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Viral dsRNA inhibitors prevent self-association and auotphosphorylation of PKR.

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SUMMARY

Host response to viral RNA genomes and replication products represents an effective strategy to combat viral invasion. PKR is a Ser/Thr protein kinase that binds to dsRNA, autophosphorylates its kinase domain, and subsequently phosphorylates eukaryotic initiation factor 2α (eIF2α). This results in attenuation of protein translation, preventing synthesis of necessary viral proteins. In certain DNA viruses, PKR function can be evaded by transcription of highly structured virus-encoded dsRNA inhibitors that bind to and inactivate PKR. We probe here the mechanism of PKR inhibition by two viral inhibitor RNAs, $EBER_I$ (from Epstein-Barr) and VA_I (from human adenovirus). Native gel shift mobility assays and isothermal titration calorimetry experiments confirmed that the RNA-binding domains of PKR are sufficient and necessary for the interaction with dsRNA inhibitors. Both $EBER_I$ and VA_I are effective inhibitors of PKR activation by preventing *trans*-autophosphorylation between two PKR molecules. The RNA inhibitors prevent self-association of PKR molecules, providing a mechanistic basis for kinase inhibition. A variety of approaches indicated that dsRNA inhibitors remain associated with PKR under activating conditions, as opposed to activator dsRNA molecules that dissociate due to reduced affinity for the phosphorylated form of PKR. Finally, we show using a HeLa cell extract system that inhibitors of PKR result in translational recovery by the protein synthesis machinery. These data indicate that inhibitory dsRNAs bind preferentially to the latent, dephosphorylated form of PKR and prevent dimerization that is required for *trans*-autophosphorylation.

Keywords

RNA; PKR; kinase; inhibition; virus

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INTRODUCTION

A hallmark of many viral infections is the presence of viral genomes and replication by products containing highly structured double-stranded RNA molecules (dsRNA). Recognition of these dsRNA molecules by cellular pattern-recognition receptors (PRRs) results in the induced interferon response, thereby modulating gene expression of enzymes that mediate innate immunity 1; 2; 3. The interferon-inducible enzyme PKR (Protein Kinase RNA-dependent) specifically serves as an intracellular sensor for viral genomic contamination to down-regulate translation initiation 4. Activated by the binding of dsRNA, PKR regulates protein synthesis by the phosphorylation of eukaryotic initiation factor 2α (eIF2α) at Ser51, thereby forming a sequestered inhibitory complex with its guanine nucleotide exchange factor eIF2B 5. As a result, the pool of active eIF2 ternary complex is reduced, causing a general decrease in both cellular and viral protein synthesis 6. Therefore, the cellular interactions between dsRNA and members of the interferon response pathway represent a fundamental anti-viral response.

Human PKR is a 551-residue enzyme comprising an N-terminal RNA binding and C-terminal kinase domain bridged by an 80-residue interdomain linker 7; 8. The N-terminal domain contains two highly basic, conserved 70-residue motifs capable of dsRNA recognition (dsRBDs); high-affinity binding requires each of the dsRBDs 9; 10; 11; 12. The C-terminal domain of PKR is a Ser-Thr Kinase domain responsible for both substrate recognition and phosphorylation activity of the enzyme 13; 14; 15. Defining characteristics of the kinase domain include the ATP coordination site required to mediate phosphoryl transfer, activation loop phosphorylation sites important for activity (T446 and T451), and substrate recognition sites 12; 13; 15. In solution, the interdomain linker is highly unstructured, resulting in a highly dynamic protein in which the domains behave as independent units 12; 16. Upon dsRNA binding, latent PKR undergoes autophosphorylation, which greatly increases the kinase activity of the enzyme 17; 18. PKR autophophorylation proceeds through a bimolecular reaction mechanism, in which the self-association of PKR is modulated by both dsRNA interactions and the phosphorylation state of the enzyme itself 12; 13; 19; 20; 21. Although the structural basis for self-association remains unclear, either conformational or electrostatic changes upon dsRNA binding are thought to unmask domains within both the interdomain linker and kinase domain to facilitate self-association 12; 13; 15. RNA-PKR complex stability is significantly reduced upon autophosphorylation of PKR 12; 22.

PKR is specifically activated by dsRNA molecules compared to other nucleic acid substrates. RNA-DNA hybrids, dsDNA, and single-stranded polynucleotides are not recognized by the enzyme 4. Single dRBDs recognize dsRNA through structure-specific, sequence independent contacts to the 2'-hydroxyl groups of the ribose sugar 23; 24; 25, and the two dsRBDs and linker likely make similar contacts. All known viral ligands of PKR are distorted dsRNA helices, possessing structural features beyond simple duplex, such as bulges and hairpin loops 26; 27; 28; 29; 30; 31. These structural features tune RNA ligand recognition by the dsRBDs, as significantly reduced affinity is observed when distortions from the dsRNA molecules are removed 10; 32. A minimum dsRNA duplex length (15−50 bp) is required for high-affinity interaction between the dsRBDs and dsRNAs 26; 32; 33; 34.

Many viruses counteract the PKR response to viral infection by encoding protein and dsRNA molecules that inhibit PKR function 3; 35; 36; 37; 38. Human adenovirus and Epstein-Barr virus encode RNA polymerase III-directed, highly structured RNAs (VA and EBER respectively) that are required for efficient translation of viral mRNAs. These inhibitory dsRNAs bind directly to PKR with similar affinity to dsRNA activators 10, but prevent subsequent activation and substrate phosphorylation, allowing protein synthesis to

proceed 10; 37; 39. The EBER and VA RNAs form roughly similar 160-nt stem-loop secondary structures containing an apical stem-loop (AS), central stem-loop (CS), and distal stem (DS) (Fig.1). Thermodynamically, a single stem-loop domain from each inhibitory dsRNA mediates the interaction with PKR; the apical stem from VA_I (VA_I-AS), and the central stem from $EBER_I$ ($EBER_I$-CS) 10, although low affinity interactions with other stem-loop domains have been reported 40. Both dsRBDs of PKR are required for high-affinity interaction with the inhibitor, and identical surfaces of the protein mediate the interaction with dsRNA whether activator or inhibitor is bound 10. Isolated dsRBD binding stem loops from inhibitors serve to activate PKR 10, indicating that dsRNA binding and inhibition are non-equivalent 41. Therefore, regions other than the dsRBD binding stem-loops must be responsible for mediating the inhibitory effect.

The mechanism of viral RNA inhibition of PKR remains poorly understood. Here we characterized viral inhibitor-PKR complexes by employing a variety of techniques, including native gel shift mobility assays, isothermal titration calorimetry (ITC), autophosphorylation assays, dynamic light scattering, and *in vitro* translation assays. These data support a model for the inactivation of PKR autophophorylation by dsRNA inhibitors in which inhibitory dsRNAs bind preferentially to the latent, dephosphorylated form of PKR and prevent dimerization that is required for efficient *trans*-autophosphorylation.

RESULTS

The dsRBDs of dephosphorylated PKR are sufficient and required for interaction with dsRNA inhibitors.

To extend our understanding of inhibition of PKR by viral dsRNA, two inhibitors of PKR were synthesized *in vitro*; adenovirus derived VA_I (Fig. 1A), and $EBER_I$ from Epstein-Barr virus (Fig. 1B). Both inhibitors contain stem-loop motifs that have been implicated in mediating the interaction with PKR 10; 40; 42. To serve as positive controls for PKR activity, two ligands of PKR were also produced that lead to activation; the apical stem-loop of VA_I (VA_I-AS, Fig. 1C) and HIV *trans*-acting response element (HIV-TAR) (Fig. 1D) 10; 32. RNA-PKR complexes were characterized biophysically to determine the mechanistic basis for inhibition.

A series of PKR domains and truncations were used to delineate regions of PKR that interact with inhibitory dsRNAs using native gel shift mobility assays (Fig. 2A). Addition of full-length PKR or truncated protein containing only the dsRBDs of PKR (dsRBD1/2) to VA_I results in formation of a higher-molecular weight species, corresponding to a 1:1 PKR:RNA complex in both cases (Fig. 2B). A functional ATP binding site in PKR is not a requirement for interaction, as mutation of the nucleotide coordination site (PKR^{K296R}) does not affect complex formation. Constructs lacking the dsRBDs ($PKR^{170-551}$, $PKR^{252-551}$ and $PKR^{170-252}$) do not discernibly interact with VA_I, indicating that the interdomain linker and/or kinase domains do not mediate the high-affinity association with PKR. Addition of the domains of PKR in *trans* (dsRBD1/2 and $PKR^{170-551}$) results only in formation of a complex with equivalent migration to a VA_I-dsRBD1/2 complex; again only the dsRBDs mediate the interaction. In contrast, phosphorylated PKR (PKR^P) does not form an observable RNA-protein complex with VA_I. Identical results were obtained when $EBER_I$ was used instead of VA_I (data not shown). In summary, these results suggest that the dsRBDs of PKR are required and sufficient for interaction with inhibitory RNAs, and that phosphorylation of PKR blocks the interaction with the inhibitors.

Gel shift mobility assays were confirmed and extended by isothermal titration calorimetry (ITC), which determines the affinity and thermodynamics of complex formation. A single, high-affinity binding-site within dsRNA inhibitors (VA_I or $EBER_I$) or activators (VA_I-AS)

(Fig. 2C) is observed for both dsRBD1/2 and full-length PKR; the affinities of inhibitor and activator RNA-protein interactions are similar. Mutations at the ATP coordination site (PKR^{K296R}) or activation loop phosphorylation sites ($PKR^{T446A/T451A}$) do not affect RNA inhibitor-PKR affinity. As expected from the gel shift assay results, phosphorylated PKR has a significantly reduced affinity for dsRNA inhibitors or activators (>15-fold decrease). Deletion mutants of PKR lacking the dsRBDs have similarly reduced affinities relative to either the full-length protein or dsRBDs alone. Thus, dsRBDs mediate interaction of inhibitors with PKR.

Inhibitors prevent trans-autophosphorylation of latent PKR

Characteristic of an autocatalytic process, a sigmoidal buildup of product with a lag phase prior to maximal rates of autophosphorylation has been observed for the bimolecular kinetics of PKR autophosphorylation 10; 12; 32. Inhibitors could be effective against the latent form of PKR, the phosphorylated form, or both. Given that inhibitors do not interact significantly with phosphorylated PKR (Fig. 2), we expected that only the latent form of the enzyme would be inhibited. To test our hypothesis, a kinase activation assay was established based on the autophosphorylation of PKR in the presence of a dsRNA activator, HIV-TAR. A buffered reaction containing ^{32}P-γATP, Mg^{2+}, HIV-TAR, and full-length PKR was incubated for a total of 2 hours, with either EDTA or dsRNA ligands added at various points in the time course. After 2 hours, reaction components were separated by SDS-PAGE under denaturing conditions, and the resulting incorporation of radiolabeled phosphate into PKR was quantified, thereby providing a direct measurement of inhibition efficiency. EDTA chelates all available Mg^{2+} in the reaction mixture and therefore quenches the reaction; EDTA acts as the idealized inhibitor of PKR as the amount of phosphorylation detected is a direct result of the bimolecular activation kinetics of PKR (Fig. 3A, dashed line).

Addition of PKR inhibitors (VA_I or $EBER_I$) is as effective as EDTA at inhibiting phosphorylation of PKR when added at earlier time points (< 15 minutes), but less effective as the pool of PKR becomes increasingly phosphorylated. This indicates that VA_I and $EBER_I$ preferentially inhibit the latent form of PKR. As expected, addition of another dsRNA activator to the reaction mixture, VA_I-AS, has no inhibitory effect (Fig. 3A). RNA inhibitors specifically block autophosphorylation by the phosphorylated form of PKR.

PKR activation proceeds through a bimolecular reaction mechanism in which one PKR phosphorylates another in *trans* 12; inhibitor-bound PKR may not be an efficient substrate for *trans*-autophosphorylation. Phosphorylated PKR (PKR^P) is capable of *trans*-phosphorylating PKR in buffered reactions containing ^{32}P-γATP and Mg^{2+} [12]. These experiments remove the need for RNA activators completely, and therefore one can dissect the ability of PKR to serve as a substrate for *trans*-phosphorylation. After 15-minute incubation, minimal phosphorylation of PKR^P itself is observed (Fig. 3B) indicating that ^{32}P incorporation in other lanes is attributable to substrate phosphorylation only. PKR^P is capable of trans-phosphorylating both latent PKR and activator-bound PKR; RNA binding to the dsRBDs does not itself preclude phosphorylation. Significantly, *trans*-autophosphorylation is strongly attenuated upon inclusion of increasing amounts of VA_I or $EBER_I$ to the reaction mixture, indicating that the inhibitors protect PKR from phosphorylation. A 3 to 10-fold excess of inhibitory dsRNA relative to PKR is required to protect PKR fully from *trans*-autophosphorylation, consistent with the known dissociation constants established for these complexes 10. Thus, RNA inhibitors block bimolecular phosphorylation by PKR.

Inhibitors prevent self-association of PKR

Multiple lines of evidence support the importance of PKR self-association in kinase activation 12; 13; 15; 19; 43; 44. As RNA inhibitors block phosphorylation of latent PKR, they may directly block self-association of PKR monomers. To probe the formation of bimolecular complexes under non-activating conditions (*i.e.* no prior incubation at 30 °C), we employed dynamic light scattering (DLS) to determine the apparent molecular weight (M_r) of complexes containing either wild-type or catalytically inactive (PKR^{K296R}) PKR at 5 μM concentration. M_r determinations for both VA_I (~55 kDa) and PKR (~83 kDa) alone were close to expected values, indicating that each molecule behaves as a monomeric species at low μM concentrations (Fig. 4A). Addition of excess ATP and Mg^{2+} did not impact the hyrdrodynamic radius of PKR; no global distortion to the protein is observed. Interaction between VA_I and PKR results in complex formation with an apparent M_r of 128 kDa, and again, addition of excess ATP and Mg^{2+} did not impact the DLS results. The catalytically inactive mutant, PKR^{K296R}, which is unable to self-associate 13, behaves in an identical manner to wild-type PKR, indicating that a 1:1 VA_I:PKR complex forms.

To examine the association state of PKR complexes, we next determined the concentration dependence (2−80 μM) of apparent M_r for wild-type PKR, PKR-VA_I, PKR-$EBER_I$, PKR-VA_I-AS and PKR-TAR complexes (Fig. 4B). No significant increase in M_r is observed at high concentration for PKR alone, whereas a significant increase in apparent M_r is observed upon increasing concentration of either PKR-VA_I-AS or PKR-TAR complex. The increase in hydrodynamic radius likely reflects an equilibrium between monomer and dimer forms of PKR. These results are consistent with previously observed PKR-activator complexes 10. Significantly, no such increase in apparent M_r is observed for PKR-VA^I or PKR-$EBER_I$; the inhibited PKR behaves remains monomeric at all concentrations examined (Fig. 4B). The hydrodynamic behavior established by DLS was confirmed using size exclusion chromatography, where molecular weight determinations were in good accordance between the two methodologies (Fig. 4C). These data indicate that PKR bound to inhibitory dsRNA is not capable of bimolecular associations required for *trans*-autophosphorylation.

dsRNA inhibitors remain associated with PKR under activating conditions

Activator dsRNAs demonstrate reduced affinity for PKR upon PKR phosphorylation, and reagents that limit phosphorylation typically prevent RNA dissociation 12; 22. As inhibitor binding to PKR prevents phosphorylation of the kinase, we hypothesized that activating conditions should not modulate the affinity of RNA inhibitors for PKR. To observe complex stability directly, native gel shift mobility assays were performed on activator (VA_I-AS) and inhibitor (VA_I or $EBER_I$) dsRNA in the presence of PKR under conditions suitable and unsuitable for autophosphosphorylation (Fig. 5A). Each dsRNA ligand is shifted to a higher molecular weight species when PKR is added, indicating successful complex formation. As expected, addition of ATP/Mg^{2+} to the reaction mixtures resulted in a marked decrease in affinity between PKR and VA_I-AS, whereas no change in complex stability is observed for complexes containing either VA_I or $EBER_I$. Stability of PKR-TAR and PKR-VA_I-AS complexes is restored using mutants of the ATP coordination site (PKR^{K296R}) or critical phosphorylation sites in the kinase domain of PKR ($PKR^{T446A/T451A}$) (Fig. 5B). As expected, neither of these mutations affected inhibitor-containing complexes (Fig. 5B). Therefore, the RNA inhibitors maintain their affinity for PKR under kinase activating conditions.

To observe the release of RNA during the progress of PKR phosphorylation, apparent M_r of complexes was next determined by DLS at specific time intervals. A 1:1 complex of PKR-VA_I-AS in the absence of reagents required for activation (ATP and $MgCl_2$) does not change with respect to time (Fig. 5C, black). Addition of ATP and $MgCl_2$ results in a time-

dependent decrease in M_r, corresponding to a transition from the fully bound state to a mixture of dsRNA and PKR (Fig. 5C, blue). In contrast, an equimolar complex of VA_I and PKR maintains a consistent M_r as a function of time (~128 kDa) regardless of reaction conditions (Fig. 5C, red and green). Similar results were obtained when $EBER_I$ was employed (data not shown). Inhibitor RNAs do not release from PKR upon incubation under kinase activation conditions.

Inhibitors of PKR result in translational recovery

We next examined the effect of dsRNA activators and inhibitors on translation initiation. PKR activation by dsRNA leads to phosphorylation of eIf2α, resulting in attenuation of translation initiation, whereas inhibitors should have the opposite effect 45. To test this hypothesis, we quantitatively measured the synthesis of luciferase in HeLa S10 cell lysate that containing functional protein translation machinery, 5'-capped luciferase mRNA, and exogenous PKR. As expected, protein synthesis is significantly reduced upon inclusion of dsRNA activators in lysates (Fig. 6A). Addition of increasing amounts of inhibitory dsRNA (VA_I) to extracts containing exogenously added PKR-activator complex results in the restoration of cap-dependent translational competency. Complete restoration is achieved when a 20-fold excess of inhibitor over activator is added; a result that is consistent with *in vitro* inhibition of autophosphorylation (Fig. 3B). As expected, restoring translational competency by addition of inhibitory dsRNA is accompanied by a decrease in eIF2α phosphorylation (Fig. 6B). Taken together, these results establish a causal link between *in vitro* phosphorylation of PKR and the inhibition of translation, with dsRNA inhibitors of PKR restoring translational competency.

DISCUSSSION

The mechanism by which dsRNA ligands modulate PKR kinase activity is central to understanding its function in innate immunity. The PKR response can be circumvented by viruses through transcription of highly-structured dsRNA inhibitors that bind to and inactivate PKR. Whereas the interaction between dsRNA inhibitors and PKR has been fairly well characterized, the mechanism of inhibition is poorly understood. Here we showed that dsRNA inhibitors interfere with the self association of PKR, thereby preventing PKR from acting as a substrate for *trans*-autophosphorylation. Numerous techniques were employed, including native gel shift mobility assays, isothermal titration calorimetry (ITC), autophosphorylation assays, dynamic light scattering, and *in vitro* translation assays.

Single stem-loops from both dsRNA activators and inhibitors mediate stable interaction with the dsRBDs of PKR 10. Here, we probed the interaction between dsRNA ligands (both activators and inhibitors) and PKR variants to delineate the requirements for binding using ITC and native gel shift mobility assays. The dsRBDs alone direct high-affinity interaction with dsRNA inhibitors, as no discernable interaction is observed with constructs of the kinase domain or interdomain linker alone. Furthermore, addition of the dsRBDs (residues 1−169) and the remainder of the protein (residues 170−551) in *trans* yields a complex containing only the dsRBDs and dsRNA; no high-affinity interaction with the interdomain linker or kinase domain is observed. Finally, mutations to critical regions in the kinase domain, including activation loop phosphorylation sites or ATP co-ordination site, have no effect on high-affinity interaction with dsRNA inhibitors.

dsRBDs of dephosphorylated PKR are sufficient and required for the interaction with either dsRNA activators or inhibitors, as previously shown. Phosphorylated PKR has minimal affinity for dsRNA inhibitors, indicating that once the phosphorylated state of the protein has been achieved, the inhibitory potential of these dsRNAs is attenuated. Kinetic studies further demonstrated that dsRNA inhibitors are preferentially effective at early points in the

sigmoidal autophosphorylation profile (<15 minutes); as PKR becomes increasingly phosphorylated, the effectiveness of the inhibitors decreases. These observations are consistent with the hypothesis that dsRNA activators function to prime PKR for activation at low cellular PKR concentrations, but once activation is achieved, PKR is capable of *trans*-autophosphorylation of PKR in an RNA-independent manner 12. Furthermore, phosphorylation of the dsRBDs is not observed under these conditions 12, indicating that communication between the kinase and dsRBD domains is responsible for disrupting the interaction.

Dissociation of dsRNA activator from PKR is coincident with activation, presumably due to a reduced affinity with the phosphorylated form of the enzyme 12; 22. RNA release closely parallels activation, as almost complete dissociation is observed at the midpoint of the sigmoidal activation progress curve at all concentrations examined. Significant attenuation of RNA release is observed when inhibitor bound PKR complexes are examined, supporting the reciprocal link between phosphorylation state and PKR-dsRNA complex stability. Thus RNA inhibitors must operate at an early stage of the PKR response, since once PKR is phosphorylated, they are ineffective.

Activation of PKR proceeds through a bimolecular reaction mechanism, in which dsRNA activators and ultimately PKR phosphorylation serve to increase the propensity for PKR self-association 12. dsRNA inhibitors of PKR oppose these processes, since the inhibitors bind to PKR but prevent self-association. Therefore, dsRNA secondary structural elements not involved in the dsRBD interaction may block the ability of PKR to self-associate. Consistent with this hypothesis, truncations of dsRNA inhibitors, containing only the dsRBDs-binding stem-loop (*i.e.* VA_I-AS or $EBER_I$-CS), behave as activators of PKR 10. dsRNA inhibitors thus function to protect latent PKR from undergoing *trans*-autophosphorylation, and therefore activation. *Trans*- autophosphorylation assays indicate that inhibitor dsRNA-PKR complexes are not competent substrates for phosphorylation. This result is in stark contrast with activator dsRNA-PKR complexes, which are suitable substrates 12. Whether dsRNA inhibitors block substrate access to the kinase active site, or mask self-association interfaces must be further investigated.

Viral dsRNA inhibitors circumvent the PKR-mediated host-cell defense mechanism. To compliment our mechanistic data, we examined the effect of dsRNA ligands in cell extracts containing all of the necessary components of the protein translation machinery. As expected, addition of dsRNA activators of PKR, which result in phosphorylation of eIF2α, resulted in a significant decrease in protein synthesis. Conversely, translational competency is restored when sufficient viral dsRNA inhibitors are added to these cell extracts. Therefore, we have established a link between the *in vitro* mechanistic effects of inhibitors and their direct impact on the protein synthesis machinery.

The results presented here, combined with prior data from other groups, allows us to assess the functional importance of various secondary structural features of the dsRNA inhibitors. Both activators and inhibitors interact using a similar stem-loop, bind with similar affinities to PKR (K_D ~ 100nM), and have similar off-rates (personal communication, C.E. Aitken). The interaction is primarily mediated by the dsRBDs of PKR, indicating that the kinase domain and interdomain linker contribute negligible thermodynamic stability to these complexes. The additional secondary structural elements in VA_I and $EBER_I$ must mediate inhibition of PKR. Mutations to the central stem of VA_I are not tolerated 37; 41; 46, indicating that this region of the RNA, while not directly involved in binding PKR, plays an important role in inhibition. Footprinting studies have also suggested that PKR engages in a strong interaction with the minor groove of the apical stem and a weaker interaction with the central stem of VA_I 40 . The dsRBDs alone protected only the apical stem from base-

specific chemical probes, leaving the central stem exposed. Specific structural features in this region must be responsible, as increased length of the dsRNA inhibitors relative to activators is not sufficient to explain inhibition. Long, idealized dsRNA polymers serve as potent activators of PKR 36; 37; 47 and the hepatitis C virus internal ribosome entry site RNA (HCV IRES), a highly structured 374-nucleotide dsRNA molecule mediates potent autophosphorylation PKR *in vitro* (personal communication, T. Shimoike).

The physical basis for inhibition remains elusive, although a dynamic interaction between central stem of VA_I and a specific region of PKR cannot be ruled out; further studies are required to explore this interaction. dsRNA inhibitors most likely prevent PKR self-association through direct steric blockage. However, an enticing possibility remains that PKR activation is mediated by the altering of the electrostatic landscape of the protein; inhibitory dsRNAs might prevent self-association through changing the electrostatic potential of the enzyme.

A simple model for the inactivation of PKR autophosphorylation by dsRNA inhibitors is consistent with the available data (Figure 7). PKR autophosphorylation proceeds through a bimolecular reaction mechanism in which *trans*-autophosphorylation occurs between PKR monomers. Interaction with dsRNA activators (and ultimately autophosphorylation) increase the propensity for PKR dimerization, thus increasing the rate of the bimolecular reaction 12. A single stem-loop from inhibitory dsRNAs interacts preferentially with the latent form of PKR via the dsRBDs; distal stem-loops of the RNA mediate the dimerization disruption. The net effect is a severe attenuation of PKR autophosphorylation and therefore inhibition of the enzyme. The data presented here provide a detailed mechanistic view of how dsRNA inhibitors function, and confirm previous insights into the mechanism of PKR activation. More detailed mechanistic and structural studies are clearly required to fully understand the function of this highly dynamic and intricately regulated enzyme.

MATERIALS AND METHODS

Sample preparation.

Expression and purification of full-length human PKR and domain constructs including dsRBD1/2 are as previously described 10; 12. Full length PKR mutant constructs (PKR^{K296R} and $PKR^{T446A/T451A}$) were generated using site-directed mutagenesis of the wild-type plasmid, and were expressed and purified identically to the wild-type protein. Phosphorylated PKR (PKR^{P}) was generated by incubating wild-type full length human PKR (15 μM) in the presence of ATP (1 mM) and $MgCl_2$ (2 mM) at 30 °C for 2 hours in a buffered solution containing Tris•HCl (pH 7.5), 100 mM NaCl, and 5 mM ß-mercaptoethanol (ß-Me), and purified as described elsewhere 12. RNA samples were prepared by *in vitro* transcription using T7 polymerase as described previously 10; 48 using a BsaI site for plasmid linearization.

Native Gel Shift Mobility Assay.

All samples were prepared in 50 mM Tris/Cl (pH 7.5), 100 mM NaCl, and incubated at room temperature for 10 minutes. Samples were mixed with non-denaturing load mix and loaded onto 5% non-denaturing TBE gels (Bio-Rad). Sample running buffer contained 0.5X TBE (50 mM Tris base, 41.5 mM boric acid, and 1 mM EDTA, final pH 8.3). Electrophoresis was performed at 60 V at 4 °C, and gels were stained with 1X SybrGreenII fluorescent dye (Molecular Probes Inc.) for quantitation. Fluorescently stained gels were scanned (GE Healthcare) and quantitated using the ImageJ software 49.

Isothermal Titration Calorimetry.

A VP-ITC microcalorimeter (Microcal) was used to analyze the stability of protein-RNA complexes. The sample cell contained the RNA ligand (10 μM RNA, 50 mM Tris•HCl, pH 7.5, 100 mM NaCl) and a concentrated protein solution (200 μM, 50 mM Tris•HCl, pH 7.5, 100 mM NaCl, 5 mM ß-Me) was placed in the syringe. Each titration was performed at 30 °C, and involved a single 2 μL injection, followed by twenty-four 10 μL injections of protein solution into the sample cell containing RNA. Titration curves were fit by a nonlinear least-squares method with a model for two binding sites using Microcal Origin (version 5.0) to extract thermodynamic parameters K_A (association constant), ΔH (binding enthalpy), and N (stoichiometry). In each case, a single high-affinity site and a second weak affinity non-specific site were observed; reported results are from the high-affinity binding site. From these data, the changes in entropy (ΔS) and free energy (ΔG) were calculated using established equations 10; 32.

PKR Autophosphorylation Assays.

All phosphorylation assays for PKR were performed in 50 mM Tris/Cl (pH 7.5), 100 mM NaCl, 1 mM ATP, 2 mM $MgCl_2$, and 1 μCi of ^{32}P-γATP. Reactions were performed at 30 °C with the concentrations and incubation times as denoted in the figure legends. Reactions were quenched with SDS-PAGE load mix. Phosphorylated proteins were separated on a 4−20% SDS-PAGE gel (Bio-Rad), dried for 30 minutes at 80 °C, and autoradiographed (GE Healthcare) to trace the incorporation of ^{32}P into PKR. Band intensities were quantitated using the ImageJ software 49.

Dynamic Light Scattering.

Protein samples were pre-filtered through a 0.22 μM Millex-GV syringe filter (Millipore) prior to analysis. Dynamic light scattering experiments were performed at 30 °C with a DynaPro-801 molecular sizing instrument (Protein Solutions Co.). A minimum of 50 data points at 10-second intervals was collected for each sample examined. The hydrodynamic radius, apparent molecular weight, and polydispersity were determined on the basis of an autocorrelation analysis of scattered light intensity data using the DYNAMICS (version 6.0) software package.

In vitro translation.

The basis for *in vitro* translation assays is a HeLa S10 cell lysate; preparation of cell extracts is discussed elsewhere 50. The lysate (50% vol.) is supplemented with 0.5 U/μl RNase inhibitor, SUPERase·In (Ambion), 25μM Amino Acid Mixture, Complete (Promega), 60mM KCl (for cap-dependent translation), and 1mM $MgCl_2$ (required for activation of PKR). Modulation of cap-dependent translation by PKR was determined by adding purified PKR and RNA ligands to the lysate containing 5'-capped *Renilla* luciferase mRNA (reporter RNA) at concentrations denoted in the figure legend. Reporter RNA is preincubated at 65 °C for 3 min, and then immediately cooled in ice-cold water prior to addition. The translation reaction is incubated at 30 °C for 60 minutes prior to Luciferase activity assay. Using a reporter construct that contains cap-Luc, the activity (intensity of luminescence from substrate) of *Renilla* luciferase is determi ned by the luciferase assay system (P romega) and luminometer (Analytical Luminescence Laboratory).

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

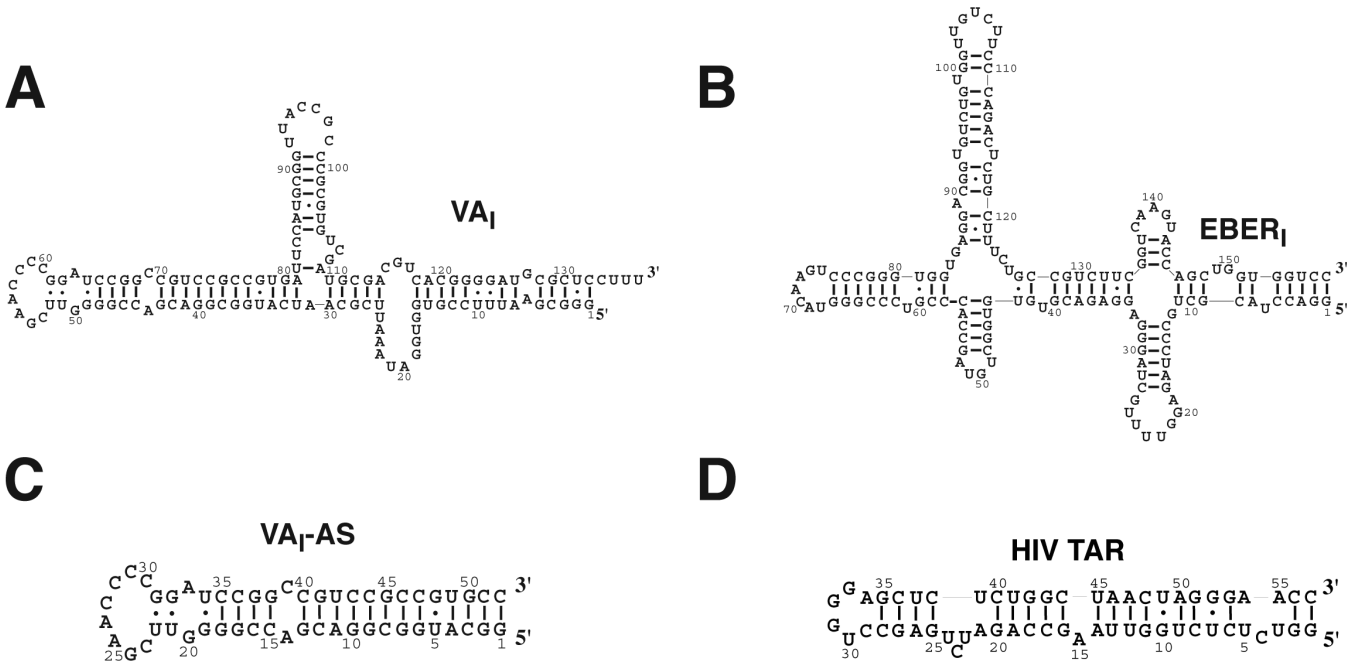
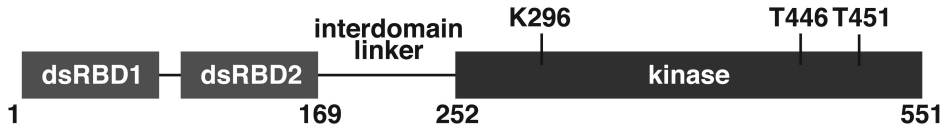


Figure 1.

Sequences and secondary structures of viral dsRNAs. Secondary structures of (A) adenovirus VA_I inhibitor 51, (B) Epstein-Barr virus $EBER_I$ inhibitor 52, (C) the apical stem truncation of VA_I (VA_I-AS), and (D) HIV TAR dsRNA 53.

A



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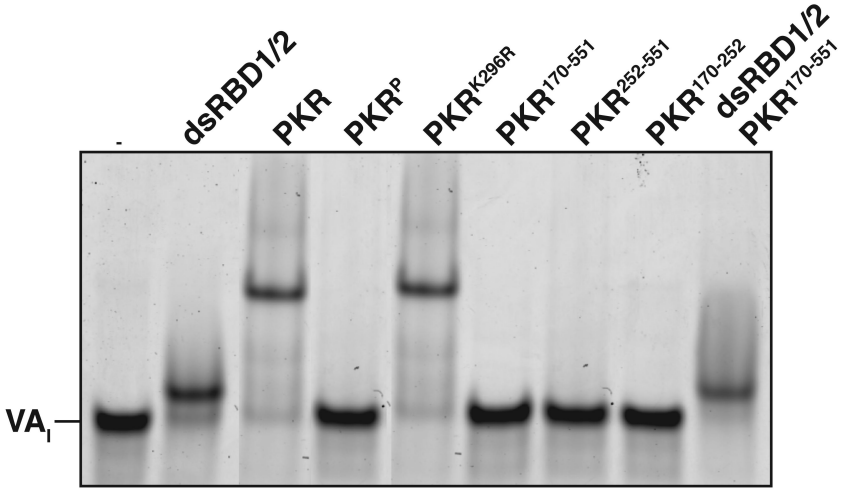

C

	VA_I	$EBER_I$	VA_I-AS
dsRBD1/2	76±9	76±9	82±8
PKR	79±9	94±10	77±9
PKR^P	>2300	>1800	>1700
PKR^{K296R}	91±7	79±7	86±8
$PKR^{252-551}$	>1400	>1400	>1900
$PKR^{170-551}$	>1300	>1700	>1600
$PKR^{T446A/T451A}$	87±9	85±8	75±7

Figure 2.

dsRBDs of PKR are sufficient and required for interaction with inhibitory dsRNAs.

(A) Domain organization of PKR. N-terminal dsRBDs, C-terminal kinase domain, and the interdomain linker are shown. Critical autophosphorylation sites (T446, T451) in the kinase domain are indicated. (B) Native gel mobility shift assay for PKR derivatives (600 nM) binding to VA_I (200 nM). (C) Summary of dissociation constants (μM) at 30 °C for titration of dsRNA (10 μM, sample cell) with PKR derivatives added *in trans* (150 μM, syringe). Thermodynamic parameters are included in the supplemental materials.

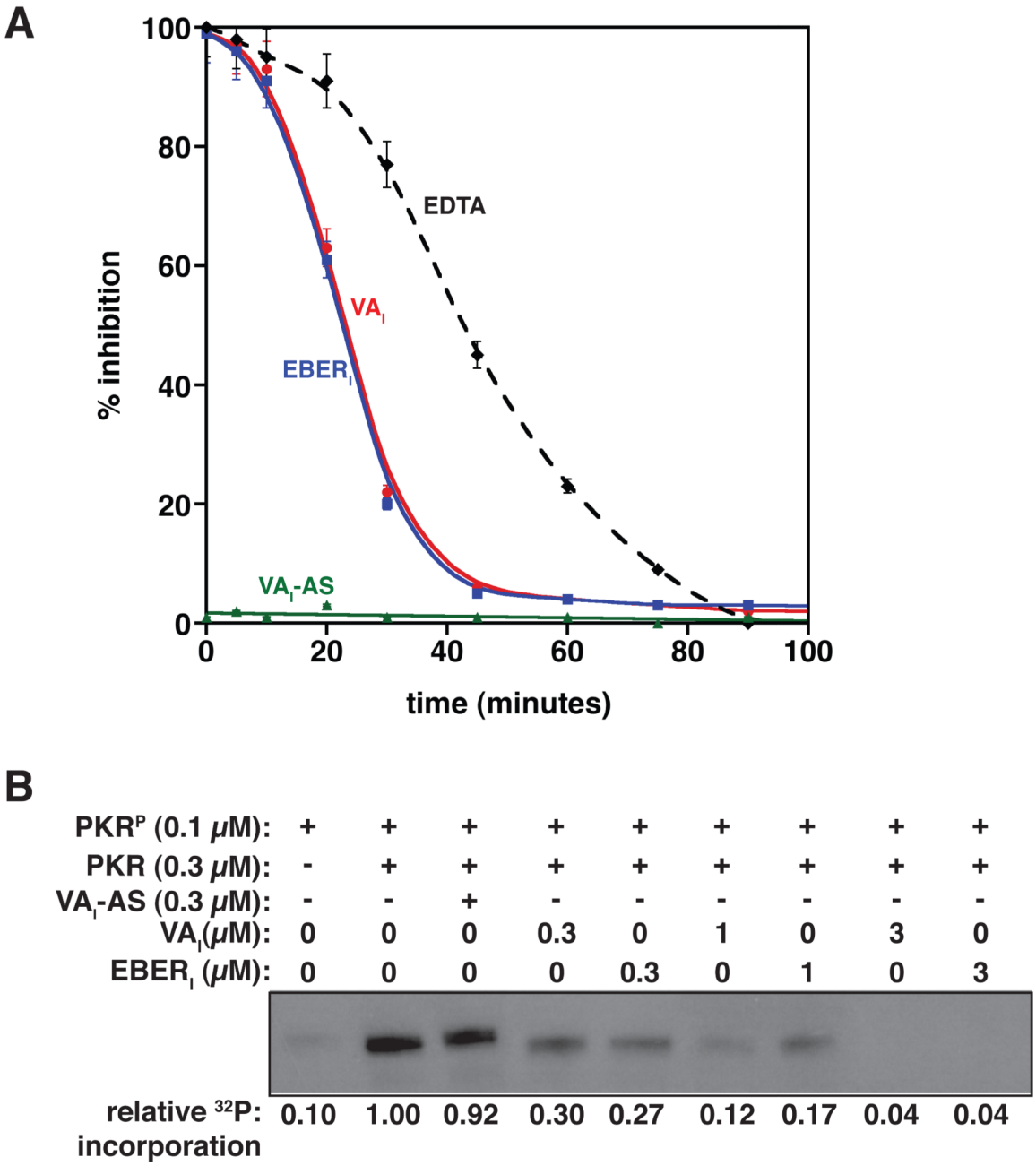

Figure 3.

Inhibitors prevent latent PKR from *trans*-phosphorylation. (A) Inhibition of PKR autophosphorylation in the presence of HIV-TAR activator. Either EDTA, VA_I, or $EBER_I$ was added to a reaction mixture containing PKR, TAR, ATP and $MgCl_2$ at various time points as indicated. The reaction was then allowed to proceed for the complete 2 hours. Each time point was performed in triplicate, resolved by SDS-PAGE, and quantitated for ^{32}P incorporation by autoradiography. (B) Trans-autophosphorylation assays in which PKR^P was used to phosphorylate PKR in the presence of increasing amounts of inhibitor (VA_I or $EBER_I$). Reaction mixtures were incubated in the presence of [γ-^{32}P]ATP at 30°C for 15 minutes, and quenched by addition of EDTA. SDS-PAGE separation followed by autoradiography is shown.

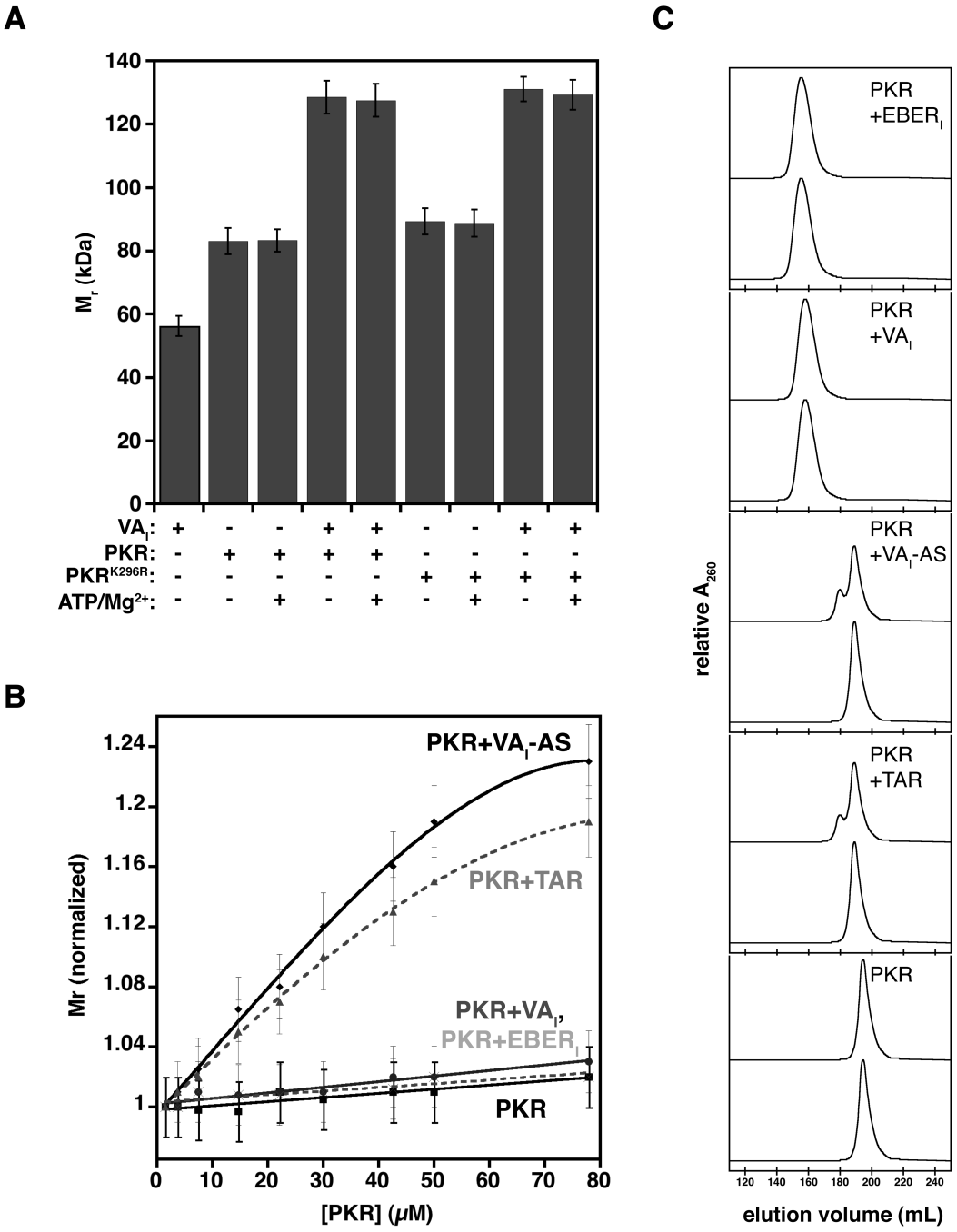

Figure 4.

Inhibitors of PKR prevent its self-association (A) Molecular weight of PKR-VA_I complexes (5 μM) as determined by DLS without incubation at 30 °C. (**B)** Concentration-dependent dimerization of PKR was examined by determining the molecular weight at the specified concentration of PKR (solid black line, squares), PKR-VA_I (solid black line, circles), PKR-$EBER_I$ (dashed grey line, crosses), PKR-VA_I-AS (solid black line, diamonds), or PKR-TAR (dashed grey line, triangles). Each data point was repeated in triplicate and error bars reflect standard deviation associated with measurements. **(C)** Superdex 200

HiLoad 26/60 size exclusion chromatography elution profiles of complexes at 5 μM (bottom) and 80 μM (top).

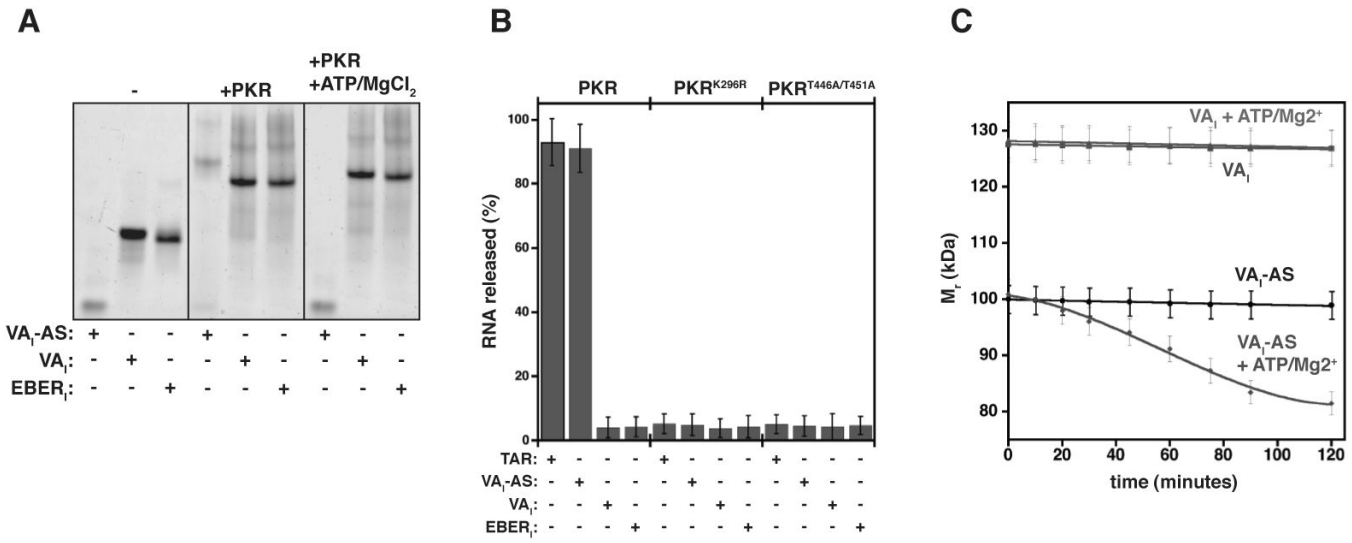

Figure 5.

Inhibitory dsRNAs remain associated with PKR under activating conditions (A) Native gel mobility-shift for VA_I, $EBER_I$, or VA_I-AS (200 nM) binding to PKR (400 nM) in reactions containing ATP (1 mM) and $MgCl_2$ (2 mM) in certain cases. Samples were incubated at 30 °C for 90 minutes, resolved on 5% TBE gels, and visualized by SybrGreenII staining. **(B)** PKR-dsRNA complexes were pre-assembled (300 nM), and incubated at 30 °C for 90 minutes in the presence or absence of ATP (1 mM) and $MgCl_2$ (1 mM)**.** RNA release was quantified by resolving reaction components on native 5% TBE gels and dsRNA staining by SybrGreenII. Each data point represents a triplicate measurement. **(C)** Time-dependent molecular weight determination PKR-dsRNA complexes. Equimolar complexes of PKR and dsRNA (2 μM) were incubated in the cuvette at 30°C for the time specified prior to acquisition. ATP (1 mM) and $MgCl_2$ (1 mM) were included in some cases. Each data point was repeated in triplicate.

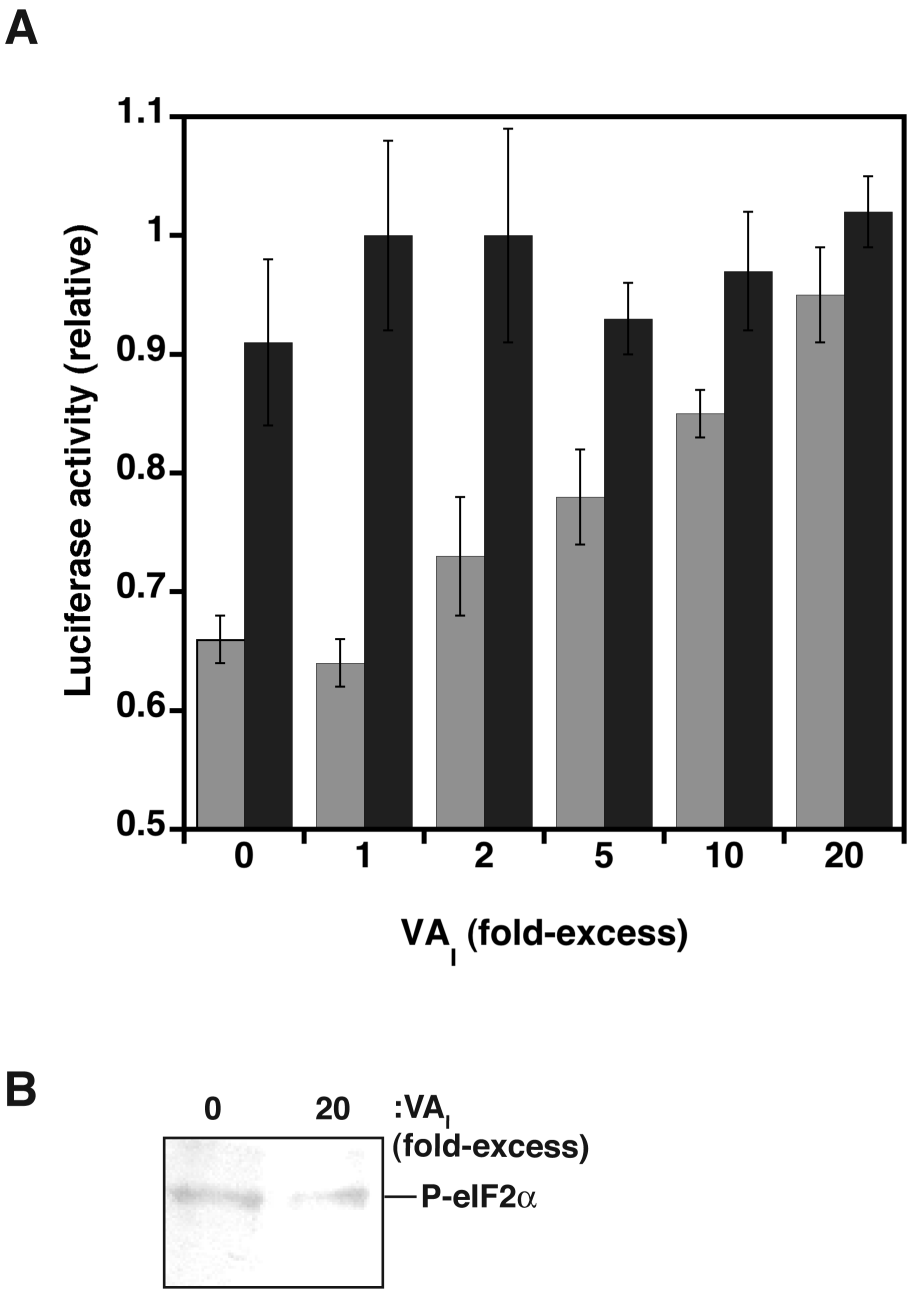

Figure 6.

Inhibitors of PKR result in translational recovery. (A) HeLa S10 cell lysates containing the protein translation machinery, exogenous PKR (10 nM), and 5'-capped luciferase mRNA (50 nM) are incubated for 1 hour at 30 °C in the presence (grey) or absence (black) of HCV-IRES activator dsRNA (50 nM) and increasing amounts of VA_I inhibitor (0−2 μM). Measurements were performed in triplicate and standardized relative to maximum Luciferase activity. **(B)** Western blot probed with an anti-(P-Ser51)-eIF2a antibody (ab4837−50, abcam) for selected reactions shown in (A).

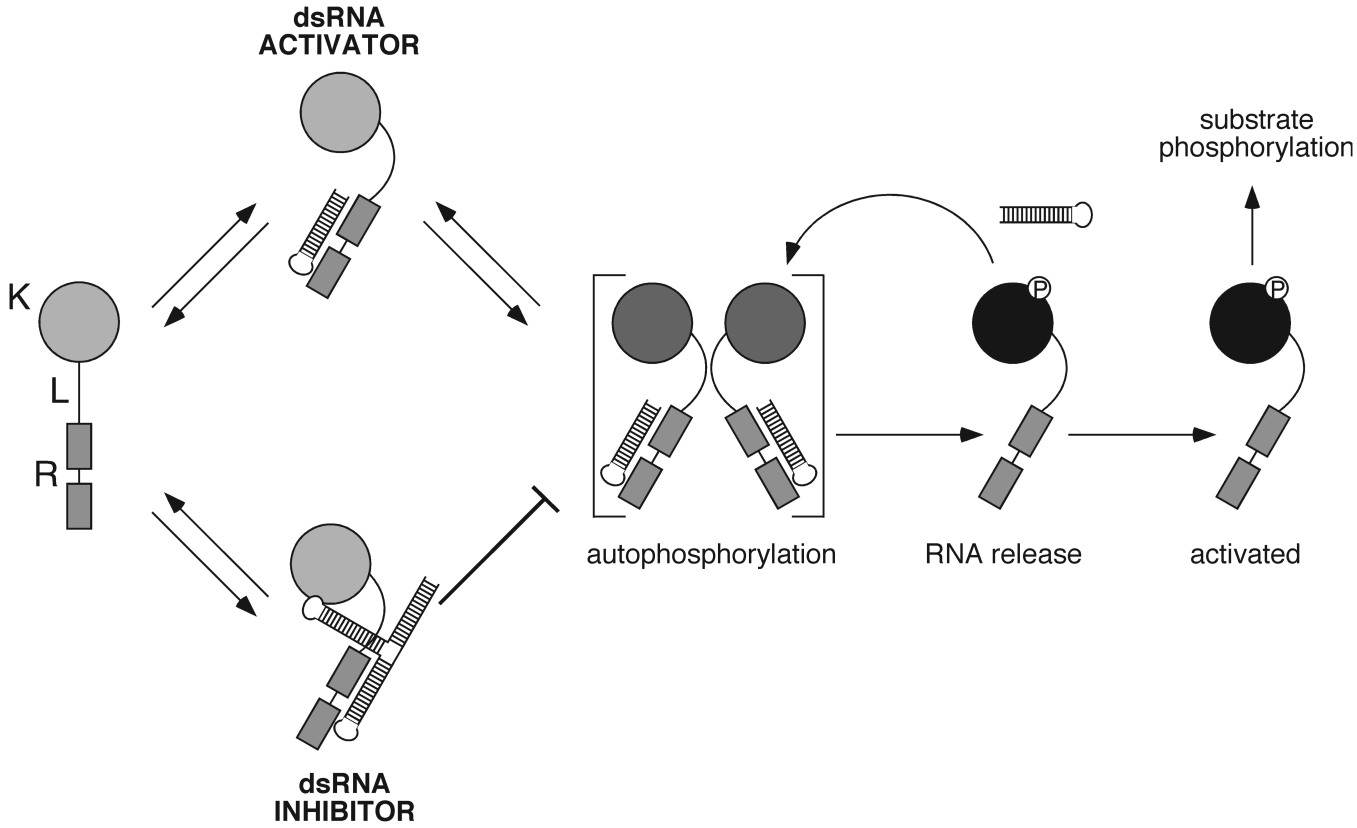

Figure 7.

Model for the inhibition of PKR kinase activity. Model summarizing the framework for the inhibition of PKR; dsRBDs (R), kinase domain (K) and interdomain linker (L). Upon binding of activator dsRNA to the dsRBDs of PKR in the latent form, enhanced bimolecular interaction between two PKR molecules is observed. In this conformation, autophosphorylation occurs, leading to RNA release and activation competency of PKR. Subsequently, activated PKR may feed back to trans-phosphorylate remaining latent PKR. Addition of inhibitory dsRNA leads to interaction with the latent form of PKR; binding prevents dimerization and prevents PKR from acting as a substrate for *trans*-autophosphorylation. Structured stem-loops not required for interaction with the dsRBDs may be responsible for mediating inhibition.