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Double-stranded-RNA-dependent protein kinase and TAR RNA-binding protein form homo- and heterodimers *in vivo*

(eIF-2α kinase/protein dimerization/yeast two-hybrid assay)

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ABSTRACT **The yeast two-hybrid system and far-Western protein blot analysis were used to demonstrate dimerization of human double-stranded RNA (dsRNA)-dependent protein kinase (PKR) *in vivo* and *in vitro*. A catalytically inactive mutant of PKR with a single amino acid substitution (K296R) was found to dimerize *in vivo*, and a mutant with a deletion of the catalytic domain of PKR retained the ability to dimerize. In contrast, deletion of the two dsRNA-binding motifs in the N-terminal regulatory domain of PKR abolished dimerization. *In vitro* dimerization of the dsRNA-binding domain required the presence of dsRNA. These results suggest that the binding of dsRNA by PKR is necessary for dimerization. The mammalian dsRNA-binding protein TRBP, originally identified on the basis of its ability to bind the transactivation region (TAR) of human immunodeficiency virus RNA, also dimerized with itself and with PKR in the yeast assay. Taken together, these results suggest that complexes consisting of different combinations of dsRNA-binding proteins may exist *in vivo*. Such complexes could mediate differential effects on gene expression and control of cell growth.**

The mammalian double-stranded RNA (dsRNA)-dependent protein kinase (PKR; also termed p68, DAI, dsI, P1 kinase) is an interferon-inducible regulator of cell growth and virus replication (1–4). PKR-mediated regulation occurs largely via the phosphorylation of eukaryotic initiation factor 2 (eIF-2) resulting in translational arrest (5–7).

Activation of PKR is dependent on dsRNA or single-stranded RNA with double-stranded regions. These effector molecules bind to the N-terminal regulatory region of PKR, which harbors two copies of a consensus motif that is common to a number of dsRNA-binding proteins (8–12). Upon dsRNA binding, PKR undergoes autophosphorylation at multiple unidentified sites (13, 14), and the phosphorylated form of PKR catalyzes the phosphorylation of the α subunit of eIF-2 (eIF-2α).

PKR-induced inhibition of cell growth has been demonstrated in yeast (15, 16) and mammalian cells (17, 18) and results from the phosphorylation of endogenous eIF-2α. PKR also behaves as a tumor suppressor *in vivo*, as expression of functionally defective PKR mutants with wild-type (WT) dsRNA-binding domains causes malignant transformation in NIH 3T3 cells (17, 19). The oncogenic transformation is presumed to result from inhibition of the endogenous PKR, either because of dsRNA sequestration by the mutant PKR or through heterodimer formation between the WT and mutant proteins. The loss of PKR activity could deregulate the expression of key protooncogenes, resulting in transformation.

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Several studies have implied the requirement of PKR dimerization for activation. For example, *in vitro* studies have shown that (*i*) the PKR activation process exhibits second-order kinetics (20), (*ii*) the phosphorylated form of the protein is eluted as a dimer in size-exclusion chromatography (21), and (*iii*) the WT enzyme phosphorylates a catalytically inactive PKR species in trans (22). However, evidence in favor of the view that PKR functions as a monomer has also been presented (23, 24).

We have utilized the two-hybrid system in yeast (25, 26) and the far-Western technique (27) to test directly for PKR dimerization. We show that PKR dimerizes *in vivo* and that truncation of the C terminus, which deletes the kinase domain but leaves the dsRNA-binding domains intact, has no effect on this interaction. PKR dimerization *in vitro* is dependent on the presence of dsRNA. In addition, we demonstrate that PKR can complex with an unrelated human dsRNA-binding protein, the TAR RNA-binding protein (TRBP), which recognizes the transactivation region (TAR) of human immunodeficiency virus (10, 28) and which has recently been identified as a modulator of PKR function (29). The biological significance of these interactions is discussed.

MATERIALS AND METHODS

Construction of Vector Inserts. Full-length human PKR containing a point mutation which replaced the lysine-296 with arginine (30) was subcloned into pBluescript KS (Stratagene) at the *Hin*dIII and *Pst* I sites to generate pKS-PKR[K296R]. PCR was used to truncate the 5′ untranslated region of the PKR insert by introducing a *Hin*dIII site 3 nt upstream of the initiator ATG codon and to simultaneously introduce an *Nco* I site at the initiator codon, generating pKS-(Δ5′)PKR[K296R]. The Δ2 deletion of PKR, in which aa 104–158 are deleted, was derived from pSRG2ΔL (9). The *Eco*NI–*Msc* I fragment was removed and subcloned into *Eco*NI/*Msc* I-digested pKS-(Δ5′)PKR[K296R], generating pKS-(Δ5′)PKR[K296R]Δ2. This construct was then digested with *Nco* I and religated to generate pKS-(Δ5′)PKR[K296R]Δ12. The Δ9 truncation of PKR (encoding p20, aa 1–184) was obtained in the pSRG5ΔLBN background (31). The *Stu* I–*Bam*HI fragment was removed and subcloned into *Stu* I/*Bam*HI-digested pKS-(Δ5′)PKR[K296R], thereby generating pKS-(Δ5′)p20. TRBP cDNA was obtained in pBluescript

Abbreviations: PKR, mammalian double-stranded-RNA-dependent protein kinase; eIF-2α, eukaryotic initiation factor 2 subunit α; TAR, transactivation region; TRBP, TAR RNA-binding protein; dsRNA, double-stranded RNA; GAL4-DB, GAL4 DNA-binding domain; GAL4-TA, GAL4 transactivation domain; HMK, heart muscle kinase recognition motif; ORF, open reading frame; WT, wild-type.

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SK (29). The open reading frame (ORF) was excised by digestion with *Bst*UI/*Kpn* I and inserted into pBluescript KS at the *Hinc*II and *Kpn* I sites to generate pKS-TRBP. Vaccinia virus K3L cDNA was provided in pTM1 (32) (a gift of B. Moss, National Institute of Allergy and Infectious Diseases).

Two-Hybrid-System Expression Vectors. Yeast expression vectors pGBT9 and pGAD.GH carry the GAL4 DNA-binding domain (GAL4-DB) and GAL4 transactivation domain (GAL4-TA), respectively, and were gifts from P. Bartel (State University of New York, Stony Brook) and G. Hannon (Cold Spring Harbor Laboratory). These vectors were linearized with *Eco*RI and *Spe* I, respectively, and the 5′ overhangs were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs). The vectors were then digested with *Bam*HI. Each of the PKR constructs in pBluescript KS was digested with either *Cla* I or *Hin*dIII and the 5′ overhangs were filled in. The ORF-containing fragments were then released with *Bam*HI and ligated into pGBT9 (for *Cla* I-digested inserts) or pGAD.GH (for *Hin*dIII-digested inserts). Similar in-frame fusions were generated for TRBP and K3L by digesting pKS-TRBP with *Hin*dIII/*Kpn* I or pTM1-K3L with *Nco* I/*Bam*HI and filling in the overhangs of the ORF-containing fragments. pGBT9 and pGAD.GH were digested with *Eco*RI and *Sma* I, respectively, and made blunt-ended before ligation of these inserts. Yeast *SNF1* and *SNF4*, inserted in pGBT9 and pGAD.GH, respectively (33, 34), were gifts of G. Hannon.

Two-Hybrid Assay. Yeast strain Y526 (35) was a gift of P. Bartel. Cells were transformed with various combinations of the yeast expression constructs by the lithium acetate method (36). Resulting colonies were replica plated onto selection medium supplemented with 200 μg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Biosynth, Staad, Switzerland) per ml and 70 mM phosphate buffer, pH 7. Development of blue colonies was assessed following 3 days of incubation at 30°C. For quantitative β-galactosidase assays, colonies were grown to midlogarithmic phase in liquid selection medium before cells were harvested and lysed by the glass-bead method (37). Enzyme activity was quantitated as described (38). Protein concentration was determined by the method of Bradford (39).

Far-Western Analysis. The bacterial expression vector pAR(ΔRI)[59/60] (27), carrying the Flag purification epitope and the heart muscle kinase recognition motif (HMK), was a gift of M. Blanar (University of California, San Francisco). This vector was linearized with *Eco*RI and the 5′ overhangs were filled in. The PKR[K296R] ORF was released from pKS-PKR[K296R] by digestion with *Cla* I/*Pst* I and the overhangs were filled in. This fragment was ligated into pAR(ΔRI)[59/60] to yield pAR(ΔRI)-PKR[K296R], consisting of an in-frame fusion with the Flag epitope/HMK at the 5′ end of the insert. A similar in-frame fusion was generated for TRBP by digesting pKS-TRBP with *Hin*dIII/*Kpn* I and blunt-ending the ORF-containing fragment. This fragment was then ligated into pAR(ΔRI)[59/60] to yield pAR(ΔRI)-TRBP. *E. coli* BL21(DE3)/pLysS was transformed with either pAR(ΔRI)-PKR[K296R] or pAR(ΔRI)-TRBP by a variation of the $CaCl_2$ method (40). Independent clones were grown in liquid culture and induced with 1 mM isopropyl β-D-thio-galactopyranoside (Boehringer Mannheim) (41). The far-Western probe was purified from *E. coli* lysates by using Flag immunoaffinity resin (IBI). The HMK domain on the PKR[K296R] or TRBP far-Western probes was radiolabeled essentially as described (27). Purified truncation mutants of PKR, designated p10 (aa 1–91) and p20 (aa 1–184) (31) were provided by C. Schmedt (Cold Spring Harbor Laboratory). The purified proteins were electroblotted onto nitrocellulose membranes for far-Western analysis (27). Polynucleotides tested in the far-Western analysis were poly(I)·poly(C) (Pharmacia), reovirus RNA (a gift from A. Craig, McGill University), poly(A)·poly(U) (Pharmacia), poly(A)·poly(dT) (Pharmacia), and sheared salmon sperm DNA (Sigma). Polyclonal antibody against recombinant PKR (42) was a gift of G. Barber and M. Katze (University of Washington).

RESULTS

Full-Length PKR[K296R] Homodimerizes *in Vivo*. The yeast two-hybrid assay (25, 26, 43) is often used to demonstrate protein–protein interactions *in vivo*. The proteins under study are expressed in a yeast reporter strain as fusions to either GAL4-DB or GAL4-TA. Interaction of the test proteins brings the two GAL4 domains into proximity and activates the transcription of a *lacZ* reporter gene under control of upstream GAL4 binding sites.

To examine the homodimerization of PKR in the two-hybrid system, we used a functionally defective PKR containing a point mutation in catalytic subdomain II (30) (designated PKR[K296R]; see Fig. 1). This mutant was used instead of the WT enzyme because WT PKR is spontaneously activated in yeast and inhibits growth through the phosphorylation of eIF-2α (15, 16). As a positive control for the assay, yeast SNF1 and SNF4 proteins were coexpressed as fusions to GAL4-DB or GAL4-TA, respectively (Table 1). It was previously demonstrated that these proteins interact in the two-hybrid system (25, 26, 34). Negative controls were generated by coexpressing either the SNF1 or the SNF4 fusion protein with PKR[K296R] fused to GAL4-TA or GAL4-DB, respectively.

Formation of PKR[K296R] homodimers in the two-hybrid system was detected by an increase in the β-galactosidase signal upon coexpression of the PKR[K296R] fusion proteins (Table 1). However, no colony color development was observed with the negative controls, and the specific activity of β-galactosidase for these samples (0.3 unit/mg) represented only the background level for this assay. To evaluate the significance of the signal observed with the GAL4-DB/PKR[K296R]–GAL4-TA/PKR[K296R] combination, quantitative measurements were determined for 10 independent samples from the negative controls and PKR[K296R] transformants. The mean β-galactosidase activity observed for PKR[K296R] cotransformants was 2.4 ± 0.62 units/mg (8-fold over background). This signal represents a statistically significant increase over the negative controls ($P < 0.001$) and compares well with the β-galactosidase activity generated by the SNF1–SNF4 interaction (Table 1). These data provide direct evidence for PKR homodimerization in an *in vivo* system and are in agreement with the conclusions of Romano *et al.* (44) deduced from genetic assays in yeast.

The N-Terminal dsRNA-Binding Domain Is Necessary and Sufficient for PKR Dimerization. To map the dimerization domain in PKR, we examined a C-terminal truncation mutant, p20. This mutant encodes the first 184 aa of PKR (9), encompassing the two dsRNA-binding motifs, but lacks the kinase domain and the basic region (domain III) between residues 233 and 271 (45). p20 formed homodimers and

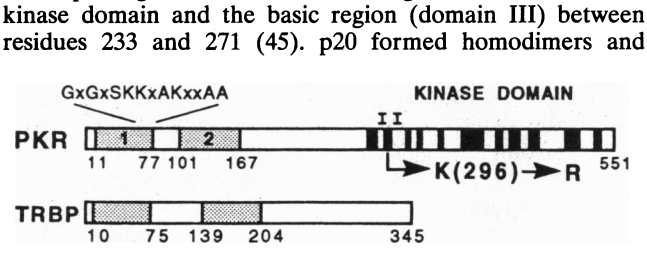

FIG. 1. Schematic diagram of PKR and TRBP. The dsRNA-binding consensus motifs and the kinase subdomains of PKR are shown as shaded boxes and black boxes, respectively. The core consensus sequence of the dsRNA-binding motifs is given in one-letter code above the schematic (x, any amino acid). Location of the K296R point mutation of PKR is also identified. Amino acid numbering is given below each diagram.

Table 1. β-Galactosidase activities displayed by GAL4 fusion proteins of full-length PKR[K296R]

Fusion proteins		Colony color*	Specific activity,† unit(s)/mg	Fold increase‡
GAL4-DB	GAL4-TA			
SNF1	SNF4	++	5.1	17
PKR[K296R]	SNF4	−	0.3 ± 0.05	1
SNF1	PKR[K296R]	−	0.3 ± 0.04	1
PKR[K296R]	PKR[K296R]	+	2.4 ± 0.62	8

Indicated combinations of vectors expressing fusions to GAL4-DB or GAL4-TA were transformed into yeast strain Y526 and assessed for their ability to activate *lacZ* transcription as described in *Materials and Methods*. SNF1 and SNF4 were transformed together as a positive control and with the two PKR[K296R] constructs as negative controls.

*Transformants were replica plated onto selection medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and evaluated for the development of color: +++, dark blue; ++, blue; +, light blue; −, white.

†Transformants were grown in liquid medium and assayed for β-galactosidase activity as described. Values represent the mean of two experiments for the SNF1/SNF4 positive control and the mean ± SD of 10 experiments for the PKR[K296R] combinations.

‡Fold increase over the values obtained for negative controls.

Table 2. β-Galactosidase activities displayed by GAL4 fusion proteins of PKR[K296R] and p20 or vaccinia virus K3L expressed in combination with PKR[K296R] or p20

Fusion proteins		Colony color	Specific activity, unit(s)/mg	Fold increase
GAL4-DB	GAL4-TA			
p20	SNF4	−	0.3	1
SNF1	p20	−	0.3	1
PKR[K296R]	p20	++	35.7	119
p20	PKR[K296R]	++	47.1	157
p20	p20	+++	234.4	781
K3L	SNF4	−	0.4	1
SNF1	K3L	−	0.3	1
K3L	PKR[K296R]	+++	75.3	188
K3L	p20	−	0.4	1
PKR[K296R]	K3L	++	6.2	21
p20	K3L	−	0.4	1

Values represent the mean of two experiments. See Table 1 for experimental details.

heterodimerized with the full-length PKR[K296R] mutant (Table 2). The signal generated for homodimerization of p20 was much stronger than that observed for PKR[K296R] (781-fold versus 8-fold over background, respectively). In comparison, heterodimerization of p20 and PKR[K296R] amplified the β-galactosidase expression to a level intermediate between these two values. It should be emphasized that the relative differences in β-galactosidase induction in these experiments could reflect potential variations in the affinity between the respective proteins in addition to differential expression, stability, folding, and transport of the fusion proteins to the yeast nucleus. These possible mechanisms have been discussed in the literature (43, 46). Thus, it is not possible to directly correlate the quantitative data with the relative affinities of PKR[K296R] and p20. However, our results demonstrate that the N-terminal region of PKR (aa 1–184) is sufficient for *in vivo* dimerization.

As an additional potent test for the specificity of PKR interactions in the yeast system, we employed the vaccinia virus K3L protein. K3L protein is a pseudosubstrate of PKR and is an inhibitor of PKR-mediated phosphorylation of eIF-2α. It is presumed that this protein inhibits PKR by binding to the active site in the C-terminal kinase domain of the enzyme (32, 47). When K3L was coexpressed with PKR[K296R] in yeast, there was an increased signal in the two-hybrid assay (Table 2). However, no signal was obtained with K3L and p20 (Table 2). Lack of interaction between K3L and p20 would be expected since p20 lacks the substrate binding site. That p20 and K3L fusion proteins interacted with PKR[K296R] as either GAL4-DB or GAL4-TA fusion proteins confirms that all three fusion proteins were expressed and transported to the nucleus. Thus, the lack of signal between K3L and p20 provides *in vivo* evidence for K3L binding to the catalytic domain of PKR and independently demonstrates the specificity of the two-hybrid assay.

While our data show that the N-terminal region encompassing the dsRNA-binding motifs of PKR is sufficient for dimerization, they do not exclude the possible occurrence of additional dimerization domains. To test this, a deletion mutant lacking both of the dsRNA-binding motifs was constructed (PKR[K296R]Δ12; deletion of residues 1–97 and 104–157, respectively). The PKR[K296R]Δ12 fusion proteins with either GAL4-DB or GAL4-TA interacted with their respective GAL4-K3L counterparts (Table 3). The signal intensities differed depending upon which GAL4 domain was fused to PKR[K296R]Δ12 and K3L; however, such differences can be attributed to a variety of factors (see above) and have been demonstrated to occur in the two-hybrid assay using other fusion proteins (46). The interaction between PKR [K296R]Δ12 and K3L indicates that the PKR[K296R]Δ12 fusion proteins were folded correctly and transported to the nucleus. In contrast, the PKR[K296R]Δ12 mutant did not homodimerize or heterodimerize with PKR[K296R] or p20 (Table 3). Thus, the N-terminal domain of PKR encompassing the two dsRNA-binding motifs is both necessary and sufficient for PKR dimerization.

PKR and TRBP Heterodimerize. To further address the question of the importance of the dsRNA-binding domain *in vivo*, we examined the ability of PKR to interact with an

Table 3. β-Galactosidase activities obtained for GAL4 fusion proteins of PKR[K296R]Δ12 expressed in combination with K3L or as a homodimer or heterodimer with PKR[K296R] or p20

Fusion proteins		Colony color	Specific activity, unit(s)/mg	Fold increase
GAL4-DB	GAL4-TA			
PKR[K296R]Δ12	SNF4	−	0.3	1
SNF1	PKR[K296R]Δ12	−	0.3	1
PKR[K296R]Δ12	K3L	++	13.6	45
K3L	PKR[K296R]Δ12	+	2.0	5
PKR[K296R]Δ12	PKR[K296R]	−	0.3	1
PKR[K296R]Δ12	p20	−	0.4	1
PKR[K296R]	PKR[K296R]Δ12	−	0.2	1
p20	PKR[K296R]Δ12	−	0.3	1
PKR[K296R]Δ12	PKR[K296R]Δ12	−	0.3	1

Values represent the mean of two experiments. See Table 1 for experimental details.

unrelated dsRNA-binding protein. A number of proteins containing dsRNA-binding motifs homologous with those of PKR have been identified (8). One example is TRBP (10, 28, 29), which contains two dsRNA-binding consensus motifs (Fig. 1). TRBP homodimerized and formed heterodimers with PKR[K296R] and p20 in the two-hybrid assay (Table 4). This suggests that PKR dimerization with TRBP *in vivo* is mediated by their respective dsRNA-binding motifs. Although we cannot exclude other cryptic functions concealed within the dsRNA-binding motifs, these data also imply that PKR dimerization occurs through dsRNA binding.

Dimerization *in Vitro*. We used a far-Western blotting assay to examine the requirement of dsRNA for dimerization *in vitro*. Purified p10 and p20 PKR truncation proteins (encoding the first 91 and 184 aa of the protein, respectively) (31) were resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were probed with ^{32}P-labeled HMK-tagged PKR[K296R] or ^{32}P-labeled HMK-tagged TRBP. The radiolabeled PKR[K296R] interacted with the membrane-bound p20 only when the blotted protein had been preincubated with poly(I)·poly(C) (Fig. 2*A*). Similar results were obtained for full-length PKR (data not shown). The p10 truncation mutant contains only the first dsRNA-binding motif of PKR and binds to dsRNA with a 100-fold lower affinity than p20 (31). Predictably, p10 did not interact with the radiolabeled PKR[K296R] probe in this assay (Fig. 2*A*). Although we cannot exclude conformational disruption in p10, taken at their face value these data suggest that PKR dimerization *in vitro* requires interaction with dsRNA. We also examined whether other natural or synthetic polynucleotides facilitated PKR dimerization. Reovirus RNA, poly(I)·poly(C), and poly(A)·polyU elicited strong signals, whereas preincubation with dsDNA or poly(A)·poly(dT) did not promote PKR interaction (Fig. 2*B*). These results correlate with the intrinsic ability of these polynucleotides to activate PKR (48) and indicate that PKR dimerization *in vitro* requires the presence of dsRNA.

As observed with the PKR[K296R] probe, ^{32}P-labeled HMK-tagged TRBP reacted with membrane-bound p20 only after preincubation with poly(I)·poly(C) (Fig. 2*A*) and failed to interact with p10. The relative ability of various polynucleotides to promote interaction of TRBP with p20 was also similar to that observed with PKR (Fig. 2*B*).

DISCUSSION

Using the two-hybrid assay, we have provided direct evidence for PKR dimerization *in vivo*. We have also mapped the interaction region to the N-terminal dsRNA-binding domain of PKR (9, 30, 49–52) encompassing two copies of a consensus motif that is shared by many dsRNA-binding proteins (8–12). These motifs are rich in basic residues and contain within them a highly conserved core sequence with predicted α-helical structure (Fig. 1). Both copies of the dsRNA consensus motif are required for efficient interaction between dsRNA and PKR (9, 31, 52).

Table 4. β-Galactosidase activities obtained for fusion proteins of TRBP as heterodimers with PKR mutants or as homodimers

Fusion proteins		Colony color	Specific activity, unit(s)/mg	Fold increase
GAL4-DB	GAL4-TA			
TRBP	SNF4	−	0.3	1
SNF1	TRBP	−	0.2	1
TRBP	PKR[K296R]	++	16.1	54
TRBP	p20	+++	109.3	364
PKR[K296R]	TRBP	+	6.0	20
p20	TRBP	++	52.1	174
TRBP	TRBP	++	33.0	110

Values represent the mean of two experiments. See Table 1 for experimental details.

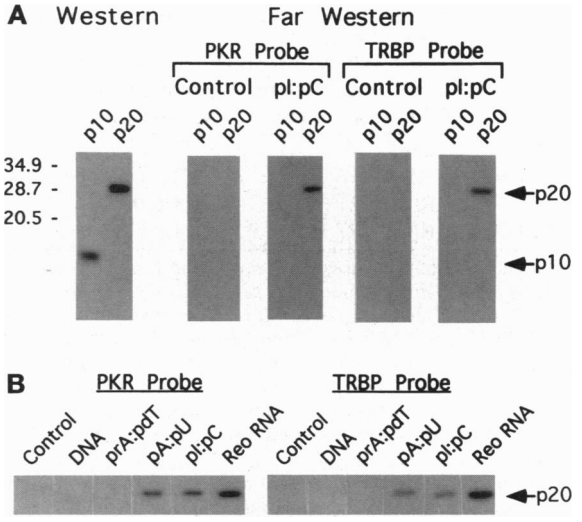

FIG. 2. Efficient dimerization of a PKR truncation mutant (p20) with PKR or TRBP *in vitro* is dependent on dsRNA and requires both dsRNA-binding domains. (*A*) Equivalent amounts (100 ng) of pure p20 and p10 truncation proteins were electrophoresed in SDS/15% polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was performed with polyclonal antibody to recombinant PKR (42). For far-Western blotting, membranes were incubated with either hybridization buffer (27) alone (control) or with hybridization buffer containing poly(I)·poly(C) (pI:pC) for 60 min and washed three times prior to addition of ^{32}P-labeled HMK–PKR[K296R] fusion protein (PKR probe) or ^{32}P-labeled HMK–TRBP (TRBP probe). Molecular size markers are indicated in kilodaltons at left. (*B*) Effect of various polynucleotides on dimerization of p20 with PKR or TRBP. Nitrocellulose blots of p20 protein were prepared as described above. Membranes were preincubated for 60 min in hybridization buffer with each indicated polynucleotide at 10 μg/ml, except for reovirus RNA (Reo RNA), which was at 0.1 μg/ml. Washing steps and addition of radiolabeled probes were as described above.

Two models have been postulated to account for the dimerization of PKR that results in its activation (2): (*i*) two PKR monomers are brought into proximity when they bind to the same dsRNA molecule; (*ii*) alternatively, dsRNA-bound PKR monomer initiates dimerization with a second PKR molecule. The second model requires protein–protein interactions as well as protein–dsRNA interactions (31, 53). Our results indicate that dimerization requires both dsRNA and the intact dsRNA-binding domain, suggesting that RNA–protein contacts are critical. However, these data do not rule out potential protein–protein interactions which could be induced by dsRNA binding to PKR.

A deletion mutant of PKR that lacked both dsRNA-binding motifs (PKR[K296R]Δ12) failed to dimerize, although it retained the ability to interact with a pseudosubstrate of PKR, the vaccinia virus K3L protein. The absence of interaction between PKR[K296R]Δ12 and either PKR[K296R] or p20 indicates that both partners require the intact dsRNA-binding domain for dimerization. More persuasive evidence for participation of the dsRNA-binding motifs in the dimerization process was the finding that PKR interacts with the unrelated dsRNA-binding protein TRBP. Since the only detectable homology between PKR and TRBP is in their dsRNA-binding motifs, it is unlikely that PKR dimerization takes place through any other domain. Furthermore, the *in vitro* binding of soluble PKR or TRBP to the immobilized p20 fragment of PKR required the presence of dsRNA, which could not be replaced by dsDNA or an RNA·DNA hybrid.

The results of our two-hybrid assays are in agreement with a recent report by Romano *et al.* (44) suggesting that PKR

dimerization occurs in yeast, based on the functional complementation of two defective mutations in the first and second repeats of the dsRNA-binding motif. These workers also demonstrated that substitution mutants in the first dsRNA-binding motif of PKR, which reduce dsRNA binding *in vitro* by >90% (9, 52), impair but do not abolish PKR activation in yeast (44). This finding suggested that the substitution mutants exhibit reduced but detectable dsRNA-binding capacity *in vivo*. Our results agree with this conclusion, as two of the mutants studied (LS4 and LS13) (44) also homodimerized in the two-hybrid system (data not shown).

The data presented here lend further support to the idea that dominant negative mutants of PKR inhibit WT activity by the formation of heterodimers (17, 44). Moreover, these data provide a mechanism for the inhibition of PKR activity by TRBP (29) through the dsRNA-dependent formation of inactive heterodimers consisting of PKR and TRBP. As previous studies underscored the importance of PKR in many aspects of cellular growth control and tumorigenesis (17–19), it is conceivable that PKR activation is tightly regulated through interactions with dsRNA or other activators. Inasmuch as TRBP was suggested to represent a group of cellular dsRNA-binding proteins that antagonize the interferon-mediated translational control mechanism (29), heterodimerization of PKR with other dsRNA-binding proteins may represent an alternative means of translation regulation.

Note. While this paper was under review, we learned of the studies of Patel *et al.* (54), who also demonstrated the dimerization of PKR. In contrast to our findings, these authors reported that the interaction was independent of dsRNA and depended on protein–protein interactions, in part because it occurred in the absence of deliberately added dsRNA. Furthermore, the interaction was detected by using a mutant (K60A) in the dsRNA-binding region of PKR. This mutant is very similar to LS4, which retains a detectable kinase activity in yeast (44) and also retained the ability to dimerize in our study.

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