The interferon-inducible double-stranded RNA (dsRNA)-activated protein kinase PKR is regulated by dsRNAs that interact with the two dsRNA-binding motifs (dsRBMs) in its N terminus. The dsRBM is a conserved protein motif found in many proteins from most organisms. In this study, we investigated the biochemical functions and cytological activities of the two PKR dsRBMs (dsRBM1 and dsRBM2) and the cooperation between them. We found that dsRBM1 has a higher affinity for binding to dsRNA than dsRBM2. In addition, dsRBM1 has RNA-annealing activity that is not displayed by dsRBM2. Both dsRBMs have an intrinsic ability to dimerize (dsRBM2) or multimerize (dsRBM1). Binding to dsRNA inhibits oligomerization of dsRBM1 but not dsRBM2 and strongly inhibits the dimerization of the intact PKR N terminus (p20) containing both dsRBMs. dsRBM1, like p20, activates reporter gene expression in transfection assays, and it plays a determinative role in localizing PKR to the nucleolus and cytoplasm of the cell. Thus, dsRBM2 has weak or no activity in dsRNA binding, stimulation of gene expression, and PKR localization, but it strongly enhances these functions of dsRBM1 when contained in p20. However, dsRBM2 does not enhance the annealing activity of dsRBM1. This study shows that the dsRBMs of PKR possess distinct properties and that some, but not all, of the functions of the enzyme depend on cooperation between the two motifs.

PKR is a serine/threonine kinase found in a latent state in most cells. It plays an important part in cellular anti-viral defense as well as apoptosis, signal transduction, cell growth, and differentiation (1, 2). Recently it was also found to be involved in RNA splicing of the tumor necrosis factor-α gene (3). The role of PKR in protein synthesis is well studied. It inhibits translation initiation by phosphorylating the α subunit of initiation factor eIF2. Phosphorylated eIF2α binds with high affinity to a second initiation factor, eIF2B, that is usually present in limiting amounts. Sequestration of eIF2B prevents it from promoting GTP/GDP exchange on the eIF2 complex, a critical step in forming the ternary complex eIF2-GTP-Met-tRNA, that is essential for initiation. Kinase activity of PKR is activated by autophosphorylation upon binding to its activators, which include most notably, perfect duplex dsRNAs (4) as well as other highly structured RNAs (5–8), polyanionic molecules such as heparin or polyglutamine, and protein regulators such as PKR-activating protein (9) and nuclear factor 90. Inhibitors of PKR are even more numerous and diverse than activators; they include viral and cellular RNAs and proteins, such as adenovirus virus-associated RNA, Epstein-Barr virus EBER RNA, human Alu RNA, human p58 protein, TAR RNA-binding protein, and vaccinia virus E3L and K3L proteins (2, 10–15).

Two dsRNA-binding motif variants, dsRBM1 and dsRBM2, occupy the regulatory N-terminal portion of PKR. The dsRBM is a ~70-amino acid motif that has been found in various proteins in almost all organisms from Escherichia coli to humans (16). Sequence analysis of completed genomes revealed 3 dsRBMs in 2 proteins of the yeast Saccharomyces cerevisiae, 24 dsRBMs in 9 proteins of the nematode Caenorhabditis elegans, and 54 dsRBMs in 14 proteins of Drosophila melanogaster (17). The three-dimensional structure of the dsRBM has been solved by nuclear magnetic resonance and crystallographic techniques (18–20). The x-ray crystal structure of the second dsRBM of Xlrpa1 in complex with dsRNA revealed three regions of dsRBM forming direct contacts with dsRNA. Sequence alignments of dsRBMs display the existence of variations in these regions, suggestive of variations in dsRNA binding. Several biochemical studies have indicated that dsRBMs can exhibit wide differences in dsRNA binding (21, 22). In particular, the deletion or mutation of dsRBM1 and dsRBM2 of PKR exerts different effects on its dsRNA binding ability and kinase activity (23–26), even though the three-dimensional folding of these two motifs is similar (19).

The dsRBMs play a key role in PKR function and regulation. Although their exact role in PKR activation is still not clear, it appears that dsRNA binding leads to autophosphorylation of PKR facilitated by protein homodimerization (27, 28). It has been suggested that latent PKR exists in an inactive dimer conformation and changes to an active dimer form upon binding to activators, possibly by releasing the kinase domain from inhibitory interactions with the protein’s N terminus (2, 29). An intrinsic dimerization activity has been mapped within this region (30–32). The dsRBMs have also been implicated in further aspects of PKR function. For example, mutations in the kinase domain of PKR usually show dominant negative effects, including the ability to enhance the expression of reporter genes (23, 28, 33). The mechanism for the dominant negative
effect is not fully understood, but it is thought that the mutants either dimerize with wild-type PKR, thereby blocking its function, or bind to and sequester cellular PKR activators such as dsRNA (23). Moreover, PKR localizes in the cytoplasm and nucleus of the cell (34–36) and associates with ribosomes (37–39). The ribosome association is tightly related to the dsRNA-binding activity of PKR, but the region of PKR that is responsible for its subcellular localization is not clear. Finally, a dsRNA-annealing activity has been reported for the dsRBMs of Xlrnba (40). Whether this feature is shared by the dsRBMs of PKR is unknown.

To understand the functional differences between the two dsRBMs of PKR, and their involvement in the biochemical activities and cytological properties of the full-length protein, we have studied protein-RNA interactions, RNA annealing, protein dimerization, reporter gene activation, and protein localization. Our results suggest that, with the exception of oligomerization, the differences between dsRM1 and dsRM2 reflect their different dsRNA binding affinities. Nevertheless, the two dsRBMs cooperate to achieve optimal dsRNA binding and to accomplish both reporter gene activation and correct protein localization.

MATERIALS AND METHODS

Plasmids—Plasmids SRG5ΔLBN and SRG5ΔLp10 (24) were used to construct 6×His-tagged p10A, p10B, and p20. SRG5ΔLBN was cut with NdeI and BamHI to give a fragment encoding p20, or with NcoI and BamHI for p10B. SRG5ΔLp10 was cut with NdeI and BamHI to give a fragment encoding p10A. Using complementary restriction sites, the fragments were ligated into pET28b (Novagen) yielding His-p10A and His-p20, and into pET30LIC for His-p10B. For EGFP constructs, the EGF expression vector pEGFP (BE) was first constructed by inserting the EGF coding fragment from pBEGFP (CLONTECH) into pcDNA3.1 (Invitrogen) using BamHI and EcoRI. pEGFP was cut with Nhel and BamHI and ligated with PKR fragments generated by PCR using the following primers: PKR-N, 5′-TAGCGCTAGCTAGTGCTG- GATCCTTCCAG-3′; PKR-C, 5′-AGCTGATCCACATGTCGTT- TCCACTTTT-3′; p10B-N, 5′-TAGCGCTAGCTAGGACAAAGCAGATTTCT- TCC-3′; p10A-C, 5′-CAGCTGATCCATTGGAATAGTTGTG-3′; and p20-C, 5′-CAGCTGATCCACACATGTCGTTTCCACTTTT-3′. p10A-EGFP, containing amino acids 1–95 of PKR, was constructed using the primer set PKR-N and p10A-C. Similarly, p10B-EGFP (amino acids 88–186) was constructed using p10B-N and p20-C; p20-EGFP (amino acids 1–186) was constructed using PKR-N and p20-C; PKR-EGFP and K296R-EGFP (full-length, amino acids 1–551) using PKR-N and PKR-C; and Δ1-EGFP (amino acid 88–551) using p10B-N and PKR-C. The primer set PKR-N and PKR-C was also used for constructing Δ2-EGFP, and ΔN-EGFP. PCR products were purified using a PCR purification kit (Qiagen), cut with Nhel and BamHI, and ligated into the pEGFP vector that was cut with the same enzymes. For His-EGFP-tagged proteins, fragments cut from various EGFP constructs were inserted into pET28b using Nhel and Nol. For luciferase reporter assays, the luciferase plasmid pBLCat (41) was constructed by ligating the luciferase fragment excised from pSPLUC (+) (Promega) by Nhel and XbaI into pcDNA3.1 (+) cut with Nhel.

Protein Purification—Transformed E. coli were grown overnight in 3–ml cultures and transferred to 500 ml of LB medium with kanamycin. Cultures were grown at 37 °C until the absorbance at 600 nm reached 0.5 then 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) was added to induce protein expression at 30 °C for 2.5 h. Cells were collected by centrifugation and resuspended in buffer A with protease inhibitors (5 mM imidazole, 0.5 mM NaCl, 0.1% Nonidet P-40, and 20 mM HEPES, pH 7.5). Cells were briefly sonicated and centrifuged at 10,000 × g for 30 min. The supernatant was used for protein purification. For His-tagged protein purification, the POROS-MC (PE Applied Biosystems) column matrix was used in a BioCAD Sprint perfusion chromatography system (PE Applied Biosystems). The column was charged with 50 column volumes of 0.1 mM NiCl2, washed, and loaded with bacterial extract. After extensive washing with buffer A, His-tagged proteins were eluted by buffer B (500 mM imidazole, 0.5 mM NaCl, and 20 mM HEPES, pH 7.5). Proteins were dialyzed overnight against dialysis buffer (20% glycerol, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and 20 mM HEPES, pH 7.5) and renatured on the column at room temperature for 90 min by gradually changing the proportions of buffer RA (from 100% to 0%) and buffer RB (150 mM NaCl, 20% glycerol, and 20 mM HEPES, pH 7.5, from 0% to 100%). Proteins were eluted with buffer DB (0.5 mM imidazole, 150 mM NaCl, 20% glycerol, and 20 mM HEPES, pH 7.5) and dialyzed as above. The denaturation step reduced the binding of 32P-labeled poly(rI·rC) with His-tagged p20-EGFP to background levels.

RNA Synthesis and Purification—RNAs for dsRNA production and annealing assays were obtained by cutting plasmid pBSKII (Strategene) with PvuII and transcribing with T3 and T7 RNA polymerases. The T3 and T7 transcripts are 245 and 335 nucleotides long, respectively, and complementary over 132 nucleotides. dsRNAs were generated and purified as described previously (43).

Gel Mobility Shift Assay—The gel mobility shift assay was described previously (24). Briefly, purified dsRNAs and proteins were mixed on ice in gel mobility shift buffer and run on 5% tris-glycine polyacrylamide gel.

Poly(rI·rC) Pull-down Assay—The poly(rI·rC) pull-down assay was described previously (43). Briefly, poly(rI·rC)-Sepharose was incubated with the proteins and washed with different concentrations of salt. Proteins were eluted by mixing with gel loading buffer, briefly boiled and loaded onto SDS-polyacrylamide gels.

Filter Binding Assay—Poly(rI·rC) at 200 μg/ml was 5′-end labeled using [γ-32P]ATP (ICN) and T4 polynucleotide kinase (New England Biolabs) at 37 °C for 45 min, heated at 68 °C for 5 min, cooled down slowly to room temperature, and passed through a Bio-Gel p-30 polyacrylamide gel column (Bio-Rad). Labeled poly(rI·rC) (about 50,000 cpm, was incubated with proteins on ice for 20 min in binding buffer (5 mM MgCl2, 1 mM DTT, 0.1 mg/ml E. coli RNA, 0.1 mg/ml bovine serum albumin, and 20 mM HEPES, pH 7.5). The filter binding assay was carried out as described previously (4) using washing buffer (50 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 20 mM HEPES, pH 7.5) and a 0.45-μm nitrocellulose membrane (Schleicher & Schuell). Retained poly(rI·rC) was quantified using an InstantImager (Packard).

Annealing Assay—For each reaction, a 15 μl solution containing proteins or KCl, labeled T3 and T7 RNA substrates, and 1× annealing buffer (200 mM KCl, 1 mM magnesium acetate, 1 mM DTT, 100 μg/ml bovine serum albumin, 10 mM HEPES, pH 7.6) was incubated at 30 °C for 1 h. Digestion mix (35 μl) containing 25 unit T1 RNase (Roche Molecular Biochemicals) and 10 μl 5× T1 digestion buffer (5 mM EDTA, 750 mM NaCl, 50 mM Tris-HCl, pH 8.0) was then added. After incubation at 30 °C for 1 h, the reaction was stopped by adding 150 μl of precipitation mix (80 μg/ml yeast total RNA and 266 μg/ml NaCl) and 200 μl of phenol:chloroform:isoamyl alcohol (1:1:1, v/v). The RNAs were precipitated with ethanol and resolved in an 8 μl urea/10% TBE polyacrylamide gel.

Protein Cross-linking—Proteins and poly(rI·rC) in phosphate-buffered saline (PBS) were incubated on ice for 20 min and then mixed with cross-linking buffer (2 mM dimethyl suberimidate (Pierce), 100 mM NaCl, 10 mM HEPES, pH 8.0). After incubation at room temperature for 90 min, the reaction was stopped by adding 1 ml glycine and 2× gel loading buffer. Samples were run in 12.5% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Millipore), blotted with anti-His-tag antibody (CLONTECH), and visualized with ECL (PerkinElmer Life Sciences).

GST Pull-down Assay—GST fusion PKR was expressed in E. coli and purified using glutathione-Sepharose 4B (Amersham Pharmacia Bio tech) according to the manufacturer’s protocol. Dialyzed purified GST-PKR was immobilized on the beads. His-tagged p20 and different concentrations of poly(rI·rC) were added, and the slurry was incubated on ice for 20 min. Then beads were washed with PBS containing 1 mM DTT and briefly boiled in 2× gel loading buffer. Samples were run in 12.5% SDS-polyacrylamide gels, followed by Western blotting using anti-His antibody.

Cell Transfection and Luciferase Reporter Assay—Human 293 cells grown in 12-well plates were transfected with plasmids using the calcium phosphate method as described previously (41). Cells were harvested 40 h after transfection by washing once with PBS and incubating
**RESULTS**

**dsRNA Binding**—Earlier mutational and deletion analysis indicated that the two dsRBMs of PKR differ in their dsRNA binding affinity and their importance for PKR function (23, 25, 26, 44). To examine their properties and behavior at the biochemical level, we cloned dsRBM1 and dsRBM2 of PKR into vectors that allowed expression in *E. coli* either as unmodified or His-tagged proteins (Fig. 1). In accordance with their approximate molecular weights and following the previous consideration of their affinities (23, 25), we conducted filter-binding assays with end-labeled poly(rI·rC). As estimated from the data of Fig. 2B, the *Kd* for p10A binding to poly(rI·rC) is about 5 × 10⁻⁶ M, in agreement with previous measurements by gel mobility shift analysis (24, 45). The affinities of p10A and p10B were lower, with *Kd* values about 5 × 10⁻⁶ and 2 × 10⁻⁷ M, respectively. The affinity of p10A for perfectly duplexed RNA measured by gel mobility shift analysis, 3.8 × 10⁻⁷ M (24), was somewhat lower than the estimate obtained here, possibly implying that single-stranded tails present in poly(rI·rC) contribute to the binding.

In gel mobility shift assays with purified dsRNA of defined size, p20 gives rise to a series of bands corresponding to the
sequential binding of p20 molecules until the RNA is fully saturated. This occurs at ~11 base pairs/p20 molecule (4, 24). Using dsRNA of 132 base pairs in the presence of competitor tRNA, low concentrations of p20 gave rise to several distinct bands (Fig. 3A, lane 5). At higher concentrations, p10A caused a mobility shift without giving rise to a very discrete banding pattern (lanes 9–12), whereas p10B did not elicit a mobility shift even at the highest concentrations tested (lanes 6–8). These assays indicate that p10A binds to dsRNA with a higher affinity than p10B and that there is cooperativity between the two dsRBMs in binding dsRNA. Mixing experiments (lanes 2–4) show that this cooperativity is achieved only when the two dsRBMs are tandemly located on the same protein molecule. The failure to detect an interaction between p10B and dsRNA by the mobility shift technique, in contrast to the matrix-binding techniques used with poly(rI:rC) to inhibit cross-linking was ineffective (lanes 2–4). Annealing activity was displayed by p10A (lanes 14–16) and p20 (lanes 8–10) but not by p10B (lanes 11–13). Thus, the annealing activity of the PKR dsRBMs correlates with their dsRNA binding affinity, suggesting that dsRNA binding is important for the annealing function, but cooperativity between the two dsRBMs is not evident in this assay.

Protein Dimerization—The activation of PKR requires that it undergo dimerization and autophosphorylation (27, 28). Dimerization has been observed both in vivo and in vitro and is attributed to sequences within (30, 46) as well as outside (31) of the dsRBMs. In the case of dimerization via sequences in the dsRBM region, it has been questioned whether the dimerization is an intrinsic activity of the protein or is mediated by dsRNA (46). To address this issue, we used DMS, a chemical cross-linking reagent reactive with primary amine groups (30). Cross-linking was carried out with p20, p10A, and p10B in the presence or absence of poly(rI:rC). Fig. 5A (top panel) shows that p20 dimerizes in the absence of poly(rI:rC), confirming the intrinsic dimerization activity of the PKR N terminus (30). p20 dimerization was inhibited by high concentrations of poly(rI:rC), suggesting a competition between dsRNA binding and protein dimerization. It could be that residues involved in dsRNA binding also participate in protein-protein interactions, or perhaps dsRNA binding causes a conformational change in the protein that disfavors dimerization. Both p10A and p10B were also cross-linked by DMS (Fig. 5A, middle and bottom panels) but with somewhat different characteristics from p20 and each other. Like p20, p10B predominantly gave rise to dimers, whereas p10A multimerized. The dimerization of p10B was not inhibited by poly(rI:rC), however, and the multimerization of p10A was only slightly reduced at high concentrations of poly(rI:rC). These observations can be interpreted in terms of the distribution of dimerization and dsRNA binding sites (see “Discussion”).

These results were obtained with proteins purified under denaturing conditions (followed by a renaturation step) to exclude possible bacterial dsRNA contaminants from the protein preparations. In a reconstruction experiment, the purification protocol reduced dsRNA binding to undetectable levels (data not shown). To verify our conclusions, we studied the cross-linking of mutant forms of p20 that are defective in dsRNA binding (44). All four mutants were cross-linked by DMS to similar extents in the absence of poly(rI:rC), but the ability of a high concentration of poly(rI:rC) to inhibit cross-linking was determined by the mutants’ ability to bind dsRNA (Fig. 5B).
The two mutants in which dsRNA binding is only mildly impaired (LS14 and LS17: dsRNA binding ~20 and 45% of wild-type, respectively) behaved like wild-type p20: Their cross-linking was sharply decreased by poly(rI·rC). In contrast, protein-protein cross-linking was unaffected by poly(rI·rC) for the two mutants in which dsRNA binding is essentially ablated (LS16 and LS19: <5% of wild-type dsRNA binding), confirming that the intrinsic capacity of p20 to dimerize is reduced by interaction with high concentrations of dsRNA.

The ability of p20 to dimerize in the absence of dsRNA appears to contradict our previous observation (46). Instead of monitoring the existence of preformed oligomers as above, we therefore conducted pull-down assays to monitor the formation of protein-protein interactions. Full-length PKR tagged with GST was immobilized on glutathione-Sepharose beads, which were incubated with p20 in the presence of various concentrations of poly(rI·rC). GST-PKR interacted weakly with p20 in the absence of poly(rI·rC). In the presence of increasing concentrations of poly(rI·rC), p20 binding increased to a maximum then declined. High concentrations of poly(rI·rC) effectively eliminated the binding of p20 to PKR (Fig. 5C). Consistent with our earlier findings (46), these results indicate that the formation of p20-PKR heterodimers is largely dependent on the presence of dsRNA. Evidently, under these conditions, the pre-existing p20 dimers efficiently dissociate and heterodimerize with GST-PKR only in the presence of dsRNA.

Activation of Reporter Gene Expression—Several inactive mutants of PKR function as dominant negative inhibitors and enhance the expression of reporter genes in trans. However, the mechanism of this effect is uncertain. We therefore examined the ability of p10A, p10B, and p20 to stimulate gene expression. EGFP-tagged dsRBMs constructs were transfected into human 293 cells together with a firefly luciferase reporter gene. At the highest concentrations of EGFP-dsRBMs fusion constructs tested, reporter gene expression was enhanced about 7-fold by p20 and 4-fold by p10A, but no effect was observed with p10B or with EGFP alone (Fig. 6). Similar results were obtained with the DHFR reporter gene by Wu and Kaufman (33), who also concluded that dsRNA binding rather than dimerization correlates with PKR activation. The enhancements seen with p20 and p10A were dose-dependent, and the maximal effect attained by p20-EGFP was similar to that obtained with the dominant negative mutant of PKR, K296R (data not shown). Thus, the results obtained in these reporter gene expression assays correlate with the dsRNA binding activity of the dsRBMs but not with their dimerization ability.

Subcellular Localization—By immunofluorescence and electron microscopy, PKR is detected in the cytoplasm and nucleoli as well as diffusely throughout the nucleoplasm (34, 35, 47). To define the contribution of the dsRBMs to the intracellular distribution of PKR, we expressed EGFP-tagged constructs in human HeLa cells (Fig. 7). As expected, PKR-EGFP elicited clear signals in both the cytoplasm and nucleoli. The inactive kinase mutant K296R-EGFP exhibited the same distribution as wild-type PKR, indicating that the kinase activity of PKR is not necessary for its proper localization. EGFP alone gave a diffuse signal throughout the cell, especially in the nucleus. This pattern of EGFP distribution was unaffected by the attachment of dsRBMs (p10B-EGFP), the kinase domain (AN-EGFP), or both (Δ1-EGFP). However, the intact N terminus of PKR (p20-EGFP) conferred a localization indistinguishable from that of the wild-type PKR construct, indicating that this region is sufficient for the proper localization of PKR. Similar distributions were seen with p10A-EGFP and Δ2-EGFP (deletion of amino acids 104–185), which contains dsRBM1 plus the kinase domain. These constructs were concentrated in the nucleus, although they gave an increased nucleoplasmic signal relative to PKR-EGFP, p20-EGFP, and K296R-EGFP. Thus, dsRBM1 plays an important role in PKR distribution, and it is nearly sufficient alone for the proper localization of PKR. Presumably, the failure of these fusion proteins to clear fully from the nucleoplasm is due to their decreased affinity for RNA relative to PKR forms containing both dsRBMs. One construct, Δ2-EGFP (deletion of amino acids 104–185), gave an anomalous pattern. This fusion protein, which lacks most of dsRBM2 but retains its C-terminal third, was restricted to the cytoplasm possibly because it is misfolded or binds preferentially to cytoplasmic components such as ribosomes (38).

Discussion

It has been reported that members of the dsRBMs motif family differ in their dsRNA binding affinity (16, 22, 48). Here we have examined the two dsRBMs of PKR to explore differences between dsRBMs with respect to several additional functions and to assess whether these functions are related to dsRNA binding. The additional functions include RNA anneal-
ing, protein dimerization, stimulation of gene expression, and intracellular localization. We confirmed that dsRBM1 has a greater affinity for dsRNA than dsRBM2 and found that it also has a much greater ability to anneal single-stranded RNAs, to enhance gene expression in transfection assays, and to participate in PKR localization. As with dsRNA binding, dsRBM2 facilitated the activity of dsRBM1 in all these assays except for RNA annealing. Cross-linking assays showed that dsRBM1 is able to oligomerize, whereas dsRBM2 dimerizes under the same conditions.

The x-ray crystal structure of dsRBM2 of Xlrbpa, a dsRBM-containing protein found in Xenopus laevis, in complex with dsRNA showed that three regions within the dsRBM are involved in dsRNA binding (20). NMR studies of PKR indicated that dsRBM1 and -2 have nearly the same three-dimensional protein folding (19). The differences in dsRNA binding between the dsRBMs of PKR are therefore attributable to differences between the side chains of the two dsRBMs. Inspection of the alignment of the two PKR dsRBMs with the consensus sequence (Fig. 1) suggests that regions 1 and 3 are similar between dsRBM1 and -2, whereas region 2 of dsRBM2 differs from that in dsRBM1 and the consensus sequence. The histi-
dine and arginine residues in region 2 participate in protein-RNA interactions (20), implying that the lack of homology in this region may contribute to the weaker activity of dsRBDM2 in dsRNA binding. dsRBMs have been divided into two groups based on their homology to the consensus sequence (21, 22). Group A dsRBMs, including dsRBM1 of PKR, are homologous to the consensus throughout the sequence, whereas group B dsRBMs like PKR dsRBM2 display conservation only in the C-terminal area where region 3 is located. Our observation that p10B exhibits weak dsRNA binding affinity correlates with its relatively low level of homology in region 2.

In various assays for dsRNA binding, we observed that dsRNA has a higher affinity for p20 than for p10A or p10B. This implies that dsRBDM2 is able to strengthen the dsRBM1:RNA interaction considerably due to the cooperativity between the two dsRBMs as suggested previously (44). Cooperation requires that the dsRBMs are covalently joined (Fig. 3A), possibly indicating that the presence of two dsRBMs on the same molecule reduces ligand off-rate. The fact that eukaryotic dsRBM-containing proteins frequently contain more than one dsRBM suggests that cooperative binding might be a commonly used device to augment the protein’s affinity for dsRNA. Another possibility is that multiple dsRBMs confer sequence specificity upon the interactions with RNAs, particularly in the case of proteins that contain more than three dsRBMs. Conceivably PKR has evolved to have two dsRBMs to allow it to bind dsRNA with high affinity yet low specificity. A notable feature of our filter binding assays is the observation that at the saturation point more end-labeled poly(rI·rC) was bound to p20 than to p10A and p10B (Fig. 2). This may indicate that p20 can bind certain structured RNAs, e.g. tailed duplexes, that exist in poly(rI·rC) and are not bound efficiently by p10A or p10B.

It was reported previously that the dsRBMs of Xirbpa can facilitate dsRNA annealing and that this annealing activity is independent of dsRNA binding, because some dsRBMs that failed to bind dsRNA still catalyzed annealing (40). In PKR, annealing activity seems to correspond more closely to dsRNA binding activity, although cooperativity between the two dsRBMs is not beneficial toward the reaction (Fig. 4). This would be expected if the protein functions by facilitating the matching up of two complementary strands. The correlation of RNA binding with annealing is supported by the observation that the binding of one dsRBM-containing molecule induces a more uniformly double-stranded conformation in an imperfect duplex, thereby facilitating the binding of further protein molecules (49). The annealing activity of the dsRBM suggests that PKR and other dsRBM-containing proteins may play a role as chaperones, facilitating the folding of cellular RNAs (40). This property of PKR may sensitize the cellular response to viral infection by annealing two complementary viral RNAs, which can then activate the PKR-mediated anti-viral defense mechanism.

The ability of PKR to dimerize has been demonstrated by several assays, including yeast two-hybrid, gel filtration, chemical cross-linking, far-Western, and various pull-down protocols (30, 46, 50). At least two regions of the molecule have been implicated in dimerization (30, 31). Dimerization involving the N-terminal region is complicated by the possibility that the interactions may be bridged by dsRNA as well as due to direct protein-protein interactions. In our experiments, we excluded dsRNA contamination by purifying the proteins under stringent denaturing conditions followed by a renaturing step: Such preparations still formed dimers (p20 and p10B) and higher multimers (p10A). Furthermore, p20 dimerization was unaffected by mutations that severely impair dsRNA binding (Fig. 5B), indicating that the N-terminal dsRBMs have an intrinsic ability to interact. Interestingly, the dimerization of p20 was inhibited by poly(rI·rC) at high concentration. Two possible explanations for this observation can be considered. One is that amino acids involved in dsRNA binding are also engaged in dimerization, resulting in competition between dsRNA binding and dimerization for the same interaction site. This view is supported by our finding that competition correlates with dsRNA binding affinity. When dsRNA binding is strong, as in the case of p20, dimerization is efficiently competed, whereas when the dsRNA binding is weaker (p10A) or very weak (p10B, LS16, LS19) there is less or no detectable competition (Fig. 5A and B). An alternative interpretation is that the binding of dsRNA changes the structure of the protein, thereby disfavoring protein-mediated dimerization. Support for this view can be drawn from mutational data such as that in Fig. 5B, indicating that the dsRNA-binding activity is separable from protein dimerization (28, 33, 51). Furthermore, residues responsible for dimerization and dsRNA binding lie on distinct faces of the C-terminal α-helix of the dsRBM (52). It is possible that dsRNA can switch PKR from an inactive dimer mediated by protein-protein interactions to an active dimer mediated by dsRNA. Comparison of p20 homodimers (whose existence is independent of dsRNA binding) with the formation of GST-PKR-p20 heterodimers (which is dsRNA-dependent: Fig. 5C) suggests that the efficiency or rate of protein-protein dimerization (p20-PKR) is considerably less than that of dsRNA-mediated dimerization. Consistent with this notion, no heterodimers were detected in pairwise cross-linking reactions between p10A, p10B, and p20 (data not shown). Apparently, pre-existing dsRBM oligomers do not readily dissociate and heterodimerize in the absence of dsRNA. This observation may help explain conflicting reports regarding the RNA dependence of PKR dimerization (30, 46).

Mutations in the kinase domain of PKR enhance the expression of reporter genes, presumably by down-regulating the activity of cellular wild-type PKR. Two mechanisms have been advanced to explain this dominant negative effect: It could result from the interaction of the mutant kinase with wild-type kinase resulting in the formation of inactive homodimers or the sequestration of PKR activators such as dsRNA in the cell (23, 53). Our results show that the N-terminal region of PKR (p20) and even the first dsRBM alone (p10A), are able to stimulate reporter gene expression, whereas dsRBM2 (p10B) lacks this activity (Fig. 6). The magnitude of the effect correlates with the ability of the dsRBMs to bind to dsRNA but not with their ability to dimerize, supporting the view that dsRNA sequestration underlies the dominant negative effect.

PKR localizes in the cytoplasm, strongly in the nucleolus, and diffusely throughout the nucleoplasm (34, 35, 47). Its cellular localization does not change after interferon induction or adenovirus infection (34). By immunoelectron microscopy, PKR is found mainly in the dense fibrillar component of the nucleolus, which is believed to be the site of nascent rRNA synthesis and processing (54), suggesting that PKR might be involved in ribosome biosynthesis (34). Our study indicates that the dsRBMs are required for PKR’s localization and that this activity correlates with dsRNA binding (Fig. 7). However, the exact mechanism for this localization is still not clear. Because ribosomes are assembled and processed in the nucleolus and PKR associates with ribosomes (35, 37, 55), it is tempting to speculate that ribosome binding is responsible for PKR’s subcellular localization. In extracts of yeast expressing human PKR, the kinase was found to be associated with the 40 S ribosomal subunit (37), although, in mammalian extracts, PKR was shown to interact with the L18 protein of the 60 S ribosome subunit and to compete for dsRNA binding (38). This discrep-
ancy notwithstanding, in both cases the dsRBMs seemed to be important for ribosome binding. Because PKR's localization correlates with its dsRNA-binding activity, its ribosome association is likely to be due to the binding of PKR to structured RNA such as ribosomal RNA (rRNA). This view is supported by the fact that the NMR structure of dsRBM has many features in common with the N-terminal domain of the prokaryotic ribosomal S5 protein (18). Furthermore, several other dsRBM-containing proteins also localize in the nucleolus (56, 57). 3

A related question raised by this study is how PKR is transported into the nucleus. Although the localization of proteins within the nucleus may be largely driven by diffusion and retention, the process of moving proteins into the nucleus is believed to be carried out by an elaborate transport system (58). For the relatively small protein molecules in this study, such as EGFP itself and its fusions with p10A, p10B and p20, movement into the nucleus may be through diffusion, whereas for larger proteins such as the fusions of EGFP with full-length PKR, Δ1, Δ2', and ΔN, nuclear penetration might be facilitated by an active transport process. No nuclear localization signal has been found in PKR, however. Remarkably, Δ2-EGFP failed to localize in the nucleus. One possible explanation is that PKR lacking dsRBM2 is misfolded in the cell and cannot pass through the nuclear pores. Consistent with this explanation, PKRΔ2 is highly deficient in dsRNA binding (42). Alternatively, it is possible that the presence of the C terminus of dsRBM2 together with an intact dsRBM1 might cause the protein to bind to some component in the cytoplasm with very high affinity, so that it is effectively sequestered in the cytoplasm. In this case, however, the RNA binding properties of the Δ2 mutant make it unlikely that the ligand is RNA.

In conclusion, the RNA-annealing, reporter gene activation, and subcellular localization properties of PKR are related to the dsRNA-binding activity of its dsRBMs, which is predominantly dependent on dsRBM1, whereas oligomerization appears to be at least partially independent of dsRNA binding. Of the RNA-related properties, only annealing is not discernibly influenced by cooperation between the two dsRBMs. Based on this study and previous findings, such as the observation that cytosolic PKR is predominantly in a dimeric form, whereas ribosome-associated PKR is monomeric (50, 59) and that PKR in the nucleus is predominantly basic (i.e. unphosphorylated) (35), we propose the model shown in Fig. 8 for the activation and localization of PKR. In the latent state, PKR exists in the cell in two forms: an inactive dimer in the cytoplasm, and an inactive monomer associated with ribosomes. It is actively transported to the nucleus by unknown mechanisms and moves to the nucleolus by binding to structured RNA, probably rRNA. When dsRNA is present, as during viral infection, PKR in the cytoplasm switches from an inactive, protein-mediated homodimer form to an active, dsRNA-mediated dimer. It is unclear whether ribosome-associated PKR can be directly activated by dsRNA. The binding of short structured RNAs, such as adenovirus VA RNA, leads to the formation of inactive PKR monomers in the cytoplasm. We also speculate that protein activators such as PKR-activating protein and nuclear factor 90, which, respectively, contain three and two dsRBMs, activate PKR by a mechanism involving dsRBM interactions, which allow PKR to switch from an inactive dimer to an active dimer.

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REFERENCES

1. Williams, B. R. (1997) Biochem. Soc. Trans. 25, 509–513
2. Kaufman, R. J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 503–528, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Osman, F., Farrus, N., Ben-Assouli, Y., and Raemder, I. (1999) Genes Dev. 13, 3280–3293
4. Manche, L., Green, S. R., Schmidt, C., and Mathews, M. B. (1992) Mol. Cell. Biol. 12, 5238–5248
5. Tian, B., White, R. J., Xia, T., Welle, S., Turner, D. H., Mathews, M. B., and Thornton, C. A. (2000) RNA (N. Y.) 6, 79–87
6. Bevilacqua, P. C., George, C. X., Samuel, C. E., and Cech, T. R. (1998) Biochemistry 37, 6303–6316
7. Circle, D. A., Neel, O. D., Robertson, H. D., Clarke, P. A., and Mathews, M. B. (1997) RNA (N. Y.) 3, 438–448
8. Bischoff, J. R., and Samuel, C. E. (1989) Virology 172, 106–115
9. Patel, R. C., and Sen, G. C. (1998) EMBO J. 17, 4379–4390
10. Clemens, M. J., and Elia, A. (1997) J. Interferon Cytokine Res. 17, 503–524
11. Fevery, T., and Mathews, M. B. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 371–424, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Mathews, M. B., and Shenk, T. (1991) J. Virol. 65, 5657–5662
13. Clemens, M. J., Lai, K. C., Jeffrey, I. W., Schoffield, A., Sharp, T. V., Elia, A., Matys, V., James, M. C., and Tillera, V. J. (1994) Biochimie (Paris) 76, 770–778
14. Chu, W. M., Ballard, R., Carpick, B. W., Williams, B. R., and Schmid, C. W. (1998) Mol. Cell. Biol. 18, 58–68
15. Melville, M. W., Katze, M. G., and Tan, S. L. (2000) Cell. Mol. Life Sci. 57, 311–322
16. Fierro-Monti, I., and Mathews, M. B. (2000) Trends Biochem. Sci. 25, 241–246
17. Rubin, G. M., Yandell, M. D., Worton, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharana, I. K., Formini, M. E., Li, P. W., Waple, R., Fleischmann, W., Cherry, J. M., Henikoff, S., Skupski, M. P., Mora, S., Ashburner, M.,

---
3 B. Tian and M. B. Mathews, unpublished data.
dsRBMs of PKR

Birney, E., Boguski, M. S., Brody, T., Brokstein, P., Celniker, S. E., Chervitz, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, B. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, P., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J., Kuehl, P. M., Lemaitre, B., Littleton, J. T., Morrison, D. K., Mungall, C., O’Farrell, P. H., Pickeral, O. K., Shue, C., Vassalli, L. B., Zhang, J., Zhao, Q., Zheng, X. H., Zhong, F., Zhong, W., Gibbs, R., Venter, J. C., Adams, M. D., and Lewis, S. (2000) Science 287, 2204–2215

Bycroft, M., Grunert, S., Murzin, A. G., Proctor, M., and St. Johnston, D. (1995) EMBO J. 14, 3563–3571

19. Nanduri, S., Carpick, B. W., Yang, Y., Williams, B. R., and Qin, J. (1998) EMBO J. 17, 5458–5465

20. Ryter, J. M., and Schultz, S. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10979–10983

21. St. Johnston, D., Brown, N. H., Gall, J. G., and Jantsch, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10979–10983

22. Krovat, B. C., and Jantsch, M. F. (1996) Virology 231, 92–99

23. Romano, P. R., Green, S. R., Barber, G. N., Mathews, M. B., and Hinnebusch, A. G. (1995) Mol. Cell. Biol. 15, 365–378

24. Schmedt, C., Green, S. R., Manche, L., Taylor, D. R., Ma, Y., and Mathews, M. B. (1995) J. Mol. Biol. 249, 29–44

25. McCormack, S. J., Ortega, L. G., Doohan, J. P., and Samuel, C. E. (1994) Virology 198, 92–99

26. McMillan, N. A., Carpick, B. W., Hellis, B., Toone, W. M., Zamanian-Daryoush, M., and Williams, B. R. (1995) J. Biol. Chem. 270, 2601–2606

27. Thomis, D. C., and Samuel, C. E. (1993) J. Virol. 67, 7695–7700

28. Ortega, L. G., McCotter, M. D., Henry, G. L., McCormack, S. J., Thomis, D. C., and Samuel, C. E. (1996) Virology 215, 31–39

29. Robertson, H. D., and Mathews, M. B. (1996) Biochemie (Paris) 78, 909–914

30. Patel, R. C., Stanton, P., McMillan, N. M., Williams, B. R., and Sen, G. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8283–8287

31. Tan, S. L., Gale, M. J., Jr., and Katze, M. G. (1998) Mol. Cell. Biol. 18, 2431–2443

32. Rende-Fournier, R., Ortega, L. G., George, C. X., and Samuel, C. E. (1997) Virology 239, 410–423

33. Wu, S., and Kaufman, R. J. (1996) J. Biol. Chem. 271, 1756–1763

34. Jimenez-Garcia, L. F., Green, S. R., Mathews, M. B., and Spector, D. L. (1993) Mol. Cell. Biol. 13, 2728–2737

35. Jeffrey, I. W., Kadereit, S., Meurs, E. F., Metzger, T., Bachmann, M., Schwemmle, M., Hovanessian, A. G., and Clemens, M. J. (1995) Exp. Cell Res. 218, 17–27

36. Schwemmle, M., Clemens, M. J., Hise, K., Pfeifer, K., Troster, H., Muller, W. E., and Bachmann, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10292–10296

37. Zhu, S., Romano, P. R., and Wek, R. C. (1997) J. Biol. Chem. 272, 14434–14441

38. Kumar, K. U., Srivastava, S. P., and Kaufman, R. J. (1999) Mol. Cell. Biol. 19, 1116–1125

39. Raine, D. A., Jeffrey, I. W., and Clemens, M. J. (1998) FEBS Lett. 436, 343–348

40. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) Mol. Cell. Biol. 18, 2262–2277

41. Green, S. R., and Mathews, M. B. (1992) Genes Dev. 6, 2478–2490

42. Green, S. R., Manche, L., and Mathews, M. B. (1995) Mol. Cell. Biol. 15, 358–364

43. McCormack, S. J., and Samuel, C. E. (1995) Virology 206, 511–519

44. Cosentino, G. P., Venkatesan, S., Serluca, F. C., Green, S. R., Mathews, M. B., and Sonenberg, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9445–9449

45. Besse, S., Rebouillat, D., Marie, L., Puvrin-Dutilleul, F., and Hovanessian, A. G. (1998) Exp. Cell Res. 239, 379–392

46. Brooks, R., Eckmann, C. R., and Jantsch, M. F. (1998) FEBS Lett. 434, 121–126

47. Bevilacqua, P. C., and Cech, T. R. (1996) Biochemistry 35, 9983–9994

48. Langland, J. O., and Jacobs, B. L. (1992) J. Biol. Chem. 267, 10729–10736

49. Patel, R. C., Stanton, P., and Sen, G. C. (1996) J. Biol. Chem. 271, 25657–25663

50. Patel, R. C., and Sen, G. C. (1998) Mol. Cell. Biol. 18, 7009–7019

51. Wu, S., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 1291–1296

52. Lamond, A. I., and Earnshaw, W. C. (1998) Science 280, 547–553

53. Kostura, M., and Mathews, M. B. (1989) Virology 175, 189–198

54. Zhang, S., Herrmann, C., and Grosse, F. (1999) J. Cell. Sci. 112, 2693–2703

55. Patel, R. C., Vestal, D. J., Xu, Z., Bandyopadhyay, S., Guo, W., Erme, S. M., Williams, B. R., and Sen, G. C. (1999) J. Biol. Chem. 274, 20432–20437

56. Lewis, J. D., and Tollervey, D. (2000) Science 288, 1576–1586

57. Patel, B. W., Graziano, V., Schneider, D., Maitra, R. K., Lee, X., and Williams, B. R. G. (1997) J. Biol. Chem. 272, 9510–9516
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