RNA helical imperfections regulate activation of the protein kinase PKR: Effects of bulge position, size, and geometry

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ABSTRACT

The protein kinase, PKR, is activated by long stretches of double-stranded (ds) RNA. Viruses often make long dsRNA elements with imperfections that still activate PKR. However, due to the complexity of the RNA structure, prediction of whether a given RNA is an activator of PKR is difficult. Herein, we systematically investigated how various RNA secondary structure defects contained within model dsRNA affect PKR activation. We find that bulges increasingly disfavor activation as they are moved toward the center of a duplex and as they are increased in size. Model RNAs designed to conform to *cis*, *trans*, or bent global geometries through strategic positioning of one or more bulges decreased activation of PKR relative to perfect dsRNA, although *cis*-bulged RNAs activated PKR much more potently than *trans*-bulged RNAs. Activation studies on bulge-containing chimeric duplexes support a model wherein PKR monomers interact adjacently, rather than through-space, for activation on bulged substrates. Last, unusually low ionic strength induced substantial increases in PKR activation in the presence of bulged RNAs suggesting that discrimination against bulges is higher under biological ionic strength conditions. Overall, this study provides a set of rules for understanding how secondary structural defects affect PKR activity.

Keywords: PKR; dsRNA; innate immunity

INTRODUCTION

PKR is a vital component of the innate immune response and has long been implicated in antiviral host defense mechanisms, where it is activated by long stretches of double-stranded RNA (dsRNA). Upon binding to long dsRNA, PKR undergoes dimerization and autophosphorylation and then phosphorylates its cellular substrate elongation initiation factor 2 α , and consequently translation is inhibited (Lemaire et al. 2005; Cole 2007). Activators of PKR include viral dsRNA replication intermediates from both DNA and RNA viruses, nonviral RNA aptamers, and a pseudoknot IFN- γ mRNA promoter (Jacquemont and Roizman 1975; Katze et al. 1987; Maran and Mathews 1988; Bevilacqua et al. 1998;

Zheng and Bevilacqua 2004; Garcia et al. 2006). PKR interacts with these RNAs in a nonsequence specific fashion, and is regulated by RNAs containing helical defects, such as bulges, internal loops, stem-loops, multistem junctions, and even more complex tertiary interactions, such as pseudoknots and kissing hairpins (Zheng and Bevilacqua 2000; Ben-Asouli et al. 2002; Cohen-Chalamish et al. 2009; Heinicke et al. 2009). Due to the diversity of these RNA structural defects, it has been difficult to predict whether a given RNA structure will activate PKR.

We recently described PKR activation by HIV-1 TAR dimer, which contains multiple bulges (Heinicke et al. 2009). Numerous variants of TAR RNA were constructed to promote dimerization. On the basis of structure mapping and PKR activation results, we found that RNAs with more symmetrical defects were better activators. This observation provided motivation for the present study wherein we investigate how diverse bulges and internal loops affect PKR activation.

The conformation and flexibility of bulges and internal loops in helical RNA and DNA have been previously characterized by various methods, including native PAGE (Hsieh

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2636911>.

and Griffith 1989; Rice and Crothers 1989; Bhattacharyya et al. 1990; Tang and Draper 1990, 1994; Wang and Griffith 1991; Wang et al. 1991), transient electric birefringence (TEB) (Kebbekus et al. 1995; Zacharias and Hagerman 1995a; Lu et al. 2001), and ligase-catalyzed cyclization (Kahn et al. 1994). Electrophoretic mobility and TEB analyses indicate that bulges are not points of flexibility, but rather are rigid (Bhattacharyya et al. 1990; Tang and Draper 1994; Zacharias and Hagerman 1995a). Electrophoretic mobility data support this conclusion on the basis of pronounced retarded gel mobility with increasing bulge size, and less linear global geometry as seen with *cis* geometry of two-bulge containing helices. Base identity also affects mobility, with more retarded mobility being associated with A_n than U_n bulges (Bhattacharyya et al. 1990; Zacharias and Hagerman 1995a). TEB analyses provide quantitative measurements for bend angles. Consistent with mobility measurements, A_n bulges induce more acute bend angles than U_n bulges. Moreover, the addition of bases opposite a bulge, to make an internal loop, reduces bending (Riordan et al. 1992).

The effect of salt on a bulge angle has also been examined. Addition of magnesium ions, which can compact the folds of certain RNA motifs, has little effect on A_n bulge angles (Zacharias and Hagerman 1995a). However, laser light scattering experiments have revealed greater flexibility (i.e., decreased persistence length) of DNA in the presence of increased ionic strength from 5 to 200 to 1000 mM NaCl (Kam et al. 1981).

Unlike bulged nucleic acids, internal loop-containing nucleic acids do not have pronounced changes in electrophoretic mobility, thus requiring a different methodological approach for study. The flexibility of internal loops in DNA has been examined by ligase-catalyzed cyclization experiments (Kahn et al. 1994). Using this approach, internal loops have been found to be quite flexible with no preference for bend orientation.

In the present study, we systematically examine how helical defect position, size, and geometry affect activation of PKR. We find that, in general, PKR activation is enhanced in the presence of less flexible and less bent RNA substrates.

RESULTS

Sequence, geometry, and native gel mobility of bulged RNAs

To investigate the effect of bulges and the role of RNA symmetry in PKR activation, we prepared a series of RNA duplexes containing symmetric or asymmetric adenosine bulges (see Table 1 for shorthand and a list of RNAs studied).

TABLE 1. Summary of RNA duplex constructs and nomenclature

Construct name	LH(center)-RH (bp)	Total bp	Total nt	Bulge size and strand
dsRNA-40 ^a	20-20	40	80	None
bent [2 × 0] ^a	20-20	40	82	[2 × 0] TS
flex [2 × 2] ^a	20-20	40	84	[2 × 2]
dsRNA-51 ^b	20-11-20	51	102	None
dsRNA-51-chi ^{b,c}	20-11(D)-20	51	102	None
dsRNA-52 ^b	20-12-20	52	104	None
dsRNA-53 ^b	20-13-20	53	106	None
<i>cis</i> 1 ^d	20-11-20	51	104	2[1 × 0] TS
<i>trans</i> 1 ^d	20-11-20	51	104	[1 × 0] TS, [1 × 0] BS
<i>cis</i> 1s ^{d,e}	20-5-20	45	92	[1 × 0] TS, [1 × 0] BS
<i>trans</i> 1s ^{d,e}	20-5-20	45	92	2[1 × 0] TS
<i>cis</i> 2 ^d	20-11-20	51	106	2[2 × 0] TS
<i>cis</i> 2-chi ^{d,c}	20-11(D)-20	51	106	2[2 × 0] TS
<i>trans</i> 2 ^d	20-11-20	51	106	[2 × 0] TS, [2 × 0] BS
bent A ^d	46-5	51	104	[2 × 0] BS
bent B ^d	41-10	51	104	[2 × 0] BS
bent C ^d	36-15	51	104	[2 × 0] BS
bent D ^d	31-20	51	104	[2 × 0] BS
bent E ^d	26-25	51	104	[2 × 0] BS
bent E [1 × 0] ^d	26-25	51	103	[1 × 0] BS
bent E [2 × 0] ^d	26-25	51	104	[2 × 0] BS
bent E [4 × 0] ^d	26-25	51	106	[4 × 0] BS
bent E [6 × 0] ^d	26-25	51	108	[6 × 0] BS

^cIn these dsRNA chimeras, the 11 bp dsRNA central helix is replaced with an RNA:DNA hybrid, where RNA is on the top strand and DNA is on the bottom strand.

^dAll bulged nucleotides are A's. The dash depicts positions of A substitutions. See Figure 1 for diagram of *cis* and *trans* orientations.

^e"s" is for shortened versions of *cis* 1 and *trans* 1; in these, the innermost 5 bp are retained, with 3 bp deleted from either side of the central helix.

Representative *cis* and *trans* RNAs containing two $[2 \times 0]$ A bulges in the context of a 51 base pair (bp) helix, termed “*cis* 2” and “*trans* 2,” are provided in Figure 1A (Bhattacharyya et al. 1990; Zacharias and Hagerman 1995a). *cis* RNAs have a geometry in which 20-bp flanking helices are on the *same* side of a central 11-bp helix, whereas *trans* RNAs have these flanking helices on *opposite* sides of this central helix. Arms of 20 bp were chosen because PKR requires at least 16 bp for binding (Manche et al. 1992; Bevilacqua and Cech 1996), but ≥ 30 bp for dimerization and activation (Manche et al. 1992; Zheng and Bevilacqua 2004; Lemaire et al. 2008). These lengths of flanking helices thus minimize defect-independent dimerization and activation of PKR. Eleven bp was chosen for the central region because this is the length of one turn of A-form dsRNA.

In general, the mobility of RNA on native PAGE is controlled by global geometry, with the following migration order: linear $>$ *trans* $>$ *cis* (Bhattacharyya et al. 1990). To determine if our constructs have appropriate mobilities, we compared native gel migration of dsRNA-53, *trans* 2, and *cis* 2. As expected, these RNAs migrated in the order: dsRNA-53 $>$ *trans* 2 $>$ *cis* 2 (Fig. 1B). As all three duplexes are 106 nucleotides (nt), the observed differences in mobility can be attributed to differences in global geometry rather than mass. The addition of KCl to native gels did not improve resolution of these RNAs, and *cis* and *trans* duplexes containing $[1 \times 0]$

bulges were found to have less distinct electrophoretic mobilities (data not shown).

Effect of bulge position on PKR activation

We next investigated how the position of the bulges affects activation of PKR. For these and all subsequent activation assays, two independent experiments were conducted: one representative set of gels is provided in each figure, whereas results from both experiments are shown in the quantitation, and their average provided in the text. We compared native gel electrophoretic mobility and PKR activation by five single-bulge containing RNAs: bent A, bent B, bent C, bent D, and bent E. Each RNA contains one $[2 \times 0]$ A-bulge positioned 5, 10, 15, 20, or 25 bp from the end of a 51-bp duplex (Fig. 2A). Mobility of these RNAs on native PAGE is progressively more retarded as the $[2 \times 0]$ bulge is moved toward the center of the duplex, as expected (Fig. 2B).

Next, activation assays were performed to assess differences in PKR phosphorylation upon changing the position of the single A₂ bulge (Fig. 2C,D). Overall, activation decreases as the bulge is moved toward the center of the duplex. At 0.02- μ M RNA, PKR activation by bent A, bent B, bent C, bent D, and bent E is, on average, 1.4-, 2.3-, 3.7-, 4.0-, and 4.0-fold less than by dsRNA-51 RNA, respectively, while at 0.1- μ M RNA, activation is, on average, 1.5-, 1.9-, 2.1-, 2.9-, and 2.9-fold less than dsRNA-51, respectively. Thus, as the bulge is moved toward the center of the duplex there is a monotonic decrease in activation, with a substantial maximal effect of fourfold, although loss in activation can be somewhat suppressed by increasing the concentration of the RNA. Overall, an A₂-bulge is a major impediment to PKR activation when presented in the proper context.

FIGURE 1. Geometry, sequence, and native gel mobility analysis of representative *cis* and *trans* RNA duplexes. (A) Global geometry and sequence of *cis* 2 and *trans* 2 duplexes. *cis* RNAs containing a dsRNA-51 core (shown in Table 1 footnote) were constructed with two $[2 \times 0]$ A-bulges on the *top* strand and a bulge-free *bottom* strand. *trans* RNAs containing a dsRNA-51 core were constructed with one $[2 \times 0]$ A-bulge on the *top* strand and one on the *bottom* strand. Nomenclature for all duplexes is detailed in Table 1. Number of A-bulges is represented by the number of concentric circles; duplex length, and bend angle in the absence of Mg²⁺ are drawn roughly to scale in this and other figures. (B) Native PAGE mobility analysis of perfect dsRNA-53, *trans* 2, and *cis* 2 RNAs. Note that all three constructs contain 106 nt. Gel was loaded running, and RNAs were intentionally loaded from *right* to *left*, the direction of increasing mobility. Gel contains 10% acrylamide (29:1), 0.5X TBE, and was run at 300 V and 16°C for 4 h.

Effect of bulge size on PKR activation

Next, we examined effects of altering the size of the bulge in the context of bent E RNA, which contains a bulge in the center of the 51-bp duplex (Fig. 3). Bent E RNA had the slowest native gel mobility and was the weakest activator of PKR (Fig. 2). We compared mobility of and PKR activation by four duplexes: bent E $[1 \times 0]$, bent E $[2 \times 0]$, bent E $[4 \times 0]$, and bent E $[6 \times 0]$ (Fig. 3A). Approximate bulge angles, based on the studies of Zacharias and Hagerman (1995a) are presented in Figure 3A. As expected, electrophoretic mobility on native PAGE becomes more retarded as bulge size

FIGURE 2. Effects of bulge position on PKR activation by bent RNAs. (A) Global geometry of dsRNA-51 core bent RNAs containing one $[2 \times 0]$ bulge located 5, 10, 15, 20, or 25 nt from the *right* helical end. RNAs are named “bent A” through “bent E” (see panel; Table 1). (B) Native gel mobility analysis of perfect dsRNA-52 and the bent RNAs from panel A. Note that all constructs contain 104 nt. Gel was loaded running, and RNAs were intentionally loaded from *right* to *left*, the direction of increasing mobility. Gel contains 15% acrylamide (29:1), 0.5X TBE, and was run at 300 V, 16°C for 12 h. (C) PKR activation by bent RNAs with different bulge positions. Representative data set provided in gel format, where percent PKR activation is normalized to dsRNA-51, and fold-effects relative to dsRNA-51 are provided. Gel is 10% SDS-PAGE. Experiments were conducted in duplicate. (D) Plot of PKR activation versus bulge position in bent RNAs for two independent data sets, depicted by filled and open symbols. RNA concentration is 0.02 μM . The average of these data sets is shown as the trend line.

increases, owing to a more acute bend (Fig. 3B; Lilley 1995).

An activation assay was then performed to assess differences in PKR phosphorylation upon increasing the size of the single bulge (Fig. 3C,D). Overall, activation decreases as bulge size increases. In particular, at 0.02- μM RNA, PKR activation by bent E $[1 \times 0]$, $[2 \times 0]$, $[4 \times 0]$, and $[6 \times 0]$ is, on average, 1.7-, 2.7-, 3.8-, and 7.0-fold less than dsRNA-51 RNA, respectively. Curiously, positioning flanking helices in closest proximity, as shown in bent E $[6 \times 0]$ with its approximate right angle bend (Fig. 3A), only serves to further abrogate activation of PKR.

Effect of bulge geometry and defect flexibility on PKR activation

In the previous two sections, PKR activation was examined in the presence of single bulges. Next, PKR activation was tested in the presence of RNAs containing *two* bulges, with either *cis* or *trans* global geometry. *cis* and *trans* geometries were engineered into 45- or 51-bp RNAs with either $[1 \times 0]$ or $[2 \times 0]$ bulges (Fig. 4A–D). In addition, bulge defect flexibility was examined in the context of 40-bp RNAs comparing $[2 \times 0]$ and $[2 \times 2]$ defects (Supplemental Fig. S1). Three perfect duplex control RNAs were also included in these assays: dsRNA-40, dsRNA-51, and dsRNA-79. Forty bp was chosen because that is the sum of the two flanking base-

paired regions in the bulged helices; 51 bp because it is the total number of base pairs in the bulged helices; and dsRNA-79 because this a standard PKR control duplex.

First, we tested PKR activation by 51-bp *cis* and *trans* RNAs with $[1 \times 0]$ or $[2 \times 0]$ bulges, termed “*cis* 1,” “*cis* 2,” “*trans* 1,” and “*trans* 2.” We found that both *cis* and *trans* geometries activate PKR significantly less than either dsRNA-40 or dsRNA-51 (Fig. 4A,B). Moreover, RNAs having a *trans* global geometry decrease PKR activation more than RNAs having a *cis* geometry, and increasing bulge size from 1 to 2 nt further decreases activation in both geometries. For example, at 0.02 μM RNA, activation by *cis* 1, *cis* 2, *trans* 1, and *trans* 2 is, on average, 9.4-, 14-, 24-, and >40-fold less than dsRNA-51, respectively (Fig. 4A,B,E). Similar effects hold at 0.1 μM RNA (Fig. 4). When compared to the shorter dsRNA-40, overall effects were more modest in magnitude, whereas general trends were unaffected (Fig. 4). In addition, we also tested *cis* and *trans* duplexes containing $[3 \times 0]$ A-bulges and found them to activate PKR similarly to $[2 \times 0]$ RNAs (data not shown).

Second, we examined effects of shortening *cis* 1 and *trans* 1 RNAs from 51 to 45 bp (Fig. 4C). The shortened duplexes, termed “*cis* 1s” and “*trans* 1s,” have a 5-bp central helix and 20-bp flanking helices (Table 1). Our hypothesis was that by shortening the central double helical segment, which moves the two 20-bp ends closer together, we might restore activation. (Due to the presence of just a half-turn of dsRNA

FIGURE 3. Effect of bulge size on PKR activation. (A) Global geometry of bent E RNAs containing one [1 × 0], [2 × 0], [4 × 0], and [6 × 0] A-bulge. Approximate bulge angles, based on the studies of Zacharias and Hagerman (1995a) are provided. (B) Native gel mobility analysis of bent E RNAs. Gel was loaded running and RNAs were intentionally loaded from *right* to *left*, the direction of increasing mobility. Gel contains 15% acrylamide (29:1), 0.5X TBE, and was run at 300 V, 16°C for 8 h. (C) PKR activation by bent E RNAs with different bulge sizes. Representative data set is provided in gel format, where percent PKR activation is normalized to dsRNA-51, and fold-effects relative to dsRNA-51 are provided. Gel is 10% SDS-PAGE. Experiments were conducted in duplicate. (D) Plot of PKR activation versus A-bulge size for two independent data sets, depicted by filled and open symbols. RNA concentration is at 0.02 μM . The average of these data sets is shown as the trend line.

in the central helix, helices were engineered with bulges positioned on strands opposite those shown in Figure 1A for 51-bp *cis* and *trans* duplexes—see Table 1.) PKR was significantly *less* activated by the shortened central helices for the *cis* constructs (Fig. 4C). Thus, contrary to our expectations, shortening *cis* RNAs significantly *decreased* activation. Shortening *trans* RNAs appears to have an effect on activation as well, however these effects are more difficult to assess because *trans* RNAs are poorer activators to begin with. Apparently, PKR activation is enhanced when the multiple defects in a helix are spaced further apart.

Next, we considered whether PKR monomers in an active complex overlap the bulges, or act through space. To test this, we constructed chimeric forms of dsRNA-51 (dsRNA-51-chi) and *cis* 2 (*cis* 2-chi) in which the central 11-bp RNA portion was replaced by an RNA/DNA hybrid (Fig. 4D), which does not support dsRBM binding (Bevilacqua and Cech 1996). Activation of PKR by dsRNA-51-chi decreased, on average, by 35-fold compared to dsRNA-51 at a 0.02- μM RNA. Moreover, activation of PKR by *cis* 2-chi was considerably weaker than by *cis* 2. These data suggest that in either perfect or bent dsRNA, activation of PKR does not occur through space, and that PKR instead has to overlap these defects (see Discussion).

Effect of incorporating a flexible [2 × 2] symmetric internal loop in a 40-bp RNA, termed “flex [2 × 2],” on activation of PKR was also tested (Supplemental Fig. S1). We reasoned that if we provided bases opposing the bulged bases, which remove bending (Riordan et al. 1992), stronger activation might be restored; as noted earlier, internal loops are points of flexibility (Kahn et al. 1994). As with *cis* and *trans* RNAs, we retained short (20-bp) terminal helices to discourage binding of more than one PKR molecule per helix terminus. To provide comparison of the effect of an internal loop on PKR activation, we included in the assay both dsRNA-40 and a 40-bp bulged RNA termed “bent [2 × 0].” At a 0.02- μM RNA, PKR activation by bent [2 × 0] and flex [2 × 2] are 3.8- and 5.7-fold less than dsRNA-40, respectively (Supplemental Fig. S1). Thus, incorporating a flexible internal loop disfavors PKR activation and has a larger effect than an asymmetric bulge.

We conducted native gel mobility shift assays of PKR’s dsRBD (P20) with five RNAs: dsRNA-51, dsRNA-51-chi, *cis* 2, *cis* 2-chi, and *trans* 2 RNAs (Supplemental Fig. S2). No appreciable differences in binding were observed among these. All RNAs gave 5–7 band shifts, as one would expect based on length of these duplexes (Manche et al. 1992), and

FIGURE 4. Effect of bulge geometry on PKR activation. (A) PKR activation by *cis* and (B) *trans* RNAs, both based on the dsRNA-51 core. Gels are 10% SDS-PAGE. (C) PKR activation by *cis* and *trans* RNAs containing two $[1 \times 0]$ bulges separated by different spacers, either 11 or 5 bp. (D) PKR activation by chimeric duplexes, 51-bp chi and *cis* 2-chi, compared to all-RNA duplexes. Chimeric region depicted by filled lines. (E) Fold-effects of bulge RNAs compared to dsRNA-51 at 0.02 μM concentration of duplex, in which fold-effects from two independent data sets are shown by filled and open bars. In all gel panels, percent PKR activation is normalized to dsRNA-51, and fold-effects relative to dsRNA-51 are provided. Reactions were stopped at 10 min except panel C, which was stopped at 20 min. Gels are 10% SDS-PAGE.

for all RNAs the concentration of P20 required to completely shift the free RNA was nearly the same. This behavior is generally consistent with our previous work on modified dsRNAs, wherein nonactivating modified RNAs gave similar binding activity as activating unmodified RNAs (Nallagatla and Bevilacqua 2008). Overall, binding and activation of PKR by the RNAs herein is not equivalent, and we conclude that orientation of PKR on the dsRNA is also of critical importance for activation (see Discussion).

Lastly, we point out that it remains possible that small and difficult to detect differences in binding are incurred due to the presence of the chimeric stretch; such differences have the potential to be quite significant for PKR activation due to orientational and positioning effects, but can be subtle to detect by a binding assay due to the many registers for P20 binding. Further studies, including high-resolution structures, will be needed to resolve such issues.

Effect ionic strength on the kinetics of PKR activation

Ionic strength affects the flexibility of helical DNA, with greater ionic strength leading to more flexibility (Kam et al. 1981). In an effort to gain insight into how helical flexibility might affect PKR activation, we investigated the effects of lowering salt concentration from the standard value of 100 to 50 mM on the kinetics of PKR activation in the presence of a few RNAs: dsRNA-51, *cis* 2, *trans* 2, and bent D RNAs. Four time points (4, 10, 30, and 60 min) were collected for activation assays in the presence of 50 or 100 mM KCl, and normalized percent activation assays were plotted (plots in Fig. 5 and gels in Supplemental Fig. S3).

PKR activation assays were first performed in 50 mM KCl, which is below biological ionic strength, and revealed only small differences between the three [2 × 0] duplexes relative to dsRNA-51, with *cis* 2 and bent D being slightly better activators than *trans* 2 (Fig. 5, open symbols). For instance, at 10 min and an RNA concentration of 0.02 μ M, *cis* 2, *trans* 2,

and bent D were reduced in activity relative to dsRNA-51 by only 1.5-, 1.9-, and 1.3-fold, respectively (Supplemental Fig. S3A). Moreover, initial rates of phosphorylation were similar for all RNAs, with bulged RNAs differing from perfect dsRNA by just 1.5- to 2.3-fold (Table 2).

Next, we considered time courses of PKR activation assays in a higher and more biologically relevant (Alberts et al. 1994) concentration of KCl. Remarkably, assays in 100 mM KCl revealed a much greater loss in activity for the three bulge-containing duplexes than perfect dsRNA-51 (Fig. 5, closed symbols). For example, at 10 min and an RNA concentration of 0.02 μ M, *cis* 2, *trans* 2, and bent D were reduced in activity relative to dsRNA-51 by 11-, >20-, and 9.2-fold, respectively (Supplemental Fig. S3B). Moreover, the time dependence for PKR activation revealed that dsRNA-51 reaches a plateau at 30 min, but that *cis* 2, *trans* 2, and bent D RNAs are still increasing in phosphorylation even at 1 h (Fig. 5, closed symbols). Also, initial rates for bulged RNAs were lower by a striking $\sim$ 30–60-fold in 100 mM KCl (Table 2).

Last, ionic strength altered the shape of the activation profiles somewhat. In particular, activation profiles for bent D, *cis* 2, and *trans* 2 at higher ionic strength reveal the optimal RNA concentrations for activation shifted from 0.02 to 0.1 μ M (Supplemental Fig. S3). In sum, a biologically relevant ionic strength disfavors activation of PKR by defect-containing RNAs in two ways: more RNA is required for activation, and the kinetics of activation are much slower.

DISCUSSION

The protein kinase PKR acts in the innate immunity pathway by sensing RNA (Robertson and Mathews 1996; Williams 1999; Nallagatla et al. 2008). However, the features of RNA that lead to activation have not been fully established. In this study, we tested effects of RNA defect position, size, and geometry on activation of PKR. All bulges examined led to decreased activation of PKR relative to perfect dsRNA, with

the largest suppression observed for RNAs with centrally positioned bulges, large bulges, and multiple bulges in a *trans* geometry. In addition, activation by bulged RNAs was suppressed by increasing ionic strength to a biologically relevant concentration.

Overall, the dependence of activation on bulge position has a sigmoidal shape (Fig. 2D). Loss in PKR activation with bulge position is steepest in bent C, which has the bulge 15 bp from the end of 51 bp, with only minor further losses in activation in going to bent D (20 bp from end) and bent E (25 bp from end) (Fig. 2C,D). Notably, bent C has a bulge-free segment of just 36 bp, which is similar to the minimal length needed

FIGURE 5. Effect of ionic strength and time on PKR activation by various helical defects. Time points were collected at 4, 10, 30, and 60 min. Different ionic strengths are shown with open (50 mM KCl) or filled (100 mM KCl) symbols. (A) Plot of PKR activation versus time for 0.02- μ M dsRNA-51 ($\circ$, $\bullet$) and bent D ($\triangle$, $\blacktriangle$). (B) Plot of PKR activation versus time for 0.02- μ M *cis* 2 ($\square$, $\blacksquare$) and *trans* 2 ($\diamond$, $\blacklozenge$). Percent PKR activation is normalized to 0.01- μ M dsRNA-79 RNA. Arrows depict the effect of increasing ionic strength. Gels are provided in Supplemental Fig. S3.

TABLE 2. Ionic strength dependence of initial rates of PKR phosphorylation

RNA construct	Slope (% activation/min) 50 mM KCl ^a	Slope (% activation/min) 100 mM KCl ^b	Fold-effect of ionic strength (50 mM/100 mM KCl)
dsRNA-51	13.3	5.3	2.5
bent D	9.0	0.3	30
<i>cis</i> 2	8.3	0.3	28
<i>trans</i> 2	5.8	0.1	58

Results are from linear fits to data plotted in Figure 5.

^aInitial rates were determined using only the first time point for all RNAs.

^bInitial rates were determined using the first two time points for dsRNA-51, and all four time points for bent D, *cis* 2, and *trans* 2.

for dimerization (Lemaire et al. 2008) and to the activating length of ~ 33 bp (Manche et al. 1992; Zheng and Bevilacqua 2004). These data strongly suggest that bent A (5 bp from end) and bent B (10 bp end) use their longer bulge-free segments (of 46 and 41 bp) to activate PKR. Thus, an A_2 -bulge is a major impediment to PKR activation when presented in a central context.

Recalling that PKR requires a minimum of 16 bp to bind (Bevilacqua and Cech 1996), we might expect bent B and dsRNA-40 to activate to the same extent. However, at 0.02 μ M RNA activation is sixfold higher for bent B than dsRNA-40 (Figs. 2C, 4A,B). Thus, short helices flanking an optimal activation helix appear to augment activation. One possibility is that PKR is capable of binding across bulges in a manner that enhances activation relative to dsRNA-40, albeit not to the same extent as a perfect 51 bp helix (Fig. 2C,D) (see model in the following).

Increasing the size of the bulge from $[1 \times 0]$ to $[6 \times 0]$ progressively decreased activation of PKR. Presumably this arises because increasing the bend angle in the RNA from 15° to 93° (Zacharias and Hagerman 1995b) disfavors productive dimerization of PKR. These observations further suggest that the two monomers of PKR in an activation complex do not interact through space, otherwise increasing the bend angle should drive association of PKR monomers (see model in the following).

A minimal model for activation of PKR by bulged RNAs is presented in Figure 6. Three panels are provided, each with the same final state for activation, on the basis of results provided herein and parsimony. Panel A presents the standard model for PKR dimerization and activation on perfect dsRNA, which is supported by a wealth of biophysical data (Lemaire et al. 2005; Cole 2007). Panel B presents a model for PKR dimerization and activation on *cis* 2 RNA, which is quite similar to the model in panel A involving PKR dimerization, as supported by bell-shaped activation profiles (Fig. 4). Straightening, diagrammed in this panel, is supported by independent studies (Zheng and Bevilacqua 2000). Weaker activation of PKR by this RNA as compared to perfect dsRNA, as observed in Figure 4, may be due in part to the thermodynamic penalty for binding to bent RNAs (Zheng

and Bevilacqua 2000), although further biophysical studies are needed to confirm this. Panel C presents a model for PKR dimerization and activation by *cis* 2-chi RNAs, which is also quite similar to panel A involving PKR dimerization. The weakest activation by this RNA, as observed in Figure 4, can be understood by the double penalty of PKR having to bind to bent RNA *and* having to contend with an RNA–DNA hybrid, for which PKR has poor affinity (Bevilacqua and Cech 1996; Zheng and Bevilacqua 2000).

PKR having to contend with this RNA–DNA hybrid region rejects through-space interaction of PKR monomers.

To address the observation that *cis* is a better activator than *trans*, it is reasonable to suggest that PKR monomers prefer to bind just one face of dsRNA, which is bulge-free in the *cis* (but not the *trans*) geometry. Observation of lesser activation by closely spaced defects further indicates importance of PKR monomer orientation. Additional experiments, including high-resolution structures, are needed to fully develop models consistent with these particular details.

Last, bulged RNAs had a large negative dependence on ionic strength, whereas perfect dsRNAs did not (Fig. 5). Reduced flexibility (i.e., increased persistence length) of nucleic acids has been reported in lower ionic strength (Hagerman 1981; Kam et al. 1981). Thus, helix stiffening might cause the various defect-containing RNA substrates to interact more productively with PKR in lower ionic strength. Likewise, gain in flexibility in 100 mM KCl supports a larger population of bent RNA states, which appears to be less compatible with PKR activation (Zheng and Bevilacqua 2000). Consistent with this notion, an RNA containing a flexible $[2 \times 2]$ internal loop was a poor activator of PKR (Supplemental Fig. S1).

FIGURE 6. Model for PKR dimerization and activation on bulge-containing RNAs. Activation by (A) perfect dsRNA; (B) *cis* 2 dsRNA; and (C) *cis* 2-chi dsRNA. Positions of equilibrium are denoted with various arrowheads.

As 100 mM KCl is similar to, or still slightly lower than, physiological ionic strength (140 mM KCl) (Alberts et al. 1994), these data suggest that the ability of PKR to discriminate between various biological RNA substrates is enhanced under physiological salt conditions, although additional physiological parameters, such as macromolecular crowding, remain to be explored. Importantly, the results herein also suggest that such poorer activation by bulged RNAs under physiological conditions can still be overcome by increasing the concentration of RNA, which certain viruses appear to do (Schneider et al. 1985; O'Malley et al. 1986).

MATERIALS AND METHODS

Protein expression and purification

Full-length PKR, containing an N-terminal (His)₆, was cloned into pET-28a (Novagen, Inc.) and transformed into *Escherichia coli* BL21 (DE3) Rosetta cells (Novagen, Inc.), as previously described (Bevilacqua and Cech 1996; Zheng and Bevilacqua 2004; Nallagatla et al. 2007). Briefly, cells were sonicated and the protein was purified by a Ni²⁺-agarose column (Qiagen, Inc.). PKR was dialyzed into storage buffer: 10 mM Tris (pH 7.6), 50 mM KCl, 2 mM MgOAc, 10% glycerol, and 7 mM β -mercaptoethanol. Isolated protein was treated with λ -PPase (NEB) before PKR activation assays as described below.

RNA preparation and purification

RNAs were prepared by transcription from a hemiduplex template by T7 RNA polymerase (Milligan and Uhlenbeck 1989), except for dsRNA-79, which was prepared from pUC19 as previously described (Zheng and Bevilacqua 2004). Chimeric bottom strand RNAs were purchased from Dharmacon. The sequences of relevant RNA duplexes are found in the figures and at the end of Table 1. All RNAs were purified by denaturing 8% PAGE, and the band corresponding to full-length transcript was excised and soaked overnight at 4°C in 1X TEN₂₅₀ (10 mM Tris [pH 7.5], 1 mM EDTA, 250 mM NaCl). Subsequently, RNAs were ethanol precipitated and resuspended in 1X TE (10 mM Tris [pH 7.5], 1 mM EDTA). Concentrations of RNAs were determined spectrophotometrically. 5'-end labeled RNAs were prepared by first treating with calf intestinal phosphatase, followed by polynucleotide kinase treatment in the presence of [γ -³²P]ATP.

Native gel mobility analysis

Native gels containing 10% or 15% of 29:1 (acrylamide: bis) cross-linking polyacrylamide were used to analyze the mobility differences between various bulged duplex RNAs. The buffer in both the gel and running electrophoresis was 0.5X TBE (50 mM Tris base, 40 mM boric acid, 0.5 mM EDTA). Native gels were run between 4 and 12 h at 300 V and 16°C.

Prior to loading onto a native gel, trace amounts (~1 nM) of 5'-end labeled top strand RNA was added to 10 μ M unlabeled top and 11 μ M unlabeled bottom strand RNA and renatured in 1X TEK₁₀₀ (10 mM Tris [pH 7.5], 1 mM EDTA, 100 mM KCl) by heating at 90°C for 3 min. This sample was then incubated at room

temperature for 10 min. Gels were exposed to a storage screen and analyzed on a PhosphorImager (Molecular Dynamics).

Binding of dsRBD (P20) to perfect dsRNAs and bulge containing dsRNAs were carried out by native gel mobility shift assays as previously described (Bevilacqua and Cech 1996). Duplex RNAs were prepared as previously described, except unlabeled top and bottom strand RNA concentrations were 2 and 3 μ M, respectively. Samples were prepared in binding buffer (BB: 25 mM Hepes [pH 7.5], 10 mM NaCl, 5% glycerol, 5 mM DTT, 0.1 mM EDTA, 0.1 mg/mL tRNA [Sigma]). In the binding reactions, RNA was kept at a limiting concentration (0.01 nM). Binding reactions were loaded onto a running 10% (29:1 acrylamide/bis) native gel. The gel and the running buffer contained 0.5X TBE and the electrophoresis was performed at 300 V, at 16°C for 2 h. All binding assays were conducted at least two times.

PKR activation assays

RNAs were tested for their ability to activate PKR. Prior to activation assays, PKR was dephosphorylated by λ -PPase (NEB) for 1 h at 30°C, and then inhibited with 2 mM sodium orthovanadate. The λ -PPase-treated PKR (0.6 μ M) was incubated with various concentrations of RNA in 20 mM HEPES (pH 7.5), 4 mM MgCl₂, 100 mM KCl, 1.5 mM DTT, 100 μ M ATP (Ambion), and 15 μ Ci [γ -³²P]-ATP. RNA duplexes were renatured at 10 μ M concentrations at 90°C for 3 min, incubated at room temperature for 10 min, and then serially diluted. Reaction mixtures were incubated at 30°C between 4 and 60 min, quenched with SDS stop buffer, and PKR phosphorylation was analyzed on 10% SDS-PAGE gels (Pierce). Gels were exposed to a storage PhosphorImager screen, and intensities of PKR bands were quantified using a PhosphorImager (Molecular Dynamics). In activation assay gels, a no-RNA lane is provided, and phosphorylation activities are normalized to perfect duplexes dsRNA-40, dsRNA-51, or dsRNA-79. PKR activation assays were conducted at least two times.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant no. GM-58709 (to P.C.B.).

Received January 20, 2011; accepted February 24, 2011.

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RNA 2011 17: 957-966 originally published online April 1, 2011

Access the most recent version at doi:[10.1261/rna.2636911](https://doi.org/10.1261/rna.2636911)

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