Molecular Framework for the Activation of RNA-dependent Protein Kinase*

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Sean A. McKenna 1,2, Darrin A. Lindhout 1,3, Insil Kim 4, Corey W. Liu 5, Vladimir M. Gelev 6, Gerhard Wagner 7, and Joseph D. Puglisi 1,7,9

From the 1 Department of Structural Biology and 9 Stanford Magnetic Resonance Laboratory, Stanford University School of Medicine, Stanford, California 94305-5126 and the 5 Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

The RNA-dependent protein kinase (PKR) plays an integral role in the antiviral response to cellular infection. PKR contains three distinct domains consisting of two conserved N-terminal double-stranded RNA (dsRNA)-binding domains, a C-terminal Ser-Thr kinase domain, and a central 80-residue linker. Despite rich structural and biochemical data, a detailed mechanistic explanation of PKR activation remains unclear. Here we provide a framework for understanding dsRNA-dependent activation of PKR using nuclear magnetic resonance spectroscopy, dynamic light scattering, gel filtration, and autophosphorylation kinetics. In the latent state, PKR exists as an extended monomer, with an increase in self-affinity upon dsRNA association. Subsequent phosphorylation leads to efficient release of dsRNA followed by a greater increase in self-affinity. Activated PKR displays extensive conformational perturbations within the kinase domain. We propose an updated model for PKR activation in which the communication between RNA binding, central linker, and kinase domains is critical in the propagation of the activation signal and for PKR dimerization.

The interferon response to viral infection provides a first line of host defense against infectious agents (1). Among a myriad of cellular effects, this induced interferon pathway can cause inhibition of host translation, slowing viral protein expression and progeny proliferation (2). The presence of highly structured RNAs of many viral genomes is a key extracellular and intracellular indicator of viral infection. Double-stranded RNA-dependent protein kinase (PKR) regulates host-mediated down-regulation of translation (3). Activated by the binding of double-stranded RNA (dsRNA), PKR responds to viral genomic contamination within the host cell (4, 5). Activated PKR regulates protein synthesis via efficient substrate phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) at Ser-51, inhibiting the guanine nucleotide exchange activity of the eIF2 heterotrimeric complex and resulting in the inhibition of translational initiation (4). PKR also plays a critical role in cellular processes such as the induction of apoptosis, immune response, regulation of dsRNA-dependent transcription, and cellular growth (6).

Human PKR is a 551-residue enzyme consisting of three distinct regions, each with specific activities. The N-terminal region contains two conserved dsRNA-binding motifs (dsRBD1/2) responsible for dsRNA binding. The NMR structure of dsRBD1/2 revealed two highly similar dsRBD domains connected by a 23-residue disordered linker (7). High affinity PKR-dsRNA interaction requires both dsRBDs of PKR, as well as specific structural and length requirements of the activator dsRNA (8–10). The C-terminal region of PKR is a serine-threonine kinase domain that is responsible for phosphorylation activity and substrate recognition. The crystal structure of the kinase domain in complex with eIF2α has revealed an ATP-binding pocket required for phosphotransfer, two phosphorylation sites required for activation (Thr-446 and Thr-451), and a putative substrate recognition site (11). The N- and C-terminal regions of PKR are connected by a 80-residue flexible linker rich in Ser and Asn residues, which may propagate the dsRNA activation signal from the dsRBD1/2 domain to the kinase domain. A large body of biochemical and biophysical data has defined the basic features of PKR function. Most importantly, latent PKR undergoes autophosphorylation upon dsRNA binding, which greatly increases kinase activity (12, 13). Mutagenesis has revealed multiple residues that mediate RNA binding and/or kinase activity (12–14). Dimerization of PKR may be required for efficient kinase activation (15–17) as both dsRNA binding and the phosphorylation state of the protein may affect dimerization equilibrium (18). Despite these data, the mechanism of PKR kinase activation remains unresolved.

An autoinhibition model has been proposed in which the latent form of PKR remains inactive in the cell through...
intramolecular association of dsRBD1/2 with the kinase domain (15, 16). In this model, binding of dsRNA to dsRBD1/2 relieves autoinhibition, allowing the released kinase domain to participate in activation and substrate phosphorylation. In support of this model, NMR experiments with isolated domains suggest that the second RNA-binding domain interacts with the kinase domain (19, 20). However, recent reports have also contradicted the autoinhibitory model, implying that dsRNA binding does not directly relieve inhibition caused by kinase domain interaction (21, 22).

Here we performed biochemical and biophysical experiments to provide a molecular framework for the activation of PKR. NMR of full-length PKR revealed that the three distinct regions of PKR behave as individual entities in solution, with no detectable evidence of interdomain autoinhibition. These findings are buttressed by NMR investigations of both RNA-bound and phosphorylated forms of full-length PKR, as well as complimentary studies of autophosphorylation kinetics, reaction molecularity, and RNA release. The results presented here support an updated mechanism of PKR activation in which RNA binding and autophosphorylation modulate protein self-association and activity.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Expression and purification of full-length human PKR and the dsRBD1/2 construct are as described previously (22). Full-length PKR mutant constructs (PKRK296R, PKRT446A, PKRT451A, and PKRT446A/T451A) were generated using site-directed mutagenesis of the wild-type plasmid and were expressed and purified as for the wild-type protein. PKR constructs of kinase domain (PKR252–551) and kinase plus linker (PKR169–551) were subcloned from full-length PKR template using standard PCR techniques. 2H15N-PKR was expressed in deuterated M9 minimal media containing 70% (v/v) D2O (Cambridge Isotopes), supplemented with 1 mM MgSO4, 0.1 mM CaCl2, 90 mM FeSO4, 0.01% (w/v) yeast extract, 1 mg of thiamine-HCl, 1 mg of biotin, 2.5 g of D-glucose, and 0.5 g of 15N-NaH4Cl (Cambridge Isotopes) per liter of media. Phosphorylated PKR (PKR*) was generated by incubating wild-type full-length human PKR (15 μM) in the presence of ATP (1 mM) and MgCl2 (2 mM) at 30 °C for 2 h in a buffered solution containing Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM β-mercaptoethanol and purified in the same buffer on a Superdex 26/60 size exclusion column (GE Healthcare). RNA-generated phosphorylated PKR (PKRTAR-P) was made by incubating wild-type full-length human PKR (1 μM) and HIV TAR RNA in the presence of ATP (1 mM) and MgCl2 (2 mM) at 30 °C for 2 h in a buffered solution containing Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM β-mercaptoethanol and purified as described above. RNA samples were prepared by in vitro transcription using T7 polymerase as described previously (22, 23) using a Bsal restriction site at the 3′ end of the transcript to terminate transcription. Predicted secondary structures of dsRNAs employed are shown elsewhere (22).

**Kinetics**—All autophosphorylation assays were performed as described previously (22). Kinetics experiments were analyzed using Berkeley Madonna X (version 8.3.12) software to fit the differential equations that describe either a unimolecular or a bimolecular model of PKR activation. We solved the differential equations for E*(t), the rate of formation of phosphorylated PKR product, using the Runge-Kutta algorithm. In constructing the bimolecular model, the following assumptions were incorporated: (i) PKR must be RNA-bound to become phosphorylated at low concentrations, (ii) phosphorylated PKR (E*) binds PKR-TAR complex (ER) with higher affinity than latent PKR, (iii) no change in affinity for ATP occurs when comparing EREA and ERE*A species, (iv) conversion to product is irreversible (k4 and k5), and (v) the on-rate of reactions controlled by K1, K2, K3, K4, and K5 are diffusion-limited (6 μM−1 min−1). Reverse rate constants were calculated based on established dissociation constants. Apparent Kcat and kcat values were calculated by globally fitting data at all complex concentrations simultaneously, as described elsewhere (24). At complex concentrations above 4 μM, data were excluded from further analysis as significant inhibition of autophosphorylation was observed.

**Dynamic Light Scattering**—Dynamic light scattering (DLS) experiments were performed at 30 °C with a DynaPro-801 molecular sizing instrument (Protein Solutions Co.). Protein samples were passed through a 0.22-μm Milllex-GV syringe filter (Millipore) to remove particulates. A total of 50 data points were collected at 10-s intervals for each sample. Using the DYNAMICS (version 6.0) software package, the hydrodynamic radius, apparent molecular weight, and polydispersity were determined on the basis of an autocorrelation analysis of scattered light intensity data.

**NMR Spectroscopy**—All NMR spectral data were obtained using a Varian Unity INOVA 800MHz spectrometer at 30 °C. All two-dimensional 1H,15N-TROSY HSQC spectra were acquired using the sensitivity-enhanced gradient pulse scheme in Biopack (Varian Inc.) using 1H and 15N sweep widths set to 12,001 Hz and 3242 Hz, 2048 points, and delay recovery of 3.5 s (25). Samples contained 2H15N-PKR in NMR buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM β-mercaptoethanol, 90%/10% H2O/D2O, pH 6.7). Spectral processing and assignment of all 1H, 15N-TROSY HSQC were performed using the software packages NMRPipe (26) and NMRView (27). The total average change in backbone amide 1H and 15N chemical shift, Δδtotal, was determined according to the following equation

\[
\Delta \delta_{\text{total}} = \sqrt{(0.1 \Delta \delta_{15N})^2 + (\Delta \delta_{1H})^2}
\]  

(1.1)

where Δδ15N and Δδ1H are the chemical shift changes in ppm for a given residue relative to free dsRBD1/2 or kinase domain.

**Native Gel Shift Mobility Assay**—All samples were prepared as described previously (22) with the exception that a sample buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl was employed.

**RESULTS**

**PKR Activation Is a Bimolecular Process**—The primary goal of this study was to determine the mechanism for activation of PKR in the framework of a molecular model. The activation of PKR was first characterized using the kinetics of autophosphorylation. We have established a kinase activation assay that follows the time course of PKR autophosphorylation in the pres-
ence of a dsRNA activator, HIV-TAR dsRNA (TAR). A buffered reaction containing [γ-32P]ATP, PKR, and TAR is incubated for a set time, quenched with EDTA, separated by denaturing SDS-PAGE, and quantified by autoradiography. The kinetics of the activation process was monitored over a 20-fold concentration range of PKR-TAR complex between 0.2 and 4 μM. A simple unimolecular or bimolecular reaction scheme was used to fit the kinetic data obtained.

A sigmoidal buildup of product is observed at all concentrations examined with a lag phase prior to maximal rates of autophosphorylation, characteristic of autocatalytic processes (Fig. 1A). The experimental data are well fit by a second order, bimolecular mechanism, whereby autophosphorylation occurs in trans between one PKR and a second bound PKR. Furthermore, these curves were non-superimposable once corrected for total protein concentration, thereby excluding a unimolecular reaction mechanism (Fig. 1B). The time required for activation is significantly reduced at higher PKR-TAR concentrations. By globally fitting all kinetic data simultaneously, an apparent Michaelis-Menten constant ($K_m$) for the autophosphorylation reaction was determined to be $4.1 \pm 0.7 \mu M$ and $0.7 \pm 0.3 \text{ min}^{-1}$, respectively. Both values are within the boundaries of similar kinase autophosphorylation reactions (24). The experiments described here were performed with purified PKR-TAR complex, but similar results were obtained if the reagents were mixed without complex purification or if an alternative dsRNA activator was employed (VA$_{13}$-AS, data not shown).

**dsRNA Binding and Phosphorylation of PKR Lead to Increased PKR Self-affinity**—The kinetics reported above support a bimolecular mechanism for autophosphorylation of PKR. To examine the PKR association state during activation, we determined apparent molecular weights ($M_r$) by DLS of complexes containing either wild-type or catalytically inactive (K296R) PKR. DLS under various conditions was performed at 2 μM PKR, which is a concentration greater than that expected in vivo (28). The validity of the approach was confirmed by reasonable $M_r$ determinations of both TAR (~20 kDa) and PKR (~81 kDa) (Fig. 1C). The molecular masses determined are slightly higher than predicted because of the elongated shape of both TAR and PKR. These results show that PKR in its latent form behaves as a monomeric protein at low μM concentrations. The addition of an excess of ATP to a sample containing PKR does not result in any change in hydrodynamic radius, indicating that ATP binding does not drastically change the conformation of PKR (Fig. 1C). TAR binding to PKR increases its apparent $M_r$ by ~20 kDa, and again, either ATP or ADP-PNP binding does not change the DLS data. The addition of MgCl$_2$ and ATP to the PKR-TAR complex does not significantly alter the apparent $M_r$ of the PKR-TAR complex alone. The catalytically inactive mutant, K296R, which remains monomeric under activating conditions, behaves similarly to wild-type PKR.

PKR-dsRNA complexes were next assembled and incubated under PKR autophosphorylation conditions. The apparent molecular weights of complexes were then determined by DLS at specific time intervals. A 1:1 complex of PKR-TAR in the absence of reagents required for activation (ATP and MgCl$_2$) does not change $M_r$ as a function of time (Fig. 1D, blue). The addition of ATP and MgCl$_2$ caused a time-dependent reduction in molecular mass from 104 to 84 kDa (Fig. 1D, black), which corresponds to a transition from fully bound PKR-TAR complex to a free PKR and TAR mixture. To confirm that the reduction in molecular weight was specific for activation, an equimolar complex of the catalytically inactive K296R and TAR dsRNA was assembled. This complex demonstrated no significant change in $M_r$ in either the presence (Fig. 1D, red) or the absence (data not shown) of ATP and MgCl$_2$.

To probe the formation of bimolecular complexes for the different species involved in PKR activation, we determined the concentration dependence of dimer formation for wild-type PKR, PKR-TAR complex, and PKR$^\text{R}$. We employed DLS at various PKR concentrations (1–80 μM). PKR alone (Fig. 1E) or PKR$^{K296R}$ (data not shown) show only a minor increase in $M_r$ at high concentration, whereas a significant increase in apparent $M_r$ is observed upon increasing the concentration of PKR$^\text{R}$. The increase of ~30 kDa is less than would be expected for a dimer of two PKR molecules (136 kDa). PKR dimerization may yield in a non-spherical complex, or more likely, the values observed may reflect an equilibrium between monomer and dimer. A 1:1 PKR$^\text{R}$:PKR complex gave a similar increase in $M_r$ when compared with PKR$^\text{R}$ alone, indicating a similar affinity for phosphorylated and dephosphorylated enzyme. A 1:1 PKR-TAR complex gave an intermediate increase in $M_r$ when compared with PKR or PKR$^\text{R}$, indicating that dsRNA-bound PKR has a greater self-association constant than unbound PKR but a weaker one than phosphorylated PKR.

The hydrodynamic behavior of PKR and complexes determined by DLS was confirmed using size exclusion chromatography (Fig. 1F). At all concentrations examined, latent PKR elutes as a 74-kDa protein. Identical results were observed for PKR$^{K296R}$ (data not shown). At lower concentrations (5 μM), PKR$^\text{R}$ is monomeric, whereas increased concentration (30 μM) leads to the identification of a 128-kDa species, which we ascribe to PKR dimerization. This molecular mass is in good accordance with that determined by DLS (123 kDa).

**FIGURE 1. Activated PKR forms a weak dimer.** A, progress curves of PKR autophosphorylation in the presence of equimolar TAR at complex concentrations of 0.2 (red), 0.5 (blue), 1.0 (green), 2.0 (black), and 4.0 μM (gray). Each time point was performed in triplicate, resolved by SDS-PAGE, and quantified for $^{32}$P incorporation by autoradiography. Each lane load was standardized to the 0.2 μM case. Error bars are not shown for clarity, but errors were typically less than 5% of the value shown. B, same as in A but corrected to actual concentration. C, molecular weight of PKR-TAR complexes (2 μM) as determined by DLS. Complexes were mixed with combinations of ATP (1 mM), ADP-PNP (1 mM), and MgCl$_2$ (2 mM). D, time-dependent molecular weight determination of PKR-TAR (blue), PKR-TAR plus ATP/MgCl$_2$ (black), or K296R plus ATP/MgCl$_2$ (red) by DLS. Equimolar complexes (2 μM) were incubated in the cuvette for the time specified prior to acquisition. Each data point was repeated in triplicate. Error bars have been omitted for clarity but reflect less than 5% of the value observed in all cases. E, concentration-dependent dimerization of PKR was examined by determining the molecular weight at the specified concentration of PKR (black), PKR$^\text{R}$ (blue), PKR-TAR (green), or an equimolar mixture of PKR$^\text{R}$ and PKR (orange). Each data point was repeated in triplicate. Error bars are not shown for clarity, but errors were typically less than 5% of the value shown. F, Superdex 200 10/300 GL size exclusion chromatography elution profiles of PKR (black) or PKR$^\text{R}$ at 5 μM (red) and 30 μM (blue).
Latent Form of PKR Exists in an Open Conformation—To explore the interplay between the RNA-binding (dsRBDs) and kinase domains, we performed NMR studies on full-length PKR. The $^{15}\text{N}$-TROSY HSQC spectra of latent full-length $^2\text{H}^{15}\text{N}$-PKR revealed a sufficiently resolved spectrum, which allowed spectral assignments of a large number of amide resonances (Fig. 2). The previously reported individual unbound dsRNA-binding domains (dsRBD1/2, residues 1–169) (29) and the K296R kinase domain (residues 252–551) (30) were used to assign resonances from the full-length protein by spectral superimposition. In total, 137 out of 169 resonances in the RNA-binding domains (81%) were unambiguously assigned based upon spectral overlay with the unbound dsRBD1/2 resonances, with the remaining 32 resonances (19%) not assigned due to peak overlap in the crowded spectral region (7.5–8.5 ppm). Spectral overlay with the assigned, unbound kinase domain (residues 252–551) revealed a superimposition that allowed for the unambiguous assignment of 82 out of 299 kinase resonances (27%), predominantly in the outlying regions of the spectra. The 80-residue linker region between the dsRBDs and kinase domain is unassigned as it is unstructured in solution, present with the bulk signal in the overlapping region of the spectra (supplemental Fig. 8).

FIGURE 2. NMR of full-length PKR. $^{15}\text{N}$-TROSY HSQC spectral of full-length PKR with resonances assigned as indicated. Circled cross-peaks correspond to tryptophan side chain amide resonances.
The near perfect spectral superimposition of free full-length PKR with either dsRBD1/2 (Fig. 3A) or the kinase domain resonances (not shown) suggests that the individual domains in the context of full-length PKR are behaving independently in solution. Resonances from both dsRBD1 and dsRBD2 are not significantly perturbed in full-length PKR (Fig. 3C). These data suggest that the RNA-binding and kinase domains do not significantly interact in latent PKR.

The conformation of dsRNA activator RNA complexes with full-length PKR was monitored in a similar manner using NMR. The chemical shift perturbations to full-length PKR upon formation of a 1:1 complex with TAR occur predominantly within the RNA-binding domains (Fig. 3B). Significant perturbations occur to resonances within both dsRBD domains (i.e., Leu-25, Leu-30, Lys-136) with minimal perturbations to kinase resonances (i.e., Lys-261, Gln-365, Ile-503) (Fig. 3D). Resonances within the dsRBD domains (1–169) closely match those previously reported for a dsRBD1/2-TAR complex (8), indicating that the binding of dsRBD1/2 to TAR in the presence or absence of the kinase domain is similar. A chemical shift perturbation map for PKR in the presence and absence of TAR RNA shown in B, with broadened resonances shown in blue.

FIGURE 3. NMR of PKR in the presence and absence of TAR RNA. $^{15}$N-TROSY HSQC spectral overlay of unbound dsRBD1/2 (red) and unbound PKR (black) with outlying resonances assigned as indicated (A) and unbound PKR (black) and TAR-bound PKR (green) (B). C, chemical shift difference map between unbound dsRBD1/2 and PKR shown in A for residues in the first two RNA-binding domains (1–169). D, detailed view of the three spectra shown in A and B, demonstrating chemical shift perturbations only to the RNA binding resonances within PKR upon TAR binding. E, chemical shift difference map for PKR in the presence and absence of TAR RNA shown in B, with broadened resonances shown in blue.
turbation map highlights this effect (Fig. 3E) as the PKR kinase resonances display superimpositions versus the unbound protein. Nearly identical results were obtained when an alternate dsRNA activator was employed (VAI-AS, data not shown). These results show that the kinase domain does not directly participate in the binding of dsRNA and suggest that no large conformational change within the kinase domain occurs upon activator binding.

To provide complementary information on full-length PKR/H18528-TAR interaction, we examined the NMR spectrum of TAR in the RNA-protein complex (supplemental Fig. 9). The imino proton resonances of free TAR and TAR bound to the dsRBD1/2 have been previously assigned (8). The imino proton NMR spectrum is relatively free from overlap with protein resonances. A comparison of the imino spectra of TAR bound to full-length PKR and TAR bound to dsRBD1/2 shows very similar chemical shifts. These data further support the hypothesis that bound RNA activators do not interact directly with kinase domain or interdomain linker.

Characterization of the Phosphorylated State of PKR—To probe the hypothesis that RNA binding affects PKR self-association affinity, we characterized the autophosphorylation properties of PKR truncations in which the dsRBDs or dsRBDs and linker domains were removed (Fig. 4A). As expected, only full-length PKR supports RNA-mediated autophosphorylation (Fig. 4B). Truncations containing only the kinase domain (PKR^{252–551}) or kinase domain plus interdomain linker (PKR^{169–551}) do not autophosphorylate in either the presence or the absence of dsRNA at nanomolar concentrations. RNA activators do not bind to either PKR^{252–551} or PKR^{169–551} at μM concentrations (data not shown). The addition of the PKR^{169–551} and dsRBDs in trans did not result in autophosphorylation.

To test the role of dsRBDs and linker in PKR self-association, we examined RNA-independent autophosphorylation of PKR truncation mutants. PKR autophosphorylation occurs at high protein concentrations in the absence of activator RNA in a concentration-dependent manner with complete phosphorylation occurring at concentrations >4 μM (Fig. 4C). At protein concentrations greater than μM concentrations, both the PKR^{252–551} and the PKR^{169–551} truncations autophosphorylate to some extent in an RNA-independent fashion. PKR lacking both the dsRBDs and the interdomain linker (PKR^{252–551}) demonstrates minimal autophosphorylation, even at extremely high protein concentrations. PKR lacking only the dsRBDs with intact linker autophosphorylates significantly (≈70%) of full-

FIGURE 4. Characteristics of phosphorylated PKR. A, domain organization of PKR. N-terminal dsRBDs, C-terminal kinase domain, and the interdomain linker are shown. Critical autophosphorylation sites (Thr-446, Thr-451) in the kinase domain are also indicated. B, purified PKR derivatives and HIV TAR RNA (0.9 μM) were incubated in the presence of [γ-32P]-ATP at 30 °C for 60 min, quenched, and resolved by SDS-PAGE. The concentration of PKR derivatives is indicated above the gel. C, increasing amounts of purified PKR derivatives were incubated in the presence of [γ-32P]-ATP at 30 °C for 30 min, quenched, and resolved by SDS-PAGE. Gels were quantified by autoradiography to quantify the extent of PKR autophosphorylation. D, autophosphorylation assays in which mixtures of PKR^{*} (100 nM), PKR^{169–551} (300 nM), TAR (300 nM), PKR (300 nM), or purified PKR-TAR complex (300 nM, boxed) were incubated in the presence of [γ-32P]-ATP at 30 °C for the time specified. E, trans-autophosphorylation assays in which PKR^{*} (100 nM) was added to a reaction including [γ-32P]-ATP and the specified PKR derivative at concentrations of 0.4, 0.8, and 2 μM, respectively, for 10 min at 30 °C.
length PKR), suggesting that the linker contributes to favorable protein–protein interactions leading to activation.

To further examine the properties of phosphorylated PKR, we produced phosphorylated PKR (PKR*1) in an RNA-independent fashion. Phosphorylated PKR is separated from ATP and Mg2+/H18528 by size exclusion chromatography and purifies as two overlapping peaks; this correlates with monomer and dimer species (data not shown). No such equilibrium exists for latent PKR. We have also purified PKR-PKRTAR-P using a similar approach. Wild-type PKR, PKR*1, and PKR-PKRTAR-P were used both as substrates for autophosphorylation and as kinases capable of trans-phosphorylating wild-type PKR (Fig. 4D). After a 15-min incubation, minimal phosphorylation of PKR*1 is observed when incubated in the presence of TAR activator, indicating that PKR*1 is unresponsive to dsRNA and does not further phosphorylate itself under these conditions. PKR*1 efficiently trans-phosphorylates wild-type latent PKR as a substrate. A 1:1 PKR-PKRTAR complex is also an efficient substrate for trans-autophosphorylation by PKR*1, indicating that dsRNA binding to PKR does not block phosphorylation. Trans-autophosphorylation of PKR by PKR*1 occurs at a 20-fold faster rate than dsRNA-mediated autophosphorylation. PKR-PKRTAR-P behaves identically to PKR*1 in the assay. Finally, PKR*1 is capable of stoichiometric trans-phosphorylation of latent PKR when compared with the extent of dsRNA-mediated autophosphorylation. Thus, purified PKR*1 serves as an extremely potent activator of latent PKR.

PKR mutations affect substrate activity for trans-autophosphorylation by PKR*1 (Fig. 4E). As expected, wild-type PKR serves as an efficient substrate, whereas PKR*1 does not. A PKR derivative containing a catalytically inactive mutation in the ATP-binding site, PKR*1E296R, is also phosphorylated efficiently by PKR*1, indicating that a functional ATP-binding and active site is not required in a substrate for phosphorylation. We observe no significant phosphorylation of the isolated dsRBBD1/2. Finally, mutations at either or both of two key phosphorylation sites in the activation loop (T446A and T451A) result in attenuation of phosphorylation by PKR*1.

The structural properties of PKR*1 were probed using NMR spectroscopy. The 2H3N-TROSY HSQC of PKR*1 reveals a homogeneous sample, potentially indicative of a single phosphorylation state of the protein (Fig. 5A); doubling of resonances was not observed, suggesting an intermediate or fast exchange regime between molecular species. Broader resonances were observed when compared with latent PKR, suggesting an increased rotational correlation time, indicative of a monomer/dimer equilibrium in solution. Identical results were obtained for PKR-PKRTAR-P, wherein PKR is phosphorylated in the presence of RNA and then separated from the RNA (supplementary Fig. 10). Spectral assignments were performed using superimposition with the latent protein, resulting in 91 unambiguous assignments (54%) for the dsRBBD1/2 resonances and 48 unambiguous assignments (16%) for the kinase domain. As with the unbound protein, there were no assignments for the linker region of the protein (170–251). dsRBBD1/2 amide resonances (1–169) undergo minimal perturbation upon PKR phosphorylation, indicating that they themselves are not likely phosphorylated or in contact with the kinase domain (Fig. 5B).

Resonances from the kinase domain are strongly perturbed in phosphorylated PKR (Fig. 5C). Backbone amide groups that exhibit the largest perturbations cluster to three contiguous surface-exposed regions of the protein: around the N-terminal β-sheet, the kinase active site, and the region proximal to the eIF2α substrate-binding site. Residues from the N-terminal lobe of PKR undergo significant chemical shift perturbations, indicating a potential conformational change to the N-terminal β-sheet upon phosphorylation. These include residues from β1 (Leu-272, Gly-274), β1–β2 loop (Ser-275, Gly-276, Gly-277, Gly-279), β2 (Phe-282, Lys-285), β2–β3 loop (His-286, Arg-287), β3 (Ile-294, Val-295), β4 (Asn-324, Gly-325, Cys-326, Gly-329), and β5 (Gln-365). Interestingly, the N-terminal α-helix of the kinase domain, which is directly connected to the interdomain linker, is also perturbed (Lys-261, Phe-263). The region surrounding the ATP-binding site, centered around Lys-296, is also significantly perturbed and includes residues from the β6–β7 loop (Arg-413, Asp-414, Lys-416, Ser-418), β7 (Ile-420, Phe-421), β7–β8 loop (Thr-425), β8 (Gln-427, Ile-430), and the loop C-terminal to β8 (Asp-432). Several of these residues have been directly implicated in ATP coordination, including Arg-413, Asp-414, and Asp-432 (11). A two-helix stretch that flanks the proposed eIF2α-binding site (11) is also perturbed and encompasses αD (Glu-375, Arg-381), αD–αE loop (Gly-383), αE (Leu-390, Gln-397, Gly-401, Asp-403, His-406, Lys-408), and αF–αG loop (Val-484). These NMR data show a potential connection between the N-terminal lobe, active site, and eIF2α-binding sites in the kinase domain. These changes may occur through chemical perturbations by phosphorylation, conformational changes in the kinase domain, or PKR-PKR bimolecular interaction.

**RNA Is Released upon Activation**—The link between dsRNA binding and PKR activation was further explored by monitoring the PKR-dsRNA complex during activation. Our DLS measurements implied a reduction in molecular size corresponding to the release of dsRNA activator over the time course of activation at concentrations below the dissociation constant for dimerization (Fig. 1D). To observe RNA release directly, native gel shift mobility assays were performed on TAR dsRNA in the presence of PKR under activating (ATP/MgCl2) and non-activating (ADPPNP/MgCl2) conditions (Fig. 6A). TAR is shifted to higher molecular weight bands in the presence of latent PKR alone. The addition of ATP/MgCl2 or ADPPNP/MgCl2 resulted in identical migration when compared with PKR alone. Upon incubation at temperatures sufficient for PKR activation (90 min), dissociation of the PKR-TAR complex is observed when both ATP and MgCl2 are present. When a non-hydrolysable ATP analogue (ADPPNP) is employed, no RNA release is observed (Fig. 6A). Inclusion of both ATP and MgCl2 is required for release; the absence of either inhibits both activation and RNA release (data not shown). RNA release occurs rapidly when compared with activation (Fig. 6B), with no lag phase and almost complete release of RNA by the midpoint of activation. This result suggests that once a critical concentration of phosphorylated PKR is established, PKR behaves in an RNA-independent manner.

Autophosphorylation of PKR is inhibited by high molar excesses of dsRNA (19). PKR autophosphorylation is indeed
attenuated at high molar excess of TAR dsRNA (Fig. 6C). dsRNA inhibition at high concentration may be related to the inability to release RNA. Using native gel shift mobility assays, we observed that RNA release was inhibited at higher concentrations, as predicted (Fig. 6D). An equilibrium dissociation constant for TAR RNA-PKR complex of ≈4 μm was determined, representing a 40-fold decrease in RNA affinity for PKR upon phosphorylation. This result was confirmed by comparing the affinity of TAR for both latent and phosphorylated PKR (Fig. 6E).

PKR phosphorylation is required for RNA release. Native gel shift mobility assays were used to quantify dsRNA release from both wild-type and mutant PKR. In all cases where ADP-PNP was used, only minor RNA release is observed (Fig. 6F). However, under activating conditions, only wild-type PKR releases RNA efficiently, whereas ATP-binding site mutants (PKR<sup>K296R</sup>) and activation loop phosphorylation site mutants (PKR<sup>T446A</sup> and PKR<sup>T451A</sup>) do not. Therefore, phosphorylation and RNA release are coupled. This coupling is a general phenomenon as other dsRNA activators (i.e., VA<sub>1</sub>-AS) behave similarly to TAR (22).

ATP Binding Does Not Affect dsRNA Binding to PKR—Since ATP is required for phosphorylation activity, we explored the potential for coupling between ATP- and RNA-binding sites in PKR. The apparent affinity of ATP for PKR was determined by measuring the initial velocity of the autophosphorylation reaction under constant PKR and MgCl<sub>2</sub> concentrations and increasing amounts of the substrate ATP. Using standard Michaelis-Menten approaches, a maximal velocity, <i>V</i><sub>max</sub>, of 10.2 ± 0.3 nM/min and a <i>K</i><sub>m</sub> of 164 ± 8 μm were determined (supplemental Fig. 11). To determine whether ATP binding induces a conformation change in the dsRBDS, leading to an
**FIGURE 6. Activator RNA is released upon PKR phosphorylation.**

A, native gel mobility-shift for TAR (200 nM) binding to PKR (300 nM) in reactions containing ATP (1 mM), ADP-PPN (1 mM), and MgCl₂ (2 mM). Samples were incubated at 30 °C for the time indicated, resolved on 5% Tris borate-EDTA gel, and visualized by Sybr Green II staining. B, PKR (200 nM) was incubated with [γ-³²P] ATP in the presence of TAR (300 nM), and aliquots were removed and resolved by either SDS-PAGE and quantified for autophosphorylation (left axis, solid black line) or RNA release by native gel mobility-shift (right axis, dashed line) as in A. C, PKR (200 nM) phosphorylation assays with increasing amounts of TAR (0–50-fold excess). D, concentration dependence of TAR release upon activation of PKR (200 nM). PKR was incubated with increasing amounts of TAR RNA at 30 °C for 90 min, resolved by native gel mobility shift, and stained with Sybr Green II. Lanes alternate between no ATP/MgCl₂ or 1 mM of each reagent. E, increasing amounts of PKR (top) or PKR (bottom) was added to TAR RNA (200 nM). Reaction components were resolved by native 5% Tris borate-EDTA gels and stained with Sybr Green II. F, PKR-TAR complexes were preassembled (200 nM) and incubated at 30 °C for 90 min in the presence or absence of ATP (1 mM) and MgCl₂ (1 mM). RNA release was quantified by resolving reaction components on native 5% Tris borate-EDTA gels and dsRNA staining by Sybr Green II. Each data point represents a quadruplicate measurement. WT, wild type.
Molecular Framework for Activation of PKR

FIGURE 7. **Molecular framework for the activation of PKR.** A model summarizing the molecular framework for the activation of PKR via the dsRBDs (R), kinase domain (K), and interdomain linker (L) is shown. Upon binding of activator dsRNA to the dsRBDs of PKR in the latent form, a conformation change in the protein may lead to enhanced bimolecular interaction between two PKR molecules. 

in kinase activation. By dynamic light scattering and size exclusion chromatography, latent PKR is primarily monomeric at concentrations examined (\(K_D \approx 500 \mu M\)), whereas the binding of HIV-TAR RNA significantly increases PKR self-affinity (\(K_D \approx 75 \mu M\)). Self-affinity is further increased when PKR is phosphorylated (\(K_D \approx 20 \mu M\)). These observations are supported by prior sedimentation equilibrium measurements that indicate that latent PKR undergoes a weak, reversible monomer-dimer equilibrium (\(K_D \approx 450 \mu M\)) and that self-association may be stabilized by autophosphorylation (18). Our kinetic data indicate a bimolecular reaction mechanism in which two PKR molecules are required for trans-autophosphorylation. Autophosphorylation reaction kinetics were only well fit once the constraints of PKR dimerization and increased affinity upon phosphorylation of PKR were included. PKR autophosphorylation can occur in an RNA-independent manner at high concentrations, indicating that once the self-associated state is significantly populated, phosphorylation can occur. The competency of latent PKR to serve as a substrate for trans-activation by phosphorylated PKR also serves to reinforce the requirement for two molecules of PKR in the reaction mechanism. These data further strengthen the argument that dsRNA does not serve as a scaffold for activation as these reactions contain no dsRNA (Fig. 4D).

Using NMR, we have probed the conformations of three PKR species involved in activation: latent, RNA-bound, and phosphorylated PKR. Previous studies of PKR have implied an auto-inhibitory interaction between the dsRBDs and kinase domain based primarily on experiments in which isolated dsRBDs and kinase domain (or kinase domain peptides) were mixed in trans (19, 20). NMR spectra of full-length protein provided high-quality data, and prior chemical shift assignments allowed us to investigate the conformations of dsRBDs and kinase domains. The interdomain linker appears to be unstructured and/or undergoes exchange processes (supplemental Fig. 8). Upon comparison of full-length PKR with each of its individual domains, no significant chemical shift perturbations were observed in either the dsRBDs or the kinase domain. Therefore, the latent form of PKR appears to exist in a conformation in which no detectable interactions between the two domains are observed. In support, recent work using atomic force microscopy also resolved individual, unbound domains of monomeric PKR in its unbound form (21). Direct displacement of the kinase domain from an inhibitory interaction with the dsRBDs is not sufficient to explain the activation of PKR (22). Dynamic interactions may occur between the dsRBDs and kinase domain that are not detected by our current approaches.

To assess directly the mechanism of dsRNA activation, we examined the PKR-TAR complex by NMR spectroscopy to observe perturbations to PKR upon dsRNA binding. As expected, chemical shift perturbations were observed in the dsRBDs, corresponding almost identically to those observed in the absence of the kinase domain (8). No significant perturbations to the kinase domain were observed upon dsRNA binding, indicating that dsRNA binding to the dsRBDs, at the level of resolution of these current experiments, does not induce a direct conformational change in the kinase domain.

Phosphorylated PKR displays significant chemical shift perturbations in the kinase domain when compared with the latent form. Changes are observed in the N-terminal α-helix and β-sheet, the active site cleft, and two α-helices proximal to the substrate binding site. Local changes in chemical environment due to phosphorylation at Thr-446 and Thr-451 alone cannot account for the chemical shift changes observed as residues distant from these sites are perturbed. Perturbations in the N-terminal α-helix (α0) and β-sheet (β1-β5) and connecting loops, near the interdomain linker, highlight residues involved in dimerization observed in the crystal structure of the kinase domain (11). Chemical shift perturbations observed in the phosphorylated kinase include residues whose mutation disrupts both PKR dimerization and kinase activity (17). Whether the observed shifts demonstrate formation of the crystallographically observed kinase domain dimer in phosphorylated PKR will require further experimentation.
Perturbations to the N-terminal lobe of the kinase domain may represent a mechanistic link between PKR dimerization and kinase activation. We observe perturbation of the β1–β2 loop, whose typical position is overhauling the active site cleft (11). This may reflect an increased accessibility to the active site upon activation without increased affinity for ATP (21). The active site cleft itself is also significantly perturbed upon activation, including key residues involved in direct coordination or maintenance of active site architecture. In contact with the active site cleft is a helix-loop-helix motif encompassing α-helix D to α-helix E (residues 375–410) in which a significant number of residues are perturbed. These helices buttress the active site and provide the backbone for eIF2α recognition (11). Our NMR results, which highlight contiguous regions of the kinase domain in phosphorylated PKR, suggest that protein dimerization, the active site architecture, and substrate recognition may be modulated upon activation.

Biochemical characterization of phosphorylated kinase confirmed trans-autophosphorylation between two PKR monomers. PKR is not responsive to typical dsRNA activators due to its 40-fold decreased affinity for dsRNA when compared with latent PKR. Kinetically, PKR is an extremely efficient enzyme when compared with PKR-TAR complex, which is consistent with a bimolecular reaction mechanism in which PKR demonstrates an increased self-affinity. Activation of PKR by phosphorylation represents a covalent modification to increase substrate affinity.

The ability to purify phosphorylated PKR in the absence of RNA enabled the assessment of various PKR derivates as target substrates for phosphorylation. PKR is competent as a target substrate, indicating that a functional ATP coordination site is not a substrate requirement for PKR. Conversely, mutation at either position Thr-446 or Thr-451, the critical phosphorylation sites required for activation, results in attenuation of trans-autophosphorylation. These residues are the only required first sites of autophosphorylation in our in vitro system. Furthermore, these results suggest potential cooperativity between Thr-446 and Thr-451, a plausible hypothesis based on their structural proximity (11). Isolated dsRBDs alone are not trans-autophosphorylated, indicating that they may not be targets for initial activation. This is consistent with the observation that no significant perturbations to the amide resonances of the dsRBDs are observed in full-length phosphorylated PKR by NMR experiments (Fig. 5A).

dsRNA release is a consequence of autophosphorylation of PKR. We demonstrated dissociation of dsRNA activator from PKR coincident with activation. RNA release closely parallels activation as almost complete dissociation is observed at the midpoint of the sigmoidal activation progress curve at all concentrations examined; RNA activators dissociate rapidly from PKR with rates >1 s⁻¹ (data not shown). Attenuation of RNA release is observed when mutations to the ATP-binding site (K296R) or crucial autophosphorylation sites (T446A, T451A) are made, supporting the link between activation and RNA release. Activated PKR has at least 40-fold decreased affinity for dsRNA activators when compared with latent kinase. An increased affinity for dsRNA has been reported when PKR is treated with phosphatase (31). RNA release upon activation explains inhibition of autophosphorylation at high activator concentrations. In support, we observed that at concentrations where activation is inhibited, RNA release is simultaneously inhibited.

dsRNA activators function to prime PKR for activation at low cellular PKR concentrations (<μM), but once activation is achieved, dsRNA is released and can be used again. The unliganded phosphorylated PKR is a more efficient kinase for latent PKR and eIF2α. PKR must sense viral RNAs at low concentration, and RNA release would allow a small quantity of dsRNA to stimulate a comparatively large amount of PKR through autocatalysis. A potential basis for RNA release is suggested by a recent report that the dsRBDs of PKR may become phosphorylated upon activation (32), although we do not observe phosphorylation or significant conformational change of the dsRBDs upon PKR activation using our approaches. The molecular basis for RNA release remains unclear but demonstrates thermodynamic and mechanistic coupling between kinase and RNA-binding domains.

The interdomain linker (15, 16) and N-lobe of the kinase domain (17) have been implicated in PKR self-association and function. The 81-amino-acid linker (residues 170–251) is acidic (estimated pI of 4.3) and hydrophilic, with 24 serines and threonines. Both the kinase and the dsRBD domains are basic and structured. Our NMR data strongly suggest that the linker region is mainly unstructured in PKR. Full-length PKR and a construct lacking only RNA-binding domains PKR can overcome the absence of dsRNA and autophosphorylate at protein concentrations >5 μM, whereas the isolated kinase domain demonstrates near basal autophosphorylation over the same concentration range. Therefore, the interdomain linker may mediate the dynamic communication between kinase and RNA-binding domains of PKR.

Electrostatic changes may be the key to PKR activation. The surfaces of both dsRBDs and the kinase domain near the active site are positively charged. The extended conformation of PKR may result from electrostatic repulsion between the two domains. Binding of RNA changes the electrostatic potential of the dsRBDs and may alter the disposition of the linker to allow initial autophosphorylation. Covalent phosphorylation leads to RNA release, potentially through reciprocal electrostatic signaling, and may stabilize an active form for PKR with high affinity for substrate. Highly charged molecules such as heparin also lead to activation of PKR (33), consistent with a change in electrostatic potential modulating PKR self-association. More detailed structural studies, coupled with investigations of inter- and intra-domain dynamics, are needed to unravel the molecular details of PKR activation.

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