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Mutants of the RNA-Dependent Protein Kinase (PKR) Lacking Double-Stranded RNA Binding Domain I Can Act as Transdominant Inhibitors and Induce Malignant Transformation

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Recently we reported that introduction of catalytically inactive PKR molecules into NIH 3T3 cells causes malignant transformation and the development of tumors in nude mice. We have proposed that PKR may be a tumor suppressor gene possibly because of its translational inhibitory properties. We have now designed and characterized a number of PKR mutants encoding proteins that retain their catalytic competence but are mutated in their regulatory double-stranded RNA (dsRNA) binding domains (RBDs). RNA binding analysis revealed that PKR proteins either lacking or with point mutations in the first RBD (RBD-1) bound negligible amounts of dsRNA activator or adenovirus VAI RNA inhibitor. Despite the lack of binding, such variants remained functionally competent but were much less active than wild-type PKR. PKR variants completely lacking RBD-1 were largely unresponsive to dsRNA in activation assays but could be activated by heparin. To complement these studies, we evaluated the effects of point mutations in RBD-1 or the removal of either RBD-1 or RBD-2 on the proliferation rate of mouse 3T3 cells. We were unsuccessful at isolating stably transformed cells expressing RBD-1 point mutants or RBD-2-minus mutants. In contrast, NIH 3T3 cells, which constitutively expressed PKR proteins that lacked RBD-1, were selected. These cells displayed a transformed phenotype and caused tumors after inoculation in nude mice. Further, levels of endogenous eIF-2α phosphorylation in RBD-1-minus cell lines were reduced, suggesting that such mutants act in a dominant negative manner to inhibit the function of endogenous PKR. These results emphasize the importance of RBD-1 in PKR control of cell growth and provide additional evidence for the critical role played by PKR in the regulation of malignant transformation.

The interferons are a family of related cytokines that can influence the regulation of cellular differentiation and viral replication through the induction of more than 30 responsive genes (33, 48). Two of these inducible proteins are regulated by double-stranded RNA (dsRNA), the activation of which leads to the inhibition of protein synthesis through separate mechanisms (19, 47). The first, designated the 2-5A-oligoadenylate synthetase, is activated by 2′-5′-phosphodiester-linked oligoadenylates and subsequently activates a novel RNase, RNase L. A second protein considered to play a key role in the effects mediated by interferons is the serine/threonine kinase referred to as PKR (for protein kinase RNA dependent). Interaction with dsRNA causes PKR to autophosphorylate and in turn catalyze phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) on serine 51 (16, 18). eIF-2α forms part of the ternary complex (eIF-2–GTP–Met-tRNA) that is responsible for transferring initiator Met-tRNA to 40S ribosomal subunits prior to the binding of mRNA. Following transfer, eIF-2–GTP is hydrolyzed to eIF-2–GDP and must be recycled into its former state by the guanine nucleotide exchange factor (eIF-2B) before it can be employed in new ternary complexes (18, 25). In sum, since phosphorylated eIF-2α sequesters eIF-2B, a limiting and essential component in the cell, activation of PKR often leads to a dramatic inhibition in translation initiation. Finally, PKR expression itself is autoregulated at the level of translation (6, 51). Following transient transfections, the wild-type kinase is poorly expressed whereas catalytically inactive PKR molecules or even mutants unable to bind dsRNA are expressed to much higher levels (6, 51).

Because of its translational inhibitory properties, it has been postulated that PKR participates in both the antiviral and antiproliferative properties of interferon. Indeed, PKR regulation is best understood in the viral systems. For example, the activation of PKR by viral RNAs has been documented extensively (8, 36, 45). Numerous viruses have devised a variety of mechanisms to downregulate PKR function and avoid a decline in protein synthesis rates that would dramatically affect their replication (reviewed in references 21 and 22). In regard to PKR's antiproliferative properties, it was found that overexpression of wild-type PKR (PKR-WT) in mammalian, insect, and yeast cells suppresses growth (4, 10, 13). Alternatively, expression of catalytically inactive PKR molecules in NIH 3T3 cells induces cellular transformation of these cells (26, 40). Such nonfunctional PKR proteins may act as dominant negative mutants that interfere with PKR-WT function (26). Further evidence for a key role of PKR in the control of cell growth came from our studies of the influenza virus-activated cellular PKR inhibitor, p58 (32). We found that cell lines overexpressing p58 lacked endogenous PKR function and became malignantly transformed (3). In addition to its translational regulatory roles, PKR may be a transducer for the

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dsRNA signalling of NF-κB and play a role in transcriptional regulation (28, 35). Moreover, it was recently shown that PKR promoter sequences contain multiple recognition elements for factors usually involved in the induction of immune and inflammatory response genes as well as those associated with the regulation of cellular differentiation (50), suggesting that PKR may be involved in multiple regulatory processes.

Despite these intensive studies on the molecular biology of PKR, little is currently known about the role of the dsRNA binding domains (RBDs) in PKR regulation and growth suppression in mammalian cells. Mutational analysis has determined that two repeated RBDs reside in the amino-terminal region of PKR and that this region is both necessary and sufficient for binding dsRNA (15, 17, 24, 37, 38, 42, 44). In PKR, the first of these motifs, which are conserved in such proteins as *Drosophila* staufen and *Escherichia coli* RNase III, occurs from amino acids 55 to 76, and the second occurs from amino acids 145 to 166 (49). RNA binding analysis has suggested that the first domain (RBD-1) is more critical for interaction with dsRNAs than the second (RBD-2) (11, 37). To learn more about the functional role of these critical PKR regulatory domains, we generated PKR mutants that are catalytically active but mutated in or lacking RBD-1. We found that PKR variants with point mutations in the location of RBD-1 bound RNA inefficiently but remained partially functional. Similarly, PKR proteins that completely lacked RBD-1 were largely unresponsive to activation by dsRNA. We were unsuccessful at establishing cell lines overexpressing PKR containing small mutations in and around RBD-1. However we were able to isolate cell lines expressing two distinct PKR variants completely lacking this domain. These cells displayed a fully transformed phenotype and had dramatically reduced levels of eIF-2α phosphorylation. These data demonstrate that in vitro, dsRNA binding properties of PKR are not necessarily predictive of kinase function in mammalian cells. More importantly, these data also emphasize the importance of the RBDs in the regulation of cell growth by PKR.

MATERIALS AND METHODS

Plasmid construction and site-directed mutagenesis. The cDNA for PKR (a *Hin*dIII-*Eco*RI fragment representing 2473 bp) was isolated from a lambda gt11 expression library and subcloned into Bluescript SK M13+ (Stratagene) as previously described (39). The cDNA was then excised by *Hin*dIII-*Bam*HI digestion and placed into the M13 series of vectors. Site-specific mutagenesis was performed as described previously (24), using the Muta-Gene M13 In Vitro kit (Bio-Rad). Oligonucleotides were used to change amino acids 38(R → Q) and 39(R → Q) for PKR-M4 and amino acids 58(R → Q), 60(K → N), 61(K → N) for PKR-M5. Mutant plaques were identified performed by sequence analysis using the dideoxy-chain termination method. *Hin*dIII-*Bam*HI fragments were retrieved from M13/PKR mutant DNA and placed into pcDNAI/NEO vectors (Invitrogen) such that the PKR mutant DNA was under control of the T7 and immediate-early cytomegalovirus promoters. PKR-M6 is identical to PKR-M5 except that nucleotide 60 has been deleted. This causes a frameshift to occur and introduces a termination codon into the open reading frame of PKR-M6 at position 70. Construction of PKR-M7, a PKR mutant that lacks the first 98 amino acids, has been described previously (4). Site-specific mutagenesis was similarly used to construct PKR-M8 and PKR-M9, which are identical to PKR-M6 except that the ATG codon at position 98 was altered to GTG and GCG, respectively. Finally, PKR-M10 was generated by digesting PKR-M6 with *Hin*dIII and *Stu*I and inserting the 434-bp fragment representing the first 97 amino acids of the PKR protein into *Hin*dIII- and *Eco*RV-cut pcDNA/NEO.

In vitro expression of PKR mutants in rabbit reticulocyte extract. PKR proteins were synthesized in vitro as previously described (24). Briefly, pcDNAI/NEO plasmids containing wild-type or mutant PKR cDNAs were linearized with *Eco*RV, and transcription of PKR mRNA was initiated from the T7 promoter. Following quantitation by 8% polyacrylamide–7 M urea gels, in vitro-transcribed RNAs were added to message-dependent rabbit reticulocyte lysates and translated in the presence or absence of [^{35}S]methionine as described previously (24). Protein products were quantitated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Protein analysis. Cells were gently rinsed with phosphate-buffered saline (PBS) and disrupted in lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, 1 mM $MgCl_2$, 0.2 mM phenylmethanesulfonyl fluoride, 100 U of aprotinin per ml, 1% Triton X-100). For immunoblot analysis, cell lysates were briefly centrifuged, and the supernatants were taken and added to protein disruption dye (5% SDS, 20% β-mercaptoethanol, 150 mM Tris-HCl [pH 6.8], 20% glycerol, bromophenol blue). Following boiling, samples were loaded onto SDS–10% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes (Amersham). After blocking in 5% milk, blots were incubated with an anti-PKR specific monoclonal antibody (30) or anti-PKR rabbit polyclonal antisera (4, 5). After incubating with the appropriate species-related conjugates, proteins were visualized with the Amersham ECL chemiluminescence system. For immunoprecipitation analysis, reticulocyte extracts (or cells lysed as described above) were diluted with buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 100 U of aprotinin per ml, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 20% glycerol). This mixture was then reacted for 1 h at 4°C with an anti-PKR monoclonal antibody (30) or anti-PKR polyclonal antisera (4). Protein G-agarose was added, and the extracts were incubated for another hour. Precipitates were subsequently washed four times in buffer I and three times in buffer II (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 100 U of aprotinin per ml, 20% glycerol). Protein disruption dye was added to the immunoprecipitates, and the samples were boiled and electrophoresed as described above. Protein gels were fixed and autoradiographed.

Analysis of PKR autophosphorylation and activity. PKR proteins synthesized in reticulocytes or obtained from cell lysates were immunoprecipitated as described above. After washing in buffer II, immunoprecipitated complexes were resuspended in kinase reaction buffer (20 mM Tris-HCl [pH 7.5], 0.01 mM EDTA, 50 mM KCl, 8 μg of aprotinin per ml, 0.3 mg of bovine serum albumin per ml, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1.25 μM [γ-^{32}P]ATP, 0.1 mM phenylmethylsulfonyl fluoride, 5% glycerol), and the appropriate activator, poly(I-C) or heparin, was added. After incubation at 30°C for 15 min, the reaction was stopped by adding 2× disruption buffer. Following boiling, the mixtures were analyzed on SDS-polyacrylamide gels. To assay for PKR substrate activity, 0.5 μg of purified eIF-2 was added to the kinase reaction mixtures prior to incubation (24).

Analysis of eIF-2α phosphorylation. Cells at similar densities were rinsed twice in ice-cold PBS and lysed in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2)–2 mM EDTA–100 mM KCl–0.05% SDS–0.5% Elugent–10% glycerol–20 μg of chymostatin per ml–50 nM microcystin–1 mM dithrothreitol. The 10,000 × *g* supernatant was clarified with BPA-1000 (Toso-Haas, Philadelphia, Pa.). Supernatant (50 μg of protein) was fractionated by vertical slab gel electrophoresis (9) to separate phosphorylated from nonphosphorylated forms of eIF-2α. Proteins were transferred to Immobilon P and subjected to immunoblotting with a monoclonal antibody to eIF-2α (3).

RNA binding analysis. In vitro-transcribed and -translated PKR variants were quantitated by laser densitometry scanning following gel electrophoresis. Equimolar amounts of PKR variants were then reacted with a PKR-specific antiserum (4) and immunoprecipitated as described above. As a negative control, the antiserum was reacted with a translation extract which was not programmed with PKR RNA as previously described (24). Viral RNAs were radiolabeled as previously described (24). Briefly VAI RNA was transcribed in vitro in the presence of [α-^{32}P]UTP, while reovirus dsRNAs of reovirus type 3 were end labeled in the presence of [γ-^{32}P]ATP. Approximately 2 to 5 pmol of PKR variant was reacted with the RNAs in binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 7 mM β-mercaptoethanol, 100 U of aprotinin per ml, 10 μM ATP, 10 μg of tRNA per ml, 0.5% Triton X-100, 20% glycerol). After incubation at 30°C for 20 min, RNA-protein complexes were washed in buffer II containing 0.5% Triton X-100 and resuspended in NET (50 mM Tris-HCl [pH 7.5], 150 mM KCl, 0.05% Nonidet P-40), carrier tRNA, and 1% SDS. After phenol-chloroform extraction and ethanol precipitation, RNAs bound to PKR were analyzed on either an 8% polyacrylamide–7 M urea gel (for VAI RNA) or an SDS–10% polyacrylamide gel (for reovirus dsRNAs).

Construction of PKR mutant-expressing cell lines. Murine NIH 3T3 cells (plated in 100-mm-diameter dishes, 5×10^4 cells per dish) were transfected with 1 to 3 μg of pcDNAI/NEO plasmid carrying PKR mutant cDNA per ml (46). After 18 h of incubation, the DNA mix was removed from the cells and fresh medium (Dulbecco modified Eagle medium [DMEM] supplemented with 10% fetal calf serum [FCS] and containing antibiotics) added to the plates. After 24 h, this medium was removed and complete medium containing 600 μg of G418 (Geneticin; GIBCO) per ml was added to the dishes. Surviving cells (those that formed colonies) were collected by trypsinization and cloned in 24-well plates, using the G418-containing medium. Individual clones from a number of transfection were screened for human PKR-specific mutant proteins by immunoblot analysis using the anti-PKR polyclonal antiserum as described above (4).

Analysis of cell line growth properties. Cells were plated at 5×10^4/60-mm-diameter dish, and cell density determined at A_{600} was recorded every 24 h. Cell medium (DMEM, 200 μg of G418 per ml, 2 or 10% FCS) was replaced daily. Anchorage-dependent growth studies were carried out as described elsewhere (3).

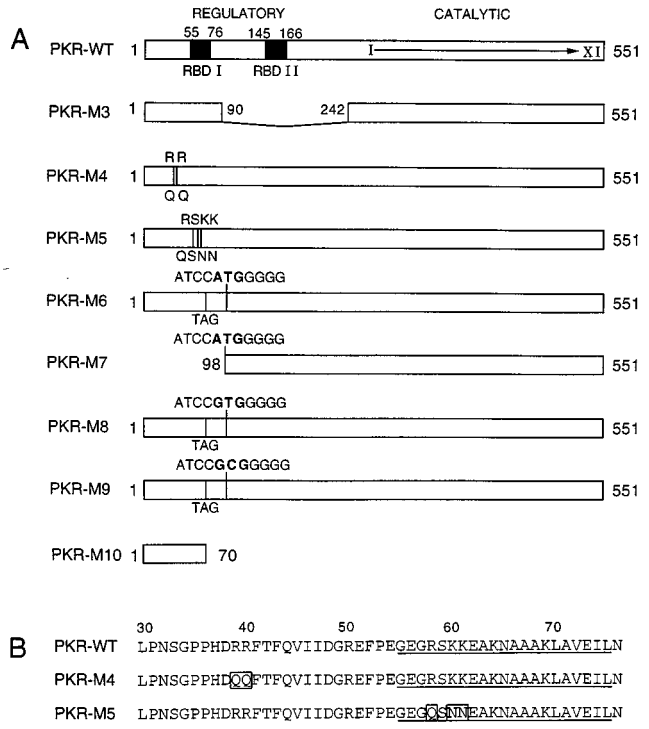

FIG. 1. Description of PKR mutants. (A) Schematic representation of PKR-WT, containing the two RBDs, and various PKR mutants. For PKR-M4 and -M5, wild-type amino acid residues are represented above the diagrams and were substituted with the amino acids depicted below. For PKR-M6, a termination codon (TAG) was introduced at amino acid 70. The ensuing ATG codon (amino acid 98) is highlighted at the top. For PKR-M8 and -M9, the ATG codon at position 98 in PKR-M6 was changed to GTG (PKR-M8) or GCG (PKR-M9) and is shown above the diagram. (B) The PKR-WT amino acid consensus motif representing RBD-1 is shown (underlined), with the amino acids substitutions boxed.

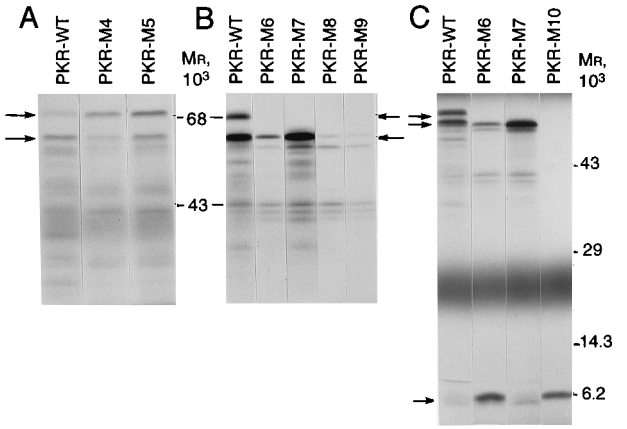

FIG. 2. In vitro expression of PKR proteins. (A and B) Approximately 100 ng of in vitro-synthesized PKR mRNA was added to rabbit reticulocyte extracts in the presence of [^{35}S]methionine. Translation products were electrophoresed on an SDS–10% polyacrylamide gel. Arrows depict full-length and shorter in vitro-synthesized PKR proteins. Positions of molecular weight markers are shown on the right. (C) In vitro-synthesized PKR products were electrophoresed on SDS–17% polyacrylamide gels prior to autoradiography.

Nude mouse studies. Athymic nude mice (*nu*/*nu*; 4 to 6 weeks old) on a BALB/c background were obtained from Seimenson Laboratories, Seattle, Wash. Mice were maintained in a pathogen-free environment and injected subcutaneously with 10^6 cells in 500 μl of DMEM.

Culture of tumor cells. Tumors were recovered under sterile conditions and after washing in PBS were diced into small pieces. Tumor fragments were then added to a solution of 0.5% collegenase-dispase (Sigma), homogenized briefly with a Dounce vessel, and incubated for 2 h at 37°C. Cells were then washed once with complete DMEM and seeded into plates for the generation of tumor cell lines.

RESULTS

Construction and in vitro expression of PKR variants mutated in RBD-1. The amino-terminal region of PKR has been shown to contain the regulatory domains comprising two RNA binding motifs (15, 17, 24, 37, 38, 42, 44). In previous studies, we found that PKR-WT molecules were poorly expressed in transiently transfected COS-1 cells as a result of autoregulation at the level of translation (6). PKR-M3 proteins, which lacked RBD-2 (Fig. 1), in contrast, did not interact efficiently with dsRNAs and were expressed to high levels in transfected COS-1 cells as a result of decreased function (6). To extend our studies, we have now developed PKR constructs with mutations or deletions in and around RBD-1. We made substitutions of a cluster of lysine and arginine residues, which are positively charged amino acids often implicated in the interaction with RNA. Two glutamines were substituted for arginines at positions 39 and 40 (PKR-M4). Similarly, asparagine and glutamine residues were substituted for arginine and two lysines at positions 58, 60, and 61, which were subsequently found to lie directly in the conserved RNA binding motif identified by St. Johnston et al. (PKR-M5) (49).

In addition to these variants, we generated PKR-M6, which contained a termination codon at residue 70, and PKR-M7, which lacked all of RBD-1 and initiated from an ATG codon at position 98 (Fig. 1). Analysis of these PKR variants following expression in a cell-free translation system revealed that PKR-WT, PKR-M4, and PKR-M5 synthesized full-length proteins of the expected size, approximately 68,000 Da (Fig. 2A). In addition, however, these constructs generated a smaller 62-kDa protein. In fact, the smaller protein was often expressed with equal or greater efficiency compared with the full-length product, depending on the experiment. Conceivably, such a 62-kDa protein may arise through degradation of the full-length kinase protein or mRNA. Curiously however, translation of mRNA derived from PKR-M6 yielded a 62-kDa protein (Fig. 2B) in addition to a 6-kDa protein (Fig. 2C). Moreover, the PKR-M6 62-kDa protein electrophoresed with a mobility identical to that of the PKR-M7 protein, although the former was expressed to lower levels, as might be predicted. These two 62-kDa proteins also comigrated with the proteins of similar size produced from the PKR-WT, PKR-M4, and PKR-M5 proteins. We hypothesize that the PKR-M6 6-kDa protein is produced from the first ATG methionine, stopping at the termination codon at position 70. Conversely, the 62-kDa protein is produced from the second ATG at position 98 as a result of leaky scanning or through reinitiation.

Because it was essential for us to confirm the origins of the 62- and 6-kDa proteins encoded by PKR-M6, the following experiments were performed. We first generated a construct termed PKR-M10 (Fig. 1), which encoded only the first open reading frame (i.e., amino acids 1 to 70). The 6-kDa protein encoded by PKR-M6 was the same size as that encoded by PKR-M10 (Fig. 2C). The 6-kDa product was not present in lanes containing in vitro-synthesized PKR-M7, which lacked the first ATG and amino-terminal 97 amino acids. To provide proof that the 62-kDa protein arose from initiation at the ATG encoding methionine 98 in PKR-M6, we altered this initiation codon present in PKR-M6 (TCCATGGCC) to valine (TCC GTGGCC; PKR-M8) or to alanine (TCCGCGGCC; PKR-

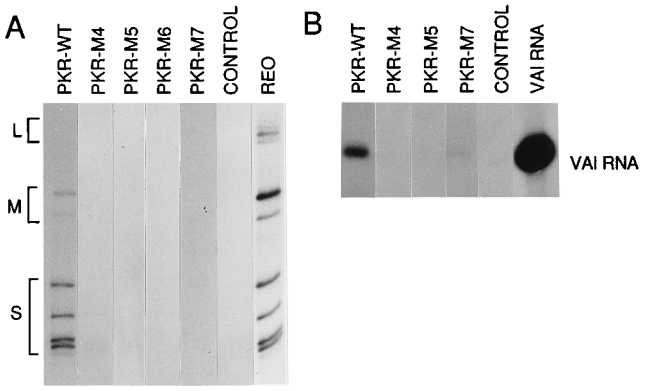

FIG. 3. Binding of reovirus dsRNA and VAI RNA to PKR proteins. In vitro-synthesized PKR proteins were immunoprecipitated with an anti-PKR polyclonal serum, and equimolar amounts were reacted with 0.1 μg of radiolabeled reovirus RNA (REO) or in vitro-transcribed ^{32}P-labeled VAI RNA. Reovirus dsRNA was analyzed on SDS–10% polyacrylamide gels (A), and VAI RNA was analyzed on 8% acrylamide–7 M urea denaturing gels (B). Lanes labeled CONTROL show binding of the RNAs to antibody reacted with a translation extract lacking PKR mRNA. Positions of migration of large (L; ca. 3.5 to 3.9 kb), medium (M; ca. 2.2 to 2.3 kb), small (S; ca. 1.2 to 1.4 kb) reovirus dsRNA species are indicated. Lanes marked REO and VAI RNA display aliquots of the radiolabeled starting material.

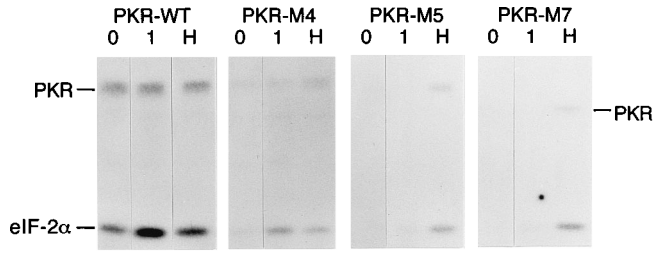

FIG. 4. Activity of in vitro-translated wild-type and mutant PKR proteins. In vitro-synthesized PKR proteins were immunoprecipitated with an anti-PKR polyclonal antiserum, and equimolar amounts were incubated in the presence of purified eIF-2α (0.5 μg) and either poly(I-C) (1.0 μg/ml) (lanes 1), or heparin (10 U/ml) (lanes H) as described in Materials and Methods. Lanes 0 depict incubation in the absence of any activator.

M9) (Fig. 1). Following in vitro synthesis and SDS-polyacrylamide gel analysis of PKR-M8 and PKR-M9 protein products, we found that 62-kDa protein was now more inefficiently synthesized by the latter two constructs than by PKR-M6. We conclude from these several experiments that it is likely the 62-kDa protein is expressed from the position 98 ATG methionine in construct PKR-M6 and is possibly identical to the smaller 62-kDa proteins expressed from the full-length PKR constructs. Although we have no direct evidence as yet that the 62-kDa protein is normally made inside the cell (from the endogenous PKR mRNA), the potent biological properties of this protein would have profound implications for the regulation of PKR in vivo, as discussed in more detail below.

RNA binding analysis. We next analyzed the ability of each of these PKR mutant proteins to interact with dsRNA activators. For these studies, we chose two types of viral RNAs. The first was reovirus dsRNAs because these RNAs are efficient activators of the kinase and can be end labeled with [^{32}P]ATP and readily analyzed on polyacrylamide gels (24). The second type of RNA tested was the adenovirus-encoded VAI RNA, which can be labeled in vitro and is known to bind to PKR and inhibit its activation (23). Although the mechanism of VAI RNA inactivation of PKR remains unclear, we and others have previously shown that both of these viral RNAs bind to similar, if not identical, regions of the PKR protein (17, 24). Following in vitro synthesis and immunoprecipitation with an anti-PKR polyclonal serum, equimolar amounts of PKR variants were reacted with the labeled viral RNAs as described in Materials and Methods (4, 24). The constructs were tested against a wide concentration of RNAs, with only the highest amount shown for simplicity. As controls, we tested binding of the viral RNAs to antibody bound to Sepharose beads. Following gel electrophoresis, the amount of RNA bound to immunoprecipitated PKR was quantified by laser densitometry (4, 24). Similar to our RNA binding analysis of PKR proteins deficient in RBD-2, none of the constructs that were mutated in or lacked RBD-1 were found to bind efficiently to adenovirus VAI or reovirus RNA (Fig. 3). We estimate the efficiency of binding to be less than 5% of that of PKR-WT. Similar findings have been reported by other groups using comparable mutations and other RNA binding assays (17, 37, 42). These results corroborate our earlier studies suggesting that both reovirus dsRNA (an activator of PKR) and VAI RNA (an inhibitor of PKR) bind to the same domain of the kinase. In addition, these data emphasize that alteration or deletion of RBD-1 largely abrogates the ability of viral RNA regulators to interact with this enzyme.

Activities of PKR variants defective for binding dsRNA. The functional activities of PKR variants that fail to bind dsRNA efficiently were tested. This was important since we have previously shown that PKR variants that did not appear to bind RNA in vitro still remained partially active and retained growth-suppressive properties (6). Following in vitro synthesis, PKR-WT or PKR variants mutated around or lacking RBD-1 were immunoprecipitated with a polyclonal antiserum raised to purified PKR (6). It is important to note that equal moles of PKR proteins were assayed for the ability to autophosphorylate and to catalyze phosphorylation of exogenous eIF-2α substrate (Fig. 4).

Our results revealed that efficient phosphorylation of substrate occurred when PKR-WT was activated in the presence of poly(I-C) and heparin, as has been previously shown (20, 24). The constructs were tested against a wide range of dsRNA concentrations, with only the data for 1 μg of poly(I-C) per ml shown since maximal activation was obtained at this concentration. We consistently find that when we assay for PKR activity with the PKR antibody, the basal levels of autophosphorylation (in the absence of activator) are relatively high compared with the phosphorylation state of the substrate (2, 5, 8, 24). Thus, addition of activator causes a more dramatic increase in substrate phosphorylation compared with PKR autophosphorylation. Since this does not occur when we assay highly purified PKR (32), we assume that the elevated autophosphorylation levels may be due to PKR interaction with its antibody. The observed background PKR activity in this experiment also may be caused by residual fragments of micrococcal nuclease-treated RNAs or by PKR mRNA itself preactivating the kinase prior to immunoprecipitation (52). PKR-M4, which was mutated outside the narrowly defined RBD-1 and bound dsRNA very inefficiently, was also found to autophosphorylate and to phosphorylate substrate, though to a much lesser extent than PKR-WT (Fig. 4). We estimate, following PhosphorImager analysis, that PKR-M4 phosphorylated exogenous eIF-2α about four- to fivefold less efficiently than PKR-WT. The PKR variant mutated directly within RBD-1 (PKR-M5) was even more unresponsive to activation by dsRNA in our assays. Similar results were obtained for PKR-M7, which entirely lacked RBD-1 (Fig. 4). As a negative control, we treated extracts which were not programmed with exogenous mRNA. As expected, no detectable levels of phos-

phorylation of PKR or eIF-2α were observed (data not shown). These data strongly indicate that PKR proteins that lack RBD-1, and perhaps PKR proteins that are mutated in key amino acids required for interaction with dsRNA in RBD-1, are particularly nonfunctional in response to dsRNA. It is noteworthy that these mutants are not catalytically dead, as evidenced by a weak response to dsRNA at very high levels and also by the fact that these PKR proteins can still be activated by other polyanions such as heparin (Fig. 4) (43).

Constitutive expression of PKR proteins deficient in binding dsRNA. We have recently shown that constitutive expression of catalytically inactive PKR proteins in NIH 3T3 cells leads to cellular transformation (26, 40). It has been speculated that the mutant forms of PKR interfere with the function of the endogenous PKR-WT in a dominant negative manner (26). Little, however, is known about the effects that PKR proteins, impaired in their regulatory domains, will have on the regulation of cellular growth. We therefore transfected NIH 3T3 cells with pcDNAI/NEO plasmids containing the cDNAs encoding PKR-M3, -M4, -M5, -M6, and -M7. Control transfections were carried out by using the pcDNAI/NEO vector alone (3). After selection for neomycin resistance, we isolated several independent clones of each construct. Cellular extracts were analyzed by immunoblotting with monoclonal antibodies or antisera which were known to react specifically with the respective PKR proteins. Somewhat surprisingly, few neomycin-resistant cell lines were generated following transfection with PKR-M3, -M4, or -M5. Moreover, none of these isolates contained any detectable product resembling human PKR protein, as determined by immunoblot analysis, despite the fact that select proteins could be efficiently expressed transiently (data not shown). For example, we have previously shown that PKR-M3 was transiently synthesized at levels 70-fold higher than those of its wild-type counterpart (6). However, we could show that PKR-M3 still phosphorylated yeast eIF-2α and was toxic when expressed constitutively at high levels in yeast cells (6). Since our analysis suggested that PKR-M4 and perhaps PKR-M5 still remain weakly functional (similar to PKR-M3), we speculate that these proteins may accumulate to levels that ensure that sufficient eIF-2α is phosphorylated to inhibit protein synthesis in the cell and thus disallow isolation of cell lines overexpressing these mutants.

Although we were unable to obtain stable cell lines expressing PKR proteins that were mutated in RBD-1 or that lacked RBD-2, we were able to obtain cell lines that expressed PKR-M6 and -M7, both of which lacked RBD-1. Several independent clones were isolated from PKR-M6-transformed cells and analyzed for protein expression by using the PKR polyclonal antibody (Fig. 5). Analysis of four independent clones revealed a PKR-specific protein of 62 kDa, confirming that the ATG at position 98 in construct PKR-M6 likely was utilized in vivo as predicted from our in vitro analysis. We were unable to detect the 6-kDa protein synthesized from the first ATG (data not shown). Several cell lines from PKR-M7 transfections were similarly isolated. It was more difficult to unequivocally measure the levels of the 62-kDa protein in these primary transformants, probably because of either low expression or the general instability of PKR proteins lacking their amino termini (data not shown). Expression levels were enhanced significantly, however, in cell lines derived from tumors overexpressing PKR-M7 (see below). Morphological examination of cell lines expressing the truncated PKR-M6 proteins revealed that they displayed a transformed phenotype, possessing a spindle-shaped morphology and increased refractility, compared with control NEO cells, which had a flat shape (Fig. 6). Transformed foci also readily formed on monolayers of

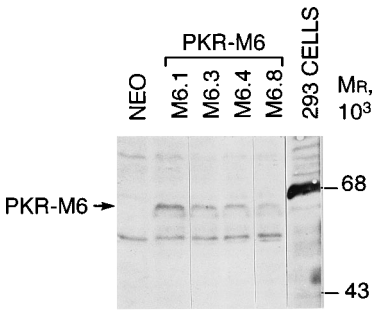

FIG. 5. Expression of PKR proteins in NIH 3T3 cell lines overexpressing PKR-M6. Cytoplasmic extracts prepared from four independent PKR-M6-expressing cell lines were analyzed by immunoblotting with an anti-PKR polyclonal serum which fails to efficiently react with the murine PKR. As a positive control, extracts from human 293 cells were examined. NIH 3T3 cells transfected with pcDNA/NEO alone (NEO) served as negative controls. Positions of molecular weight markers are shown on the right.

cells that expressed PKR-M6 and -M7 in contrast to control cells. Consistent with their transformed phenotype, all of the PKR-M6- and PKR-M7-transformed cells displayed anchorage-independent growth and formed colonies in soft agar. No colony formation was observed with control cells (Fig. 6; Table 1). In addition, we examined the growth properties of the transformed clones. Cells expressing PKR-M6 and -M7 grew faster than the NEO control cells and to a higher saturation density in growth medium containing 10% FCS (Fig. 7A and B) or 2% FCS (Fig. 7C). The cell line growth data shown in Fig. 7 are representative of all cell lines obtained, whether primary transformants or cell lines established from tumors. For unknown reasons, we consistently found that the PKR-M6-transformed cell lines grow faster and to higher densities than the PKR-M7-transformed cell lines. We next injected cells expressing PKR-M6 and PKR-M7 into athymic nude mice. All nude mice injected with two independent PKR-M6- and PKR-M7-transformed cell lines rapidly developed tumors within 20 days (Table 1). In contrast, cells containing vector alone developed only one tumor within the 6- to 8-week period of observation. We also derived cell lines from tumors as described in Materials and Methods following selection in medium containing G418. As mentioned above, the tumor cell lines derived from the PKR-M7-induced tumors were shown to express the 62-kDa protein (Fig. 8). Furthermore, cells derived from mutant-induced tumors gave rise to tumors with a shorter latency period when reinjected into nude mice (Table 1). Taken together, these data suggest that constitutive expression of PKR proteins lacking RBD-1 can cause malignant transformation, similar to those expressing catalytically inactive PKR molecules.

Analysis of eIF-2α phosphorylation levels in cell lines expressing PKR proteins lacking RBD-1. One possible mechanism by which PKR mutant proteins may induce transformation of NIH 3T3 cells is through the transdominant downregulation of the endogenous PKR. Recently, we showed that accurate measurement of PKR activity can be obtained through analysis of endogenous eIF-2α phosphorylation (3, 9). We examined endogenous levels of eIF-2α phosphorylation by subjecting extracts of cells (harvested at similar cell densities) to isoelectric focusing and immunoblot analysis using a monoclonal antibody specific to eIF-2α (Fig. 9A). We found a severalfold reduction in endogenous eIF-2α phosphorylation levels in both PKR-M6- and PKR-M7-transformed cell lines compared with NIH 3T3 cells carrying vector alone. Indeed, phosphorylation levels were barely detectable in cell lines con-

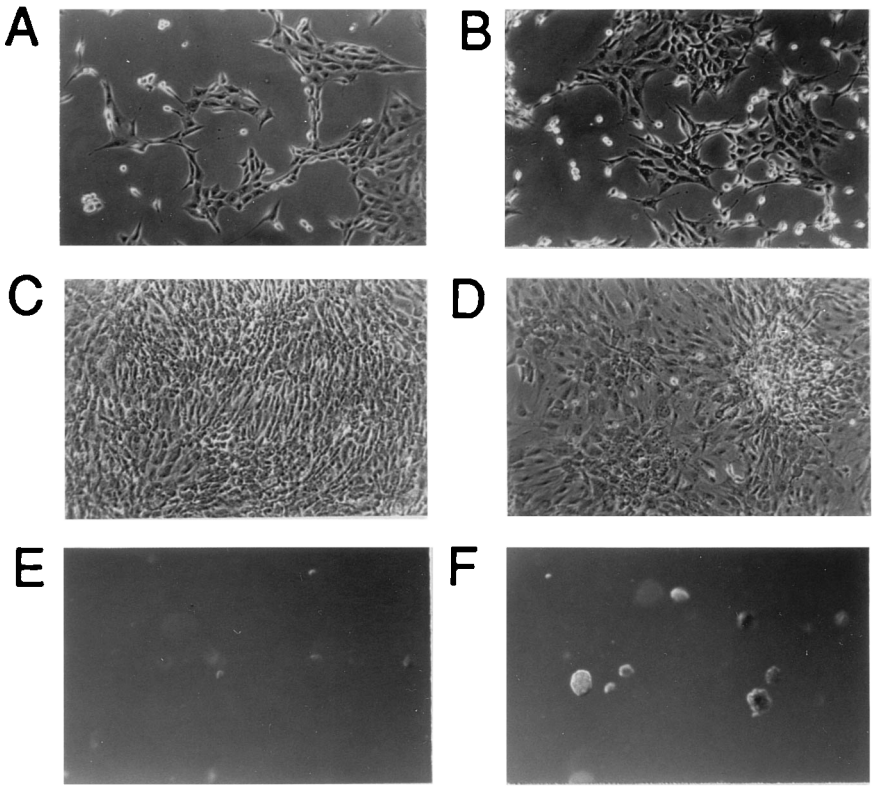

FIG. 6. Morphological characteristics of NIH 3T3 cells expressing PKR-M6 proteins. Cells from control NIH 3T3 clones transfected with pcDNAI/NEO alone (A, C, and E), and NIH 3T3 clones expressing PKR-M6 (B, D, and E) were plated at 10^5 cells per 100-mm-diameter dish in DMEM supplemented with 10% FCS and G418. (A and B) Cells in exponential growth; (C and D) cells maintained in culture 3 days after reaching confluency; (E and F) anchorage-independent growth of clones maintained in soft agar. Magnification, ×50.

taining PKR molecules lacking RBD-1. These observations were consistent whether mutant cells were grown under low- or high-serum conditions (Fig. 9B). These data provide evidence that one potential mechanism leading to PKR-M6- and PKR-M7-induced malignant transformation could be through reduced activity of the murine PKR leading to increased levels of functional eIF-2. One may argue that perhaps all transformed cell lines will have reduced levels of eIF-2α phosphorylation because of the enhanced requirement for higher protein synthetic rates. However, adenovirus-transformed 293 cells do not have reduced phosphorylation levels (Fig. 9B). Perhaps more relevant are our observations that the highly tumorigenic PKR catalytic domain II mutant-overexpressing NIH 3T3 cells have only modest reductions in eIF-2α phosphorylation levels compared with the drastic reductions in the PKR-M6- and PKR-M7-overexpressing cell lines (2, 41).

TABLE 1. Transforming properties of cells expressing PKR variants

Clone	Cloning efficiency (%)[a]	No. of animals with tumors/ no. injected[b]	Latency (days)
NEO	0	1/9	>30
PKR-M6.1	7.9	10/10	15–21
PKR-M6.3	9.8	10/10	7–9
PKR-M7.8	12.5	5/5	15–21
PKR-M7.9	8.5	5/5	15–21
PKR-M6.1 (TCL)	ND[c]	2/2	7–9
PKR-M6.3 (TCL)	ND	2/2	7–9

[a] Cells (10^4) were suspended in 0.35% agar solution in DMEM containing 20% FCS and overlaid onto a 0.5% agar solution in the same medium. Cells were overlaid with 0.5% agar–medium solution prior to addition of 2 ml of DMEM–10% FCS. Colonies were scored 3 weeks after plating. Each experiment was done in triplicate. Cloning efficiency is the number of colonies times 100 divided by the number of cells plated.

[b] Four- to six-week-old nude mice (BALB/c *nu/nu*) were injected subcutaneously with 2×10^6 cells resuspended in 500 μl of serum-free DMEM. The time required to produce tumors of at least 5 mm was considered the latency period. Mice that did not produce tumors were examined for 6 weeks. Cell lines derived from PKR-M6-producing tumors (TCL) were injected in a similar manner.

[c] ND, not done.

DISCUSSION

PKR has been implicated as an essential component of the interferon response to viral infection and cellular proliferation and may also play an important role in the induction of certain signalling pathways (33, 35). To gain further insight into the regulation of PKR, we have examined the functional importance of the PKR regulatory domains. As part of these studies, we were interested in determining the consequences of expressing PKR variants, mutated in the regulatory domains, in mammalian cells. As stated earlier, PKR contains two consensus RNA binding motifs in the amino-terminal region of the protein, the cores of which are approximately 21 amino acids long (RBD-1, residues 55 to 75; RBD-2, residues 145 to 166). Similar to other groups, we have shown that mutations directly in or around RBD-1 largely inhibit the ability of PKR variants to interact with dsRNA. One construct (PKR-M4) consisted of a double-point mutation at amino acids 39 and 40 that was proximal to the consensus motif. A second construct (PKR-M5), consisting of a triple-point mutation at amino acids 58,

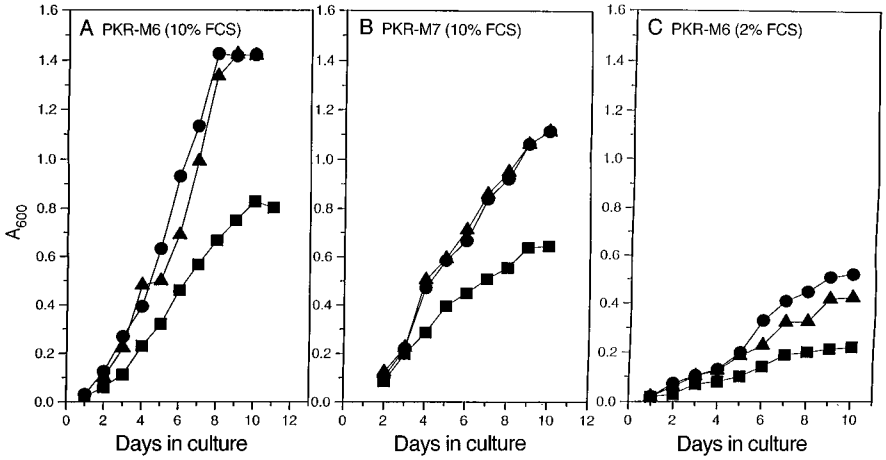

FIG. 7. Growth properties of cells expressing PKR proteins. Cells were grown in DMEM supplemented with 10% FCS (A and B) or 2% FCS (C) and G418. The medium was changed and cell densities were determined every 24 h by measuring the A_{600}. (A and C) Squares, NEO controls; circles, PKR-M6.1; triangles, PKR-M6.3. (B) Squares, NEO controls; circles, PKR-M7.8; triangles, PKR-M7.9.

60, and 61, resided in the heart of the consensus motif. We estimate that both constructs bound less than 5% of dsRNA compared with PKR-WT. PKR variants lacking all of RBD-1 (i.e., PKR-M6 and PKR-M7) gave similar results. It is therefore likely that even conservative amino acid changes in these regions affect the conformation of the molecule and alter the efficiency of interaction with dsRNA. These relatively minor amino acid changes could potentially disrupt an alpha-helical structure that is predicted to reside in this region and that may be critical for PKR activation (17, 37). Despite the defect in dsRNA binding, PKR proteins with point mutations around the RNA binding motif (PKR-M4 and PKR-M5) retained activity, though considerably less so than PKR-WT. Complete deletion of RBD-1 resulted in a PKR variant that also was largely defective in autophosphorylation and the ability to phosphorylate exogenous substrate in response to dsRNA (but the variant remained responsive to heparin).

It is noteworthy that clear-cut phenotypic differences between the point and deletion mutants were obtained only when we tried to establish cell lines overexpressing the mutants. Thus, the dsRNA binding properties and even analysis of kinase activity in vitro were not reliably predictive of PKR function inside a mammalian cell. Somewhat surprisingly, we could not establish stable cell lines that constitutively expressed PKR-M3, -M4, and -M5, even though some of the variants were efficiently expressed transiently (2). In contrast, we were able to isolate cells that constitutively expressed PKR proteins that lacked RBD-1 (PKR-M6 and -M7). One possible explanation is that the threshhold of eIF-2α phosphorylation is exceeded in cell lines expressing the point mutants which are accordingly still toxic to cell growth. Alternatively, it is possible that these kinase variants are unable to inactivate the endogenous murine PKR as effectively as those lacking the entire RBD-1. It was recently shown that point mutants in the cata-

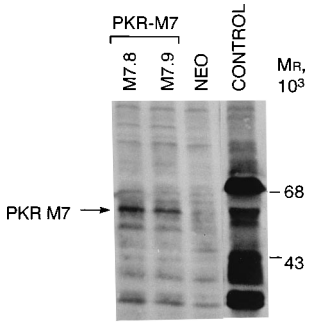

FIG. 8. Analysis of PKR-M7 proteins in cell lines derived from tumors. Cell lines were prepared from the two PKR-M7-induced tumors and cultured in DMEM containing 10% FCS and G418. Cell extracts were prepared and analyzed by immunoblotting with a PKR-specific monoclonal antibody recognizing the COOH terminus of the protein (2). Cell extracts from NEO and 293 cells (CONTROL) were analyzed in parallel as negative and positive controls, respectively.

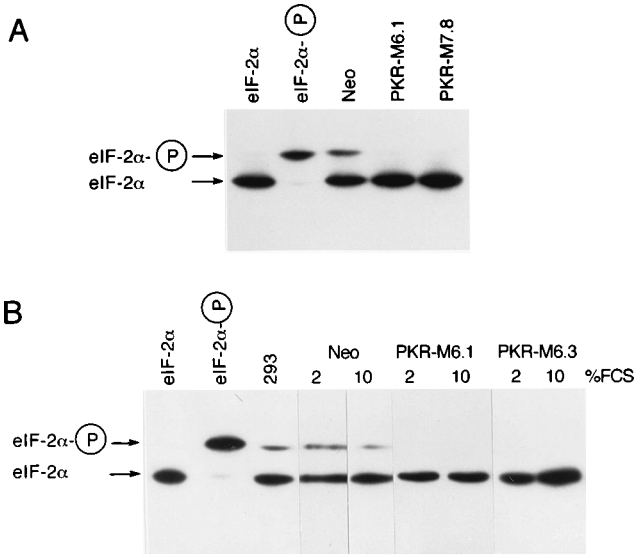

FIG. 9. Analysis of eIF-2α phosphorylation levels in NIH 3T3 cells expressing mutant PKR proteins. Cell extracts were analyzed by isoelectric focusing gel electrophoresis and immunoblot analysis. Blots were incubated with an anti-eIF-2α monoclonal antibody (3, 9). Lanes marked eIF-2α and eIF-2α-P are standards of purified eIF-2α incubated in the presence of the heme-regulated eIF-2α kinase to demonstrate the positions of the phosphorylated and nonphophorylated forms of eIF-2α. (A) Extracts were prepared and analyzed from NEO, PKR-M6-expressing, and PKR-M7-expressing cells (at equal cell densities) grown in 10% FCS (day 5). (B) Extracts were prepared and analyzed from cells grown in 2% as well as 10% FCS. Lane marked 293 refers to the analysis of extracts prepared from interferon-treated 293 cells grown in 10% FCS.

lytic PKR domains were unable to act as transdominant inhibitors in yeast cells, whereas deletions within the same domain were able to do so (44). As suggested by Romano et al. (44) for the catalytic PKR variants, it is possible that heterodimers, which may form between either the PKR-M4 or -M5 variant and the endogenous murine PKR, remain active. There is evidence in the literature that PKR may indeed dimerize to become functionally active (29, 34). In contrast, we know that the RBD-1-minus mutants, PKR-M6 and -M7, can inactivate PKR in vivo (Fig. 9). Furthermore, we now have direct evidence that truncated variants lacking RBD-1 can act as efficient transdominant inhibitors of PKR-WT in vitro as well (2). Since these PKR mutant proteins are severely defective in the binding of dsRNA and since roughly stoichiometric amounts of the variants are required for inactivation, we conclude that the RNA binding variants are functioning not as sequesters of activator but as transdominant inhibitors which may form inactive heterodimers with PKR-WT.

In addition to this report, two other studies recently have examined the functional activity of PKR variants lacking amino acids 1 to 97, mutants identical to PKR-M6 and -M7. Using a vaccinia virus expression system, Lee et al. (31) have claimed that PKR encoded by such a construct retained full activity in a reporter gene assay, although expression levels of the PKR variant were not measured. In a separate report, Romano et al. (44) examined the expression and phenotype of this PKR mutant in yeast cells. Consistent with our findings, these authors demonstrated that PKR containing amino acids 98 to 551 had dramatically reduced activity and was minimally growth suppressive. In contrast to our data, however, PKR encoded by this construct was not able to act transdominantly to inhibit PKR-WT, leading the authors to suggest that dsRNA binding domains are required for efficient heterodimer formation and for a mutant to act as a dominant negative inhibitor in yeast cells. We currently have no concrete explanation for these and other differences found in the yeast system regarding PKR regulation. It is clear, however, that in mammalian cells (this report) and in in vitro experiments (2), the RBD-1 mutants can act as effective dominant negative inhibitors of PKR function.

This work documents another example of the key role played by PKR in the regulation of cell growth and malignant transformation. The data also provide additional support for a role of PKR as a tumor suppressor gene, although no alterations of the PKR gene in a known human tumor have yet been identified. The chromosomal locations of PKR have been isolated to locus 2p21 in humans and to mouse 17E2 (1). Earlier we demonstrated that NIH 3T3 cells overexpressing two different catalytically inactive PKR mutants become transformed (26, 40). Moreover, a cellular PKR inhibitor, p58, also caused the transformation of these cells (3). It is nonetheless unclear what constitutes the actual molecular mechanisms that trigger transformation. Since endogenous eIF-2α phosphorylation is diminished in cell lines expressing p58 (3), PKR-M6 and PKR-M7 (this report), and the catalytically inactive PKR delta 6 mutant (26), it is tempting to speculate that transformation arises from an enhanced capacity for protein synthesis resulting from increased levels of functional eIF-2. For example, it is known that many proto-oncogenes and growth factors have multiple upstream AUGs, open reading frames, and/or 5′ untranslated regions that appear to be translationally regulated (27). The enhanced translation of such cellular RNAs may participate in the stimulation of cellular growth. In contrast, however, cell lines overexpressing the catalytically inactive PKR with a mutation in catalytic domain II (PKR-M1), although tumorigenic (40), do not appear to have dramatically decreased levels of eIF-2α phosphorylation, thus demonstrating that large reductions in eIF-2α phosphorylation are not a general property of all malignantly transformed NIH 3T3 cells (2, 41). This does not rule out the possibility that minor decreases in eIF-2α phosphorylation do occur as a result of malignant transformation and the requirement for enhanced protein synthetic rates. It has been recently shown that expression of a similar catalytic domain II mutant can alter the activation state of NF-κB (28), raising the possibility that variant PKRs may trigger malignancy through multiple mechanisms, including mechanisms that may be independent of their acting as dominant negative inhibitors of the endogenous PKR. This latter scenario may occur even though both PKR-M7 and PKR-M1 are strong transdominant inhibitors of PKR-WT autophosphorylation and activity when tested in vitro, either in reticulocyte lysate or in in vitro kinase assays with highly purified reagents (2).

Finally, it is worth commenting on the potential implications of the production of a truncated PKR protein initiated at the ATG encoding methionine at amino acid position 98. We found that in vitro translation of PKR-WT, PKR-M4, and PKR-M5 resulted in the synthesis of two products, one full-length and one smaller 62-kDa protein. Expression of PKR transcripts containing a stop codon at amino acid 70 (PKR-M6) still yielded the 62-kDa product, suggesting that it was synthesized as a result of reinitiation at the alternate downstream ATG codon. Moreover, the migration of the 62-kDa product was identical to that of the PKR product encoded by PKR-M7, which begins at methionine 98. We concede that our studies provide no direct evidence that the smaller 62-kDa product is made in vivo even though a protein of that size is often detected in our PKR immunoprecipitations from cellular extracts (2). However, there are several instances of the usage of alternative initiation codons in eukaryotic mRNAs, including the cyclic AMP-responsive-element modulator and the ErbA/thyroid hormone receptor (7, 12). In both cases, the smaller products possessed properties opposite those of the full-length protein and functioned as dominant negative autoregulators. It remains to be seen whether PKR encodes both products in vivo and whether the smaller protein that lacks RBD-1 plays a general role in PKR regulation when both are expressed.

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