolishes its association with WT PKR but partially diminishes its self-association (Fig. 4). These findings are in accord with previous observations by Wu and Kaufman (44) that dimerization of intact PKR with p20 requires the dsRNA binding activity. Consistent with this, recent studies by Tian and Mathews have shown that the efficacy or rate of p20/PKR dimerization through a protein/protein interaction is considerably less than that of their dsRNA-mediated dimerization (42).

Our data show that self-association of PKRΔE7 can also take place independently of dsRNA, and this is in line with previous data showing that dimerization of p20 is independent of RNA (45). In accord with this, the intrinsic ability of RNA-free preparations of p20 to dimerize in the absence of dsRNA has been recently reported (42). Therefore, PKRΔE7 dimerization in vitro may take place in the absence of dsRNA, whereas the presence of dsRNA may induce conformational changes that facilitate heterodimerization and/or heterodimer stabilization between PKRΔE7 and WT PKR (42, 45).

We have seen that self-association of PKRΔE7 is stronger than its association with full-length PKR, in agreement with a previous study showing that homodimerization of p20 in yeast two-hybrid assays is better than its heterodimerization with
the full-length PKR (32). The requirement of dsRNA for PKRΔE7 binding to PKR might give the specificity for PKRΔE7 to selectively associate with PKR that is bound to dsRNA supporting the notion that dsRNA binding is required for the dominant negative activity of the N terminus truncated form of PKR (44). In regard to this, the dominant negative function of PKRΔE7 could be mediated through its interaction with the WT PKR, resulting in the formation of inactive heterodimers (Fig. 9). This may be limited to those latent PKR molecules that are accessible to dsRNA, and this could relieve possible localized inhibitory effects of PKR on protein synthesis. Alternatively, expression of PKRΔE7 may lead to the sequestration of cellular activator dsRNA, resulting in the inhibition of WT PKR activation (Fig. 9). Consistent with this notion, Tian and Mathews have recently shown that the dominant negative function of p20 correlates with the ability of the two dsRBMs to bind to dsRNA but not with their ability to dimerize, supporting the view that dsRNA sequestration may underlie the dominant negative effect (42).

PKR localizes in the cytoplasm, strongly in the nucleolus, and diffusely throughout the nucleoplasm (46–48). Studies by Tian and Mathews (42) recently showed that the dsRBMs are required for the localization of PKR, and this activity correlates with dsRNA binding. The same authors demonstrated that p20 exhibits a localization indistinguishable from that of WT PKR, suggesting a similar function for PKRΔE7 (42). Interestingly, nuclear localization of PKR was recently shown to be rapidly induced upon treatment with DNA-damaging agents (49), but it is not known whether this process requires dsRNA binding. The two dsRBMs of PKR were reported to be required for its association with ribosomes, and targeting to ribosomes may bring PKR closer to the translation machinery, thus facilitating phosphorylation of eIF-2α (50). In the same study, a PKR mutant with deletion of the entire kinase catalytic domain from amino acid residue 271 to 551 was found to associate strongly with ribosomes (50). Based on this finding, it is reasonable to speculate that PKRΔE7 competes with WT PKR for binding to ribosomes, thus preventing the accessibility of PKR to eIF-2α. However, expression of PKRΔE7 compared with PKR is less than 10% in most tissues, suggesting that the competition between the two molecules for binding to ribosomes could be local. In fact, several observations have supported the theory of localized activation of PKR in regulation of translation of specific genes (2).

Expression of PKRΔE7 RNA was detected in a broad range of human tissues at variable levels (Fig. 8). Heart, brain, pla-

cents, liver, and skeletal muscle were among the top five tissues that exhibited expression over 5% compared with WT PKR RNA, with the highest levels of PKRΔE7 RNA expression in the skeletal muscle (8.4%). These five tissues are all energy-demanding, and this could possibly indicate a role of PKR in energy/intermediate metabolism pathways. The other tissues contained less than 5%, whereas expression of PKRΔE7 RNA in spleen was undetectable. Interestingly, expression of PKRΔE7 was higher in Jurkat cells than in normal PBMCs (Fig. 3).

Whether or not this difference is a cause or an effect of the transformed phenotype of Jurkat cells is an issue that requires further investigation.

We have shown that PKRΔE7 exhibits a dominant negative function in PKR activation in mouse, human, and yeast cells, which results in growth inhibition through the induction of eIF-2α phosphorylation. Whether the dominant negative function of PKRΔE7 plays a role in pathways other than inhibition of eIF-2α phosphorylation is currently under investigation. For example, we have shown that PKR phosphorylates human p53 on serine 392 in vitro, which may account for some of the translational properties of p53 (51). Therefore, PKRΔE7 may also exhibit a dominant negative function in p53 phosphorylation through its capacity to physically associate with PKR and p53 (data not shown). Also, PKRΔE7 contains the two dsRBMs, which have been found in many dsRNA-binding proteins (18). These proteins bind dsRNA in a largely sequence-independent fashion and are involved in a myriad of biological processes such as RNA editing (52), RNA trafficking (53), RNA processing (54), transcriptional regulation (55), and the interferon antiviral response (56). Also, the dsRBMs of PKR and other dsRBM-containing proteins have been shown to possess dsRNA annealing activity and may play a role as chaperones by facilitating the folding of cellular RNAs (42, 57). Therefore, the possibility that PKRΔE7 exhibits functions that are independent of PKR cannot be excluded (Fig. 9).

Given the potential importance of the tight regulation of PKR activity in growth control, it is not surprising that several cellular inhibitors of PKR have been identified and characterized. For example, the human immunodeficiency virus-1 TAR
RNA-binding protein (TRBP) is a dsRNA-binding protein that inhibits PKR (58) by binding to dsRNA and forming heterodimers with endogenous PKR (32). Interestingly, TRBP overexpression can transform mouse NIH3T3 cells in culture through the inactivation of endogenous PKR (59). However, unlike TRBP, PKRΔE7 does not exhibit a dominant negative effect on mouse PKR (data not shown), providing evidence for differences in the specificity between the two dsRNA-binding inhibitors of PKR.

The question arises as to what is the physiological significance of inactivation of PKR by PKRΔE7 and how the inhibitory function of PKRΔE7 differs from that of other dsRNA-binding PKR inhibitors (e.g. TRBP). One possibility is that association of each of these dsRNA-binding proteins with PKR requires a specific RNA structure, resulting in a local and specific inhibition of PKR activation and eIF-2α phosphorylation. Another possibility is that each of the heterodimers between PKR and dsRNA-binding proteins plays a role in RNA-mediated biological processes other than translation (i.e. RNA trafficking, editing, splicing, or transport). In this regard, activation of PKR has been implicated in the splicing of human Tnf-α mRNA (60).

In conclusion, the data presented here demonstrate the expression and the dominant negative function of a dsRNA-binding alternatively spliced product of human PKR. Further understanding of the basis of regulation and function of alternatively spliced PKR products may yield important insights into biological function of the kinase in regard to RNA metabolism, transcription, translation, and regulation of signaling pathways that affect cell proliferation.

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