Activation of PKR by short stem–loop RNAs containing single-stranded arms

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ABSTRACT

Protein kinase R (PKR) is a central component of the innate immunity antiviral pathway and is activated by dsRNA. PKR contains a C-terminal kinase domain and two tandem dsRNA binding domains. In the canonical activation model, binding of multiple PKR monomers to dsRNA enhances dimerization of the kinase domain, leading to enzymatic activation. A minimal dsRNA of 30 bp is required for activation. However, short (~15 bp) stem–loop RNAs containing flanking single-stranded tails (ss-dsRNAs) are capable of activating PKR. Activation was reported to require a 5′-triphosphate. Here, we characterize the structural features of ss-dsRNAs that contribute to activation. We have designed a model ss-dsRNA containing 15-nt single-stranded tails and a 15-bp stem and made systematic truncations of the tail and stem regions. Autophosphorylation assays and analytical ultracentrifugation experiments were used to correlate activation and binding affinity. PKR activation requires both 5′- and 3′-single-stranded tails but the triphosphate is dispensable. Activation potency and binding affinity decrease as the ssRNA tails are truncated and activation is abolished in cases where the binding affinity is strongly reduced. These results indicate that the single-stranded regions bind to PKR and support a model where ss-dsRNA induced dimerization is required but not sufficient to activate the kinase. The length of the duplex regions in several natural RNA activators of PKR is below the minimum of 30 bp required for activation and similar interactions with single-stranded regions may contribute to PKR activation in these cases.

Keywords: dsRNA; innate immunity; PKR; protein kinase

INTRODUCTION

Protein kinase R (PKR) is a key component of the interferon-induced viral response pathway (Toth et al. 2006). PKR is synthesized in a latent state and is activated via autophosphorylation upon binding to duplex regions present in viral RNAs (Nallagatla et al. 2011). Activated PKR then phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2α), leading to inhibition of protein synthesis in infected cells. The large number and varieties of virally encoded PKR inhibitors highlights the importance of the PKR antiviral pathway (Langland et al. 2006). PKR also phosphorylates insulin receptor substrate (Nakamura et al. 2010) and participates in pathways regulating stress response, cellular growth and proliferation, nutrient signaling, and metabolism (García et al. 2006; Pindel and Sadler 2011).

PKR contains two tandem dsRNA binding domains and a C-terminal kinase domain connected by a flexible ~90-residue linker. This linker allows PKR to sample multiple compact and extended conformations in solution (Vanoudenhove et al. 2009). A complex of the PKR kinase domain and eIF2α crystallizes as a dimer with the N-terminal lobe of the kinase domain forming the dimer interface (Dar et al. 2005). Dimerization plays a key role in the PKR activation mechanism (Robertson and Mathews 1996; Cole 2007). A potential allosteric pathway has been identified linking the dimer interface with the kinase active site (Dar et al. 2005; Dey et al. 2005). PKR dimerizes weakly in solution and dimerization is sufficient to activate PKR in the absence of RNA (Lemaire et al. 2005). Activation of PKR by dsRNA shows a characteristic bell-shaped dependence on dsRNA concentration (Hunter et al. 1975; Manche et al. 1992). This behavior is rationalized in a model where high dsRNA concentration reduces the number of PKR monomers bound to the same dsRNA and thus inhibits dimerization (Kostura and Mathews 1989). A minimum length of 30 bp of dsRNA is required to induce PKR autophosphorylation (Manche et al. 1992; Lemaire et al. 2008; Husain et al. 2012) and dimerization of PKR on dsRNA (Husain et al. 2015). Consistent with the dimerization model, dsRNAs capable of inducing PKR activation bind at least two PKR monomers (Lemaire et al. 2005). This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
A novel family of RNA activators of PKR has been described that contains a 16-bp imperfect duplex stem capped by a variable length loop with flanking ssRNA regions on the 5′- and 3′-ends (ss-dsRNAs) (Zheng and Bevilacqua 2004; Nallagatla et al. 2007). The presence of a 5′-triphosphate (5′-ppp) and minimal ssRNA tails of 9 nt on the 5′- and 10 nt on the 3′-side are crucial to PKR activation. Activation is also observed for longer (≥47 nt) ssRNAs containing short (5 bp) stems. A 5′-ppp is an important structural feature for activation of RIG-I (Hornung et al. 2006; Pichlmair et al. 2006), an intracellular sensor of viral RNAs. The 5′-ppp binding site lies within a positively charged pocket at the C-terminus of RIG-I (Cui et al. 2008; Takahasi et al. 2008; Lu et al. 2010; Luo et al. 2012). However, sites for ssRNA or triphosphate binding have not been identified in PKR (Toroney et al. 2012). The duplex regions in all of these RNAs are too short to mediate PKR activation alone and the mechanism by which the 5′-ppp and ssRNA regions elicit activation is not known.

Here, we characterize the structural features of ss-dsRNAs that contribute to PKR binding and activation. We have designed a model ss-dsRNA PKR activator containing two single-stranded tails of 15 nt and a 15-bp stem (15–15–15) and made systematic truncations of the tail and stem regions. Our studies confirm that stem–loops containing flanking ssRNA regions function as PKR activators. However, we find that the 5′-ppp is dispensable for activation and does not significantly contribute to RNA binding affinity. In contrast, both 5′- and 3′- ssRNA arms are required for PKR activation and affect binding affinity. The binding affinity and activation potency generally decrease as the tails are truncated and weakly binding RNAs do not activate. However, activity is not correlated with binding for ss-dsRNAs with intermediate affinities. These data support a model where ss-dsRNA induced dimerization is required but not sufficient to activate the kinase.

RESULTS

Characterization of ss-dsRNAs

We have designed a series of ss-dsRNAs to characterize the length dependence of ssRNA tails and stem–loop region and contribution of the 5′-triphosphate to the binding energetics and activation of PKR. Truncations were made to a model ss-dsRNA with a 15-bp duplex stem capped by a stable UCCG tetraloop (Dale et al. 2000) flanked by 15-nt single-stranded tails. The sequences of the ssRNA tails have been designed to prevent formation of extraneous secondary structures. Tail truncation constructs were designed by deletion of nucleotides immediately flanking the stem. Additional mutations within the single-stranded regions were required in some cases to disrupt formation of alternative secondary structures. The sequences are indicated in Supplemental Table S1. Analysis of the sequences using MFOLD (Zuker 2003) indicate the absence of alternative folded conformations (data not shown). Enzymatic structure probing experiments confirm the predicted secondary structure of the model 15–15–15 ss-dsRNA (Fig. 1). Thermal denaturation measurements indicate a single cooperative transition associated with unfolding of the 15-bp stem in both 15–15–15 and in the deletions constructs (Supplemental Fig. S1). Thus, the tails do not adopt unwanted secondary structure. Self-complementary RNA hairpins have the potential to dimerize, which can complicate analysis of their interactions with PKR (Heinicke and Bevilacqua 2012; Heinicke et al. 2009). Sedimentation velocity analysis indicates that all of the ss-dsRNAs are homogeneous and monomeric (Supplemental Table S2).

Effect of tail deletions

The PKR binding stoichiometries and affinities of the ss-dsRNAs were measured using a sedimentation velocity analytical ultracentrifugation method (Wong et al. 2011). Initial experiments were performed in a buffer containing 200 mM NaCl (AU200) to correlate with our previous PKR–RNA binding measurements (Lemaire et al. 2008; Heinicke et al. 2009; Launer-Felty et al. 2010). The data were first analyzed by the time derivative method (Stafford 1992; Philo 2006) to determine the qualitative behavior of the system and to define the correct association model to use for subsequent global analysis (Wong et al. 2011). As shown in Supplemental Figure S2, the peak of the g^A(s*) distribution for ppp-15–15–15 shifts to the right from ~3 S to ~5 S when PKR binds due to formation of a protein–RNA complex with a higher sedimentation coefficient. The shift is saturated upon addition of six equivalents of PKR. Assuming a 1:1 binding stoichiometry, a sedimentation coefficient of ~5 S corresponds to a frictional ratio f/f0 ∼1.5 (Supplemental Table S3), which is in the range that we typically observe for PKR–RNA complexes (Wong et al. 2011). Thus, the sedimentation data are consistent with binding of a single PKR to ppp-15–15–15 in AU200.

PKR dissociation constants were obtained by globally fitting the sedimentation velocity data using SEDANAL (Stafford and Sherwood 2004). We obtain an excellent fit of the ppp-15–15–15 data to a 1:1 binding model with Kd = 360 nM and a low RMS deviation of 0.00587, which is on the order of the noise level of the absorbance optics (Table 1; Supplemental Fig. S2). Hydrodynamic calculations indicate that each of the tail deletion RNAs also binds a single PKR in AU200 (Supplemental Table S3). Deletion of the 5′-tail has a negligible effect on Kd, whereas deletion of the 3′-tail or both tails decreases binding affinity by about threefold (Table 1).

Activation of PKR by ss-dsRNAs was assayed by measuring PKR autophosphorylation in AU200. Deletion of either of
the flanking ssRNA tails abolishes the ability to activate (Fig. 2A). At the PKR concentration used in the assay, the maximum level of activation occurs at 1 µM and is inhibited at higher RNA concentrations, giving rise to a bell-shaped curve similar to activation by simple dsRNAs (Fig. 2B). Our previous analysis of a truncation series of dsRNAs revealed a correlation between the ability to activate PKR autophosphorylation and the binding of two or more monomers in AU200 (Lemaire et al. 2008; Husain et al. 2012). Thus, it is noteworthy that ppp-15–15–15 functions as a potent activator without detectable formation of a 2:1 species in 200 mM NaCl. The bell-shaped activation curve suggests that like duplex RNAs, ss-dsRNAs activate PKR by facilitating dimerization of PKR on the RNA. The amount of 2:1 species populated may be below the detection limits for the sedimentation velocity analysis.

The affinity of PKR binding to dsRNA is strongly dependent on monovalent ion concentration (Clarke et al. 1994; Bevilacqua and Cech 1996) and the stoichiometries can increase upon decreasing [NaCl] from 200 to 75 mM (Lemaire et al. 2008; Husain et al. 2012). Figure 3 shows the normalized g(∗) distributions of a PKR titration against ppp-15–15–15 in 75 mM NaCl (AU75 buffer). Addition of a large molar excess of PKR causes a large increase in the sedimentation coefficient to ∼8 S, whereas the maximal sedimentation coefficient in 200 mM NaCl is only ∼5 S (Supplemental Fig. S2). A sedimentation coefficient of 8 S is larger than expected for a 1:1 complex of PKR and ppp-15–15–15 but is consistent with formation of a 2:1 complex (Supplemental Table S3). Reducing the NaCl concentration to 75 mM also induces a large increase in the maximum sedimentation coefficient for PKR binding to the nonactivating RNAs ppp-0–15–15, ppp-15–15–0, and ppp-0–15–0 (Supplemental Table S3). Again, the limiting sedimentation coefficients of the protein–RNA complexes indicate that two PKR monomers also bind to these RNAs under these conditions. Despite the ability to bind two PKR monomers, the tail deletion constructs are deficient in enzymatic activation measured at 75 mM NaCl (Figs. 4, 5). Thus, the binding stoichiometries alone cannot explain the difference in PKR activation among these ss-dsRNAs.

Global analysis reveals significant differences in the PKR binding affinities for the activating and nonactivating ss-dsRNAs measured in AU75. PKR binds strongly to ppp-15–15–15 with KD1 and KD2 lying in the low and mid nanomolar region, respectively (Table 2). Deletion of the 3′-tail increases KD1 by about sixfold and KD2 by about threefold. Deletion of the 5′-tail results in a 30-fold increase in KD1 and a 2.5-fold increase in KD2. The largest penalty is observed when both tails are deleted.

**TABLE 1.** PKR binding to tail deletion constructs measured in AU200 buffer at 20°C

| RNA                  | s(RNA)   | KD (nM) | s(RP)  | RMSD    |
|----------------------|----------|---------|--------|---------|
| ppp-15–15–15         | 3.19 (3.17, 3.21) | 360 (304, 420) | 5.17 (5.09, 5.26) | 0.00587  |
| ppp-0–15–15          | 2.66 (2.66, 2.67) | 449 (409, 491) | 4.72 (4.67, 4.77) | 0.00652  |
| ppp-15–15–0          | 2.90 (2.90, 2.91) | 1240 (1169, 1319) | 4.96 (4.91, 5.01) | 0.00483  |
| ppp-0–15–0           | 2.43 (2.42, 2.45) | 935 (840, 1042) | 4.52 (4.49, 4.56) | 0.00607  |

Parameters obtained by global nonlinear least square analysis of sedimentation velocity experiments. The values in parentheses represent the 95% joint confidence intervals obtained using the F-statistic.

aUncorrected sedimentation coefficient of RNA in Svedbergs.
bUncorrected sedimentation coefficient of RNA–protein complex in Svedbergs.
cRoot mean square deviation in absorbance units.
For a panel of dsRNAs of increasing length, the rate of PKR autophosphorylation is proportional to the maximum population of PKR that is contained in RNA complexes containing two PKR monomers, denoted RP2 (Lemaire et al. 2008; Husain et al. 2012). Table 2 shows the maximum percentage of RP2 for the ss-dsRNAs calculated using the experimentally determined dissociation constants. The activating ppp-15′-15′-15′ RNA produces a maximum of ∼13% RP2, and the non-activating tail deletion constructs induce about half as much.

Further analysis of the ss-dsRNA structure-activity relationships was carried out in 75 mM NaCl so that correlations could be made between the population of the RP2 species and activation potency. Magnesium is required for kinase activity and is present in our autophosphorylation assays at 5 mM. However, we observe a significant decrease in binding affinities upon addition of 5 mM Mg2+, which complicates measurement of the second dissociation in a background of 75 mM NaCl (Supplemental Table S4). Although PKR binding to dsRNAs is not regulated by divalent ion (Bevilacqua and Cech 1996), the interaction of PKR with a large, complex RNA, Adenovirus VAI, is modulated by Mg2+ (Launer-Felty et al. 2010). We asked whether Mg2+ affects PKR’s interaction with ss-dsRNAs by comparing binding affinities in AU75 and AU60/5 Mg buffers, where the increase in ionic strength conferred by the addition of 5 mM Mg2+ to AU75 is compensated for by reducing the NaCl concentration to 60 mM. For both ppp-15′-15′-15′ and ppp-0′-15′-0′, which lacks single-stranded tails, the binding affinities decrease strongly in AU60/5 Mg (Supplemental Table S4). This observation is consistent with previous studies indicating that Mg2+ is ~100-fold more efficient at charge screening for RNAs compared to monovalent cations (Bizarro et al. 2012; Chen et al. 2012). As indicated by a similar reduction in the maximum RP2 for the two RNAs, there are no specific Mg2+ effects conferred by the single-stranded tails. Furthermore, thermal denaturation measurements indicate that Mg2+ does not induce formation of additional secondary structure in ss-dsRNAs (data not shown).

**Effect of tail truncations**

We further examined the contribution of each single-stranded tail to binding affinity and activation by creating a series of ss-dsRNAs with 5-nt incremental symmetric and asymmetric truncations of the tails. The portfolio of ss-dsRNAs was designed to resolve the minimum length requirements for activation and test whether the 5′ - and 3′-tails are equivalent in their contribution to affinity and activation. Figure 4 shows the RNA concentration dependence of activation for a series of 5-nt incremental truncations made to either 5′ or 3′ tails while maintaining the 15-nt opposing tail length. Some RNAs exhibit a bell-shaped activation profile while other activation profiles show a monotonic increase with RNA. The absence of a maximum in the activation profile is correlated with weaker binding (vide infra). Activity measurements at ss-dsRNA concentrations >1.5 µM are precluded by potential dimerization of the self-complementary sequences. Truncation of either tail leads to a gradual decrease in activation potency indicating a direct relationship between tail length and stimulation of enzymatic activity. Truncations made to the 5′ tail have a slightly greater effect on PKR activation than those made to the 3′ tail. In particular, ppp-15′-15′-5 retains its ability to activate PKR where little or no activation is detected for ppp-5′-15′-15′.

Binding affinity, and maximum %RP2, decrease in parallel with activation potency as the 3′-tail is reduced in 5-nt increments (Table 2). However, the correlation does not hold for the 5′ tail truncation series. ppp-10′-15′-15′ exhibits weaker
PKR binding than the full-length ss-dsRNA, removal of an additional 5 nt from the 5′-tail results in enhanced affinity such that the ppp-5–15–15 has a maximal %RP2 close to ppp-15–15–15. Despite the similar population of the RP2 species, ppp-15–15–15 is a potent activator whereas ppp-5–15–15 fails to activate, indicating that these parameters are not well correlated for ss-dsRNAs.

The next series of truncations were made to both tails. Figure 5 shows the activation by ss-dsRNAs grouped according to the symmetry of the truncations. Asymmetric truncation to produce ppp-5–15–10 shifts the maximum to slightly higher RNA concentration, whereas ppp-10–15–5 shows weaker activation. The effects of symmetric truncation of both tails are more dramatic: Deletion to 10 nt or less essentially abolishes activation. Within the asymmetric truncation group the relative binding affinity and activation potency are somewhat correlated: ppp-5–15–10 populates a higher percentage of RP2 and is a more potent activator than ppp-10–15–5 (Table 2). The correlation is less clear for the symmetric constructs. PKR binding to ppp-10–15–10 is only slightly reduced relative to the parent ppp-15–15–15 yet it does not activate.

Stem deletions

The effects of decreasing the length of the duplex stem were investigated by creating ppp-15–10–15 and ppp-15–5–15. PKR is potently activated by ppp-15–10–15 with a maximum

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**FIGURE 3.** Sedimentation velocity analysis of PKR binding to ppp-15–15–15. (A) Normalized g*(s*) distributions for 0.7 μM 15–15–15 alone (black) and 0.7 μM 15–15–15 plus 0.5 eq. (blue), 1 eq. (green), 1.5 eq. (red), 3 eq. (brown), and 6 eq. PKR (purple). The distributions are normalized by area. (B) Global analysis of the sedimentation difference curves. Scans within each data set were subtracted in pairs to remove time-invariant background and fit to a sequential 2:1 binding model using SEDANAL (Stafford and Sherwood 2004). The top panels show the data (points) and the fitting results using the parameters presented in Table 2 (continuous lines). The bottom panels show residuals. Only every other difference curve is shown for clarity. Measurements were performed in AU75 buffer at 20°C and 40,000 rpm using absorbance detection at 260 nm.
with T4 polynucleotide kinase and [γ-32P]ATP because of the presence of a 5′-ppp, but the corresponding dephosphorylated RNAs are substrates (Fig. 7B). Mass spectroscopy analysis indicates that dephosphorylation results in a mass decrease of 300–315 Da, consistent with a predicted change of 300 Da (removal of the 5′-ppp and the 2′–3′ cyclic phosphate produced by the HDV ribozyme cleavage) (Fig. 7C). Additionally, an ss-dsRNA labeled during in vitro transcription in the presence of [γ-32P]GTP displays complete removal of the 5′-ppp after phosphatase treatment (Supplemental Fig. S4), indicating that the activity of the 5′-OH RNAs is not due to the presence of residual triphosphate-containing RNA. Removal of the 5′-ppp does affect PKR binding affinity (Table 4). $K_{d1}$ increases about 10-fold and $K_{d2}$ increases slightly. However, the maximum %RP2 does not change substantially and the binding parameters remain in the range of ss-dsRNAs that activate, such as ppp-15-15-5 and ppp-5-15-10.

**DISCUSSION**

We have systematically dissected a model ss-dsRNA activator to determine the contributions of the single-stranded tails, stem-loop, and the 5′-triphosphate to PKR activation and binding. The model ss-dsRNA activates PKR level about equal to the control ss-dsRNA (Fig. 6). Further reduction of the stem to 5 bp abolishes RNA-induced autophosphorylation. In the case of the stem deletions, the binding affinities correlate with activation (Table 3). For the 10-bp stem construct, $K_{d1}$ is increased somewhat but $K_{d2}$ is essentially unchanged, resulting in a similar %RP2. For the 5-bp construct, both $K_d$ values are significantly increased, consistent with the loss of measureable activation.

5′-Triphosphate

It has been reported that a 5′-ppp is crucial for activation of PKR by the canonical ss-dsRNAs as well as ssRNAs containing short duplexes (Nallagatla et al. 2007; Toroney et al. 2012). However, dephosphorylation of ppp-15-15-15 and ppp-5-15-10 by treatment with calf intestinal alkaline phosphatase (CIP) has no significant effect on the extent of PKR activation (Fig. 7A). Control experiments verify that the 5′-ppp is removed by the phosphatase. ppp-15-15-15 and ppp-5-15-10 cannot be labeled with a 5′-32P by treatment with T4 polynucleotide kinase and [γ-32P]ATP because of the presence of a 5′-ppp, but the corresponding dephosphorylated RNAs are substrates (Fig. 7B). Mass spectroscopy analysis indicates that dephosphorylation results in a mass decrease of 300–315 Da, consistent with a predicted change of 300 Da (removal of the 5′-ppp and the 2′–3′ cyclic phosphate produced by the HDV ribozyme cleavage) (Fig. 7C). Additionally, an ss-dsRNA labeled during in vitro transcription in the presence of [γ-32P]GTP displays complete removal of the 5′-ppp after phosphatase treatment (Supplemental Fig. S4), indicating that the activity of the 5′-OH RNAs is not due to the presence of residual triphosphate-containing RNA. Removal of the 5′-ppp does affect PKR binding affinity (Table 4). $K_{d1}$ increases about 10-fold and $K_{d2}$ increases slightly. However, the maximum %RP2 does not change substantially and the binding parameters remain in the range of ss-dsRNAs that activate, such as ppp-15-15-5 and ppp-5-15-10.

**FIGURE 4.** Activation of PKR by single tail truncation constructs in 75 mM NaCl. Autophosphorylation assays were performed as previously described (Lemaire et al. 2005) in AU75 buffer at 32°C. The signals quantified by phosphorimager analysis were normalized to the 32P-incorporation in control samples containing 100 nM ppp-15-15-15. Error bars represent the standard deviation of three experiments. (A) 5′ tail truncations: ppp-15-15-15 (black), ppp-10-15-15 (blue), ppp-5-15-15 (red), ppp-0-15-15 (green). (B) 3′ tail truncations: ppp-15-15-15 (black), ppp-15-15-10 (blue), ppp-15-15-5 (red), ppp-15-15-0 (green).

**FIGURE 5.** Activation of PKR by double tail truncation constructs in 75 mM NaCl. Experiments were performed and analyzed as described for Figure 4. (A) Asymmetric truncations: ppp-15-15-15 (black), ppp-10-15-10 (blue), ppp-5-15-5 (red), ppp-0-15-0 (green). (B) Symmetric truncations: ppp-15-15-15 (black), ppp-10-15-10 (blue), ppp-5-15-5 (red), ppp-0-15-0 (green).
autophosphorylation strongly and the presence of both flanking 5′- and 3′-ssRNA tails are required for activation. When either tail is truncated, the activation potency drops with tail length and truncation of the 5′-tail is more deleterious to activation than the 3′-tail. However, the effects of tail truncations are not simply additive as the lengths of the tails are reduced. For example, the symmetric construct containing 10 nt 5′ and 3′ tails does not activate PKR, yet ppp-10–15–5 and ppp-5–15–10 both stimulate autophosphorylation. Activation is also sensitive to stem length: The titration is shifted to higher RNA concentrations for the 10-bp construct and the 5-bp construct is inactive. Finally, the 5′-ppp is not required for activation.

The ss-dsRNA activation motif was originally identified and characterized by the Bevilacqua group (Zheng and Bevilacqua 2004; Nallagatla et al. 2007). It was reported that either the 5′- or the 3′-ssRNA tails were dispensable for activation, with minimal length requirements of 9 and 10 nt for the 5′ and 3′ tails, respectively (Zheng and Bevilacqua 2004). Here, we find that both tails are required for activity and symmetric deletion to 10 nt 5′ and 3′ tails blocks activation. It has been reported that a 5′-ppp is crucial for activation of PKR by the canonical ss-dsRNAs as well as ssRNAs containing short duplexes (Nallagatla et al. 2007; Toroney et al. 2012). In contrast, we find that this moiety does not significantly contribute to PKR activation and only slightly affects binding affinity. We also find that PKR activation by a previously described ss-dsRNA (Zheng and Bevilacqua 2004; Nallagatla et al. 2007) does not require a 5′-triphosphate (C Mayo and JL Cole, unpubl.). snoRNAs, which contain short duplexes interspersed with single-stranded regions, were recently reported to activate PKR. In some cases activation is dependent on a 5′-ppp, whereas others are active in 5′-OH and 5′-p states (Youssef et al. 2015), suggesting that the structural context can modulate the triphosphate dependence.

The bell-shaped curve for activation by ss-dsRNAs supports a model in which lower RNA concentrations activate by inducing PKR dimerization and high RNA concentrations dissociate active, RNA-bound PKR dimers onto separate molecules of dsRNA (Kostura and Mathews 1989; Cole 2007). However, at high salt (200 mM NaCl), our AUC experiments detect the binding of only one PKR monomer to both activating and nonactivating RNAs. Possibly, the observed activation is mediated by very weak binding of a second PKR monomer. At lower salt (75 mM NaCl), ss-dsRNAs bind two PKRs. In cases where the binding affinity is strongly reduced, such that the maximal RP2 <10%,
activation is abolished or greatly attenuated. However, it is not possible to predict activation based on $K_{d1}$, $K_{d2}$ or maximal % RP$_2$ for the ss-dsRNAs with intermediate binding affinities: Some (e.g., ppp-5–15–15) are inactive and others (e.g., ppp-5–15–10) are quite active. Thus, a threshold concentration of RP$_2$ is required, but not sufficient, to elicit PKR autophosphorylation by ss-dsRNA. Potentially, specific sequences in the tail regions may contribute to the relative activation potencies.

Our results implicate the 5′- and the 3′-ssRNA regions in the mechanism of activation of PKR by ss-dsRNAs. Although it has been reported that dsRNA binding domains do not bind to ssRNA (St Johnston et al. 1992; Bass et al. 1994), and PKR is not activated by ssRNAs (Hunter et al. 1975; Sen et al. 1978), we have observed that PKR binds to a 30-nt ssRNA and have identified a possible binding site near the kinase domain (C Mayo and JL Cole, unpubl.). Furthermore, the presence of 5′- and 3′-tails in ss-dsRNAs enhances PKR binding affinity, suggesting that the tails directly bind to PKR. Note that tethering of the ssRNA region of a ss-dsRNA to PKR via the stem–loop would enhance ssRNA binding to PKR by increasing the effective local concentration of the ligand (Zhou and Gilson 2009).

Conventional binding assays and analytical ultracentrifugation measurements can determine how many PKRs are bound to an RNA but do not directly probe dimerization via the kinase domain interface. Therefore, we recently developed a sensitive homo-FRET assay to directly probe PKR kinase domain dimerization on RNAs (Husain et al. 2015). Activating duplex RNAs containing 30 bp bind two PKRs and induce dimerization. However, some nonactivating RNAs that also produce a high population of the RP$_2$ species either fail to induce dimerization or produce an alternative, inactive dimer configuration. This assay indicates that the activating ss-dsRNA ppp-15–15–15 induces a comparable extent of kinase domain dimerization as obtained with a 40-bp duplex RNA (C Mayo, B Husain, and JL Cole, unpubl.). We propose that the flanking ssRNA arms in activating ss-dsRNAs may directly engage a basic region near the kinase domain, orienting two PKR monomers into productive kinase domain dimers.

There is evidence that the single-stranded regions may contribute to PKR activation in complex, natural RNAs. In the context of perfect duplex dsRNA, a length of at least 30 bp of dsRNA is required to activate PKR autophosphorylation (Manche et al. 1992; Lemaire et al. 2008). Interestingly, the length of the duplex regions in several natural RNA activators of PKR, including HCV IRES (Shimoike et al. 2009; Toroney et al. 2010), TNFα mRNA (Osman et al. 1999), interferon-γ mRNA (Ben-Asouli et al. 2002) and the 3′-UTR regions of several cytoskeletal muscle mRNAs (Nussbaum et al. 2002) are below the 30 bp required for PKR activation. Similarly, influenza B ribonucleoprotein is a potent PKR

**FIGURE 6.** Activation of PKR by stem truncation constructs in 75 mM NaCl. Experiments were performed and analyzed as described for Figure 5. ppp-15–15–15 (black), ppp-15–10–15 (blue), ppp-15–5–15 (red).

**FIGURE 7.** Activation by ss-dsRNAs does not require a 5′-triphosphate. (A) Activation by 5′-triphosphate and 5′-OH ss-dsRNAs. 5′-OH RNAs were prepared by treatment with CIP as described in Materials and Methods. Samples contain 300 nM RNA. Autophosphorylation activity is indicated under the gel as a percentage normalized to activation by ppp-15–15–15. (B) Assay for dephosphorylation by 5′-32P labeling by T4 PNK. RNAs containing a 5′-OH are substrates for T4 PNK, whereas 5′-ppp prevents phosphorylation. (C) MALDI-ToF analysis of dephosphorylation of ppp-15–15–15. (D) MALDI-ToF analysis of dephosphorylation of ppp-5–15–10. ppp-15–15–15 exhibits a mass loss of 300 Da and ppp-5–15–10 exhibits a mass loss of 315 Da. The predicted mass loss is 300 Da (removal of the 5′-ppp and the 2′-3′-cyclic phosphate resulting from HDV ribozyme cleavage).
activator that contains only a short 14- to 16-bp “panhandle" (Dauber and Wolff 2009). Thus, the single-stranded regions may contribute to PKR activation in these complex natural RNAs.

MATERIALS AND METHODS

All buffers were made from reagent grade chemicals with deionized, distilled water (Mili-Q, Millipore). Unphosphorylated PKR was expressed and purified as previously described (Lemaire et al. 2008; Anderson et al. 2011). To produce homogeneous 3′-ends, RNAs were generated by incubating 100 µL of 2 µM in vitro transcribed RNA with 10 U calf intestinal alkaline phosphatase (CIP, New England BioLabs) in 50 mM Tris pH 7.9, 100 mM NaCl, 10 mM MgCl2, 0.1 mM EDTA, 0.1 mM TCEP) at 32°C for 10 min. Phosphorylation reactions were initiated by addition of ATP to a final concentration of 0.4 mM containing 2 µCi [γ-32P]ATP (Perkin Elmer). Reactions were quenched with sample loading buffer after 20 min and resolved on SDS-PAGE. The gel was then dried and exposed to a phosphor screen then scanned with a Typhoon phosphorimager.

Unless otherwise indicated, autophosphorylation assays were carried out by preincubating 100 nM PKR with various concentrations of RNA in AU75/Mg2+ buffer (20 mM HEPES pH 7.5, 75 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM TCEP) at 32°C for 10 min. Phosphorylation reactions were initiated by addition of ATP to a final concentration of 0.4 mM containing 2 µCi [γ-32P]ATP (Perkin Elmer). Reactions were quenched with sample loading buffer after 20 min and resolved on SDS-PAGE. The gel was then dried and exposed to a phosphor screen followed by quantification on a Typhoon phosphorimager (GE Healthcare). Each gel contained an autophosphorylation reaction performed with ppp-15–15–15 at 100 nM and a 40mer dsRNA at 50 nM. Activation plots were normalized to the ppp-15–15–15 control and the 40mer dsRNA served as an internal standard.

Enzymatic structure probing assays were conducted by incubating 2 µg 5′-32P labeled RNA with varying concentrations of RNases T1, V1, or A (Ambion) and 4 µg yeast RNA in 10 mM Tris pH 7.0, 100 mM KCl, 10 mM MgCl2 at room temperature for 15 min. An alkaline hydrolysis ladder was produced by incubating 5′-32P labeled RNA and yeast RNA in 50 mM sodium carbonate pH 9.2, 1 mM EDTA at 95°C for 2.5, or 15 min. The RNase T1 sequencing ladder was produced by first incubating 5′-32P labeled RNA and yeast RNA in 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 7 M Urea at 50°C for 5 min. Either 1 or 0.1 U of RNase T1 was then added and incubated for 15 min at RT. The cleavage reactions were stopped by ethanol precipitation and resolved by 12% TBE-Urea PAGE. The gel was dried and exposed to a phosphor screen, then scanned with a Typhoon phosphorimager.

| RNA       | KD1 (nM) | KD2 (nM) | Maximum % RP2 | RMSDa |
|-----------|----------|----------|---------------|-------|
| ppp-15–15–15 | 4 (2, 6) | 294 (252, 343) | 12.8 | 0.00821 |
| HO-15–15–15 | 36 (26, 49) | 411 (357, 473) | 9.9 | 0.00861 |

Parameters obtained by global nonlinear least square analysis of sedimentation velocity experiments. The values in parentheses represent the 95% joint confidence intervals obtained using the F-statistic.

*Root mean square deviation in absorbance units.

Activiation of PKR by stem–loop RNAs

Unless otherwise indicated, autophosphorylation assays were carried out by preincubating 100 nM PKR with various concentrations of RNA in AU75/Mg2+ buffer (20 mM HEPES pH 7.5, 75 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM TCEP) at 32°C for 10 min. Phosphorylation reactions were initiated by addition of ATP to a final concentration of 0.4 mM containing 2 µCi [γ-32P]ATP (Perkin Elmer). Reactions were quenched with sample loading buffer after 20 min and resolved on SDS-PAGE. The gel was then dried and exposed to a phosphor screen followed by quantification on a Typhoon phosphorimager (GE Healthcare). Each gel contained an autophosphorylation reaction performed with ppp-15–15–15 at 100 nM and a 40mer dsRNA at 50 nM. Activation plots were normalized to the ppp-15–15–15 control and the 40mer dsRNA served as an internal standard.

Sedimentation velocity analysis of PKR-RNA binding was carried out at 20°C and 40,000 RPM in either AU200 buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM TCEP) or AU75 buffer as previously described (Wong et al. 2011). Initial data analysis was performed using the time derivative method (Stafford 1992) with DCDT+ (Philò 2006) to obtain g^4(s^4) distributions. Distributions were normalized by area (absorbance) for presentation. Multiple data sets were globally fit to association models using SEDANAL (Stafford and Sherwood 2004). Confidence intervals were obtained using the F-statistic to define a statistically significant increase in the variance upon adjusting each parameter from its best-fit value. Correction of sedimentation coefficients to standard conditions was performed using Equation 1.

\[
s_{20,w} = \frac{(1 - \bar{b}p)_{20,w}}{(1 - \bar{b}p)_{20,w}} \eta_{20,w},
\]

where the subscript 20,w refers to the parameter measured at 20°C in pure water, the subscript Tb refers to the parameter measured at temperature T in buffer, s is the sedimentation coefficient, \( \bar{b} \) is the partial specific volume, \( \rho \) is the buffer density, and \( \eta \) is the buffer viscosity. Frictional ratios (f/R0) for RNAs and RNA-PKR complexes were calculated using Equation 2.

\[
f/R_0 = \frac{M(1 - \bar{b}p)_{20,w}}{N_A 6 \pi \eta R_{20,w}^{3/2} M^{1/3}},
\]

where \( N_A \) is Avogadro’s number and M is the molecular weight.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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