Specific Mutations Near the Amino Terminus of Double-stranded RNA-dependent Protein Kinase (PKR) Differentially Affect Its Double-stranded RNA Binding and Dimerization Properties

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The amino-terminal region of the double-stranded (ds) RNA-dependent protein kinase, PKR, has been shown to mediate both dsRNA binding and protein dimerization. To critically examine if PKR dimerization is dependent on dsRNA binding, we generated a series of mutants that are incapable of binding dsRNA. Some, but not all, of these mutants retained the ability to dimerize, as shown by a two-hybrid transcriptional activation assay in vivo and a chemical cross-linking assay in vitro. These mutants were used further to demonstrate that the translational inhibitory activity of PKR in vivo requires dsRNA binding; PKR mutants that dimerized but did not bind dsRNA could not inhibit the translation of a transfected reporter gene.

The double-stranded (ds)1 RNA-activated protein kinase (PKR) is an interferon-inducible, serine/threonine kinase (1). Most mammalian cells contain a low constitutive level of this enzyme. Other than dsRNA, small polyanionic molecules such as heparin can also activate PKR (2, 3). Activation of PKR is accompanied by autophosphorylation of the latent enzyme, an event that enables PKR to phosphorylate the α subunit of the eukaryotic protein synthesis initiation factor (eIF2α) at serine 51 (4–6). This phosphorylation of eIF2α leads to a global block in protein synthesis (7, 8). Other substrates of PKR have been described recently, which include eIF2β, the inhibitor of the kinase subdomain, and human immunodeficiency virus Tat protein (9). The most well studied effect of PKR phosphorylation is on the translational inhibitory activity of PKR (10). The most well studied effect of PKR activation is inhibition of translation as a result of eIF2α phosphorylation. Since the replicative intermediates of many viruses can lead to PKR activation, several viruses have evolved strategies to evade PKR activation (11, 12). In addition to its role in interferon-mediated immunity against viruses, PKR has been implicated in diverse cellular processes such as signal transduction (13–16), cell growth (17–19), and differentiation (20, 21). Overexpression of the enzymatically inactive R296R mutant of PKR has been shown to lead to oncogenic transformation of NIH3T3 cells (18, 22).

The primary structure of PKR deduced from its cDNA sequence identified that the conserved kinase subdomains are located in the carboxyl-terminal part of the molecule (23). We and several other groups have previously mapped the dsRNA binding domain (DRBD) of PKR to the amino-terminal 170 residues (17, 24–28). This region contains two groups of dsRNA binding motif found in a number of different RNA-binding proteins (29). The carboxy-terminal part of this motif is more conserved and has been shown by nuclear magnetic resonance solution structure to form an α-helix (30, 31).

Mutations of some of the positively charged residues within this motif have been shown to abolish its dsRNA binding activity (32, 33). It has been suggested that PKR activation by dsRNA is achieved by mutual autophosphorylation of the PKR molecules bound to each other (34, 35). In support of this model, we have recently provided direct evidence for PKR dimerization (36). Our studies revealed that the dimerization domain and the dsRNA binding domain of PKR overlap. These studies also indicated that dsRNA binding is not required for dimerization. In contrast to our conclusions, Cosentino et al. (37) suggested that PKR dimerization is a consequence of dsRNA binding. In this report, we have critically examined this point of difference. For this purpose, we have generated a large number of PKR mutants and studied their dsRNA binding and dimerization properties. The results presented here demonstrate that the dsRNA binding and the dimerization properties of PKR are genetically separable. Several mutants of PKR that were completely devoid of the ability to bind dsRNA could dimerize efficiently. Thus, dsRNA binding is not a prerequisite for PKR dimerization.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The point mutants were generated by oligonucleotide-directed mutagenesis of the p68/BS (25) using the Mutagen phase in vitro mutagenesis kit (Bio-Rad) (3). To generate these mutants, the following oligonucleotides were used: Q19L, 5′-GG-AACCTAATACATTACCTGCTGAAAGCAGAGGAGTACGGA-3′; P35,36L, 5′-CTAATTCCAGGATCCTACATGAGGAACATGTCAG-3′; P141A, 5′-GATAGAGGGCG- TACATTAGA-3′; K60A, 5′-GATAGATCGCGAGGAGGAGCA-3′; K157Q, 5′-GATACAGGAAGGAGCAAA-3′; K146E, 5′-GAGGAGCGAGAAATGTCCCA-3′; V72Y, 5′-CAATTGACTTATGAGATACCT-3′; Q110L, 5′-GAATTGGCCTTGAGAAGAAG-3′; F131A, 5′-CCAGAAGGAGCTCA- TTATGAA-3′; K150A, 5′-GATGTCACTGCAGCAGGAGCA-3′; K154E, 5′-CAGAAGCAGAACAACTTGG-3′; and a deleted gene phagemid (25) clone digested with ScaI and StuI and religated to generate a deletion of motif 1. This mutant was termed D1.

dsRNA Binding and PKR Activity Assay—The in vitro-translated, 35S-labeled proteins were synthesized using the TNT T7 coupled reticulocyte lysate system from Promega. The dsRNA binding activity was measured by poly(I)-poly(C)-agarose binding assay performed with 35S-labeled, poly(I)-poly(C) probe.

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§5S-labeled proteins were synthesized using the TNT T7 coupled reticulocyte lysate system from Promega. The dsRNA binding activity was measured by poly(I)-poly(C)-agarose binding assay performed with 35S-labeled proteins (25). Four μl of translation products diluted with 25 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 0.3 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 10% glycerol) were mixed with 25 μl of poly(I)-poly(C)-agarose.
beads and incubated at 30°C for 30 min with intermittent shaking. The beads were then washed with 500 μl of binding buffer four times. The proteins bound to beads after washing were analyzed by SDS-PAGE followed by fluorography. The kinase activity assay of in vitro-translated wild type (wt) PKR and mutant proteins was performed as described previously (3). Six μl of the in vitro-translated, 35S-labeled proteins were incubated with 1 μl of antisera in 200 μl of high salt buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 10 μg of aprotinin/ml, 0.1 mM phenylmethylsulfonyl fluoride, and 5% glycerol). The PKR assay was performed in an activity buffer (500 ng of purified eIF2, 0.1 mM ATP, and 10 μCi of [γ-32P]ATP at 30°C for 10 min. One μg/ml poly(I)-poly(C) was used as activator for the enzyme. Labeled proteins were then analyzed by SDS-PAGE on a 12% gel. Autoradiography was performed at -80°C with intensifying screens.

In Vivo Protein-Protein Interaction Assay—The in vitro protein-protein interaction assay was performed as described previously (36). The proteins were in vitro-translated and [35S]methionine labeled. Four μl of the translation mix were incubated with 1 μg of hisK296R and 20 μl of Ni-charged his-bind resin at 30°C for 2 h in binding buffer (5 mM imidazole, 200 mM NaCl, 20 mM Tris-HCl, pH 7.9, and 0.5% Nonidet P-40). After binding, the beads were washed with 500 μl of wash buffer containing 500 ng of purified eIF2, 0.1 mM ATP, and 10 μCi of [γ-32P]ATP at 30°C for 10 min. One μg/ml poly(I)-poly(C) was used as activator for the enzyme. Labeled proteins were then analyzed by SDS-PAGE on a 12% gel. Autoradiography was performed at -80°C with intensifying screens.

Chemical Cross-linking with Dimethylsuberimidate—The DRBD of A68D and V72Y was subcloned in PET15b vector (Novagen) and hexahistidine-tagged mutant DRBD proteins were expressed and purified. The purified DRBD proteins were dialyzed against 2000 volumes of buffer (20 mM HEPES, pH 7.5, and 10% glycerol) at 4°C for 17 h. Four μg of DRBD was then cross-linked in 100 μl with 1 mM dimethylsuberimidate in cross-linking buffer (10 mM HEPES, pH 8.0, and 100 mM NaCl) at 25°C for 2 h. Ten μl aliquots were removed at the times indicated, and the reaction was stopped by adding 1 mM glycine to a concentration of 100 μl. Proteins were then analyzed by SDS-PAGE on a 12% gel followed by Western blot analysis using a polyclonal antibody raised against bacterially produced DRBD.

Mammalian Two-hybrid Protein-Protein Interaction Assay—The DRBD portions of the point mutants were amplified using the PCR as described previously (36) and were subcloned into pSG424 (GAL4/mutant DRBD). DRBD of each point mutant was subcloned into the pSG424 as a GAL4 DNA binding domain fusion protein and tested for interaction with VP16 activity domain fusion of wt DRBD. The GAL4/VP16 DRBD and VP16/DRBD were described as (36). COS-1 cells were transfected with 200 ng of each of the four (two test plasmids encoding proteins to be tested for interaction, the reporter plasmid pG5Luc, and pRSV-β-galactosidase plasmid to normalize the transcription efficiency) plasmid DNA's by the Lipofectamine procedure (LifeTechnologies, Inc.). Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for the transcription efficiency by measuring the β-galactosidase activity.

In Vivo Translation Inhibition Assay—The point mutants were subcloned into pcDNA3, an eukaryotic expression vector. COS-1 cells were transfected with 200 ng of each of the two (expression construct and the reporter plasmid pGL2-Control from Promega) plasmid DNA's by the Lipofectamine procedure. Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for the transcription efficiency by measuring the total protein.

RESULTS

We and others have previously characterized the DRBD of PKR within the amino-terminal 170 residues (17, 24–28). This region contains two copies of a 65-amino acid motif (Fig. 1A) that is also shared by many other RNA-binding proteins (29). The most conserved regions within these motifs are the residues 55–75 and 145–165 of human PKR. Others have identified these regions as motifs 1 and 2 and have shown their importance in dsRNA binding (27, 28). However, the region of homology among various dsRNA-binding proteins extends beyond these short stretches of strong conservation. We have shown previously by deletion and point mutation analysis that the amino acids 1–24 are involved in dsRNA binding (3). Point mutation of residues 18 and 19 significantly reduces binding to dsRNA and subsequent activation of PKR. These results strongly indicate that the regions beyond the most conserved stretches (55–75) are important for dsRNA binding. It was observed in our previous studies that a deletion of motif 2 results in loss of dsRNA binding activity, illustrating that motif 1 is necessary but is not sufficient for dsRNA binding (25). As observed in Fig. 1C, a deletion of motif 1 also results in loss of binding to dsRNA, thereby demonstrating that both motifs play an essential part for dsRNA binding activity. We have demonstrated recently that the DRBD contributes to dimerization of PKR as well. To test the role of the extended motifs 1 and 2 and to assay the contribution of each copy of the motif in dsRNA binding and dimerization, several point mutations (Fig. 1B)
were introduced in them. The mutated amino acids were chosen for their conservation and location. Mutations were introduced in the highly conserved residues, which may be important for the function of this domain. Five pairs of equivalent point mutations in two motifs were made, which are distributed throughout the entire length of the motifs. Conserved amino acids at positions 19, 41, 60, 64, and 68 in motif 1 and amino acids at equivalent positions 110, 131, 150, 154, and 158, respectively, in motif 2 were mutated. Certain residues conserved in dsRNA-binding proteins but unique to motif 1 were mutated to the corresponding residue of motif 2 than motif 1 (K64E and K154E). These results indicate that the mutations could be grouped into three classes: those which were equally detrimental either in motif 1 or in motif 2 (K60A-K150A and A68D-A158D); mutations that are more drastic in motif 1 (Q19L and F41A) than equivalent motif 2 mutations (Q110L and F131A); and mutations that are more drastic in motif 2 than motif 1 (K64E and K154E). These results indicate that both motifs contribute to dsRNA binding, and the role played by conserved amino acids at identical positions in two motifs do not follow a simple pattern.

The deleterious effect of mutating certain nonbasic residues such as Q19, F41, and A68 indicate that the binding of PKR with dsRNA is not a simple ionic interaction. Mutations K61Q and V72Y at residues not conserved in both motifs retained about 50 and 80% of the activity, and mutant P35,36L, in which the secondary structure of the domain is expected to be altered, showed more than 90% loss of activity. The importance of basic lysine residues at positions 60, 150, and 154 but not at positions 61 and 64 indicate that not all basic residues contribute to the dsRNA binding equally.

Functional Assay of the Mutants—To test whether the loss of ability to bind to dsRNA correlated with the loss of activation, some of the most severely affected mutants were tested for kinase activity, along with the wt PKR. These proteins were translated in vitro in rabbit reticulocyte system and immunoprecipitated using a polyclonal antibody against PKR. Equal amounts of immunoprecipitated proteins (Fig. 3A) were tested for eIF2α phosphorylation activity (Fig. 3B). All the mutants were defective in eIF2α phosphorylation, indicating that the ability to bind dsRNA is essential for activation. These results
**Fig. 4. In vitro dimerization activity of point mutants.** In A, $^{35}$S-labeled wt PKR and mutant proteins were synthesized in vitro and analyzed for binding to PKR$^{34}$-Sepharose. Total lanes, the total proteins from the reticulocyte lysate; Ni-Sepharose lanes, the proteins bound to Ni-charged resin; PKR$^{34}$-Sepharose lanes, the proteins bound to PKR$^{34}$ immobilized on the resin. PKR$^{34}$, hexahistidine-tagged, purified, catalytically inactive mutant protein K296R. In B, the in vitro dimerization assay in A was quantitated on a PhosphorImager by analyzing the signal in Total and PKR$^{34}$-Sepharose lanes and plotted as relative binding activity of mutants with respect to the binding activity of wt PKR taken as 100%. Bars, S.E.

are in agreement with those of Romano et al. (38) and McMillan et al. (33), who analyzed the dsRNA binding and activation of PKR in yeast.

**Dimerization Activity of the Mutants—**We have showed recently that PKR self-associates both in vitro and in vivo (36). The main dimerization domain of PKR overlaps with its dsRNA binding domain. We, therefore, tested the dimerization activity of two of the mutants using an in vitro assay. $^{35}$S-Labeled wt and mutant proteins were assayed for binding to purified, hexahistidine-tagged PKR immobilized on Ni-charged affinity column as described previously. Both mutants (Q19L and F41A) retained dimerization activity (Fig. 4A), confirming our previous observation that dsRNA binding is not necessary for dimerization. Quantitation of the dimerized mutant proteins by PhosphorImager analysis (Fig. 4B) showed that although the mutation F41A was completely devoid of dsRNA binding, it retained 85% of dimerization activity. Q19L, which has lost 80% of its dsRNA binding activity, also retained 80% of its dimerization activity. These results suggest that PKR can dimerize independently of its dsRNA binding activity.

The dimerization ability of the dsRNA binding-defective mutants was further confirmed by using a in vivo interaction assay. We have reported previously that PKR-PKR, PKR-DRBD, and DRBD-DRBD interaction could be observed in mammalian cells using a two-hybrid transcriptional activation assay (36). DRBDs of 11 point mutants defective in dsRNA binding were studied for their ability to dimerize with wt DRBD. As presented in Fig. 5, most of the mutants retained their ability to dimerize, further strengthening our earlier conclusion that dsRNA binding is not required for dimerization. Two point mutants, A68D and A158D, showed severely reduced dimerization.

The dimerization property of two of the mutants was further analyzed by chemical cross-linking of the mutant proteins (Fig. 6). The DRBD regions of the mutants were subcloned into a prokaryotic expression vector, the hexahistidine-tagged proteins were expressed in *Escherichia coli* and purified by affinity chromatography on Ni-agarose. Since it is quite an extensive amount of work to purify and cross-link all of the point mutants, we selected two representative mutants for this purpose: one with dimerization activity similar to wild type (V72Y), and one with severely reduced dimerization activity (A68D). Dimethylsuberimidate cross-linking of the purified DRBDs confirmed the in vitro dimerization and in vivo two-hybrid data that both of them retained their ability to form dimers. Dimerization activity of A68D was severely reduced as compared to that of the wt and V72Y. Although exact quantification of dimerization activity in this assay was not possible, it supports the results obtained using the mammalian two-hybrid system.

**Translational Inhibition by PKR Mutants in Vivo—**Transient transfection of cells with an expression construct encoding a reporter gene such as luciferase has been shown to result in activation of PKR, resulting in phosphorylation of eIF2α and inhibition of translation of the reporter mRNA derived from the expression construct (39–42). In this assay system, a variety of agents that can suppress the activation of PKR can stimulate the synthesis of the reporter protein. Inhibition of PKR activation by cotransfection with plasmids coding for adenovirus VAI RNA, revirus sigma 3, and transdominant negative mutant K296R, or by treatment of cells with 2-aminopurine, results in stimulation of synthesis of the reporter protein. Expression of a nonphosphorylatable form of eIF2α has been shown to have a similar effect (43). We, therefore, decided to test some of the PKR mutants for their ability to inhibit wt PKR activation. Six of the PKR mutants severely defective in dsRNA binding were subcloned into the eukaryotic expression vector pCB67. In
A Western blot analysis was performed with a polyclonal anti-DRBD antibody. The ability to bind dsRNA correlates with activation of kinase activity. The triple mutant with dsRNA binding defect (A68D, A158D) and K296R combined behaves similarly to the wt PKR. Cotransfection with wt PKR resulted in a drastic reduction of luciferase activity, as expected from previous reports (44). However, cotransfection with the various point mutants defective in dsRNA binding resulted only in a slight reduction of the luciferase activity. These results indicate that the translation inhibitory action of wt PKR requires the dsRNA binding and kinase activity. Since some of these mutants are defective in both dimerization and dsRNA binding, either property, in principle, may be required for the translation inhibition phenotype. However, since mutants such as F41A and A158D, which did not bind dsRNA but can dimerize, fail to inhibit translation like wt PKR, the dsRNA binding property seems to be the crucial determinant. Cotransfection with the transdominant negative mutant K296R resulted in stimulation of luciferase activity as reported previously (44). This stimulation of reporter protein synthesis has been shown to be due to the ability of K296R mutant to inhibit the wt PKR activation. The triple mutant with dsRNA binding defect (A68D, A158D) and K296R combined behaves similarly to the dsRNA binding-defective mutant and not like the K296R mutant. This strongly suggests that the translation stimulatory effect of the K296R mutant requires dsRNA binding.

**DISCUSSION**

The amino-terminal 170 amino acids of PKR constitute its dsRNA binding domain (17, 24–28). A 65-amino acid motif is repeated twice in this domain and shares homology with a number of RNA-binding proteins (29). Although the region of homology extends all through the motif, most of the conserved residues are present in the carboxy terminus of this domain in a region that is shown to form a positively charged α-helix by nuclear magnetic resonance analysis (30, 31). The 65-amino acid motif is present in multiple copies in most of the proteins belonging to this class, but it is not known if all of the copies function analogously for dsRNA binding. In this study, we have analyzed several point mutations throughout the two motifs, and several important conclusions can be drawn from it: (a) it establishes that two copies of this motif are required for efficient binding to dsRNA since deletion of one copy or introduction of point mutation in one copy can result in loss of binding; (b) the residues at the amino-terminal end of the motifs play an essential role in dsRNA binding, because mutations in conserved residues 19 and 110 result in reduction of activity; (c) the ability to bind dsRNA correlates with activation of kinase activity; (d) we have identified several residues that are absolutely required for dsRNA binding as well as some other residues that contribute significantly to this activity; and (e) we have evidence that the two activities of PKR, dsRNA binding and dimerization, are not interdependent because several point mutants completely defective for dsRNA binding can dimerize efficiently.

The point mutations were specifically designed to test the contribution of individual residues at identical positions in the two conserved motifs involved in dsRNA binding. Our results show that lysine residues at positions 60, 64, 150, and 154 are important for dsRNA binding. Non-basic residues in this region, such as 68 and 158, were also found to be indispensable for dsRNA binding. Two groups have recently reported the structure of the DRBD of RNase III from *E. coli* and Staufen protein from *Drosophila melanogaster*, which share homology with dsRNA binding motifs of PKR. The nuclear magnetic resonance technique has revealed that this motif has an α-β-β-α topology in which a three-stranded, anti-parallel β-sheet packs on one side against the two helices (30, 31). The residues 60, 64, 68, 150, 154, and 158 are in the region of PKR that is predicted to form an α-helical structure. The two residues F32 and K50 are required for the dsRNA binding activity of the staufen DRBD3 domain and correspond to residues F31 and K60 in PKR motif 1. A mutation in either one of these residues was also found to lead to complete loss of dsRNA binding. Helical wheel projections of residues 60 to 75 indicate that the side chains of lysines 60 and 64 and alanine 68 would all be oriented on one side of the putative helix. McCormack et al. (27) have postulated that the presence of positive charges on one side of the helix would facilitate dsRNA binding. Our results support this hypothesis. The secondary structure of the dsRNA binding domain also seems to be important since a mutant with both proline residues at 35 and 36 replaced with leucine, which is expected to have altered secondary structure, shows a loss of dsRNA binding.

It has been proposed by other groups that motif 1 plays a major role in dsRNA binding, and motif 2 functions by stabilizing the secondary structure (27, 28). This conclusion was primarily drawn from three pairs of mutants at equivalent positions of the molecular weight markers.

**FIG. 6. Chemical cross-linking of the DRBD.** Four μg of purified DRBDs (wt and the point mutants A68D and V72Y) was cross-linked with 1 mM dimethylsuberimidate in (10 mM HEPES, pH 8.0, and 100 mM NaCl) at 25 °C for 2 h. Aliquots were removed at the times indicated, and the reaction was stopped by adding 1 M glycine to a concentration of 100 mM. Proteins were analyzed by SDS-PAGE on a 12% gel. A Western blot analysis was performed with a polyclonal anti-DRBD antibody. Left, positions of the molecular weight markers.

**FIG. 7. In vivo translation inhibition assay.** COS-1 cells were transfected with 200 ng of each of the two (the reporter plasmid pGL2-Control and different PKR expression constructs in pCB6) plasmid DNAs by the Lipofectamine procedure. Cells were harvested 48 h after transfection and assayed for luciferase activity after normalizing for total protein. The wt construct and different point mutants that were assayed are indicated below the respective columns. (−), negative control, which represents the relative luciferase activity obtained with only reporter plasmid pGL2-Control. Each experiment was repeated six times; bars, S.E. The relative luciferase activity obtained is represented on the Y-axis.
positions (Q19L-Q110L, F31A-F141A, and A68D-A158D) in the two motifs. When additional mutational pairs were examined by us, their simple conclusion no longer holds true. Both motif 1 and 2 mutations at K60A and K150A were equally drastic, and the motif 2 mutation K154E was more drastic than motif 1 mutation K64E. These results indicate that both motifs are involved in dsRNA binding, and the relative contribution of individual residues cannot be predicted by their position in either motif 1 or 2. F131A mutation has been reported to cause only 10% reduction in binding (28), whereas in our assay, binding was reduced by 95%. At present, we do not know the reason for these differences, but it may result from the different methods used to measure dsRNA binding. Green and Mathews (28) have measured dsRNA binding by binding of 32P-labeled dsRNA to PKR immobilized on antibody-protein A-Sepharose beads. The antibody-PKR complex may not behave exactly in the same way as native PKR in solution as used in our binding assay.

We have shown previously that PKR can self-associate to form dimers, and this activity is also mediated through DRBD (36). We further showed that the dimerization of PKR was not dependent on its dsRNA binding activity. Our results are in agreement with Ortega et al. (45) and Wu and Kaufman (44), who reported recently that dimerization of PKR is not dependent on binding to dsRNA. Their results were primarily based on a point mutation K64E. However, Cosentino et al. (37) have reported contradictory observations supporting a view that PKR dimerization occurs on dsRNA, and therefore, dsRNA binding activity is required for its dimerization (37). We have, therefore, investigated in this report if the dsRNA binding and dimerization activities of PKR are interdependent, using many different point mutants. Several of the mutants devoid of dsRNA binding were found to retain their dimerization activity, thereby confirming our earlier observation that dimerization is not dependent on dsRNA binding. As represented in Fig. 8, we were able to dissociate the dsRNA binding and dimerization activities of PKR. Mutations that result in a loss of dsRNA binding, such as F41A and A158D, show a differential effect on dimerization property. A158D mutation caused complete loss of dimerization, whereas F41A mutation resulted in a slight increase in dimerization. Mutation K64E, which results in a partial loss of dsRNA binding, also showed slightly increased dimerization. It is, therefore, possible that although the same domain of the protein contributes to its dsRNA binding and dimerization properties, specific residues within this domain contribute differentially to these two activities.

In the COS-1 cell transfection assay, we have found that unlike the K296R mutant, several of the dsRNA binding-defective mutants that also lacked kinase activity in the in vitro assay failed to stimulate translation of a cotransfected reporter gene. The stimulation of luciferase translation by K296R mutant is attributed to its ability to inhibit activation of the endogenous PKR during transfection. Our results indicate that, although capable of dimerizing with endogenous PKR, our mutants defective in dsRNA binding are unable to inhibit its activity. Thus, the dsRNA binding activity seems to determine the ability of the mutants to stimulate the translation of a cotransfected reporter gene. Also, the triple mutant (A68D, A158D, K296R) behaves like the dsRNA binding-defective mutants and not like the K296R mutant, confirming that it can no longer inhibit activation of PKR.

Although our studies show that dimerization is not dependent on dsRNA binding, it remains to be determined if the dimerization-defective mutants can retain their dsRNA binding activity. It is possible that the high affinity dsRNA binding domain is formed by dimerization of the protein, as in the case of human immunodeficiency virus rev (46, 47). This also has important implications on the mechanism of activation of kinase activity upon binding to dsRNA. Two models have been proposed thus far, one of which involves a conformational change brought about by binding to dsRNA leading to activation (2). A second model suggests that the dsRNA binding can form two molecules of PKR together and results in intermolecular phosphorylation, leading to activation of the enzyme (34). Our data shows that mutants of PKR defective in dsRNA binding can associate to form dimers, thereby confirming that PKR forms dimers, even in the absence of dsRNA. Our results favor the first model that invokes dsRNA-dependent conformational change leading to activation. This conclusion is also supported by the reports that ATP can be photo-cross-linked to PKR only in the presence of activators such as dsRNA or heparin (48, 49). In the absence of the activators, the ATP-binding site of PKR seems to be masked by protein folding. A detailed analysis of the mutants defective in either the dimerization and/or dsRNA binding and elucidation of the structure of dsRNA-DRBD complex is required to sort out these possibilities.

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