Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8283–8287, August 1995
Biochemistry

The interferon-inducible double-stranded RNA-activated protein kinase self-associates *in vitro* and *in vivo*

(chemical crosslinking/dimerization)

R. C. PATEL*, P. STANTON*, N. M. J. MCMILLAN†, B. R. G. WILLIAMS†, AND G. C. SEN*‡

Departments of *Molecular Biology and †Cancer Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195

Communicated by Robert T. Schimke, Stanford University, Stanford, CA, May 15, 1995

ABSTRACT **The interferon-inducible double-stranded (ds) RNA-activated protein kinase (PKR) exhibits antiviral, anticellular, and antitumor activities. The mechanisms of its enzymatic activation by autophosphorylation and of the observed transdominant inhibitory phenotype of enzymatically inactive mutants have invoked PKR dimerization. Here we present direct evidence in support of PKR–PKR interaction. We show that radiolabeled PKR can specifically interact with matrix-bound unlabeled PKR in the absence of dsRNA. The self-association activity resides, in part, in the N-terminal region of 170 residues, which also constitutes the dsRNA-binding domain (DRBD). DRBD can bind to matrix-bound PKR or to matrix-bound DRBD. Dimerization of DRBD was directly demonstrated by chemical crosslinking. Affinity chromatography and electrophoretic mobility supershift assays demonstrated that mutants that fail to bind dsRNA can still exhibit protein–protein interaction. The PKR–PKR interaction could also be observed in a two-hybrid transcriptional activation assay in mammalian cells and consequently is likely to be an important feature of PKR activity *in vivo*.**

The double-stranded (ds) RNA-activated protein kinase (PKR) is the product of an interferon-inducible gene (1). Most mammalian cells, however, contain a low constitutive level of this enzyme. Besides dsRNA, small polyanionic molecules such as heparin can also activate PKR (2). The dsRNA-binding domain (DRBD) of PKR has been mapped to its N-terminal region (3–8). This domain, however, does not appear to mediate the activation by heparin. PKR mutants that are not activated by dsRNA because of a loss of dsRNA-binding activity can still be activated by heparin *in vitro* (9) and by other unknown activators *in vivo* (10). What is common between the various activation processes, however, is autophosphorylation of PKR. Activation of PKR by any activator causes its autophosphorylation and renders PKR enzymatically active and able to phosphorylate other proteins such as the translation initiation factor eIF-2α (11–13) and the transcriptional inhibitory factor IκB (14). Although the mechanism of activator-induced autophosphorylation of PKR is unclear, evidence has been presented to suggest that intermolecular phosphorylation can occur between two PKR molecules (15, 16).

The most well-studied cellular effect of PKR is inhibition of translation as a result of phosphorylation of eIF-2α, which inhibits its function in translation initiation (17, 18). dsRNA production during the replication cycle of many viruses triggers this pathway by activating PKR. Since the resultant translational inhibition is detrimental to viral protein synthesis, many viruses have evolved strategies to inhibit PKR action (19, 20). These strategies include production of other dsRNA-binding proteins (21, 22), production of decoy substrates with structural similarity to eIF-2α (23), production of RNAs that inhibit PKR activation (24, 25), sequestration of PKR (26), degradation of PKR (27), and induction of cellular inhibitors of PKR (28). In addition to its role in cellular immunity against viruses, PKR has been implicated in several other cellular processes. PKR is thought to be involved in the signal transduction process leading to transcriptional induction of some cellular genes by dsRNA (29-31). One mechanism of such involvement is by promoting activation of the transcription factor NFκB. Targeted ablation of PKR has been shown to prevent cellular activation of NFκB in response to dsRNA (32). The activation of NFκB is a result of dissociation of IκB, which is phosphorylated by dsRNA-activated PKR (14). PKR may also be involved in the control of cell growth (5) and differentiation (33, 34). Overexpression of enzymatically active PKR has been shown to be growth suppressive in mouse (35), insect (36), and yeast cells (5). On the other hand, expression of catalytically inactive mutants of PKR transforms mouse cells (35, 37). Cells expressing such mutants can grow in soft agar and form tumors in nude mice. The mutant PKR has been postulated to inhibit the action of normal cellular PKR, which may act as a tumor suppressor protein. The proposed mechanism of transdominant inhibition of PKR by an enzymatically inactive PKR mutant has invoked PKR dimerization. Thus, the postulated mechanisms for PKR autophosphorylation and the observed tumor-promoting activity of inactive PKR mutants require that PKR interacts with itself. The PKR–PKR interaction either may be mediated by dsRNA or may be a result of direct protein–protein interaction. If the interaction is mediated by dsRNA, mutants lacking dsRNA-binding activity are expected to be defective in PKR–PKR interaction.

In this paper, we provide direct experimental evidence for such PKR–PKR interaction. Here we demonstrate direct PKR–PKR interaction mediated in part by the same domain to which dsRNA binds. The dsRNA-binding and the dimerization properties of PKR are, however, distinct and dissociable. The PKR–PKR interaction observed *in vitro* could also be demonstrated *in vivo* by using a two-hybrid transcriptional activation assay. These results, therefore, support an intermolecular autophosphorylation mechanism for PKR activation and suggest that the transdominant inhibition by inactive mutants occurs through the formation of inactive heterodimers.

METHODS

Purification of hisK296R, hisDRBD, hisK60A, and his2′5′-Oligoadenylate Synthetase. Plasmids pK296R/pET15b and DRBD/pET15b were constructed such that there is an in-frame fusion of K296R (3) and DRBD (4) sequences to the histidine tag coding sequences from pET15b (Novagen). The constuction of K60A/pET15b was as described (38). The expression and purification of histidine-tagged proteins were done as described (39, 40). The K60A mutant protein was

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Abbreviations: ds, double-stranded; DRBD, dsRNA-binding domain; DMS, dimethyl suberimidate; PKR, dsRNA-activated protein kinase; 2′5′ AS, 2′,5′-oligoadenylate synthetase; wt, wild-type.

‡To whom reprint requests should be addressed.

further purified by gel filtration chromatography on a Superose-12 FPLC column (Pharmacia).

***In Vitro* Transcription and Translation.** The *in vitro* transcription and translation of PKR and the truncated PKR proteins were done using the coupled rabbit reticulocyte *in vitro* translation kit from Promega (9).

***In Vitro* Protein–Protein Interaction Assay.** The proteins were *in vitro* translated from 2 μg of plasmid DNA using the coupled rabbit reticulocyte *in vitro* translation kit from Promega. Four microliters of the translation mix was incubated with 1 μg of either hisDRBD or hisK296R and 20 μl of Ni-charged His·Bind resin at 30°C for 2 hr in binding buffer [5 mM imidazole/200 mM NaCl/20 mM Tris·HCl, pH 7.9/0.5% Nonidet P-40 (NP-40)]. After binding, the beads were washed with 500 μl of wash buffer (60 mM imidazole/200 mM NaCl/20 mM Tris·HCl pH 7.9/0.5% NP40) six times. The washed beads were then boiled in 2× Laemmli buffer (150 mM Tris·HCl, pH 6.8/5% SDS/5% 2-mercaptoethanol/20% glycerol) for 2 min and analyzed by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) on a 12% gel. Fluorography was performed at −80°C with intensifying screens.

Chemical Crosslinking with Dimethyl Suberimidate (DMS). The purified DRBD was dialyzed against 2000 vol of buffer (20 mM Hepes, pH 7.5/10% glycerol) at 4°C for 17 hr. Four micrograms of DRBD was then crosslinked in 100 μl with 1 mM DMS in crosslinking buffer (10 mM Hepes, pH 8.0/100 mM NaCl) at 25°C for 2 hr. Aliquots (10 μl) were removed at times indicated and the reaction was stopped by adding 1 M glycine to a concentration of 100 mM. Protein was then denatured by boiling in Laemmli buffer for 2 min and analyzed by SDS/PAGE on a 12% gel followed by Western blot analysis using a polyclonal antibody raised against bacterially produced DRBD (40).

dsRNA-Binding Assay. The polyI·polyC binding assay was performed with ^{35}S-labeled proteins as described (4).

Electrophoretic Mobility Shift Analysis. The mobility shift analysis of dsRNA was performed as described (40, 41).

Transfections. The VP16/K296R and VP16/DRBD were constructed by joining the filled-in 1.6-kb or 525-bp *Nde* I–*Bam*HI fragment from K296R/pET15b or DRBD/pET15b to filled-in *Eco*RI-cut pVP16AASV19N (42). The GAL4/K296R and GAL4/DRBD were constructed by joining the filled-in 1.6-kb or 525-bp *Nde* I–*Bam*HI fragment from K296R/pET15b or DRBD/pET15b to *Sma* I-cut pSG424 (42). COS-1 cells were transfected with 200 ng of each of the four (two test plasmids encoding proteins to be tested, the reporter plasmid pG5Luc, and pRSV-β-galactosidase plasmid to normalize the transfection efficiency) plasmid DNAs by the Lipofectamine procedure as described by the manufacturer (GIBCO/BRL). Cells were harvested 48-hr after transfection and assayed for luciferase activity after normalizing for the transfection efficiency by measuring the β-galactosidase activity.

RESULTS

Demonstration of PKR–PKR Interaction by Affinity Chromatography. To determine whether PKR can bind to itself, we performed affinity chromatography using bacterially expressed hexahistidine-tagged enzymatically inactive K296R mutant (PKR^M). Since wild-type (wt) PKR is toxic to *Escherichia coli* and cannot be produced in large quantities, we used the enzymatically inactive K296R mutant in these experiments. Hexahistidine-tagged PKR^M was bound to nickel-Sepharose and *in vitro* translated labeled PKR was tested for binding to PKR^M-Sepharose (Fig. 1). Full-length PKR bound specifically to PKR^M-Sepharose (lane 3). It did not bind to uncharged Ni-Sepharose (lane 2) or to Sepharose to which another dsRNA-binding protein, hexahistidine-tagged 2′5′ AS, had been bound (lane 4). Another *in vitro* translated protein, luciferase, did not bind to either uncharged Ni-Sepharose or PKR^M-Sepharose (lanes 5–7). These results established that the observed binding of PKR to PKR^M-Sepharose was specific for the labeled protein and the matrix-bound protein. It should be noted that about 40% of radiolabeled PKR bound to the affinity matrix. This could be due to partial self-association of *in vitro* translated PKR before it was exposed to PKR^M-Sepharose or due to incomplete folding of the protein to the self-associating conformation. Nonetheless, the data shown in Fig. 1 demonstrated that PKR can specifically bind to itself.

Multiple Domains Contribute to PKR–PKR Interaction. We used the affinity matrix binding assay of *in vitro* translated PKR to map the self-binding domains. We expressed various PKR-derived proteins *in vitro* and tested their abilities to bind to PKR^M-Sepharose. These experiments were done under conditions of excess affinity resin. The N-terminal fragment containing residues 1–170 bound to PKR^M, although its binding was less efficient than full-length PKR (Fig. 2*A*). Elimination of 68 residues from the C terminus of 1–170 protein completely abolished its binding. The protein containing residues 35–371 bound efficiently as did the protein containing residues 146–551. Another protein containing residues 146–239, on the other hand, failed to bind to PKR^M-Sepharose. The binding data (summarized in Fig. 2*B*) indicate that PKR contains more than one self-association domain. One of these domains resides within the N-terminal 170 residues with a C-terminal limit between residues 102 and 170 and an N-terminal limit downstream of residue 35. The second binding domain resides in the C-terminal half of PKR. It appears that the C-terminal limit of this domain is not beyond residue 371 since 1–551 and 35–371 proteins bound equally well to PKR. Although protein 146–551 bound to PKR^M, the binding was somewhat less than that of the full-length PKR, indicating that it may have lost the N-terminal binding domain. Protein 146–239 did not bind at all, indicating that the C-terminal domain has not been retained. Thus, our data clearly indicate

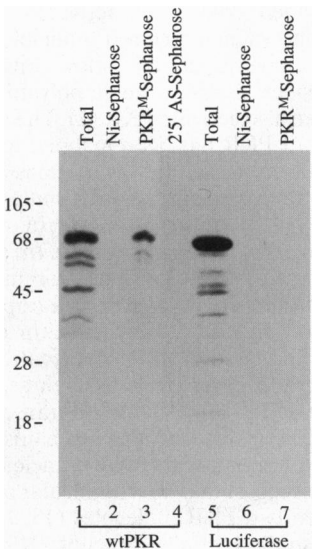

FIG. 1. *In vitro* assay for PKR–PKR interaction. *In vitro* translated PKR and luciferase were tested for interaction with PKR. Four microliters of the reticulocyte lysate containing ^{35}S-labeled PKR or luciferase was mixed with 1 μg of purified hexahistidine-tagged PKR^M or hexahistidine-tagged 2′,5′-oligoadenylate synthetase (AS) and 20 μl of the Ni-charged Sepharose in binding buffer and the mixture was incubated at 30°C for 1 hr. After extensive washing with wash buffer, the Sepharose beads were boiled in Laemmli buffer and the proteins were analyzed by SDS/PAGE. The lanes indicate to which beads the proteins were bound. "Total" lanes, 2 μl of the reticulocyte lysate; lanes 1–4, analysis of binding of wtPKR; lanes 5–7, analysis of binding of luciferase as a negative control. Positions of molecular weight markers are indicated as $M_r \times 10^{-3}$. The uppermost band in the wtPKR lanes is the full-length protein and all other smaller translation products are due to initiations at internal methionines.

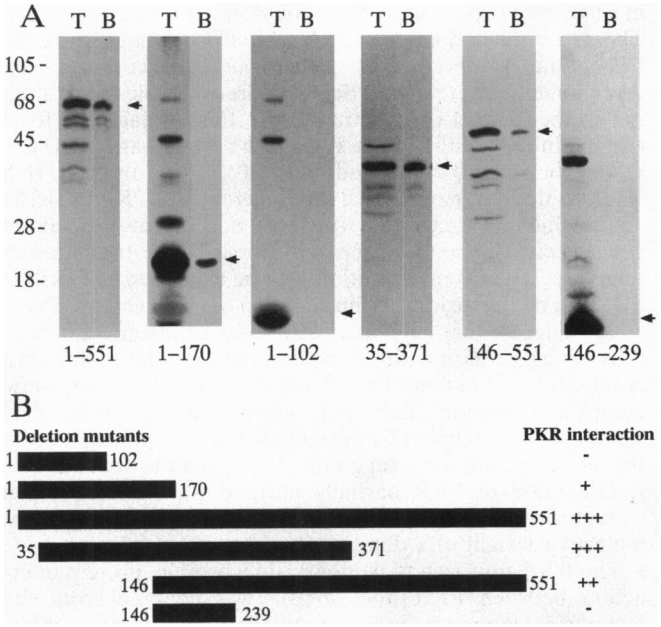

FIG. 2. Analysis of interaction of deletion mutants of PKR with PKR^M. (*A*) Binding of deletion mutants with PKR^M. ^{35}S-labeled truncated PKR proteins with various deletions were synthesized *in vitro* and analyzed for binding to PKR^M-Sepharose. *In vitro* synthesis of these proteins has been described earlier (4). In addition to the expected PKR-derived proteins, several additional bands were observed; they are the endogenous reticulocyte lysate band (M_r 44,000) and the products of internal initiations or of the residual nonlinearized plasmid. T lanes, total proteins from the reticulocyte lysate; B lanes, proteins bound to PKR^M-Sepharose. The arrows indicate the expected positions of different deletion mutants. The residues included in the protein are indicated below the lanes. Positions of molecular weight markers are indicated as $M_r \times 10^{-3}$. (*B*) Schematic representation of the PKR interaction properties of deletion mutants.

that PKR–PKR interaction involves at least two linear binding domains on the PKR molecule that can function independent of each other.

Dimerization of DRBD. The self-association properties of the N-terminal domain comprising residue 1–170 containing the DRBD of PKR (4) were further investigated using hexahistidine-tagged DRBD expressed in bacteria and purified by affinity chromatography. The purified DRBD was 95% pure as judged by silver staining analysis (40). *In vitro* translated DRBD was able to specifically bind to PKR^M-Sepharose and DRBD-Sepharose (Fig. 3*A*). Thus, the N-terminal domain of PKR can associate with itself.

The above conclusion was further supported by exposing the purified bacterially produced DRBD to DMS, a protein crosslinking agent (43), for different lengths of time followed by analysis by SDS/PAGE (Fig. 3*B*). As expected, the untreated protein migrated as a monomer of apparent M_r 22,000. Increasing lengths of exposure to DMS resulted in the formation of increasing amounts of a protein whose mobility was what is expected of a dimeric DRBD of M_r 45,000. Even after prolonged incubation with DMS, however, no larger complexes were observed. These results convincingly demonstrated that DRBD exists in solution primarily as dimeric molecules.

Dissociation of the Dimerization and the dsRNA-Binding Properties. Since a part of PKR's self-association activity is mediated by the same domain that binds dsRNA, we were curious to determine if the same structural motifs within this domain are responsible for both activities. To test this, a mutant PKR, whose residue 60 had been mutated from Lys to Ala, resulting in the loss of its ability to bind to dsRNA (38), was chromatographed on PKR^M-Sepharose. The results (Fig. 4*A*) show that this mutant retains its ability to bind to PKR^M. The same was also true for the 35–371 derivative of wt-PKR (Fig. 2*A*), which has previously been shown to lack the dsRNA-binding ability (4). These data clearly show that the dimerization and the dsRNA-binding properties of PKR are distinct. The same conclusion is true for the DRBD. Thus, an N-terminally truncated DRBD, containing residues 35–170, could still bind to PKR^M, although it failed to bind to dsRNA (Fig. 4*A*).

To confirm DRBD–PKR interactions we also employed an electrophoretic mobility shift assay using a radiolabeled 82-bp dsRNA whose mobility was retarded upon its interaction with DRBD or PKR (Fig. 4*B*). This experiment was done under dsRNA-excess conditions. Ten nanograms of DRBD yielded two protein–RNA complexes that probably contained either one or two DRBD molecules bound to one dsRNA molecule. As expected, K60A PKR could not produce any shifted complex even at a high concentration. However, when 10 ng of this protein was added to the mixture containing 10 ng of DRBD, several supershifted complexes were formed. Similar supershifted complexes were also formed when, instead of K60A PKR, an additional 10 ng of DRBD was included but not when 10 ng of bovine serum albumin was included in the reaction mixture (ref. 40; data not shown). Since K60A PKR does not interact with dsRNA, these observations can be explained only by postulating its direct interaction with DRBD.

Demonstration of PKR–PKR Interaction *In Vivo*. Although the experimental results reported so far clearly demonstrated that PKR can self-associate *in vitro*, to provide biological relevance, similar interactions needed to be tested *in vivo*. For this purpose, we used the two-hybrid transcriptional activation assay developed by Fields and Song (44). The two proteins, whose interaction was being tested, were produced as fusion proteins. One was fused to the DNA-binding domain of the bacterial GAL4 protein and the other was fused to the

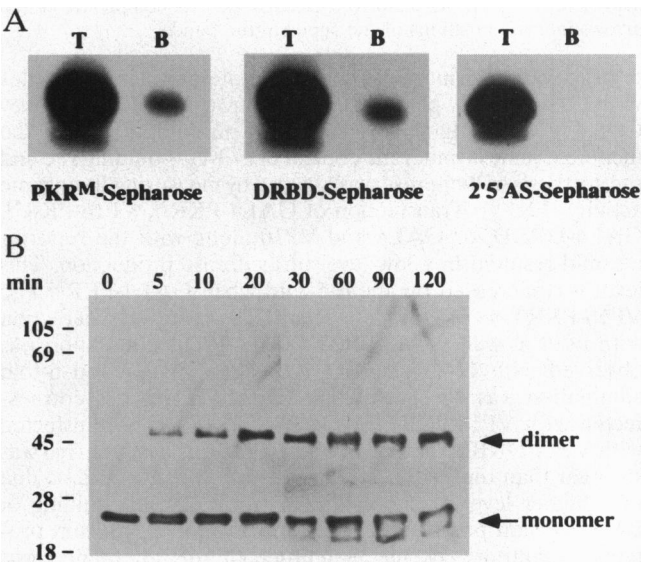

FIG. 3. Demonstration of protein–protein interaction of the DRBD. (*A*) Self-association of the DRBD. ^{35}S-labeled DRBD was synthesized *in vitro* and assayed for binding to hexahistidine-tagged purified DRBD, hexahistidine tagged PKR^M, or hexahistidine-tagged 2′-5′ AS on Ni-Sepharose. Only the relevent part of the gel showing the DRBD region is shown. T lanes, total proteins from *in vitro* translation; B lanes, proteins bound to the different matrix-bound proteins. (*B*) Chemical crosslinking of the DRBD. Four micrograms of purified DRBD was crosslinked with 1 mM DMS in 10 mM Hepes, pH 8.0/100 mM NaCl at 25°C for 2 hr. Aliquots (10 μl) were removed at times indicated and the reaction was stopped by adding 1 M glycine to a concentration of 100 mM. Protein was then denatured by boiling in Laemmli buffer for 2 min and analyzed by SDS/PAGE on a 12% gel. A Western blot analysis was performed with a polyclonal anti-DRBD antibody. Positions of molecular weight markers are indicated as $M_r \times 10^{-3}$.

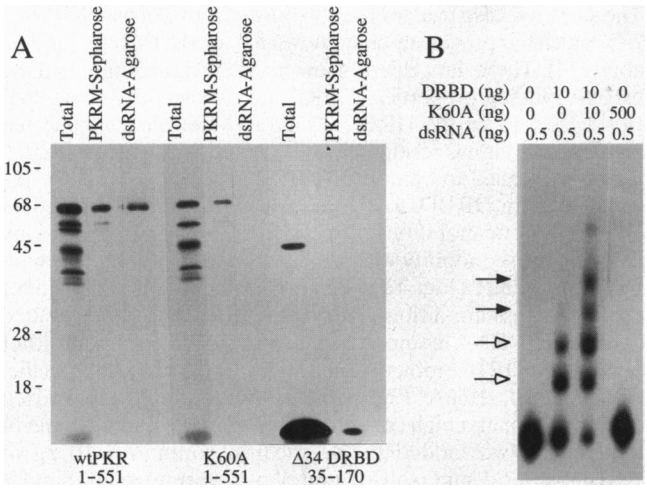

FIG. 4. Analysis of the protein–protein interaction property of PKR mutants defective in dsRNA binding. (*A*) Binding of PKR and DRBD mutants to PKR^M and dsRNA. ^{35}S-labeled wtPKR, K60A mutant, and Δ34 DRBD (residues 35–170) mutant were synthesized *in vitro* and tested for binding to PKR^M-Sepharose and polyI·polyC-agarose. PKR^M-Sepharose lanes, proteins bound to PKR^M; dsRNA-agarose lanes, proteins bound to polyI·polyC. The residues included in the protein are indicated below the lanes. Positions of molecular weight markers are indicated as $M_r \times 10^{-3}$. (*B*) Electrophoretic mobility shift assay for detecting DRBD–K60APKR interaction: The binding of 10 ng of DRBD with 0.5 ng of end-labeled 82-bp dsRNA was carried out in either the presence or the absence of 10 ng of K60APKR. The extreme left lane shows only the dsRNA probe without any protein added and the extreme right lane shows dsRNA probe with 500 ng of K60APKR protein alone. The second lane from left is dsRNA with 10 ng of DRBD alone and the next lane shows dsRNA with 10 ng each of DRBD and K60APKR protein. Open arrows represent positions of the DRBD–dsRNA complexes; closed arrows indicate positions of the supershifted bands.

transactivation domain of the VP16 protein of herpes simplex virus. These two plasmids were cotransfected in monkey COS-1 cells along with a reporter plasmid in which the luciferase gene is under the control of a GAL4-binding site and the synthesis of luciferase was assayed by measuring its enzyme activity (Fig. 5). Transfection of GAL4-PKR^M, VP16-PKR^M, GAL4-DRBD, or GAL4 and VP16 along with the reporter plasmid resulted in a low level of luciferase production. This level was increased by about 5-fold when GAL4-PKR^M and VP16-PKR^M were cotransfected. DRBD showed interaction with itself as well as with PKR^M. An 18-fold stimulation was observed with GAL4-DRBD and VP16-DRBD and 6-fold stimulation was observed when GAL4-DRBD was cotransfected with VP16-PKR^M or GAL4-PKR^M was cotransfected with VP16-DRBD. The DRBD–DRBD interaction *in vivo* was stronger than the PKR–PKR interaction, which could be due to a higher level of expression or more efficient folding of DRBD fusion proteins as compared to the PKR fusion proteins. Quantitatively, the magnitude of this interaction was about half of that observed for the interaction between RAP74 and RAP30, the two subunits of the transcription initiation factor TFIIF (42). These results, nonetheless, clearly demonstrated that PKR and DRBD can self-associate in the environment of a mammalian cell.

DISCUSSION

The mechanisms of PKR activation and phenotypes resulting from overexpression of mutant forms in mammalian cells have led to suggestions that PKR must dimerize to be active. In this investigation we have used four independent assays to provide direct evidence in support of PKR oligomerization. First, the affinity chromatography data not only demonstrated that PKR can interact with itself but also led to the conclusion that there must be more than one interaction domain on the molecule. The N-terminal domain, which partially overlaps with the DRBD and which contains at least one interaction domain, was studied in more detail. Second, direct chemical crosslinking of the DRBD demonstrated that this domain can form dimers. In view of this result, it is worth noting that DRBD can inhibit the activity of purified PKR (40). It was observed that when added to monoclonal antibody-bound PKR, DRBD could inhibit its kinase activity even at concentrations that are not sufficient to soak up the activator dsRNA. Inhibition at these low DRBD concentrations can be explained by DRBD–PKR interaction leading to inactivation of PKR activity. These results suggest that PKR may also exist as dimers, although thus far there is no direct experimental evidence for this. Attempts to crosslink PKR^M resulted in large oligomeric complex formation (data not shown) whose physiological significance remains to be evaluated. There is information in the literature, however, suggesting PKR dimer formation. Size fractionation of PKR partially purified from a mouse cell extract showed that a part of the PKR pool has the apparent molecular weight of a dimer (45).

The third approach to demonstrate a protein–protein interaction between PKR and DRBD was adapted from the techniques commonly used for studying DNA–protein interactions. To confirm the presence of an alleged protein in a shifted DNA–protein complex, a specific antibody to the protein is added to the mixture. The antibody binds to the protein–DNA complex and retards its mobility further causing a "supershifting." We used the same principle to demonstrate that a mutant PKR, which cannot bind dsRNA, could supershift DRBD–dsRNA complexes. Thus, the interaction must be directly between the two proteins. This and other results also established clearly that the dsRNA-binding and the dimerization properties of PKR are separable. Mutants defective in dsRNA binding could still dimerize. Whether the converse is also true will have to await a more accurate definition of the dimerization domain. In the case of another RNA-binding protein, human immunodeficiency virus type 1 rev (46, 47), several mutants defective in RNA binding can still dimerize. rev mutants defective in oligomerization, however, fail to bind RNA, indicating that the RNA-binding domain may be formed

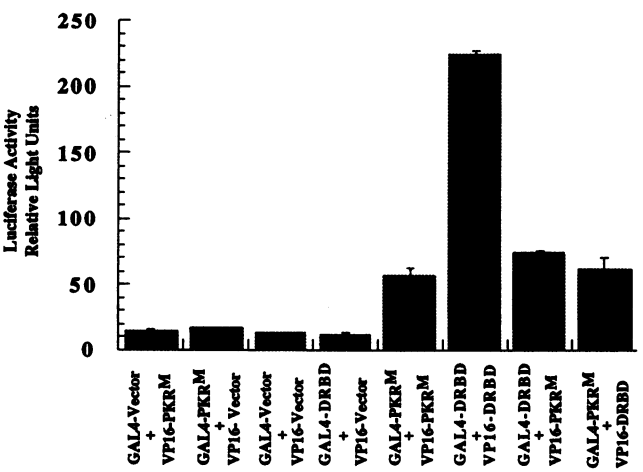

FIG. 5. Demonstration of PKR–PKR, PKR–DRBD, and DRBD–DRBD interactions *in vivo*. COS-1 cells were transfected with 200 ng of each of the four (two test plasmids encoding proteins to be tested, the reporter plasmid pG5Luc, and pRSV-β-galactosidase plasmid to normalize the transfection efficiency) plasmid DNAs by the Lipofectamine procedure. Cells were harvested 48 hr after transfection and assayed for luciferase activity after normalizing for the transfection efficiency by measuring the β-galactosidase activity. The different combinations of plasmids used are indicated below the respective bars. Each experiment was repeated six times and the averages of individual values with standard error bars are presented.

by protein–protein interactions. It would be interesting to test this model for DRBD in the future.

The final evidence for PKR–PKR interaction was provided by the two-hybrid transcriptional activation assay. Because such an interaction has far-reaching implications in cellular physiology, we decided to test for this interaction *in vivo* in mammalian cells, rather than in yeast, which is commonly used for such assays. Our results from COS cells convincingly demonstrated PKR–PKR, PKR–DRBD, and DRBD–DRBD interactions *in vivo*. This assay can also be potentially used for identifying other cellular proteins that interact with PKR. Such experiments may lead to the discovery of new substrates or new inhibitors of this enzyme, which appears to have multiple unrelated physiological effects.

Dimerization of PKR has been suggested as a possible mechanism for its autophosphorylation (15, 16, 48). It is known that dsRNA binding causes PKR autophosphorylation. Phosphorylated PKR is an active kinase that can phosphorylate substrates, such as eIF-2α, in the absence of dsRNA. The autophosphorylation reaction kinetics suggest that it is an intermolecular reaction (15) that, by definition, requires a close proximity of two PKR molecules. Binding of two PKR molecules to the same dsRNA molecule may promote such juxtaposition, thus explaining the basis of PKR activation by dsRNA. Alternatively, PKR may exist as dimers prior to its activation by dsRNA, as suggested by the evidence presented in this paper. In this scenario, dsRNA or other activators of PKR change its conformation to an active state so that it can phosphorylate its bound partner. Experimental evidence presented in this paper strongly favors the second model. It may also explain how small molecules such as heparin, which are unlikely to bind two PKR molecules simultaneously, can promote phosphorylation of PKR. The observed loss of the activator effect of dsRNA at a high concentration can be explained by postulating that if two dsRNA molecules can bind to the two partners of a PKR dimer, the two monomers are pulled apart because their affinity for dsRNA is much higher than their mutual affinity.

Much of the information in the literature in support of various cellular roles of PKR has come from the use of enzymatically inactive mutants of PKR. Studies using this approach have indicated antiviral (49), antiproliferative (6, 36, 37), and transcriptional signal transducer roles of PKR (ref. 14; R.C.P. and G.C.S., unpublished observations). More dramatically, it has been shown that cells expressing PKR inactive mutants are highly tumorigenic (35, 37), thus suggesting that PKR may be a tumor suppressor (12). Although these observations are well documented, the underlying mechanism of action of the inactive PKR mutants remains unclear. Three, not mutually exclusive, possibilities exist that all lead to the inactivation of cellular PKR. The inactive mutants may sequester the natural cellular activators of PKR (50), which may or may not be dsRNA. They may also sequester the potential substrates of PKR, such as eIF-2α and IκB, thereby protecting them from being phosphorylated by PKR. Finally, they may act as a trans-dominant inhibitor of PKR by forming heterodimers. For the third mechanism to operate, such heterodimers containing one active PKR molecule and one inactive PKR molecule should be enzymatically impaired for phosphorylating cellular substrates, a postulation yet to be demonstrated. This third model of heterodimer formation has been widely used to explain the observed trans-dominant inhibition by inactive mutants of PKR. This paper provides strong experimental support in favor of the heterodimerization model. Our experiments demonstrated PKR–PKR, PKR–DRBD, and DRBD–DRBD interactions *in vitro* and *in vivo*. The final test of the heterodimerization model for explaining trans-dominance of inactive PKR *in vivo* will come from examining the phenotypes of PKR mutants that fail to dimerize but still bind the activators and the known substrates.

We gratefully acknowledge Drs. Adrienne Takacs and Amiya Banerjee for providing the vectors and the reporter plasmids for the two-hybrid system. We also thank Laura Tripepi for assistance in preparing the manuscript. This work was supported in part by National Institutes of Health Grants AI-22510 and CA-62220.

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