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ABSTRACT

The innate immune response is the first line of defense against invading pathogens, thus it is of very critical importance to human health and medicine. There are many dangerous pathogens and microorganisms that threaten the human cell at all times and without the innate immune response’s recognition of patterns from these pathogens life would not be the same, and perhaps not even possible for humans. In 2011, the Nobel Prize in medicine was awarded to Drs. Bruce Beutler and Jules Hoffmann for their discoveries on the activation of the innate immune system.

The protein kinase PKR is an essential protein in the innate immune response that upon recognition of pathogenic patterns is activated and eventually leads to apoptosis of this infected cell. PKR recognizes molecular patterns through unique pathogenic RNA patterns, and classically by long stretches of double-stranded RNA that a virus or other pathogen could release upon infection. It is important that innate immune sensors detect broad non-specific patterns among these pathogens, and in recent years understanding of the mechanism of PKR’s regulation in the cell has grown tremendously. Although classical activation is by long double-stranded RNA, PKR recently has been shown to be more permissive, binding other functional, biological and non-conventional RNAs, as well as some proteins.

RNA molecules can adopt an array of structures and perform many different functions in the cell. It is important to understand both sides of the interaction between PKR and RNA because they are both functional molecules. Some pathogens are not recognizable by the innate immune response, and it seems as
though they have developed a way of disguising themselves from the innate immune sensors. The objective of this thesis is to provide a better understanding on how PKR recognizes a broad spectrum of RNAs and more importantly how it recognizes the difference between self and non-self RNA.

Much is already known about how PKR recognizes pathogenic RNA from viruses, but recently PKR has been shown to be activated in cells infected with RNA from bacteria. There are many levels of RNA structure found in the bacterial transcriptome where much of these RNAs regulate genes and functions. It was our hypothesis that PKR activation by bacterial RNA was through these highly structured functional RNAs including riboswitches and ribozymes. Through this idea, I published the first study that a discrete functional bacterial RNA, the *trp* 5′UTR, which binds a protein (TRAP) and regulates L-tryptophan levels in *Bacillus subtilis*, potently activates PKR in both ligand (TRAP/L-trp) –free and –bound states. I found that many structural features of this RNA, which are common to functional bacterial RNAs, activated PKR in manners that are typical to PKR activation and that this activity was maintained when lowering Mg$^{2+}$ concentrations to human physiological conditions, which are known to affect RNA structure.

I also expanded this into a detailed mechanistic study that different types of functional bacterial RNAs with extensive tertiary structure activate PKR including a riboswitch, a riboswitch-ribozyme, and a ribozyme. I showed that these bacterial RNAs maintain tertiary structure upon binding to PKR and activate PKR as strongly as classical viral dsRNA. A structural investigation by ribonuclease mapping and protein footprinting revealed that PKR is able to dimerize onto the native structures
of these bacterial RNAs. Overall, these studies show how PKR acts as a signaling protein for bacteria and suggests the apparent absence for such type of naked RNAs in the human genome in that they would activate innate immunity.

Taking a step back and investigating PKR’s interaction with RNA at the primary and secondary levels of RNA structure led to some findings that helped us understand how PKR can detect a broad range of RNA structure. At the primary level, our lab earlier showed that PKR activation by small single-stranded RNAs that required a 5’-triphosphate. In my work, we hypothesized that PKR had an exclusive method of recognizing such RNAs, but instead our studies revealed that the binding site for 5'-triphosphorylated ssRNA overlapped with that of double-stranded RNA but was mutually exclusive from the ATP binding site of PKR. And lastly, some recent discoveries in how PKR interacts with viral RNAs with long extended base-pairing regions and helical deformations, yet still led to PKR activation was further investigated by mechanistically looking at how much helical deformation PKR can handle.

In recent years, much has been learned regarding how PKR interacts with many different types of RNA structure and even proteins; however, it is still not well understood how PKR functions in vivo. For this reason, I attempted crosslinking and immunoprecipitation (CLIP) to gain a library of total RNAs that PKR interacts with in healthy and infected cells, as well as to look at PKR activity in human cells infected with bacteria. Both of these results led to a foundation for how to develop methods to work with PKR in such in vivo systems that can be pursued by future studies in the lab. We made a shift to look at how PKR interacts with RNA in more
“in-vivo-like” systems by adding in a molecular crowder or total RNA extracted from *E. coli*. I found that total *E. coli* RNA activates PKR potently and this activation surprisingly increases after degrading this RNA down into smaller fragments.

My dissertation work has made advancements in the molecular understanding of how PKR and the innate immune system identify the difference between self- and non-self. The classical model of activation of PKR by long-dsRNA has been greatly expanded, and the mechanisms of activation by all levels of RNA in the hierarchy have been investigated. I have established novel ways in which bacterial RNA is recognized by PKR under *in vitro* and *in vivo-like* conditions, which lays the foundation for *in vivo* studies of these interactions.
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ABBREVIATIONS

bp, base-pair

cdiGMP, cyclic di-guanosine monophosphate

CIP, calf intestinal phosphatase

CLIP, crosslinking and immunoprecipitation

dsRBD, dsRNA binding domain

dsRBM, dsRNA binding motif

dsRNA-79, perfectly double-stranded 79 bp RNA

dsRNA, double-stranded RNA

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

eIF2α, eukaryotic initiation factor 2α

GlcN6P, glucosamine-6-phosphate

HEK293, human embryonic kidney cells

HELA, cervical cancer cells

HEPES, 10 mM Tris, 1 mM EDTA (pH 7.5)

Huh-7, human hepatoma cells

IFN, interferon

λPP, lambda protein phosphatase

nt, nucleotide

NTPs, nucleotide triphosphates

p20, 20 kDa dsRBD of PKR

PACT, PKR activating protein

PAMP, pathogen associated molecular pattern

PEG, polyethylene glycol

PKR, protein kinase R

PNK, polynucleotide kinase

ppp, triphosphate

PRR, pattern recognition receptor

RNP, ribonucleoprotein

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssRNA, single-stranded RNA

TBE, 100 mM TRIS, 83 mM boric acid, 1 mM EDTA (pH 8.3)

TE, 10 mM Tris, 1 mM EDTA (pH 7.5)

TEN_{100}, 10 mM TRIS (pH 7.5), 1 mM EDTA, 100 sodium chloride

TEN_{250}, 10 mM TRIS (pH 7.5), 1 mM EDTA, 250 mM sodium chloride

TLR, toll-like receptor

TRIS, tris(hydroxymethyl)aminomethane

UTR, untranslated region

VA_{1}, virus associated non-coding RNA I from adenovirus

Vc2, aptamer domain of the cdiGMP riboswitch

Vc2FL, full-length 5’UTR of the cdiGMP riboswitch gene
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Chapter 1

Introduction

1.1 Biological importance of PKR and the innate immune system

The innate immune system is the first line of defense against invading organisms.\(^1,2\) This is in contrast to the adaptive immune system that the body acquires throughout a lifetime by exposure to pathogens that the innate immune system could not detect. The innate and adaptive immune systems are of great interest to the science community and were the topic of the Nobel Prize in medicine in 2011. The adaptive immune system has received a lot of attention with it being composed of antibodies and “killer-cells”, but the innate immune system is actually more ancient and general. Because of these features, the innate immune system could potentially be a better target for drugs.\(^3\) In host cells, pattern recognition receptors (PRR’s) trigger the innate immune response upon recognition of conserved structures in pathogens.\(^4\) The three main categories of PRRs are toll-like receptors (TLRs), which recognize bacteria, viruses, fungi and protozoa; NOD-like receptors (NLRs), which recognize bacteria; and RIG-I-like receptors (RLRs), which recognize viruses.\(^1\) Each of these receptors recognizes specific pathogen-associated molecular pattern (PAMP) motifs in order to induce production of the signaling proteins, inflammatory cytokines, and type-I interferons (IFN-I), which ultimately limit viral replication in cells.\(^5\) PAMPs can include any molecular motif associated with a pathogen like gycans, lipopolysaccharides and flagellin, but here we focus on the key component of infection for viruses, which is RNA. Viral replication only occurs inside a living cell, thus making RNA an important PAMP.
One of the major functions of innate immune proteins is their ability to distinguish these PAMPs as self versus non-self. Notably, it is interesting that certain viruses and other pathogens are not recognizable by the immune response, for example the poxvirus protein K3L inhibits the RNA-activated protein kinase, PKR by mimicking eIF2α, not allowing the cell to terminate translation. These pathogens have developed a mechanism to disguise themselves from the immune sensors.

**Figure 1.1: PKR’s role in the cell.** PKR lays dormant in the cell in an inactive conformation. Upon infection, interferon production occurs, which leads to an increase in PKR levels. PKR is then available to bind pathogenic RNA and it can bind either an inhibitor RNA, which is too short to allow PKR to dimerize, or an activator. An activator RNA will allow PKR to dimerize along this RNA, bind ATP and autophosphorylate. PKR then phosphorylates the eukaryotic initiation factor 2α which leads to inhibition of protein synthesis and ultimately apoptosis.
The RNA-activated Protein Kinase, PKR is an essential sensor in the innate immune system that is known to recognize dsRNA. The protein is found primarily in the cytoplasm, but about 20% of it is nuclear. The function of PKR in the cell is represented in Figure 1.1 where upon binding of long stretches of dsRNA, PKR dimerizes through its kinase domain, autophosphorylates and in turn phosphorylates the translation initiation factor, eIF2α, blocking viral replication in the cell.

**Figure 1.2: Levels of structure of PKR and its interaction with dsRNA.** (A) The primary structure of PKR contains an N-terminal dsRNA-binding domain, which contains two tandem dsRNA-binding motifs, and a C-terminal catalytic kinase domain. (B) Structural biology of PKR includes an NMR structure of the dsRBD (PDB: 1QU6) and a crystal structure of the kinase domain (PDB: 2A1A) shown to form a dimer through the N-terminal region of this domain (N-lobe), this was co-crystallized with eIF2α (not shown). (C) Structure of *X. laevis* Rbpa in complex with dsRNA (PDB: 1D12) where it can be seen that the dsRBMs interact with the wide minor groove of A-form RNA, non-sequence specifically and primarily with 2’-hydroxyls. Adapted from Nallagatla, S. R et. al. (2011) *Curr. Opin. Struc. Biol.* with permission.
PKR is composed of two domains, which are shown in Figure 1.2A, an N-terminal double-stranded RNA binding domain (dsRBD), composed of two tandem dsRNA binding motifs (dsRBM 1 and 2), and a C-terminal catalytic kinase domain. At present there are no structures of PKR bound to dsRNA, probably because of the non-specific nature of the interactions, but there is an NMR structure of the dsRBD, and a crystal structure of the kinase domain dimerized and in complex with its substrate eIF2α, shown in Figure 1.2B. The dsRBD and kinase domains are connected by a charged, 110 residue, flexible linker. The primary or “classical” substrate of PKR is dsRNA of 16 or greater base pairs (bp) for binding, 30 bp for dimerization and 33 or greater bp for potent activation of the innate immune response. The interaction is non-sequence specific, primarily in the wide accessible minor groove of A-form RNA, through interactions with the 2'-hydroxyls and phosphate groups (shown in Figure 1.2C).

1.2 RNA Biology: folding, structure and function

RNA is historically known as the intermediary of DNA and protein but has become an interesting topic to scientists studying biological processes in the past few decades. In contrast to DNA, which carries the genetic information, RNA has many more biological functions. For instance, RNA is involved in transcription, translation and splicing, as well as more complex phenomenon such as RNA interference, gene regulation and catalysis. One of the reasons RNA is so important in science is that it adopts diverse structures while having decodable sequences, yet it is still quite simple in nucleotide sequence, as it
is comprised of only four canonical bases: adenosine (A), cytosine (C), guanine (G) and uracil (U).

The primary, secondary and tertiary structure of RNA as well as its interactions with other molecules (of all sizes) is significant in understanding its function and vice versa. RNA folds hierarchically where a primary sequence of RNA is transcribed then secondary structure (base-pairing) will form, followed by tertiary interactions (Figure 1.3A). RNA often has end and internal modifications at the primary structure level as shown in Figure 1.3B, which are important signatures in both self and non-self RNA.

![Figure 1.3: Hierarchy of RNA folding.](image)

(A) RNA folds from its primary sequence (blue) to secondary structure (red) to tertiary interactions (green). (B) RNA primary structure can be modified either on its ends or internally. (C) RNA secondary structure can be somewhat diverse: it can form perfect dsRNA between two different strands or can have helical defects such as bulges or internal loops, and it can also form a hairpin within a single-strand. (D) RNA tertiary interactions can be very complex where multiple strands can come together to form an A-form like helix, RNA can fold back on itself to form a pseudoknot and two hairpins can come together to form kissing hairpins. (E) RNA has an additional level of function where it can also bind certain ligands that regulate processes in the cell, this can be anywhere from a metal to a small molecule to a protein. Adapted from Nallagatla, S. R et. al. (2011) Curr. Opin. Struc. Biol. with permission.
Figure 1.3C and D show a variety of secondary structural motifs (base-pairing level) and how they can interact with each other to form tertiary interactions (pseudoknots, kissing hairpins, etc.). As shown in Figure 1.3E, even more complexity exists in RNA, where these already complex motifs are capable of binding metals, ligands and even proteins in order to regulate functions in the cell.24

As earlier mentioned, RNA can serve as a very important PAMP in the innate immune system and this is primarily due to its ability to form the broad range of structures shown in Figure 1.3. Some of these structures have the ability of triggering the innate immune response and others have a way of hiding themselves. For these reasons it’s important to study how the innate immune system recognizes these RNA PAMPs.

1.3 Regulation of PKR and its many cellular functions

As described in the previous section, the classical activators of PKR are long stretches of double-stranded RNA (dsRNA). It has been found in the past few years that PKR is activated by an array of RNA structures beyond perfect dsRNA including 5’-triphosphorylated single-stranded RNA (ppp-ssRNA), RNA with secondary motifs such as bulges, as well as functional RNAs such as the HDV ribozyme, HCV IRES and HIV-I TAR RNA.25-29 In this section I will discuss regulation of PKR by the three levels of RNAs in the hierarchy from Figure 1.3.

Though PKR interacts with RNA non-sequence specifically, it has been shown to be regulated by single-stranded RNA with a 5’-triphosphate. In fact, it was found by our lab in collaboration with the Cameron lab that 5’-triphosphorylated ssRNA (ppp-ssRNA) activates PKR with very little secondary structure,21,28 making ppp-ssRNA a PAMP for
PKR. The necessity for this triphosphate was confirmed both in vitro and in vivo by also testing the activation of PKR by 5’-ppp, 5’-p, 5’-OH and a 7mG cap where only the 5’-ppp form of ssRNA activated.\textsuperscript{28} In this same study, the necessity of a 5’-ppp on dsRNA was also tested and was found to not be required for activation, indicating that there may be two exclusive methods of activation of PKR by ppp-ssRNA and dsRNA. These observations led to further investigation on the mechanism of activation of PKR by ppp-ssRNA, which is discussed in Chapter 4 and was my first work performed in this thesis.

This key 5’-triphosphate element is often found in pathogenic RNA but not in human RNA offering a potential explanation for one way in which PKR can recognize the difference between self and non-self RNA. Activation of PKR by an external RNA modification led to an investigation on the activation of PKR by internal RNA modifications in ssRNA, which are commonly found in self-RNA. It was found that most internal modifications in ssRNA abrogated activation of PKR even when a 5’-ppp was present, where there was less of an effect (of triphosphate) on modified dsRNAs.\textsuperscript{28}

Regulation of PKR by RNA secondary structure expands the types of activating RNAs beyond just perfectly double-stranded RNA. Many functional viral RNAs have been shown to activate PKR including HIV-TAR RNA and HCV IRES RNA. HIV-TAR RNA contains many bulges and internal loops, but also many regions of base-pairing (which are favorable for PKR binding) and it has been shown by our lab to dimerize in order to activate PKR, where the monomeric form of HIV-TAR RNA inhibits PKR. The dimerization is believed to create enough base pairs to accommodate two PKR monomers, which is required for PKR activation.\textsuperscript{30} The IRES of HCV contains long extended base-paired regions that have many secondary structure deformations. It was also found by our
lab that domain II of the IRES most potently activated PKR and was believed to do so by mimicking A-form dsRNA. These studies led us to a systematic investigation into the role of RNA secondary structural elements on PKR activation that is presented in **Chapter 5**, which is the second part of my thesis work that I contributed to chronologically.

RNA tertiary interactions also play a role in regulation of PKR and have been shown to both activate and inhibit PKR. For instance, PKR is activated by a pseudoknot that forms in the 5’UTR of the cellular mRNA for interferon-γ, which is also responsible for PKR upregulation in the cell. In contrast, PKR is inhibited by tertiary interactions in viral VA RNA. Results from our lab showed that the natural HDV ribozyme, with extensive tertiary structure activates PKR, albeit as a dimer. To link back to the finding that RNA modifications suppressed activation of PKR by small ssRNAs at the primary level, the biological importance of these modifications in their native tertiary structures was investigated in our group by looking at activation of PKR by naturally modified tRNA. It was found that unmodified tRNAs activated PKR potently, but when heavily modified this activity was abrogated.

In addition to the RNAs listed above, PKR is activated by the anionic glycosaminoglycan heparin, which involves recognition by PKR’s kinase domain. PKR activation by heparin is important because it can yield insight into drug design and targeting. Other regulators of PKR are cellular and viral proteins including PACT, which is phosphorylated during cellular stress and forms heterodimers with PKR leading to PKR activation. In addition, other proteins, such as TRBP, can inhibit PKR activation by binding PKR directly, by sequestering dsRNA or by degrading PKR.
In recent years, many important biological functions have been discovered for PKR in addition to its classical antiviral function. Several neurodegenerative diseases have been linked to PKR regulation including Huntington’s, Alzheimer’s and Parkinson’s diseases. PKR has also been referred to as a cell-stress kinase where it is activated in stressed conditions such as oxidative stress, which is probably linked to its regulation in diseases. Several other recent studies include the necessity for PKR activation in order for the inflammasome to function, as well as implications in cancer including elevated levels of PKR in cancer cells. PKR also has been shown to play an important role in the nutrient/pathogen sensing interface acting as a modulator of metabolic inflammation, insulin sensitivity and glucose homeostasis in obesity. Careful studies have shown that PKR could actually be a potential drug-target for patients suffering from type-2 diabetes. PKR was also recently shown to be crucial for the integrity of newly synthesized mRNA for production of interferons, making it even more essential in antiviral defense.

1.4 Structural Regulatory RNAs: Riboswitches and Ribozymes

There are many regulatory mechanisms in the cell, most of which are controlled by protein enzymes. However, over the last few decades key functional RNAs have been discovered including riboswitches and ribozymes. As described in section 1.2, RNA can adopt many complex structures where some of these key structures have been shown to regulate cellular functions. Riboswitches are structured domains usually residing in the non-coding regions of mRNA that control gene function by binding a metabolite that can either turn on or off a downstream gene. Such metabolites can include proteins, small
molecules and ions.\textsuperscript{43} Riboswitches are typically composed of two functional domains: first is the aptamer domain that is the “molecular sensor” responsible for binding of the metabolite, and second is the expression platform, which is usually located immediately downstream of the aptamer, where typically these two domains overlap.\textsuperscript{44}

Riboswitches can function in multiple ways as shown in Figure 1.4. A riboswitch has the possibility of turning off a downstream gene by binding a metabolite and forming an aptamer, which leads to formation of a terminator that inhibits the ribosome from binding and turns the gene off (Figure 1.4A). Secondly, a riboswitch can do the exact opposite where upon binding a metabolite and formation of the aptamer, the terminator is sequestered by an antiterminator and the downstream gene is able to be turned on (Figure 1.4B). And lastly, riboswitches can control at the translational level as well by formation of the aptamer leading to the Shine Dalgarno sequence being sequestered, not allowing the 3’-end of 16s rRNA to bind and initiate translation (Figure 1.4C).

A ribozyme is another type of structured domain that under certain cellular conditions can self-cleave in order to regulate downstream function. Initially, the fact that RNA could be catalytic was surprising because it lacks the complexity and diversity of functional groups that are present in protein enzymes. In most instances, ribozymes cleave the RNA backbone by a phosphodiester intermediate reaction, but each ribozyme ranges in their exact mechanism of cleavage.\textsuperscript{45}
Figure 1.4: Mechanisms of regulation by RNA aptamers. (A) The most common way an aptamer regulates a downstream gene is by binding a metabolite (M), rearranging structure and forming a terminator which turns “off” the gene. (B) In contrast to termination, the reverse can occur where binding of a metabolite to an aptamer can result in a terminator rearranging into an antiterminator, which turns “on” the downstream gene. (C) The third mechanism of regulation by aptamers is that of translation control where the Shine-Dalgarno sequence is part of the metabolite-bound form of the aptamer, so when the aptamer forms, the ribosome cannot bind therefore blocking translation from occurring.

Riboswitches and ribozymes regulate an abundant range of processes in the cell. A few examples include: the thiamine pyrophosphate (TPP) riboswitch, which is located in an intron and is involved in splicing regulation, the glmS riboswitch-ribozyme, which self-cleaves upon binding the small molecule glucosamine-6 phosphate (GlcN6P) in order to function as a genetic switch in regulation of GlcN6P levels in the cell, and
the hepatitis delta virus (HDV) ribozyme, which self cleaves in order to regulate RNA replication in this viral genome.\(^{48}\)

Riboswitches and ribozymes are much more abundant in prokaryotes than eukaryotes where a very small amount have been found despite extensive searches.\(^{49-51}\) Transcriptome-wide structure mapping of RNA has greatly advanced our understanding of RNA structure in multiple organisms.\(^{52-54}\) These data reveal that the majority of the RNA found in the human genome is either unwound by helicases or bound up with proteins.\(^{53,55}\) To date, no riboswitches have been found in the human genome and only a few ribozymes are present, but these are either part of RNPs like the spliceosome and ribosome, or poorly reactive.\(^{49,56}\)

It was found that PKR is activated by total RNA from bacteria, which led to my most recent thesis work in Chapters 2 and 3.\(^{57,58}\) The apparent absence of riboswitches and ribozymes in the human cell gives an insight into how the innate immune system recognizes the difference between self and non-self. It is possible that human RNAs have developed mechanisms to disguise themselves from the innate immune system either by unwinding or protection by proteins. In such case, the human cell would never allow the presence of naked RNAs unless they are predisposed to be degraded.

1.5 Thesis Objectives

Each year more cellular functions of PKR are discovered and over the last decade the classical-model of PKR as just a viral dsRNA-binding protein has greatly evolved. The focus of my thesis work has been to investigate the mechanism of how PKR is activated by primary, secondary and tertiary levels of RNA structure as well as expansion to understanding how PKR interacts with functional bacterial RNA and approaches
toward studying PKR *in vivo*. The ultimate goal of my research was to better understand the mechanism of how PKR recognizes the difference between self and non-self RNA. As already mentioned above, the timeline that the work was actually performed was in the order of chapters 4, 5, 6 then 2 and 3, but the chapters will be described in numerical order below.

In order to further investigate literature findings that PKR is activated by total bacterial RNA *in vivo*,\(^{57,58}\) Chapter 2 focuses on characterizing activation by a discrete bacterial riboswitch, the *trp* 5′UTR. This highly structured bacterial RNA contains many of the features commonly found in regulatory bacterial RNA including a protein/small molecule (L-trp) binding aptamer, blocking hairpins, and a terminator. I present the first evidence that multiple structural features in this RNA activate PKR in the ligand-free and –bound states. I also showed that this functional bacterial RNA continues to activate PKR under human physiological Mg\(^{2+}\) conditions. Lastly, I show that total RNA extracted from *E. coli* also activates PKR at both prokaryotic and eukaryotic Mg\(^{2+}\) conditions.

To follow up and expand on Chapter 2, I sought to understand if additional functional bacterial RNAs were capable of activating PKR and in Chapter 3 I investigate this by a riboswitch, a riboswitch-ribozyme and a small self-cleaving ribozyme. I characterized the mode of activation of PKR by bacterial RNAs with pseudoknots and extensive tertiary structure: the cyclic-diGMP riboswitch, the *glmS* riboswitch-ribozyme and the twister ribozyme, and show that these bacterial RNAs have a common and general mode in activating PKR both in the presence and absence of their corresponding ligands and do so with a high potency. Overall, both Chapters 2 and 3 provide new insights into how PKR acts as an innate immune signaling protein for the presence of
bacteria and suggest a reason for the apparent absence of such protein-free functional structured RNA’s in the human genome.

In Chapters 4 and 5, I looked at more of the primary and secondary RNA level regulation of PKR, first by further investigating an earlier breakthrough in PKR regulation from our lab and the Cameron lab, which was that ssRNA with a 5’-triphosphate activate PKR, and second by investigating how the level of secondary structural defects in model dsRNA affected the activation of PKR. In Chapter 4, I conducted experiments to better understand the mechanism of activation of PKR by ssRNA with a 5’-triphosphate. It was determined that the binding site for such ssRNA is separate from the ATP binding site, but overlapping with the site for dsRNA activation of PKR. My specific work involved crosslinking this ssRNA to wtPKR, to just the dsRBD of PKR (p20), and to a double-mutant containing full-length construct of PKR (dmPKR) incapable of binding dsRNA. It was found that ppp-ssRNA was capable of crosslinking to only the WT and dmPKR but not p20, indicating that the full-length protein is necessary for interaction with this RNA. In Chapter 5, I conducted experiments that led to understanding that PKR can overcome bulges in dsRNA to a certain extent and more-so at lower ionic strength (similar to that of human physiological concentrations). This all led to a set a rules that could be used to understand how secondary structural defects affected the activity of PKR.

For the last portion of my thesis, Chapter 6, I spent a considerable amount of time investigating how PKR functions and interacts with RNA in vivo. My main goal was to use crosslinking and immunoprecipitation (CLIP) of PKR in healthy and infected human cells in order obtain high-throughput sequencing data on a library of all the RNAs
PKR interacts with in these conditions. At the time, CLIP was a relatively new method developed in Prof. Robert Darnell’s lab and I spent the majority of my time developing the method to work on PKR and unfortunately was never able to acquire high-throughput sequencing data. Also, in collaboration with Dr. Eric Harvill’s lab at PSU, I looked at PKR levels in human cells infected with different stages of the bacteria with the potential to secrete nucleic acids during different stages of infection. Again, I was unable to get background phospho-PKR levels down presumably because PKR is so sensitive to the health of a cell. In an attempt to look at in vivo-like conditions in an in vitro PKR activity assay, I tested PKR activation by dsRNA-79 in various molecular crowders, but I was again unsuccessful in obtaining clear data. Lastly I looked at the activity of PKR by total bacterial RNA extracted from E. coli. As previously stated in Chapter 2, this total RNA did indeed activate PKR. Interestingly upon partial digestion of this pool of RNA, the activity of PKR was enhanced suggesting that smaller pieces of this total RNA pool may be better capable of activating PKR.
1.6 References


Chapter 2
Mechanistic analysis of activation of the innate immune sensor PKR by bacterial RNA

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2.1 Abstract

The protein kinase PKR is a sensor in innate immunity. PKR autophosphorylates in the presence of dsRNA enabling it to phosphorylate its substrate, eIF2α, halting cellular translation. Classical activators of PKR are long viral dsRNAs, but recently PKR has been found to be activated by bacterial RNA. The features of bacterial RNA that activate PKR are unknown, however. We studied the B. subtilis trp 5’-UTR, which is an indirect riboswitch with secondary and tertiary RNA structures that regulate gene function. Additionally, the trp 5’-UTR binds a protein, TRAP, which recognizes L-tryptophan. We present the first evidence that multiple structural features in this RNA, which are typical of bacterial RNAs, activate PKR in TRAP-free and TRAP/L-Trp-bound forms. Segments from the 5’-UTR, including the terminator, 5’-stem-loop, and Shine-Dalgarno blocking hairpins, demonstrated 5’-triphosphate and flanking RNA tail dependence on PKR activation. Disruption of long-distance tertiary interactions in the 5’-UTR led to partial loss in activation, consistent with highly base-paired regions in bacterial RNA activating PKR. One physiological change a bacterial RNA would face in a human cell is a decrease in the concentration of free magnesium. Upon lowering the
magnesium concentration to human physiological conditions of 0.5 mM, the \textit{trp} 5'-UTR continued to activate PKR potently. Moreover, total RNA from \textit{E. coli}, depleted of rRNA, also activated PKR under these ionic conditions. This study demonstrates that PKR can signal the presence of bacterial RNAs under physiological ionic conditions and offers a potential explanation for the apparent absence of riboswitches in the human genome.

2.2 Introduction

The first line of defense to pathogens is the innate immune system. One of the key ways the body discriminates self and non-self is through molecular patterns involving RNA. PKR is the RNA-activated protein kinase and it plays a key role in innate immunity \textsuperscript{1-3}. In the presence of pathogenic RNA, which serve as a pathogen-associated molecular pattern (PAMP), PKR dimerizes and autophosphorylates, which activates it to phosphorylate translation initiation factor eIF2\textalpha{}, blocking viral replication and shutting down translation \textsuperscript{4}.

PKR is comprised of a C-terminal kinase domain and two N-terminal dsRNA-binding motifs (dsRBMs) \textsuperscript{5}. Long stretches of viral dsRNA bind to the dsRBMs in a sequence-independent fashion and activate PKR \textsuperscript{6}. In addition, we and others have shown that many other RNA motifs serve as PAMPs to activate PKR. In particular, complex tertiary structures including pseudoknots \textsuperscript{7}, IRESs \textsuperscript{8}, and UTRs \textsuperscript{9} activate PKR, as well as a number of misfolded or dimerized functional RNAs \textsuperscript{10-12}. Multiple RNA secondary structures have been reported to promote activation of PKR including stem-loops containing defects such as smaller bulges \textsuperscript{13}. Moreover, certain unstructured RNA regions promote activation. In particular single-stranded regions with a 5' triphosphate activate
PKR, especially when fused to short stem-loops\textsuperscript{14,15}, and they do so without specificity to the 5’-base to offer broad-spectrum protection\textsuperscript{16}. We also found that activation of PKR is abrogated by many covalent modifications of RNA, especially in single-stranded regions of RNA\textsuperscript{17,18}.

Recent reports indicate that bacterial RNAs activate PKR in human cells\textsuperscript{19,20}. For instance, transfection of human cardiac myocytes with total RNA from \textit{E. coli} and \textit{S. aureus} leads to potent activation of PKR, while transfection with human RNAs does not. Similarly, others reported and we confirmed\textsuperscript{15} that PKR from \textit{E. coli} cells is purified in a phosphorylated form that must be dephosphorylated to make PKR responsive to RNA\textsuperscript{21}. These observations support the ability of bacterial RNAs to activate PKR, although this could also arise from high PKR concentrations that may be present during expression\textsuperscript{22}. However, the molecular features of bacterial RNA that activate PKR have not been identified, which motivated our present study.

The \textit{trp} 5’-UTR from \textit{B. subtilis}, which regulates the biosynthesis of tryptophan, is known to switch between several different conformations to regulate gene expression at the transcription and translational levels (Fig. 2.1). At the transcription control level, an antiterminator forms in the \textit{trp} 5’-UTR. In the presence of excess tryptophan, TRAP binds L-Trp. The Trp-bound TRAP then binds the 11 GAG/UAG single-stranded triplet repeats to drive formation of an intrinsic terminator to promote attenuation (Fig. 2.1a). At the translation control level, a large hairpin forms in the 5’-UTR that allows ribosomes access to the Shine-Dalgarno (SD) sequence (Fig. 2.1b). Upon binding TRAP, the 5’-UTR refolds to form the SD blocking hairpin to inhibit translation (Fig. 2.1)\textsuperscript{23}. In regulating expression at the transcription and translational levels, the \textit{trp} 5’-UTR serves
Figure 2.1: TRAP-free and TRAP-bound models of the RNA folds present in the B. subtilis trp 5'-UTR transcription and translation control mechanisms. (a) RNA folds of trp 5'-UTR present in the transcription control construct \(^{23,24}\). Left is the TRAP-free construct that forms in tryptophan-limiting conditions where TRAP is unable to bind, which leads to formation of an antiterminator resulting in transcription readthrough and eventually L-Trp biosynthesis. Right is the TRAP/L-Trp-bound construct wherein TRAP is able to bind to the GAG/UAG triplet repeats (bold) as they are synthesized. TRAP/L-Trp binding leads to the formation of the terminator (green) resulting in termination of
transcription. (b) RNA fold of the full-length *trp* 5'-UTR present in the translation control construct. The AUG (bold) start codon is the last three nucleotides shown. Left is the TRAP-free construct that forms in tryptophan-limiting conditions where the *trp* 5'-UTR transcript adopts a structure in which the SD sequence is single-stranded allowing the ribosome to bind and translation to occur. The structure below this one is a magnesium-dependent tertiary structure model where 24 nt from both the 5'-(magenta) and 3'-(blue) pyrimidine-rich internal loop base pair with upstream residues 36-60. Right is the TRAP/L-Trp-bound construct that forms a structure where the SD sequence is sequestered, which inhibits ribosome binding. In all structures, the 11 identical subunits of TRAP are shown in blue, yellow, red and orange, and L-Trp molecules are shown in green as per PDB 1C9S. Numbering is from the start of transcription and specific numbers labeled include the boundaries for sequence truncations used in this study.

as an indirect riboswitch, sensing tryptophan levels through binding of L-Trp to TRAP protein. In addition, the *trp* 5'-UTR has a long-distance pseudoknot with a well-characterized Mg$^{2+}$ response that helps promote translation (Fig. 2.1b, lower). Given the diversity of elements in the *trp* 5'-UTR—multiple stem-loops, a long-range RNA tertiary structure, extensive dsRNA and ssRNA regions, and an RNA-binding protein—it is an ideal system to investigate activation of PKR by bacterial RNAs. Moreover, some of these RNA elements are quite unique to bacteria, such as the terminator, SD blocking hairpin, and 5'-triphosphate, making our findings applicable to other bacterial RNAs.

In the present study, we conducted PKR activation studies on the *trp* 5'-UTR in the presence and absence of TRAP and investigated the secondary structural features of *trp* 5'-UTR responsible for activating PKR. We also tested various RNA segments for 5'-triphosphate dependence to activation and investigated the contribution of the long-distance tertiary structure to activation. To generalize our findings, we conducted experiments under physiological ionic conditions and investigated the ability of total bacterial RNAs to activate PKR. Our study provides the first evidence that specific
bacterial RNA motifs activate PKR very strongly. These results support the ability of
bacterial RNAs to activate PKR under physiological conditions.

2.3 Materials and Methods

2.3.1 Design of PCR Primers, RNAs and Blocking Oligonucleotides

The 79 bp control RNA, dsRNA-79, was prepared by transcribing opposing
strands of a portion of the pUC19 vector using two separate hemi-duplex templates. The
transcripts were purified by denaturing PAGE and annealed to yield a perfectly double-
stranded 79 bp dsRNA, which we previously used as a positive control for PKR
activation14.

All trp 5’-UTRs were transcribed from a template prepared by PCR. The region of
interest was amplified from a plasmid that contained the full-length 1-206 sequence,
inserting a T7 promoter on the 5’- end. PCR primers used for the trp 5’-UTR truncations
are provided below, where the T7 promoter is underlined and any transcribed G’s not
present in the native trp 5’UTR sequence in bold font. Such G’s were added to enhance
transcription efficiency. Numbers correspond to the first and last nucleotides of the
resultant transcript.

1-206 (FL)

TS primer (1):
5’GAAATTAATACGACTCACTATA
BS primer (206): 5’CATTGCTCTCACTCCTTATGGC

61-188 (LH)
TS primer (61):
5’GAAATTAATACGACTCACTATAAGGTAGCAGAGAATGAGTTTA

BS primer (188): 5’GGCAAGGAGAATGAGAAGATGGC

89-159 (T + tails)

TS primer (89):
5’GAAATTAATACGACTCACTATAAGGAGACATTATGTTTATTTCTA

BS primer (159): 5’AGGATAAAAATACTATATAACAAATAAAACCC

142-206 (SD SL + tails)

TS primer (142):
5’GAAATTAATACGACTCACTATAAGGATAGTATTTTATCCTCTCATGCC

BS primer (206): see above

1-60 (5’-SL + tails)

TS primer (1): see above

BS primer (60): 5’CTATTCTCTAACTCAACTCATTC

108-159 (T + 3’ tail)

TS primer (108):
5’GAAATTAATACGACTCACTATAAGGACCCAAAAGAAGTCTTTC

BS primer (159): see above

108-133 (T)

TS primer (108): see above

BS primer (133): 5’ACCCAAAAGAAAGACTCTTTTTGG
Due to inefficient transcription from the PCR products, trp 5’-UTR truncations 172-200 and 89-133, were transcribed from hemi-duplex templates, where the bottom strand is complementary (underlined) to the T7 promoter. Bold C’s denote where G’s were added to the nascent transcript in order to enhance transcription.

172-200 (SD SL)

BS template (172-200)

5’CATTGCTCTCACCCTTATGGCAAGGAGAATGAGACCTATAGTGAGTCGTA
TTAATTTC

89-133 (T + 5’ tail)

BS template (89-133)

5’ACCCAAAAGAAAAGACTTCTTTTGGTAGAATAACATAATGTCTCTATAG
TGAGTCGATTAATTTC

Sequences of the DNA/LNA chimeric blocking oligonucleotides (BOs) are provided below, where the LNA (locked nucleic acid) substitutions, which were added to strengthen binding, are in bold and DNA bases are non-bold (Exiqon). The LNA content was designed as recommended by the manufacturer.

5’BO: 5’AGAATAAAAACATAATG
3’BO: 5’GGATAAAATACCTATAAACAATAA
2.3.2 RNA preparation and purification

*In vitro* T7 transcription reactions of ~500 mL were carried out on the purified templates described above. Reactions were fully submerged in a water bath at 37°C for 3-4 h, quenched with an equal volume of 2x formamide loading buffer (95% v/v formamide, 20 mM EDTA), and fractionated on a 10% denaturing PAGE gel. The RNA bands were visualized via UV-shadowing, excised with a razor blade, and extracted overnight into 1x TEN$_{250}$ [10 mM Tris (pH 7.5), 1 mM EDTA, 250 mM NaCl]. RNA was then ethanol precipitated and resuspended in 1x TE [10 mM Tris (pH 7.5), 1 mM EDTA] and stored at -20 °C. Concentrations of RNA were determined using a Nanodrop spectrophotometer.

As appropriate, dephosphorylated RNAs (‘HO-RNAs’) were prepared by treatment with calf-intestinal phosphatase ‘CIP’ (New England Biolabs, NEB). RNAs were then phenol-chloroform extracted, ethanol precipitated, and otherwise handled as described above.

For PKR activation assays, the highest concentration of RNA tested was prepared in 1xTEN$_{100}$ and renatured at 90 °C for 1 min followed by room temperature for 10 min. If LNA blocking oligonucleotides were present, they were included in this renaturation in 2-fold excess over the trp 5’-UTR. For assays that included TRAP (+/- L-Trp), TRAP and L-Trp were added after renaturation and incubated at room temperature for 60 min, which is sufficient to allow complete binding$^{24}$, followed by serial dilution. TRAP was present in five-fold excess over the RNA concentration, and L-Trp was present at a final concentration of 1 mM. The $K_d$ between L-Trp-saturated TRAP and the trp 5’-UTR is 93 nM under conditions of 4 mM Mg$^{2+}$ and 100 mM K$^{+}$$^{24}$, which is similar to the ionic conditions in the standard PKR activation assay. These reaction conditions thus assured
that the RNA was saturated with L-Trp-bound TRAP throughout the activation assays; the only exception was the lowest concentration of RNA (0.02 µM) where L-Trp-bound TRAP was present at 0.1 mM, which is equal to the $K_d$. TRAP in the absence of L-Trp does not bind to RNA 26.

2.3.3 Protein expression and purification

Full-length wtPKR containing an N-terminal (His)$_6$ tag was cloned into pET-28a vector and transformed into E. coli BL21 (DE3) Rosetta cells (Novagen) as previously described 15,27. Cells were sonicated and purified on a Ni$^{2+}$ -NTA agarose column (Qiagen) run over a high-salt imidazole gradient on a Bio-Rad FPLC. Purified protein was dialyzed into storage buffer [10 mM Tris (pH 7.6), 50 mM KCl, 2 mM Mg(OAc)$_2$, 10% glycerol and 7 mM β-mercaptoethanol]. Protein concentration was determined spectrophotometrically, aliquotted, flash-frozen, and stored at -80°C. TRAP was a gift from Prof. Paul Babitzke’s lab, which was purified as previously described 28.

2.3.4 PKR Activation Assays

RNAs were tested for their ability to mediate PKR autophosphorylation. PKR is purified from E. coli in a phosphorylated form so it was first dephosphorylated by lambda protein phosphatase λPP (NEB). PKR and λPP were incubated at 30°C for 1 h followed by inhibition of the λPP by freshly made 2 mM sodium orthovanadate 15,21. Subsequently, PKR was incubated at a final concentration of 0.8 mM with various concentrations of RNA, RNA/BO, or RNA/TRAP (+/- L-Trp). Reactions were in PKR activation buffer [20 mM HEPES (pH 7.5), 4 mM MgCl$_2$, 50 mM KCl] and 1.5 mM DTT, 100 mM ATP.
(Ambion) plus 15 mCi [γ-\textsuperscript{32}P]-ATP. Standard incubation conditions were 30 °C for 10 min, followed by quenching with SDS loading buffer. Samples were fractionated on a 10% Bis-Tris (Novex) SDS PAGE gel, dried, and exposed to a storage PhosphorImager screen (Molecular Dynamics). Phosphorylated PKR bands were detected using a Typhoon PhosphorImager and quantified with ImageQuant. Each activation assay gel contains one negative control, a sample containing 1x TEN\textsubscript{100} instead of RNA, as well as a positive control, a sample containing 0.1 µM dsRNA-79, which was used to normalize all phosphorylation activities. Each new preparation of dsRNA-79 was titrated to find the optimal concentration for activating PKR, which was 0.1 µM dsRNA-79 (Fig. A1). For activation assays performed at varying Mg\textsuperscript{2+} concentrations and reaction times, the appropriate concentration of free Mg\textsuperscript{2+} was added to the RNA alone and incubated at 30°C for 5 min prior to the addition of PKR activation buffer (Mg\textsuperscript{2+} omitted) and PKR. For varying time assays, PKR was added to the RNA reaction mixture and aliquots were removed and quenched after 2, 5, 10 or 20 min.

2.3.5 Preparation of total E. coli mRNA

Wild-type MG1655 E. coli cells (a gift from Prof. Paul Babitzke’s lab) were grown overnight in LB broth and pelleted. Cells were treated with the RNAProtect bacterial reagent (Qiagen) to help prevent RNA degradation and then purified by the RNEasy Qiagen kit. A Ribozero kit (Epicentre) was used to deplete rRNAs, which was confirmed by a Bioanalyzer run (Agilent). Ribosomal RNA was depleted to avoid introducing naked rRNA, which is non-physiological.
### 2.4 Results

**2.4.1 The trp 5’-UTR activates PKR in both its TRAP-free and TRAP-bound states**

In an effort to assess whether the trp 5’-UTR can activate PKR, we conducted activation assays with the RNA alone, the RNA with TRAP protein, or the RNA with TRAP plus L-Trp. We began with the full-length translation control trp 5’-UTR shown in Figure 1b, which we term ‘FL RNA (1-206)’. As shown in Figure 2.2, the trp 5’-UTR activated PKR with a bell-shaped dependence on RNA concentration. The level of activation in the lowest concentration of added RNA (0.02 µM) was similar to that in the negative control of no-added RNA, as was the level of activation in the highest concentration of added RNA (10 µM) (Fig. 2.2a). These data indicate that there is a low background overall and support the bell-shaped dependence of PKR activation by RNA, where high concentrations of RNA titrate PKR protein out into inactive monomers. At intermediate concentrations of RNA (~1.0 µM), PKR activation occurred with a maximum value of ~130% the level of the reference activator, dsRNA-79 (Fig. 2.2d). The trp 5’-UTR is thus a potent activator of PKR in its ligand-free form.

Next we tested whether TRAP (+/- L-Trp) altered the activation of PKR. When we added TRAP in 5-fold excess over RNA to the activation mixture in the absence of L-Trp, there was little to no effect on the activation profile. Maximal activation remained at ~125% (Fig. 2.2b and 2d). TRAP is known to be unable to bind the 5’-UTR in the absence of L-Trp, consistent with no measurable effect on PKR activation.
Figure 2.2: Activation of PKR by the trp 5'-UTR translation control RNA (1-206) in the absence and presence of TRAP and L-Trp. PKR activation assays on trp 5'-UTR (1-206) (a) alone, (b) in the presence of TRAP, and (c) in the presence of TRAP/L-Trp. RNA was serially diluted ~2-fold from 10 to 0.02 µM. In each lane, TRAP was present in five-fold excess over the RNA, and L-Trp was present at a final concentration of 1 mM. A buffer-only negative control is included and PKR activation is normalized to dsRNA-79 in each panel. Activation values are provided under each gel and the position of phosphorylated PKR is indicated as ‘p*PKR’. Gels shown are raw data from one representative trial. (d) Graphical representation of percent PKR activation from panels (a)-(c) as a function of the concentration of FL RNA (1-206). Plotted is the average of two independent trials and error bars represent the range of these two trials. The lines for FL RNA(1-206)/TRAP and FL RNA(1-206)/TRAP/L-Trp also average points immediately adjacent on the x-axis. All forms of the RNA activated PKR with a bell-shaped dependence on RNA concentration.
As a control we also tested whether TRAP alone can activate PKR, and no activation was observed (Fig. A2), in agreement with the observations above.

When TRAP was added to the activation mixture in the same five-fold excess over the RNA but in the presence of 1 mM L-Trp, the maximum activation shifted to ~150% with a sharper profile (Fig. 2.2c and 2.2d). These conditions were assure saturation of the RNA with TRAP (see Materials and Methods). Overall, these data indicate that PKR is activated by the trp 5’-UTR both in its ligand-free and ligand-bound states. Furthermore, these results suggest that TRAP does not compete with PKR for binding to the RNA, which is consistent with the opposite RNA binding preferences of these two proteins—TRAP is known to bind to single-stranded segments of the RNA while PKR tends to bind double-stranded segments. We next investigated which of the structured regions of the trp 5’-UTR are capable of activating PKR.

2.4.2 Multiple secondary structural features in the trp 5’-UTR are capable of activating PKR

The previous section showed that PKR is activated potently by the trp 5’-UTR in both its TRAP-free and TRAP-bound states. There have been no investigations in the literature, however, of which bacterial RNA elements can activate PKR. We therefore did a systematic analysis to determine which elements of the trp 5’-UTR are capable of activating PKR. As described above, the full-length trp 5’-UTR activated PKR ~130% relative to dsRNA-79. Knowing that PKR generally prefers RNA substrates that are structured, we assessed whether the large hairpin or ‘LH’ (nt 61-188) in the TRAP-free 5’-UTR is capable of activating PKR.
Table 2.1: Multiple structural features of the *trp* 5'-UTR lead to activation of PKR

<table>
<thead>
<tr>
<th>RNA</th>
<th>Shorthand Notation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Maximal PKR Activation (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>[RNA] at Max. Act. (µM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Max. Act. Fold Effect&lt;sup&gt;c&lt;/sup&gt;</th>
<th>[RNA] Fold Effect&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Combined Effect&lt;sup&gt;e&lt;/sup&gt;</th>
<th>ppp Contribution&lt;sup&gt;f&lt;/sup&gt;</th>
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</tr>
<tr>
<td>61-188</td>
<td>LH</td>
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<td>0.625</td>
<td>0.7</td>
<td>1.6</td>
<td>1.2</td>
<td>1.1</td>
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<tr>
<td>89-159</td>
<td>T + Tails</td>
<td>88.9</td>
<td>0.625</td>
<td>0.7</td>
<td>1.6</td>
<td>1.1</td>
<td>4.7</td>
</tr>
<tr>
<td>89-133</td>
<td>T + 5' Tail</td>
<td>12.6</td>
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<td>0.1</td>
<td>0.01</td>
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<td>T + 3' Tail</td>
<td>100</td>
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<td>0.8</td>
<td>2.6</td>
<td>2.0</td>
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<td>108-133</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<td>SD SL + Tails</td>
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<td>1.3</td>
<td>0.4</td>
<td>0.5</td>
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<td>0.05</td>
<td>0.2</td>
<td>0.01</td>
<td></td>
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<tr>
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<td>0.1</td>
<td>0.78</td>
<td>10</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations for shorthand notation: FL: full-length, LH: long hairpin, T: terminator, SD: Shine-Delgarno, SL: stem-loop.

<sup>b</sup>Each of the truncated *trp* 5'-UTRs tested for PKR activation are listed at the left and the effect in activating PKR is calculated in two ways: the highest percentage of activation relative to dsRNA-79 and the concentration of RNA at the max activation. All experiments were repeated at least twice.

<sup>c</sup>An activation fold-effect is calculated for each truncation with respect to the full-length (1-206) RNA by dividing the maximal PKR activation of the corresponding truncation by that of the full-length.

<sup>d</sup>An RNA concentration fold-effect is calculated for each truncation with respect to the full-length (1-206) RNA by dividing the RNA concentration at the maximum activation of the full-length by that of the corresponding truncation.

<sup>e</sup>The combined effect is calculated by multiplying the activation and concentration fold effects together.

<sup>f</sup>Triphosphate (ppp) fold-effects were calculated for the four RNAs tested for triphosphate dependence. The ppp-effects are calculated by dividing the 5'-ppp maximum activation by those of the 5'-OH values corresponding to the same RNA (rather than full-length). Actual quantities used to calculate the ppp contribution values can be found in figure 2.3.
The results are summarized in Table 2.1 and the activation gels are provided in Figure A3. The LH (61-188) potently activated PKR at a maximum value ~95% the level of 0.1 μM dsRNA-79. Moreover, less of the LH (61-188) than full-length *trp* 5’-UTR was required to activate PKR, with the maximum activation occurring at ~0.625 μM RNA versus 1.0 μM. This gave a combined effect that was similar (1.2-fold) to full-length 5’-UTR (Table 2.1). The combined effect is calculated by multiplying maximal PKR activation and minimal RNA concentration to yield the overall potency of PKR activation, which accounts for the bell-shaped profile of PKR activation. Knowing that certain short hairpins with single-stranded tails can also activate PKR\textsuperscript{14,15}, we next investigated activation of PKR by the terminator hairpin, ‘T’ both with and without its flanking tail sequences, which we termed ‘tails’. The RNA segment from 89-159 contains the 11 bp terminator hairpin and both its 5’- and 3’-tails. This construct activated PKR potently, at a maximum value ~90% relative to that of dsRNA-79. Again, less of T + tails (89-159) RNA than full-length *trp* 5’-UTR was necessary to activate PKR at this maximum value, at only ~0.625 μM RNA. Together, the maximum activation and RNA requirement gave a combined effect of approximately equal to full-length *trp* 5’-UTR. Next, the dependence of activation on the tails flanking the terminator hairpin was determined, where construct 89-133, termed ‘T + 5’ tail’, contains the terminator hairpin and the 5’ tail, and construct 108-159, termed ‘T + 3’ tail’, contains the terminator hairpin and the 3’ tail. It was found that T + 5’ tail (89-133) only slightly activated PKR, at ~10% maximum activity, and that this occurred only at the highest RNA concentration of 10 μM, giving a very weak total combined effect of ~0.01-fold relative to the full-length *trp* 5’-UTR. Conversely, the T + 3’ tail (108-159) construct displayed a high maximum
activation of PKR was at ~100% relative to dsRNA-79, and the concentration of RNA at this maximum activation was low ~0.39 µM. This leads to a total combined effect that was 2.6-fold greater than the full-length 5’-UTR. It was also determined that when both tails were removed from the terminator hairpin, in the 108-133 (T) construct, all PKR activity was lost (Table 2.1). Apparently, the 3’-tail is an important and essential contributor to activation by the terminator hairpin. Promotion of activation by a single stranded 3’-tail is consistent with previous observations [15], although preference for the 3’-tail is stronger in this bacterial RNA. This may be due to the stem being only 11 bp in the present study, while it was 16 bp in the earlier work.

There are several other hairpins that can form in the trp 5’-UTR. Upon binding of Trp-bound TRAP, the full-length trp 5’-UTR can switch its conformation between two very different folds (Figure 2.1b). In this conformational switch, the large hairpin (61-188) region refolds to release a complement to the Shine-Dalgarno (‘anti-SD’) that sequesters the SD sequence to form an 11 bp stem-loop termed the ‘SD-blocking hairpin’ or ‘SD SL’. We first tested an RNA segment that spans from 142-206 that contains the SD blocking hairpin, a long 5’-tail (30 nt) and a shorter 3’ tail (6 nt), termed ‘SD SL + tails’. This RNA potently activated PKR at a maximal level of ~170% that of dsRNA-79. The relative amount of this RNA necessary to activate PKR was 2.5 µM, however, which was one of the higher values of all the RNAs tested. This led to a slightly diminished combined effect of 0.5-fold that of the full-length 5’-UTR. One possibility is that such an element could be a potent activator of PKR if it accumulates to high concentrations. We also tested the contribution of the tails to activation of PKR by the SD blocking hairpin. The construct SD SL (172-200) has both the 5’- and 3’-tails removed. This RNA was an
exceptionally poor activator of PKR with a maximal activation level of just \( \sim 6\% \) and a very high RNA requirement at 6.25 \( \mu M \). The inability of the SD blocking hairpin alone (172-200) to activate PKR is similar to the inability of the terminator hairpin alone (108-133) to activate PKR.

The final hairpin that we studied is the 5'-stem-loop (5’-SL). The 5’-SL is comprised of 9 bp and resides at the very 5’-end of the transcript. One of its functions is to increase the affinity of the interaction between TRAP-Trp and the 5’-UTR. We prepared an RNA segment from 1-60 that contains the 5’-SL and 28 downstream nucleotides, termed ‘5’-SL + tails’. This RNA activated PKR very well, at a maximum value that was \( \sim 70\% \) that of dsRNA-79. The relative amount of 5’-SL + tails (1-60) RNA necessary to activate PKR at this maximum value was the lowest of any trp 5’-UTR segments at just \( \sim 0.16 \mu M \). These two values lead to a combined effect of \( \sim 3.2\)-fold greater than that of the full-length 5’-UTR, which is the most potent of the trp 5’-UTR segments studied. Furthermore, this combined effect for the 5’-SL + tails (1-60) RNA is within 2.5-fold the combined effect of dsRNA-79.

### 2.4.3 Various trp 5’-UTR segments display a 5’-triphosphate dependence for PKR activation

We previously reported that short hairpins with single-stranded tails activate PKR in a 5’-triphosphate dependent fashion. Given that several of the PKR-activating trp 5’-UTR segments discussed in the preceding section are hairpins that depend on single-stranded tails for activation, we tested whether these also activate PKR in a 5’-triphosphate dependent fashion. The terminator with both 5’- and 3’-tails, ‘T + tails (89-
159)’, was prepared with and without treatment by calf intestinal phosphatase (CIP), which removes the 5’-triphosphate (Fig. 2.3a) \(^{14,31}\), and tested for PKR activation. As shown in Figure 3b, upon treatment with CIP, T + tails (89-159) became a poor activator of PKR, with a ~5-fold loss in activation. Even larger effects were obtained with the SD blocking hairpin with 5’- and 3’-tails, SD SL + tails (142-206), in which activation diminished by nearly 25-fold upon CIP treatment (Fig. 2.3c, Table 2.1). A much more subtle triphosphate effect was seen with the 5’-SL and tails (1-60) where the CIP-treated construct still activated PKR at around 40% of untreated, which yielded only a 1.7-fold loss in activation of PKR (Fig. 2.3d, Table 2.1).

We previously reported that RNA activators with extensive double-stranded structure do not show dependence on a 5’-triphosphate \(^{14}\). For instance, activation of PKR by dsRNA-79 is not diminished upon CIP-treatment \(^{29}\). Consistent with this, the more structured activating RNA constructs of full-length trp 5’-UTR (1-206) and large hairpin construct (61-188) continued to activate PKR potently after CIP treatment (Fig. 2.3b,c and Table 2.1). Importantly, observation of full activation by CIP-treated 1-206 and 61-188 RNA indicates that loss of activity upon CIP treatment in the 89-159 and 142-206 constructs is not due to inhibition of PKR activity by CIP treatment. Overall, the above results indicate that the 5’-UTR of a bacterial RNA can activate PKR both with and without a 5’-triphosphate.
Figure 2.3: Dependence of activation of PKR on 5’-triphosphate for full-length and truncated trp 5’-UTRs. (a) Model of activation by 5’-triphosphate RNA (left) and 5’-hydroxyl RNA (right). Calf-intestinal phosphatase (‘CIP’) is used to remove the 5’-triphosphate and leave a 5’-hydroxyl. In all RNA constructs tested, the 5’-triphosphate form activated PKR, but activation after CIP treatment was dependent on the size and structure of the RNA, as indicated by the question mark. (b-d) PKR activation assays of CIP-treated trp 5’-UTR constructs (b) FL (1-206) and T + tails, (89-159), (c) LH (61-188) and SD SL + tails (142-206), and (d) 5’-SL + tails (1-60). A buffer-only negative control is included and PKR activation is normalized to dsRNA-79 in each panel. Activation values are provided under each gel and the position of phosphorylated PKR is indicated as ‘p*PKR’. All assays were performed twice.
2.4.4 Tertiary structure in the trp 5’-UTR contributes to PKR activation

Prior investigations from our lab identified a long-distance Mg\(^{2+}\)-dependent pseudoknot in the trp 5’-UTR that functions to limit binding of TRAP\(^{24}\). The 5’-half of the purine-rich single-stranded triplet repeat region (nt 36-60) was shown to base pair with both strands of the pyrimidine-rich internal loop in the large hairpin (Fig. 2.4a). The base pairing of these triplet repeats is more extensive with the 5’-strand of the internal loop than the 3’-strand. Pairing with the 5’-strand of the internal loop consists of 13 base pairs with just one single-nucleotide bulge, while pairing with the 3’-strand has only 11 base pairs and these are interrupted by two defects—a single-nucleotide bulge and a 1x1 internal loop (Fig. 2.4a). We also demonstrated that this tertiary structure could be disrupted by DNA blocking oligonucleotides (BOs) directed to either the 5’- or 3’-pyrimidine-rich internal loops, with more potent disruption by the 5’-BO, as expected given the stronger long-distance base pairing of this element\(^{24}\). Controls clearly showed binding of both BOs to the trp 5’-UTR\(^{24}\). Given that this long-distance tertiary structure substantially increases the number of base pairs in the trp 5’-UTR, it seemed possible that activation of PKR would increase upon its formation.

We began by investigating the effect of the BOs on the full-length trp 5’-UTR. Representative activation gels are provided in Figure A4 and the average data are plotted in Figure 2.4. Addition of the 3’-BO had little to no effect on activation of PKR (Fig. 2.4b). This is consistent with our earlier studies that the 3’-BO is not highly effective in disrupting the pseudoknot. Addition of the 5’-BO, on the other hand, led to a flattened profile with a ~40% reduction in activation at the RNA concentration corresponding to the activation maximum (Fig. 2.4b).
Figure 2.4: Activation of PKR by the trp 5'-UTR in the presence and absence of a known long-distance tertiary interactions. (a) Model of the full-length (1-206) trp 5'-UTR in the TRAP-free form. (Left) RNA-fold with long-distance tertiary interaction between residues 36-60 and both the 5’- and 3’- regions of the downstream internal loop. (Right) Addition of either a 5’- or 3’- chimeric DNA/LNA blocking oligonucleotide (5’-BO and 3’-BO) disrupts these tertiary interactions. (b-d) Percent PKR activation as a function of RNA concentration for trp 5'-UTR constructs (b) FL (1-206), (c) LH (61-188), and (d) T + tails (89-159) with and without 5’- or 3’-BOs (legends provided in figures). Blocking oligonucleotides are in two-fold excess over the trp 5’-UTR. The average of three independent trials is plotted, connected by trend lines. The average standard deviation was ~20% PKR activation.
Because the 5’-BO fully disrupts the pseudoknot, this finding suggests that the long-distance tertiary structure aids in activating PKR.

Next, we describe control experiments for any direct effects of the BOs on PKR activation. Addition of either the 3’- or 5’-BO’s to PKR in the absence of any RNA did not activate PKR (Fig. A2). Furthermore, addition of either BO to just the large hairpin structure (61-188) led to minimal effects on activation, with perhaps slight enhancement rather than the loss of activation seen with the full-length trp 5’-UTR (Fig. 2.4c). These controls suggest that the loss of activation upon addition of 5’-BO to the full-length trp 5’-UTR is not due to direct effects of the BO with PKR.

Lastly, we tested the effect of the BOs on the terminator hairpin plus its 5’- and 3’- tails (89-159). As shown in Figure 2.4d, addition of either BO led to a ~50% reduction in activation. As presented above, activation of PKR by the terminator is dependent on single-stranded tails. Observation of substantial loss in activation upon addition of the BOs is consistent with this single-stranded tail requirement along with possible blockage of 5’-triphosphate accessibility by the 5’-BO. Moreover, addition of blocking oligonucleotides leads to RNA-DNA hybrids in the tail regions, and such hybrids have been shown to not activate PKR.

2.4.5 Bacterial RNAs activate PKR under physiological magnesium concentrations

The concentration of Mg$^{2+}$ in human cells, where PKR would be exposed to bacterial RNAs, is relatively low at ~0.5 to 1.0 µM. Standard PKR activation assays in the literature have been typically conducted over a range of 4-10 mM Mg$^{2+}$. This is much higher than the human physiological concentration of Mg$^{2+}$ as well as bacterial
physiological concentration of Mg$^{2+}$, which is ~2 mM$^{35,36}$. It is known that many structured RNAs refold with Mg$^{2+}/2$ values in the broad range of 0.5 to 10 mM$^{24,37-41}$. We thus wanted to test whether PKR could be activated at biological Mg$^{2+}$ concentrations.

We began by testing the Mg$^{2+}$- and time-dependence of PKR activation by dsRNA-79. Activation assays are provided in Figure A5 and the data are plotted in Figure 5. As shown in Figure 2.5a (bottom), PKR underwent autophosphorylation in Mg$^{2+}$ concentrations ranging from 0.5 to 4 mM. Under our standard PKR activation assay condition of 4 mM Mg$^{2+}$, maximal activation was reached in 10 min, which remained at a plateau out to 20 min. As the Mg$^{2+}$ concentration was lowered to 2.0, 1.0, and 0.5 mM, the level of activation lowered. The difference in extent of activation between lowest and highest Mg$^{2+}$ concentrations changed as a function of time, increasing and then decreasing (Figure 2.5a, top). By the longest time point of 20 min, there was little difference in extent of PKR activation between the lowest and highest Mg$^{2+}$ was sufficient to allow full extent of PKR phosphorylation independent of Mg$^{2+}$ concentration, we conducted subsequent assays at this time.

Next, the dependence of PKR activation by the FL trp 5’-UTR (1-206) on Mg$^{2+}$ concentration was tested. Given that PKR autophosphorylates to a similar extent in both 0.5 and 4 mM Mg$^{2+}$ concentrations at 20 min, this experiment reports on the ability of different Mg$^{2+}$-dependent folds of the trp 5’-UTR to activate PKR. Previous studies from our lab revealed that the long-distance tertiary structure of the trp 5’-UTR has a Mg$^{2+}/2$ of ~0.5 mM and Hill constant of ~1.5 for folding$^{24}$. Thus the trp 5’-UTR should be only ~50% folded in 0.5 mM Mg$^{2+}$ but fully formed at 4 mM Mg$^{2+}$. 
Figure 2.5: Dependence of activation of PKR on time and Mg\textsuperscript{2+} concentration. (a) PKR activation by dsRNA-79 was monitored at various concentrations of free Mg\textsuperscript{2+} as a function of time. The upper panel displays the range of PKR activation between 0.5 and 4 mM Mg\textsuperscript{2+} as a function of time where it can be seen that the range of activation is much larger for the 5 and 10 min time points. Spacing along the y-axis is the same in the lower and upper panels. (b) The 5, 10 and 20 min assays are plotted versus Mg\textsuperscript{2+} concentration. At 20 min, activation is approximately the same for all Mg\textsuperscript{2+} concentrations tested. In both panels, two individual trials are plotted. For panel (a), lines are drawn through the average of these two trials, and for panel (b) curves are from fits to a Hill equation. Colors of time match for panel (a) upper and panel (b).

As shown in Figure 2.6a,b, PKR was potently activated by the trp 5’-UTR at both 0.5 and 4 mM Mg\textsuperscript{2+} concentrations, with small differences in extent of activation at low and high Mg\textsuperscript{2+} proportionate to small differences seen in the control lanes with dsRNA-79 at these two Mg\textsuperscript{2+} concentrations. These data are consistent with observations above that the trp 5’-UTR potently activates PKR in the presence and absence of the long-distance tertiary contacts (Fig. 2.4). Importantly, these data indicate that bacterial RNA can activate PKR under physiological ionic conditions.
Figure 2.6: Activation of PKR by bacterial RNAs in different concentrations of Mg$^{2+}$. (a) PKR activation assays by full-length trp 5’-UTR (1-206) in low (0.5 mM) and standard (4 mM) Mg$^{2+}$ concentrations. The RNA was incubated in 0.5 or 4 mM Mg$^{2+}$ at 30°C for 5 min followed by a 20 min incubation with PKR. A buffer-only negative control is included and PKR activation is normalized to dsRNA-79 under 4 mM Mg$^{2+}$ conditions. The position of phosphorylated PKR is indicated as ‘p*PKR’. (b) Graphical representation of percent PKR activation from panel (a) as a function of full-length trp 5’ UTR RNA (1-206) concentration. Plotted are two independent trials of each set of data where lines are connecting the averages. (c) PKR activation assays by E. coli total RNA in low (0.5 mM) and normal (4 mM) Mg$^{2+}$ concentrations. The E. coli total RNA was rRNA depleted and incubated in 0.5 mM or 4 mM Mg$^{2+}$ at 30°C for 5 min and then incubated with PKR for 20 min. A buffer-only negative control is included and PKR activation is normalized to dsRNA-79 under 4 mM Mg$^{2+}$ conditions. The position of phosphorylated PKR is indicated as ‘p*PKR’. Experiments were performed twice and a representative gel is provided here.
Lastly, the ability of a mixture of *E. coli* RNAs to activate PKR was tested under both 0.5 and 4 mM Mg$^{2+}$ concentrations. We isolated total RNA from MG1655 *E. coli* and depleted rRNAs using standard laboratory procedures (see Materials and Methods). As shown in Figure 2.6c, total *E. coli* RNA activated PKR under both 0.5 and 4 mM Mg$^{2+}$ conditions and did so to a similar extent, with ~30% activation (relative to 0.1 µM dsRNA-79) at a total bacterial RNA concentration of 22 ng/mL. This activation is within ~10-fold that of dsRNA-79: the concentration of RNA is ~4-fold greater than 0.1 µM dsRNA-79 and the activation level is ~3.3-fold lower. We note that this modest level of activation could indeed be significant because the *E. coli* RNA preparation consists of a vast number of RNAs, present at various levels and sizes, some of which are likely potent activators of PKR. Thus, a mixture of bacterial RNAs has the potential to evoke an innate immune response through PKR.

### 2.5 Discussion

The innate immune system is the body’s first line of defense against pathogens. Factors in this pathway recognize general PAMPs, some of which are comprised of RNA features that differ between pathogens and humans. Indeed, distinction between self and non-self is a key feature of innate immunity and perception of RNA signatures plays a key role. Two proteins that play key roles in innate immunity at the level of RNA recognition are 2′,5′- oligoadenylate synthase (OAS) and PKR. It is well known that 2′,5′- OAS is activated by the viral PAMP of long dsRNA to synthesize 2′, 5′- linked adenylate oligonucleotides. The oligoadenylates activate latent RNase L which then degrades viral and cellular RNAs to block viral infection. In addition, dsRNAs activate PKR to
undergo autophosphorylation, which then phosphorylates eIF2α, thereby inhibiting translation and blocking viral infection\textsuperscript{43}. Two additional proteins that serve as receptors in the innate immune response to RNA are RIG-I and MDA5, which are helicases that bind dsRNA and are expressed and functional in the presence of viral or bacterial infection\textsuperscript{44}.

Recent reports indicate that bacterial RNAs activate PKR in mice\textsuperscript{19,20}. We found that the\textit{trp} 5’-UTR, which has structured RNA elements representative of many bacterial mRNAs such as a terminator, 5’-stem-loop, SD hairpin, and Mg\textsuperscript{2+}-dependent tertiary structures is a potent activator of PKR both in the absence and presence of bound TRAP. Multiple secondary structures drive activation of PKR including the full-length\textit{trp} 5’-UTR as well as its large hairpin, and furthermore that these RNA elements do so at a maximal level similar to that of perfect dsRNA-79 (Table 2.1). The fact that activation of these pieces adds up to more than activation by full length 5’-UTR may be due to the elements overlapping in their PKR binding sites. What is most significant is that any of several elements can lead to activation, indicating that if one element is absent, due to say protein binding or conformational switching, another can still activate PKR. We found that all of the bacterial hairpins that we examined required flanking tails to activate PKR, with a preference for a 3’-tail. Most bacterial RNAs are found in this context, however, especially those at the 5’-end of RNA, supporting the general ability of bacterial mRNAs to activate PKR.

Observation that a high level of PKR activation occurs when TRAP/L-Trp binds the 5’-UTR is consistent with TRAP binding primarily to single-stranded RNA regions, and TRAP/L-Trp-bound\textit{trp} 5’-UTR still having multiple helical regions of RNA
available for recognition by PKR. Additionally, the pseudoknot that forms in the 5′-UTR in the absence of bound TRAP promotes activation of PKR, which is supported by partial loss of activation upon disruption of the long-distance base pairing by the blocking oligonucleotides (Fig. 2.4). This observation is agrees with earlier reports that base pairing within RNA pseudoknots can promote PKR activation\(^7\). In addition to the large hairpin, the terminator and SD blocking hairpins potently activated PKR in the presence of single-stranded tails and a 5′-triphosphate. These findings indicate that the mode of PKR activation involving ssRNA tails and a 5′-triphosphate\(^{14,15}\) is important in sensing bacterial RNA.

The 5′-SL construct activated PKR the strongest. On the basis of earlier studies from our lab, this likely arises from this construct having the longest single-stranded tail\(^{15}\). From a biological standpoint, this observation indicates that the very 5′ end of the 5′-UTR can activate PKR. Moreover, the 5′-SL continued to activate PKR at a very high level after CIP treatment indicating activation of PKR both with a 5′-triphosphate and a 5′-OH. It has been reported that a 5′-triphosphate is sometimes processed to a 5′-monophosphate in bacteria by action of a pyrophosphatase\(^{45}\). We previously showed that 5′-OH, -p, and –pp are recognized similarly by PKR\(^{14}\). The ability of the 5′-SL construct to activate PKR both with and without a 5′-triphosphate supports PKR’s ability to recognize a bacterial RNA as foreign no matter its modification status. Given that most cellular RNAs have a m7G cap at their 5′-ends, activation of PKR by an RNA with both a 5′-OH and a 5′-triphosphate supports broad spectrum recognition of this bacterial RNA element as foreign. From a molecular standpoint, the mild 5′-triphosphate dependence of the 5′-SL may be due to interactions of PKR with the very long 28 nt 3′tail.
We also investigated the effect of using physiological Mg\(^{2+}\) concentration on PKR activation using a variety of RNAs. The concentration of Mg\(^{2+}\) in human cells is \(~0.5\) mM, which is much lower than in classical PKR activation assays or in bacteria. It thus seemed possible that the ability of bacterial RNAs to activate PKR is altered in the cytoplasm of human cells. We found, however, that lowering the concentration of Mg\(^{2+}\) had little effect on PKR activation either in the presence of the classical activator of dsRNA-79 or with the full-length trp 5’-UTR. Lastly, we found that a rRNA-depleted preparation of E. coli total RNA activated PKR under both 0.5 and 4 mM Mg\(^{2+}\) conditions. These data indicate that bacterial RNA can activate PKR under the Mg\(^{2+}\) conditions found in the cell.

In sum, we observed potent activation of PKR by a representative bacterial mRNA from B. subtilis and a total RNA preparation from E. coli at human physiological Mg\(^{2+}\) concentrations. Many of the features that PKR recognizes in the B. subtilis trp 5’-UTR are general and present in many bacterial RNAs. It thus appears that activation of PKR by bacterial RNAs is general and applies to Gram-positive and Gram-negative bacteria. Interestingly, activation of PKR by this mixture of bacterial RNA is within \(~10\) -fold that of perfectly dsRNA-79. During bacterial infection of human cells, bacteria are known to lyse and release their RNA contents. Before this step, certain bacteria are known to secrete factors into human cells\(^{46}\). Recent reports that Listeria activates RIG-I and MDA5 by secreting RNA and DNA into mice are consistent with this notion. Thus, it is possible that PKR is also activated by RNA during various stages of bacterial infections.
Several approaches for examining RNA structure transcriptome-wide have been developed recently, including ones that assay RNA folds in vivo \(^{47-50}\). Furthermore, studies from the Weissman lab suggest that human RNAs are significantly less structured in vivo than previously thought. Low amounts of structured, protein-free RNA in healthy human cells may be key to understanding how bacterial RNAs, which are generally quite structured, are potent activators of PKR and the innate immune system, and may suggest an explanation for the recently noted absence of riboswitches in the human genome \(^{51}\). Future experiments are needed on a transcriptome-wide level on bacterially infected human cells to determine the identity and structure of bacterial RNAs that can activate PKR.

### 2.6 Acknowledgements

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2.7 References


Appendix A: Supporting Information for Chapter 2

Sequences of trp 5’UTR Truncations (5’ to 3’)

1-206 (FL)
AGCTTAGAAATACACAAGAGTGTGTATAAAAGCAATTAGAATGAGTTGAGTTA
GAGAATAGGGTAGCAGACAATGAGTTTATGTGAGCTGAGACATTATTTAT
TCTACCCAAAAAGAAGTCTTTTCTTTTGGGTTATTTATATAGATATTTAT
TCTCATGCCCATCTCTTCATGCTTGGCCATAAGGAGTGAGAGCAATG

61-188 (LH)
GGTAGCAGAGAATGAGTTGAGCTGACATTATATTTATTTCTACCCAA
AAGAAGTCTTTTCTTTTGGGTTATTTATATAGATATTTATCTCTCAGGCC
ATCTCTCTCTCTCCTCCTGCC

89-159 (T + tails)
GAGACATTATGTCTTTTATCTACCCAAAAAGAAGTCTTTTCTTTTGGTATTAT
ATATAGATATATAGCCT

142-206 (SD SL + tails)
GGATAGTATTTTTATGCTCTCTCATGCCATCTTTCTCTCCTTGCCATAAGGAGT
GAGAGCAATG

1-60 (5’-SL + tails)
GGAGCTTAGAAATACACAAGAGTGTGTATAAAAGCAATTAGAATGAGTTGAGTTA
TAGAATGAG

108-159 (T + 3’ tail)
GGACCCAAAAAGAAGTCTTTTCTTTTGGGTTATTTATATAGATATTTATCCT
108-133 (T)
ACCCAAAAGAAGTCTTTCTTTTGGGT

172-200 (SD SL)
GGTCTCATTCTCCTTGCCATAAGGAGTGAGAGCAATG

89-133 (T + 5’ tail)
GAGACATTATGTTATTTCTACCCAAAAGAAGTCTTTCTTTTGGGT

Figure A1: PKR activation assays for dsRNA-79 for two different preparations of wtPKR protein. Concentrations of dsRNA-79 tested for activation were 0.01, 0.05, 0.1 and 0.5 µM. The dsRNA-79 concentration used for the PKR activation assay in the main paper (i.e. the positive control) is 0.1 µM. This figure confirms that this concentration is at or very near the maximum for activation. A no-RNA (-) lane is provided and all concentrations are normalized to 0.1 µM dsRNA-79. All gels are 10% SDS-PAGE.
Figure A2: PKR activation assay controls for contributing activity by TRAP and blocking oligonucleotides. (a) PKR was tested for activation by TRAP protein alone from 0.025 to 25 µM TRAP in 10-fold dilutions where no detectable activity was found. (b) PKR was tested for activation by both the 5' and 3' blocking oligonucleotides alone from 0.02 to 20 µM concentrations in 4-fold dilutions and no detectable activity was found. No-RNA (-) lanes are provided as well as 0.1 µM dsRNA-79 as a positive control. All gels are 10% SDS-PAGE.
Figure A3: Representative gel images for all RNA truncations tested in Table 2.1 including (a) long hairpin (61-188) and terminator and tails (89-159), (b) terminator and 5’ tail (89-133) and Shine-Dalgarno blocking hairpin and tails (142-206), (c) terminator and 3’ tail (108-159) and 5’ stem-loop and tails, and (d) Shine Dalgarno blocking hairpin (172-200) and terminator (108-133). In panel d, the gel image is a composite but all lanes are from the same gel and processed with the same color range. Each gel has a no-RNA (-) lane provided and all samples are normalized to 0.1 µM dsRNA-79. All gels are 10% SDS PAGE.
Figure A4: Representative gel images for PKR activation assays on (a) full-length (1-206), (b) long hairpin (61-188) and (c) terminator and tails (89-159) bound to the indicated blocking oligonucleotide. Each gel has a no-RNA (-) control and all samples are normalized to 0.1 µM dsRNA-79. All gels are 10% SDS-PAGE.
Figure A5: Representative PKR activation assays of Mg\(^{2+}\) and time dependence of activation by dsRNA-79. Each gel shows time points for both no-RNA ‘negative controls’ and 0.1 \(\mu\)M dsRNA-79 ‘positive controls’. Each gel has a constant concentration of Mg\(^{2+}\) present: (a) 0.5 mM Mg\(^{2+}\), (b) 1.0 mM Mg\(^{2+}\), (c) 2.0 mM Mg\(^{2+}\), and (d) 4.0 mM Mg\(^{2+}\). All gels were normalized to 0.1 \(\mu\)M dsRNA-79 at 4.0 mM Mg\(^{2+}\) and 10 min. All gels are 10% SDS-PAGE.
Chapter 3

Bacterial riboswitches and ribozymes potently activate the human innate immune sensor PKR

[Submitted as a paper entitled “Bacterial riboswitches and ribozymes potently activate the human innate immune sensor PKR.” By Chelsea M. Hull, Ananya Anmangandla, & Philip C. Bevilacqua to ACS Chemical Biology.]

Contributions: AA (undergraduate student) performed PKR activation assays on the twister ribozyme

3.1 Abstract

The innate immune system provides the first line of defense against pathogens through the recognition of non-specific patterns in RNA to protect the cell in a generalized way. The human RNA-activated protein kinase, PKR, is a dsRNA binding protein and an essential sensor in the innate immune response, which recognizes viral and bacterial pathogens through their RNAs. Upon activation via RNA-dependent autophosphorylation, PKR phosphorylates the eukaryotic initiation factor eIF2α, leading to termination of translation. PKR has a well-characterized role in recognizing viral RNA, where it binds long stretches of double-stranded RNA non-sequence specifically to promote activation; however, the mechanism by which bacterial RNA activates PKR and the mode by which self RNA avoids activating PKR are unknown. We characterized activation of PKR by three functional bacterial RNAs with pseudoknots and extensive tertiary structure: the cyclic-di GMP riboswitch, the glmS riboswitch-ribozyme, and the twister ribozyme, two of which are ligand-activated. These RNAs were found to activate PKR with comparable potency to long dsRNA. Enzymatic structure mapping in the absence and presence of PKR reveals a clear PKR footprint and provides a structural
basis for how these bacterial RNAs activate PKR. In the case of the cyclic di-GMP riboswitch and the \textit{glmS} riboswitch-ribozyme, PKR appears to dimerize on the peripheral double-stranded regions of the native RNA tertiary structure. Overall, these results provide new insights into how PKR acts as an innate immune signaling protein for the presence of bacteria and suggest a reason for the apparent absence of protein-free riboswitches and ribozymes in the human genome.

3.2 Introduction

The first line of defense to invading organisms is the innate immune system, which involves pattern recognition receptors (PRR’s) that detect molecular patterns in order to discriminate between self and non-self.\cite{1} Many proteins are involved in the innate immune system including the RNA-activated Protein Kinase, PKR.\cite{2} This essential sensor is comprised of an N-terminal double-stranded RNA binding domain (dsRBD) that contains two tandem double-stranded RNA binding motifs (dsRBMs), as well as a C-terminal catalytic kinase domain.\cite{3} The dsRBM is found in a number of key proteins, including Dicer, TRBP, and ADAR, and binds dsRNA in a non-sequence-specific fashion primarily in the minor groove.\cite{3,4} The classical model of PKR activation involves its binding to long stretches of dsRNA, where at least 16 bp are needed for PKR to bind and 30 bp for it to dimerize and become active.\cite{5,6,7} Upon dimerization, PKR can autophosphorylate followed by phosphorylation of eIF2\alpha leading to inhibition of translation initiation.\cite{8} Because PKR must dimerize to become active, too much RNA shuts off PKR activity by stranding PKR monomers onto RNA islands.\cite{7}
PKR is known to be classically activated by long dsRNA from viruses, but it is also known to be activated by other structured RNAs. More recently, PKR has been shown to be activated by bacterial RNA, both from total RNA in vivo as well as a discrete bacterial RNA leader in vitro. In vivo concentrations of Mg^{2+} have a substantial effect on RNA folding and function. Bacteria have physiological Mg^{2+} concentrations of ~2-3 mM, while human cells have physiological Mg^{2+} concentrations of ~0.5-1 mM suggesting that bacterial RNAs could refold in eukaryotic cells depending on local Mg^{2+} concentrations. In addition, standard PKR activation assays in the literature have been performed typically over the range of 4-10 mM Mg^{2+}. As such, the effects of prokaryotic and eukaryotic Mg^{2+} levels on PKR activation are important to test.

Key functional RNAs discovered in the last few decades include riboswitches and ribozymes. Riboswitches are aptamers that control gene function by binding a metabolite, which can be anything from a protein to a small molecule or ion. Ribozymes are catalytic RNAs that can make and break chemical bonds under certain cellular conditions, typically to regulate downstream gene function. Riboswitches and ribozymes are known primarily in prokaryotes. An outstanding question is the reason for the apparent absence of riboswitches and ribozymes in eukaryotes.

Three representative functional bacterial RNAs are studied here: a 110 nt cyclic di-GMP (cdiGMP) riboswitch from Vibrio cholerae, a 145 nt glmS riboswitch-ribozyme from Bacillus anthracis, and a 61 nt twister ribozyme from Clostridia bolteae. These RNAs span different classes and sizes of functional RNAs. In addition, we tested PKR activation by the full-length 5’UTR of the cdiGMP riboswitch, which is 350 nt in length and contains both the aptamer and expression domains. All of these RNAs have
pseudoknots and both of the ribozymes are double-pseudoknotted, allowing the importance of RNA tertiary structure in activating PKR to be tested.\textsuperscript{19-21} These particular bacterial RNAs were also chosen in part on the basis of their diversity and their pathogenesis to humans: \textit{V. cholerae} is a gram-negative bacterium that causes cholera, \textit{B. anthracis} is a gram-positive bacterium that causes anthrax, and \textit{C. bolteae} is a gram-positive bacterium that causes bowel-related issues associated with autism.

Cyclic-diGMP is a bacterial second messenger that activates extracellular polysaccharide production and biofilm formation in addition to regulating motility and virulence.\textsuperscript{22} Classically, cdiGMP was known to perform this function by binding protein enzymes, but more recently it has been shown to bind certain riboswitches.\textsuperscript{23,24} The cdiGMP riboswitch from \textit{V. cholerae} is a class-I cdiGMP riboswitch that controls the \textit{tfoX} gene by an antitermination mechanism (Figure 3.1).\textsuperscript{24}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Full length 5'UTR (Vc2FL) and aptamer-only (Vc2) constructs of the cdiGMP riboswitch from \textit{Vibrio cholerae}. (A) Effect of Mg\textsuperscript{2+} and cdiGMP on docking of the aptamer region of the cdiGMP riboswitch leading to the formation of an antiterminator, ‘AT’, from a terminator, ‘T’. Structure mapping and prediction of the terminator and antiterminator are provided in Figure B4. The upper left, upper right and lower right states are referred to in the literature as ‘undocked’, ‘pre-docked’, and ‘docked’, respectively;\textsuperscript{25} the latter two states contain the pseudoknot. Brackets in lower left figure indicate structural dynamics. (B) Secondary and tertiary structure models of Vc2.\textsuperscript{20,25} Nucleotides in the dashed region of the secondary structure were replaced with the U1A binding site during crystallization.}
\end{figure}
The cdiGMP signaling pathway is important in controlling expression of the gene in a host cell, as cdiGMP levels are often decreased upon infection allowing the bacteria to express virulence factors necessary to survive. Both cdiGMP and Mg\(^{2+}\) affect the fold of the riboswitch (Figure 3.1). In limiting Mg\(^{2+}\) conditions (0.5 mM Mg\(^{2+}\)) in the absence of cdiGMP, the aptamer adopts an undocked state. In excess Mg\(^{2+}\) conditions, (4.0 mM Mg\(^{2+}\)) the aptamer assumes a pre-docked, but not fully formed fold that upon addition of saturating cdiGMP forms a docked structure with an antiterminator that allows the downstream gene to turn on. The \textit{glmS} riboswitch-ribozyme regulates levels of glucosamine-6 phosphate (GlcN6P), which is the ligand that it binds. The twister ribozyme was recently discovered and is one of the fastest self-cleaving ribozymes, found in a large range of organisms (see structures below). Crystal structures are available for both of these RNAs.

In the present study, we conducted activation assays on the \textit{V. cholerae} cyclic diGMP riboswitch aptamer domain (Vc2) and its full-length 5’UTR (Vc2FL) in the absence and presence of cyclic-diGMP. We also removed key tertiary interactions in the Vc2 and Vc2FL riboswitches by a G83C mutation to test whether tertiary RNA interactions play a role in activation of PKR. Lastly we tested activation of PKR by the post-cleavage product of the \textit{glmS} riboswitch-ribozyme and twister ribozyme. To understand the potent activation of PKR by these riboswitches and ribozymes, we conducted structure mapping and PKR footprinting, which led to a structural model for how these functional bacterial RNAs interact with and activate PKR.
3.3 Materials and Methods

3.3.1 Design of RNAs

The control RNA, dsRNA-79, was prepared as previously described. This is a perfect dsRNA of 79 bp that is a classical activator of PKR. The Vc2FL and Vc2 constructs were prepared by transcription off a PCR product from a plasmid (gift from Scott Strobel’s lab). The glmS ribozyme was also prepared by transcription from a PCR product from a plasmid (gift from Michael Been’s lab). The ribozyme cleaved nearly to completion during transcription. RNA products were purified by denaturing PAGE. The twister ribozyme constructs were made by transcription from a hemi-duplex template. The +5, +10 and +15 twister constructs designed have the natural nucleotides present in the C. bolteae genome. Twister RNAs were also purified by denaturing PAGE and found to cleave during transcription. All sequences are provided in the Supplementary Methods in appendix B.

3.3.2 RNA preparation and purification

In vitro T7 transcription reactions of 0.5-1 mL were carried out on the templates described above. Reactions were submerged in a water bath at 37°C for 2-4 h, quenched with equal volume of 2X formamide loading buffer (95% v/v formamide, 20 mM EDTA), and fractionated on a 10% denaturing PAGE gel. RNA bands were detected via UV-shadowing, cut out with a razor blade, and eluted overnight into 1x TEN_{250} [10 mM Tris (pH 7.5), 1 mM EDTA, 250 mM NaCl]. RNA was then ethanol precipitated and
dissolved in 1x TE [10 mM Tris (pH 7.5), 1 mM EDTA], quantified by NanoDrop, and stored at –20°C.

For PKR activation assays, the highest concentration of RNA tested was prepared in 1xTEN100 and renatured at 90°C for 2 min followed by room temperature (~22°C) for 10 min. When binding cdIGMP to these riboswitches, a 2-fold excess cyclic di-GMP (Axxora) was incubated with the riboswitch in the desired concentration of Mg²⁺ in folding buffer [10 mM sodium cacodylate (pH 6.8), 10 mM KCl, 4 mM (or 0.5 mM) MgCl₂] at 70°C for 5 min followed by room temperature for 30 min. Twister and glmS ribozymes were renatured at 90°C for 2 min followed by room temperature for 10 min before each experiment.

3.3.3 Protein expression and purification

Full-length PKR with an N-terminal (His)₆ tag was cloned into pET-28a and transformed into BL21 (DE3) Rosetta cells (Novagen). Sonicated cells were passed over a Ni²⁺-NTA agarose column (Qiagen) then eluted with imidazole gradient in the background of 750 mM NaCl on a Bio-Rad FPLC. Protein was dialyzed into storage buffer [10 mM Tris (pH 7.6), 50 mM KCl, 2 mM Mg(OAc)₂, 10% glycerol and 7 mM β-mercaptoethanol]. Concentration was determined by spectroscopy and the protein was aliquotted, flash-frozen, and stored at -80°C.

3.3.4 PKR Activation Assays

RNAs were assessed for activation of unphosphorylated PKR. Upon purification from *E. coli*, PKR is phosphorylated. We dephosphorylated it by treatment with lambda
protein phosphatase at 30˚C for 1 h followed by inactivation of the phosphatase with freshly made 2 mM sodium orthovanadate.6,30 Immediately after dephosphorylation, PKR, at a final concentration of 0.8 μM, was incubated with the RNA or RNA-ligand complex of interest at 30˚C for 10 min (normal conditions) or 20 min (for Mg^{2+} dependence). RNAs were diluted in 1xTEN_{100} at 4-fold dilutions. Reactions were performed in PKR activation buffer [20 mM HEPES (pH 7.5), 50 mM NaCl] and 1.5 mM DTT, 100 μM ATP (Ambion) plus 15 μCi [γ-^{32}P]-ATP. Magnesium chloride was added at either 4 or 0.5 mM Mg^{2+} and RNAs were incubated for 5 min at 30˚C to aid folding. Reactions were stopped with addition of SDS loading buffer and fractionated on a 10% Bis-Tris (Novex) SDS PAGE pre-cast gel, dried and exposed to a storage PhosphorImager screen (Molecular Dynamics). Phosphorylated PKR bands were detected with a PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software. Each activation assay gel contains a no-RNA negative control, as well as a 0.1 μM dsRNA-79 positive control used for normalization.

3.3.5 Structure Mapping and PKR Footprinting

RNAs were 5-end labeled with [γ-^{32}P]-ATP using T4 PNK (NEB) and purified by denaturing PAGE. Labeled RNAs were renatured in 1xTEN_{100} at 90˚C for 2 min and room temperature for 10 min and if applicable, pre-incubated in binding buffer with 8 μM cdiGMP ligand at 70˚C for 5 min followed by room temperature for 30 min. To minimize background, nonspecific RNA (tRNA^{phe}) and protein (BSA) were added to the reactions at final concentrations of 130 ng and 1.5 μM respectively. RNA was subjected to limited digestion by single-stranded specific nucleases (RNase T1 or RNase A) or a
double-stranded specific nuclease (RNase V1) under native conditions in structure buffer [20 mM HEPES (pH 7), 100 mM NaCl, and 4 mM MgCl₂]. Conditions that gave single-hit digestion were 0.001 U/µL RNase T1 (Ambion), 37°C, 15 min; 0.005 ng/µL RNase A (Ambion), room temperature, 15 min; and 0.001 U/µL RNase V1 (Ambion), room temperature, 15 min.

To generate a hydrolysis ladder, labeled RNA was incubated in 100 mM Na₂CO₃/NaHCO₃ and 2 mM EDTA for 8 min at 90°C. To generate a T1 ladder (=all Gs), labeled RNA was incubated under denaturing conditions in 0.1 U/µL RNase T1, 18 mM Na-citrate (pH 3.5), 0.9 mM EDTA, and 6 M urea for 20 min at 50°C. For PKR footprinting, the RNA and master mix [1x TEN, 130 ng tRNAṭhe, 1.5 µM BSA, 4 mM Mg²⁺] was incubated with increasing concentrations of PKR (0.44, 1.75, and 7 µM) for 30 min at room temperature prior to nuclease digestions. Controls were performed on structure mapping and footprinting experiments by incubating the RNA with each component of the reaction in the absence of nuclease at 37°C for 15 min. All samples were quenched with 2x EDTA/formamide/0.2% SDS loading buffer (pH 11) and loaded onto a 12% polyacrylamide, 8.3 M urea sequencing gel, dried and exposed to a storage PhosphorImager screen overnight.

3.4 Results and Discussion

3.4.1 PKR is activated by both the Vc2 and Vc2FL forms of the cdiGMP riboswitch

The first of the three functional bacterial RNAs that was tested for activation of PKR is the cdiGMP riboswitch from Vibrio cholerae. We tested both the aptamer domain alone (Vc2) and the full-length 5’-UTR (Vc2FL), which contains both Vc2 and the terminator and antiterminator hairpins, among other possible regulatory structure. These
experiments were conducted under both standard activation conditions of 4 mM Mg$^{2+}$, similar to prokaryotic cell conditions, and human cellular salt conditions of 0.5 mM Mg$^{2+}$ in the presence and absence of saturating amounts of the metabolite, cdiGMP. These concentrations were chosen to populate the undocked, pre-docked, and docked states, as described in Figure 3.1. Results are summarized Tables B1 and B2 and representative activation gels are provided in Figure 3.2.

**Figure 3.2:** Activation of PKR by Vc2 & Vc2FL at 0.5 and 4 mM Mg$^{2+}$ in the absence and presence of cdiGMP. (A,B) SDS-PAGE gels for (A) Vc2 at 0.5 and 4 mM Mg$^{2+}$ and (B) Vc2FL at 0.5 and 4 mM Mg$^{2+}$. Bands indicate phosphorylated PKR, ‘p*PKR’. Percent PKR activation was normalized to that of 0.1 µM dsRNA-79 in 4 mM Mg$^{2+}$ conditions. (C,D) Percentage of PKR activation versus the concentration of RNA for (C) Vc2 and (D) Vc2FL at 0.5 mM and 4 mM Mg$^{2+}$ and in the absence and presence of cdiGMP (denoted cdiG in legends). Plotted are the averages of two trials and all values can be viewed in Appendix B. Note Vc2 +cdiGMP, 0.5 mM Mg$^{2+}$ 2.5 and 0.625 µM points were offset slightly so they were not hidden behind the 4 mM Mg$^{2+}$ data points.
Under standard activation conditions of 4 mM Mg\(^{2+}\), which populates the pre-docked state (Figure 3.1A), Vc2 potently activated PKR with a maximal activation level at \(~140\%\) that of 0.1 \(\mu\)M dsRNA-79 (Figure 3.2A). Activation was completely dependent on the presence of RNA. As the concentration of RNA was increased, activation increased and then decreased, with a bell-shaped dependence on RNA concentration, consistent with RNA-dependent dimerization of PKR (Figure 3.2C). Upon lowering the Mg\(^{2+}\) concentration to a physiological value of 0.5 mM, which populates primarily the undocked state, activation was still potent, at \(~110\%\) that of 0.1 \(\mu\)M dsRNA-79. Upon adding 20 \(\mu\)M cdiGMP, the activation decreased slightly for both 4 and 0.5 mM Mg\(^{2+}\) conditions, with both requiring more RNA to gain activation and having a somewhat lower maxima of \(~90\%\) dsRNA-79 but which was strong nonetheless. Note that cdiGMP alone does not activate PKR nor compete with activation by dsRNA-79 (Figure B1). (For evidence of cdiGMP binding, Vc2 folding, and PKR binding to the natively folded riboswitch, see structure mapping data below.) These ligand containing results, especially those in 4 mM Mg\(^{2+}\), reveal that the fully docked state also activates PKR. Overall, these results indicate that the bacterial Vc2 riboswitch is a potent activator of PKR under prokaryotic and eukaryotic salt conditions and in the presence and absence of bound ligand.

Next, the ability of the full length 5’UTR (Vc2FL) to activate PKR was tested. As shown in Figure 3.2D, the results were quite similar to Vc2. Potent activation was observed in the absence of ligand and under standard activation conditions of 4 mM Mg\(^{2+}\), which populates the pre-docked state, with a bell-shaped dependence on RNA concentration. Lowering the Mg\(^{2+}\) concentration to 0.5 mM, which populates primarily
the undocked state, decreased activation ~2-fold, somewhat more than for Vc2 but still with strong activation. Upon adding cdiGMP in the presence of 4 mM Mg$^{2+}$ to force the fully docked state, activation was affected only slightly, with maximal activation at ~110% dsRNA-79. (For evidence of cdiGMP binding and accompanying changes in the secondary structure of the expression domain, see Figure B4). At eukaryotic Mg$^{2+}$ concentration, activation was affected more significantly by ligand, where activation fell to ~20% that in dsRNA-79. All four conditions for Vc2FL revealed bell-shaped activation curves that were ‘left-shifted’ compared to the aptamer alone: a lower molar concentration of RNA was needed to gain activation and a lower concentration of RNA led to loss of activation. The bell-shape to the RNA activation profile is again consistent with RNA-dependent dimerization of PKR, and the leftward shift of the profile supports the longer Vc2FL (350 nt) requiring fewer moles of transcript to both activate and inactivate PKR. We also note that both ligand-free and ligand-bound Vc2 and Vc2FL compete with dsRNA-79 for activation (Figure B1), indicating that the riboswitches bind to the same, or overlapping, site(s) as dsRNA on PKR. Overall, in the majority of the cases tested, the Vc2 and Vc2FL 5’-UTR strongly activate PKR by RNA-dependent dimerization under diverse salt and ligand conditions and with several different RNA constructs.

3.4.2 Tertiary interactions in Vc2 and Vc2FL contribute to PKR activation

As described in the previous section, both the aptamer domain alone (Vc2) and full-length 5’-UTR (Vc2FL) of the cdiGMP riboswitch activate PKR potently under prokaryotic and eukaryotic Mg$^{2+}$ conditions, in both the absence and presence of the
cdiGMP ligand. The aptamer domain contains tertiary interactions that form to various extents in so-called undocked, pre-docked, and docked states under the Mg$^{2+}$ and cdiGMP concentrations studied herein (Figure 3.1).$^{25}$ In the previous section, we showed that under conditions in which the ribozyme is fully folded and ligand bound, PKR is strongly activated. We wished to test the extent to which PKR activation is affected by riboswitch tertiary interactions because they assemble three A-form helical segments. To achieve this, we prepared G83C mutants of Vc2 and Vc2FL and tested them for activation of PKR in both 0.5 and 4 mM Mg$^{2+}$ and in the absence and presence of 20 µM cdiGMP. The G83 residue is located at the center of the aptameric domain (Figure 3.1B) and changing it to a C has been shown to prevent the pseudoknot-containing ligand-free pre-docked structure in the upper right-hand corner of Figure 3.1A from forming.$^{20,25}$

As shown in Figure 3.3, the four combinations of Mg$^{2+}$ concentration and cdiGMP for Vc2 and Vc2FL G83C constructs led to WT-like results of bell-shaped activation with maximal activation at ~60-90% that of 0.1 µM dsRNA-79. These results indicate that removing tertiary interactions does not enhance activation of PKR in either the aptamer alone or the full length 5’-UTR. On the contrary, activation is more potent with wild-type than G83C mutants for Vc2 and Vc2FL in 4 mM Mg$^{2+}$ with or without ligand, conditions under which tertiary interactions are known to form in wild-type (Figure 3.2).
Figure 3.3: Activation of PKR by Vc2 & Vc2FL G83C mutants at 0.5 and 4 mM Mg$^{2+}$ in the absence and presence of cdiGMP. (A,B) Percentage of PKR activation versus the concentration of RNA for (A) Vc2 and (B) Vc2FL at 0.5 mM and 4 mM Mg$^{2+}$ and in the absence and presence of cdiGMP (denoted cdiG in legends). Plotted are the averages of two trials and all values are provided in Tables B3 and B4. Percent PKR activation was normalized to that of 0.1 µM dsRNA-79 in 4 mM Mg$^{2+}$ conditions.

3.4.3 The \textit{glmS} riboswitch-ribozyme activates PKR

We next investigated regulation of PKR by two bacterial ribozymes, the larger \textit{glmS} riboswitch-ribozyme and the smaller twister ribozyme. In both cases, one-piece versions were used since these represent the forms of the ribozymes as they occur in nature; in addition, multiple crystal structures are available for each ribozyme.\textsuperscript{19,21} Like the cdiGMP riboswitch, the \textit{glmS} riboswitch-ribozyme binds a metabolite, here GlcN6P, to regulate expression, but the \textit{glmS} riboswitch-ribozyme also self-cleaves to regulate gene expression.\textsuperscript{28} Since the one-piece version of the ribozyme self-cleaved to a significant extent during transcription (see Methods), we worked with the self-cleaved form of this RNA, which is missing just a small single-strand extension on the 5'-end. The secondary structure of the \textit{glmS} riboswitch-ribozyme is shown in Figure 3.4A where
the nucleotides involved in the double pseudoknot are highlighted, and long base-paired regions are apparent.

The self-cleaved glmS riboswitch-ribozyme activated PKR potently, at 100% the level of 0.1 µM dsRNA-79, and did so similarly at 0.5 and 4 mM Mg$^{2+}$ (Figure 3.4). The dependence of activation on RNA concentration was bell-shaped, consistent with dimerization of PKR on the glmS RNA (Figure 3.4). The shapes of the PKR activation curves for the glmS riboswitch-ribozyme were similar to Vc2, with onset of activation at ~0.1 µM RNA, maximal activation ~95% of 0.1 µM dsRNA-79 at ~1 µM RNA, and loss of activation beginning at ~10 µM RNA (Figures 3.2A and 3.4B).

Figure 3.4: Dependence of activation of PKR on Mg$^{2+}$ concentration for the glmS ribozyme. (A) Secondary structure of the 145 nt self-cleaved B. anthracis glmS ribozyme. The double pseudoknot interactions are shown in green. Nucleotides were inserted into the loop in P1 between nt 16-17 for a U1A binding site to aid in crystallization (dashed circle). (B) Percentage of PKR activation versus concentration of glmS RNA at 0.5 and 4 mM Mg$^{2+}$. Plotted are the averages of two trials and all values can be viewed in the Appendix B. Percent PKR activation was normalized to that of 0.1 µM dsRNA-79 in 4 mM Mg$^{2+}$ conditions.
Consistent with this similar behavior, the crystal structures of Vc2 and the glmS riboswitch-ribozyme reveal similar ‘Y-shaped’ overall architectures and extensive tertiary structure. (For folding of the glmS riboswitch-ribozyme and binding of PKR, see structure mapping data below.)

3.4.4 PKR is activated by the small self-cleaving twister ribozyme by extending its 3’-end

The third and final bacterial RNA investigated for activation of PKR is the recently discovered twister ribozyme. Unlike the prior two bacterial RNAs, twister is not a riboswitch; moreover, it is a smaller RNA of just 66 nt that does not form an extensive and homologous ‘Y-shaped’ tertiary structure. A one-piece version of the twister from Clostridium bolteae was studied, along with various 3’-end extensions. Like the glmS riboswitch-ribozyme, all of the twister ribozyme constructs cleaved during transcription. The secondary structure of the twister ribozyme is provided in Figure 3.5, and the double pseudoknot is highlighted.

We began with a self-cleaved version of twister with the minimal 3’-end needed to form the secondary structure of the active ribozyme and tested its ability to activate PKR in 4 mM Mg$^{2+}$ (Figure 3.5A). This construct, which we denote as ‘T’, did not significantly activate PKR (Figure 3.5B). We then lengthened the 3’-end of the ribozyme by 5, 10, or 15 nucleotides. The T+5 RNA also did not significantly activate PKR. Results on T and T+5 indicate that not all RNAs activate PKR.

The T+10 RNA, on the other hand, showed significantly more activation, up to 30% the level of dsRNA-79, but at a high RNA concentration for maximal activity of ~3 µM. Strikingly, the T+15 RNA gave substantial activation, at ~80% that of 0.1 µM
dsRNA-79 and a lower RNA concentration for maximal activation, of ~0.6 µM. This T+15 RNA has the ability to form a simple stem-loop at the 3’end. The T+15 RNA continues to activate PKR under eukaryotic Mg\(^{2+}\) conditions of 0.5 mM, albeit ~2-fold lower (Figure 3.5C). In sum, the majority of the riboswitches and ribozymes tested activate PKR, and do so using a variety of RNA secondary and tertiary structures, ranging from complex tertiary structures to simple stem-loops.

Figure 3.5: Activation of PKR by a twister ribozyme is dependent on flanking-tail length and concentration of Mg\(^{2+}\). (A) Secondary structure of the *C. bolteae* twister ribozyme (black) with the double pseudoknot interactions shown in yellow and orange. (B) Percentage of PKR activation versus concentration of twister RNA for varying length flanking tails under standard PKR activation conditions (4 mM Mg\(^{2+}\), 10 min). (C) Percentage of PKR activation for the highest activating twister construct, T +15, at 0.5 and 4 mM Mg\(^{2+}\). Plotted are the averages of three trials, and all values can be viewed in the Appendix B. Percent PKR activation was normalized to that of 0.1 µM dsRNA-79 in 4 mM Mg\(^{2+}\) conditions.

3.4.5 Structure mapping and PKR footprinting reveal binding modes of PKR

In an effort to gain insight into the structural basis for activation of PKR by functional RNAs, we performed structure mapping on the 5’-end labeled aptamer domain of the Vc2 riboswitch and the *glmS* riboswitch-ribozyme in the absence and presence of
PKR. These two RNAs were chosen because crystal structures are available and they are large and complex enough to potentially lock PKR into specific sites to offer footprinting.

As shown in Figure 3.6, structure-mapping experiments on the Vc2 aptamer in the absence of cdiGMP and PKR were consistent with the Vc2 crystal structure. Single strand-specific RNases T1 and A largely cleaved in the expected single-stranded regions, whereas the double strand-specific RNase V1 cleaved expected double-stranded regions. Under native conditions, G-specific single-strand RNase T1 cleaved G67, G70, G105, and G110, which are either in L3 or at the 3’-single-strand overhang (Figure 3.1), consistent with the crystal structure whereas all of the other Gs are base paired. Pyrimidine-specific single-strand RNase A cleaved C17, C22, C46, and C72, along with several positions in the 3’-single-strand overhang. These cleavages are consistent with the crystal structure, except for C22 and C46, which are in the lower part of P2 in the crystal structure. However, this part of P2 is likely weak as it is only 2 base pairs in length and C22 and C46 are also cleaved by RNase V1 suggesting pairing in a fraction of the strands. The double-strand specific RNase V1 cleaved at C17, C22, C27, A28, C46, C59, C64-C65, C72, U90, C92, C93, and A100, which are in helical regions. Addition of cdiGMP gave rise to several changes in protection consistent with published effects; in particular, G32 and G45 became protected from RNase T1 cleavage, while A48 and C59 gained protection from RNase V1.27 These data support native folding of the Vc2 riboswitch aptamer domain and proper binding of its cdiGMP ligand.
Figure 3.6: Structure mapping and PKR footprinting of Vc2. (A) 5’-end labeled RNA at 4 mM Mg$^{2+}$ was incubated alone or with cdiGMP or PKR, subjected to limited nuclease digestion, and fractionated by 12% denaturing PAGE. Leftmost lanes are controls in the absence of nuclease; underlined lanes were incubated at 37˚C for 15 min to simulate the RNase activity. ‘OH’ is a limited alkaline hydrolysis ladder and ‘den T1’ is RNase T1 in denaturing conditions; these yield a ladder of all nucleotides and all Gs, respectively. The right side of the gel displays RNase digestion in native conditions for RNases T1, A and V1 for 15 min at the temperature noted. The following were probed: Vc2 RNA bound to cdiGMP (‘RNA+CDG’), RNA only, and RNA with PKR titrated from 0.44 to 7 µM. Gray dashed line depicts gel defect. Black dots denote PKR footprinting. (B) Structure mapping and PKR footprinting data placed on the crystal structure of Vc2 (PDB ID: 3MXH). Spheres are 2’-hydroxyls that are either always protected (light pink) or PKR protected (black). cdiGMP is shown as a red stick model. Figure 3.1 provides the secondary structure. Dashed line represents the U1A binding site. (C) 90˚ rotation.
Effects of PKR on cleavage by RNases T1, A, and V1 were tested. PKR conferred significant protection from RNase V1 cleavage, but only small protections from RNase T1 and no changes for RNase A, consistent with PKR’s known preference to bind dsRNA. For instance, PKR protected from RNase V1 cleavage at positions C27, A28, A48 C59, C64-C65, C72, U90, and U92, in pairings P1, P2, and P3. Protections from RNase T1 were found at G32, G45, and G67, while no protections from RNase A were observed. Moreover, there were no enhancements of RNA cleavage in the base pairing regions of the aptamer in the presence of PKR, consistent with absence of large-scale conformational changes or disruption of secondary structure, supporting the notion that PKR binds the natively folded Vc2 aptamer structure. Interestingly, a subset of the protections--those at G32, G45, A48, and C59--are the same as those with cdIGMP binding, suggesting that PKR also enforces RNA tertiary structure. PKR may thus clamp down on the native tertiary structure of the ribozyme. There are additional protections that are unique to PKR versus cdIGMP including those at A48, C64-C65, G67, C72, U90, U92, and A100. These results indicate that PKR footprints to the aptamer.
Figure 3.7: Structure mapping and PKR footprinting of the \textit{glmS} ribozyme. (A) 5'-end labeled RNA at 4 mM Mg\textsuperscript{2+} was incubated alone or with PKR, subjected to limited nuclease digestion, and fractionated by 12% denaturing PAGE. Leftmost lanes are controls in the absence of nuclease; underlined lanes were incubated at 37˚C for 15 min to simulate the RNase activity. ‘OH’ is a limited alkaline hydrolysis ladder and ‘den T1’ is RNase T1 in denaturing conditions; these yield a ladder of all nucleotides and all Gs respectively. The right side of the gel displays RNase digestion under native conditions for RNases T1, A and V1 for 15 min at the temperatures noted. The following were probed: \textit{glmS} RNA only, and RNA with PKR titrated from 0.44 to 7 µM. The lane with an asterisk was not used in our data analysis because it seemed to be
overdigested. Black dots denote PKR footprinting. (B) Structure mapping and PKR footprinting data mapped onto the crystal structure of self-cleaved glmS (PDB ID: 3G9C) using the cleavage data. Spheres are 2'-hydroxyls that are either always protected (light pink) or PKR protected (black). Figure 3.4 provides the secondary structure. Dashed line represents the U1A binding site. (C) A 180° rotation. (D) 90° rotation. region to some extent, consistent with partial melting of P4. RNase V1 cleaved position C44, A46, G68, U70, C85, U91, and U105 in P2.1, P3, and P4, consistent with their base pairing in the crystal structure, and showing that the P4 is also folded at least some of the time. Overall, these structure mapping data support native folding of the glmS riboswitch-ribozyme.

Given that there were no significant changes in tertiary structure, we modeled the RNase protections onto the crystal structure of the Vc2 riboswitch aptamer domain. Nucleotides that were protected from RNases in both the absence and presence of PKR, denoted with pink spheres on the 2’OH, are in the pseudoknotted region of helical docking, further supporting PKR binding the natively folded aptamer. Positions of RNase protection dependent on the presence of PKR are denoted with black spheres. These positions line the periphery of the riboswitch in the double-stranded regions (Figure 3.6B, C) and suggest several PKR footprints as depicted in Figure 3.8 and discussed below. Native RNA tertiary structure appears to be required for site binding of PKR, as the G83C mutant, which disrupts tertiary structure, has no clear PKR footprint (Figures B2 and B3) even though it activates PKR (Figure 3.3). Apparently, loss of tertiary structure in G83C allows PKR to access multiple activating helical regions, the average of which reveals no distinct footprint.

The glmS riboswitch-ribozyme was also subjected to structure mapping in the absence and presence of PKR (Figure 3.7). In the absence of PKR, the single and double strand-specific RNases cleaved in the single and double stranded region expected from the crystal structure. RNase T1 cleaved only two Gs significantly, G19 and G112, consistent with the crystal structure of this highly structured RNA (Refer to Figure 3.4 for
secondary structure). RNase A strongly cleaved residues U41, C85, and C123, which are exposed in the crystal structure. In addition, RNase A cleaved the AU- and GU-rich P4

Upon addition of PKR, significant protections from RNase V1 but not RNases T1 and A were observed, supporting PKR binding to double-stranded regions of this functional bacterial RNA. In particular, RNase V1 protections by PKR were found at C44 and A46 in P2.1, A68 and U70 in P3, and C85 in J3/4. There were also no clear enhancements of RNA cleavage in the presence of PKR, consistent with PKR binding to the fully folded *glmS* riboswitch-ribozyme. Modeling of the RNase protections onto the crystal structure of the *glmS* riboswitch-ribozyme was then performed as described above. Positions of protection in both the absence and presence of PKR are again found in helical docking area (pink spheres), while positions of protection dependent on PKR are found on the peripheral double-stranded regions of the riboswitch (Figure 3.7B-D, black spheres) suggesting several PKR footprints (Figure 3.8).

### 3.4.6 Conclusions

We tested three different functional bacterial RNAs for their ability to activate PKR: the Vc2 riboswitch, the *glmS* riboswitch-ribozyme, and the twister ribozyme. Most constructs derived from these RNAs activated PKR, provided they were long enough to form sufficient RNA structure. In the case of the Vc2 aptamer and *glmS* riboswitch-ribozyme, RNA folding and PKR protection were examined by structure mapping. Footprints of PKR are provided in Figure 3.8, along with a model of 16 bp dsRNA. Prior studies revealed that 16 bp is the minimal length of dsRNA required to bind one protomer of PKR, but that 30 bp is the minimal length needed for potent activation of PKR.5,6
Work from Cole and co-workers showed that 30 bp is the minimal length to dimerize PKR,\textsuperscript{7} consistent with a PKR dimerization model of autophosphorylation. Along these lines, the 23 bp hairpin inhibitor of PKR, HIV-1 TAR RNA, becomes an activator when it dimerizes, as it is then long enough to dimerize PKR.\textsuperscript{32}

Figure 3.8: Footprints of PKR (lilac transparent cylinders) mapped onto 16 bp of A-form dsRNA, the Vc2 riboswitch, and the glmS riboswitch-ribozyme. Positions of PKR-mediated RNase protection for the Vc2 riboswitch and the glmS riboswitch-ribozyme are from the structure mapping experiments displayed in Figures 3.6B and 3.7C, respectively. The 16 bp of A-form dsRNA are modeled as a cylinder that is superposed onto the Vc2 and glmS structures covers the majority of PKR-mediated RNase protections. Two footprints are possible for the Vc2 riboswitch and glmS riboswitch-ribozyme which may drive PKR dimerization and activation. Note that glmS is large and rotating the bottom cylinder counterclockwise by $\sim45^\circ$ may afford an additional helical binding site.

Two footprints of PKR are found on the Vc2 aptamer and glmS riboswitch-ribozyme that are A-form-like and similar in length to 16 bp dsRNA (Figure 3.8). This suggests that PKR dimerizes on functional bacterial RNAs, which leads to its activation,
a notion further supported by the bell-shaped dependence of PKR activation on the concentration of these RNAs (Figures 3.2 and 3.4). The two PKR footprints on these functional RNAs are parallel to one another, or nearly so. Observation that PKR is activated by these two functionally distinct RNAs, which are structurally similar only in their ‘Y-shaped’ architecture, is consistent with PKR having a broad-spectrum response to accessible structured RNA. Moreover, activation of PKR by RNAs with unrelated tertiary structures—those from cdiGMP riboswitch, glmS ribozyme, and twister ribozyme in this study, and that of the \textit{trp} 5’UTR from \textit{B. subtilis} provides overwhelming support that a specific tertiary structure is not needed to activate PKR. Rather, it appears that duplexed regions, exposed upon tertiary folding, are the key elements for activating PKR. This is as expected for an innate immune sensor and consistent with the non-specific recognition of bacterial RNA by the dsRBD.

Transcriptome-wide mapping of RNA structure has been recently developed in several labs.\textsuperscript{33-35} Comparison of \textit{in vitro} and \textit{in vivo} transcriptome-wide RNA structure mapping data sets in yeast and human reveals that much of the RNA in the human genome is either unwound by ATP-dependent helicases or bound with proteins.\textsuperscript{34,36} Other studies show that RNA secondary structures that are nearby on a transcript exchange faster \textit{in vivo} than \textit{in vitro},\textsuperscript{37} and that RNA secondary structure is destabilized in the presence of many cosolutes, which may be related to the cosolute-rich cytoplasm of a cell.\textsuperscript{38,39} Despite extensive efforts,\textsuperscript{17,18} no riboswitches have been identified in the human genome\textsuperscript{21,22} and the few known ribozymes are present either in RNPs, as in the case of the ribosome and spliceosome,\textsuperscript{16} poorly reactive, as in the CPEB3 HDV-like ribozyme with a one-base pair P1.1 pseudoknot,\textsuperscript{40} or discontinuous, as in the hammerhead ribozyme
Internal RNA modifications, including pseudouridine\textsuperscript{42,43} and m6A,\textsuperscript{43-45} have been identified transcriptome-wide in numerous mRNA and ncRNAs. In particular, m6A modification, which is the most common modification of mRNA and ncRNA, has been shown to destabilize model RNA duplexes,\textsuperscript{46} affect mRNA stability,\textsuperscript{47} destabilize mRNA and ncRNA secondary structure \textit{in vivo} to facilitate protein binding,\textsuperscript{48} and abrogate PKR activation both \textit{in vitro} and \textit{in vivo}.\textsuperscript{11-15,49-51} External modifications, such as the 7mG cap, also prevent PKR activation by the default 5’-triphosphate present in certain viral and bacterial transcripts.\textsuperscript{29} The innate immune system may thus distinguish self RNA from non-self RNA by a combination of unwinding cellular RNAs, destabilizing them with modifications and cellular conditions, and cloaking structured self-RNAs with proteins and modifications. Non-self RNAs may then be detected as those naked RNAs with appreciable secondary and tertiary structure. This may account, at least in part, for the apparent absence of protein-free riboswitches and ribozymes in the human genome.

\textbf{3.5 Acknowledgements}

We thank Scott Strobel, Kathryn Smith and Robert Meehan for cdiGMP riboswitch constructs and Michael Been for the \textit{glmS} plasmid as well as Jamie Bingaman for providing purified cleaved \textit{glmS} RNA. We also thank Jamie Bingaman, Kathleen Leamy, Erica Frankel and Dr. David Mitchell for helpful comments on the manuscript. This research was supported by National Institutes of Health Grant R01GM110237.
3.6 References


Appendix B: Supporting Information for Chapter 3

Supplementary Methods

PCR primers, hemiduplex templates and RNA sequences

All Vc2 and Vc2FL transcripts were made by amplifying the region of interest by PCR and fusing a T7 promoter at the 5’-end. PCR primers for the Vc2 and Vc2FL transcripts are listed below, followed by the complete RNA sequences. G83 is colored red, T7 promoters are in bold, and an additional guanosine inserted to promote transcription is shown by a lowercase ‘g’. In Vc2FL the italicized region corresponds to the Vc2 RNA sequence.

Vc2 TS primer: 5’ TAATACGACTCACTATAGGAAAAATGTCA
g
Vc2 BS primer: 5’ GTATGCATTTTGCCATCGTAA
g

Vc2 RNA:
5’ GGAAAAUGUCACGCACAGGGCAACCAUUGCAGAGUGGAGCGCAAAGCCUCGGCUCUAAACCAGACAUUGGAGGUA

Vc2FL TS primer: 5’ TAATACGACTCACTATAgGAAAAATGTCA
Vc2FL BS primer: 5’ TTNTATACGACTCACTATAgGAAAAATGTCA

Vc2 FL RNA:
5’ gGAAAAUGUCACGCACAGGGCAACCAUUGCAGAGUGGAGCGCAAAGCCUCGGCUCUAAACCAGACAUUGGAGGUA

95
Vc2 and Vc2FL G83C sequences have the G highlighted above changed to a C using QuikChange (Agilent) and the following PCR primers,

**G83C TS:** 5’ CCAGAAGACATGGTAGGTA C CGGGGTTACCGATGGC

**G83C BS:** 5’ GCCATCGGTAACCCCGGTACCTACCATGTCTTCTGG.

All sequences were confirmed by standard Sanger sequencing.

The cleaved *glmS* ribozyme was generated using the same PCR method described above. PCR primers and final RNA product are provided below. The top strand primer is ~100 nt upstream (site not shown) of the T7 promoter found in the plasmid, and the bottom strand primer abuts the very end of the *glmS* sequence. The asterisk indicates the cleavage site of the ribozyme.

**glmS TS primer:** 5’-TCACTCATTAGGCACCCCAG

**glmS BS primer:** 5’-TCTCTCATCACACTTCTTACCTTTG

**glmS RNA:**

5’ ggGAAUCAAUGUAAAUUAJAGAA*GCGCCAGAAACUAAGUAGUGUAGUGACGAG GUGGGGUUUAUCGAGAUUUCGGCGGAUGACUCCCGGUUGUUGUCAUCACACCCGCAAGCUU UUACUUAAUCUAAGGUGACUUAGUGGACAAAAAGUGGAAGUGUGAGAGAGA

Twister ribozyme transcripts were made using hemiduplex templates as shown below. Templates are listed 5’- to 3’ in which the product is the reverse complement to the template starting after the boldface T7 binding site. Bottom strand (BS) templates were annealed with a top-strand T7 promoter. The asterisk indicates the cleavage site of the ribozyme. Colors indicate flanking nucleotides that were added in from the *C. bolteae* gene in order to extend the constructs and correspond to those in Figure 3.5. Those
nucleotides added upstream of the cleavage site were not present in any of the experiments because we worked the self-cleaved form that populated during transcription.

Twister (T) BS template:

5' TTCCACCTCTGCATTGATCAGGGCTTTGTGACCTGCACCGGCTATAGGCCGGTGCTG CATT*AGGAAGGTATAGTGAGTCGTAATTAATTT

T+5 BS template:

5' CTCCTTTCCACCTCTGCATTGATCAGGGCTTTGTGACCTGACCGGCTATAGGCCGGTGCTG CATT*AGGAAGGGCGGTTAGTGAGTCGTAATTAATTT

T+10 BS template:

5' ATTTTCCTCCTTTCCACCTCTGCATTGATCAGGGCTTTGTGACCTGCACCGGCTATAGGCCGGTGCTG CATT*AGGAAGGGCGTCGTAATAGTGAGTCGTAATTAATTT

T+15 BS template:

5' CATGATTTCCTCCTTTCCCACCTCTGCATTGATCAGGGCTTTGTGACCTGCACCGGCTATAGGCCGGTGCTG CATT*AGGAAGGGCGTCGTCGTAATAGTGAGTCGTAATTAATTT
RNA products were in the cleaved form lacking the 5’-RNA tail and are listed below.

T RNA:
5’ AAUGCAGCCACCGCCUAUAGCCGGUGCAGGCAACAAGCCUCGAUCAAUGCAGAGUGGGAA

T+5 RNA:
5’ AAUGCAGCCACCGCCUAUAGCCGGUGCAGGCAACAAGCCUCGAUCAAUGCAGAGUGGGAAAGGAG

T+10 RNA:
5’ AAUGCAGCCACCGCCUAUAGCCGGUGCAGGCAACAAGCCUCGAUCAAUGCAGAGUGGGAAAGGAGGAAAU

T+15 RNA:
5’ AAUGCAGCCACCGCCUAUAGCCGGUGCAGGCAACAAGCCUCGAUCAAUGCAGAGUGGGAAAGGAGGAAUCCAU
Figure B1: Additional activation and competition assays for Vc2 and Vc2FL. (A) Activation of PKR by Vc2FL in the absence and presence of cdiGMP, as well as cdiGMP alone. (B) Activation of PKR by Vc2 in the absence and presence of cdiGMP. (C) Competition for dsRNA-79-mediated activation of PKR by Vc2FL in the absence and presence of cdiGMP, as well as cdiGMP alone. (D) Competition for dsRNA-79-mediated activation of PKR by Vc2 in the absence and presence of cdiGMP. Bands indicate phosphorylated PKR, "p*PKR". Percent PKR activation was normalized to that of 0.1 µM dsRNA-79. Shown are SDS-PAGE gels and all experiments were in 4 mM Mg²⁺.
Figure B2: Structure mapping and PKR footprinting of the Vc2 G83C mutant riboswitch. (A) 5’-end labeled RNA at 4 mM Mg$^{2+}$ was incubated alone or in the presence of cdiGMP or PKR, subjected to limited nuclease digestion, and fractionated by 12% denaturing PAGE. Leftmost lanes are controls performed in the absence of nuclease; underlined lanes were incubated at 37°C for 15 min to simulate the RNase activity (note some lanes were skipped due to defects in the gel). ‘OH’ is a limited alkyline hydrolysis ladder and ‘den T1’ is RNase T1 in denaturing conditions. The right side of the gel displays RNase digestion in native conditions for RNases T1, A and V1 for 15 min at the temperature noted. The following were probed: Vc2 G83C RNA bound to cdiGMP (‘RNA+CDG’), RNA-only, and RNA with PKR titrated from 0.44 to 7 µM. (B) Secondary structure of Vc2 with G83C labeled and tertiary interactions that involved this nucleotide removed.
Figure B3: Vc2 G83C structure mapping comparison to WT Vc2.
(A) Excerpts from the RNase V1 lanes in Figure 3.6 in Chapter 3 (left side of the above gel) and Figure B2 (right side of the above gel) for direct comparison of nucleotides that have changes in cleavage between WT Vc2 and G83C Vc2, marked to the right of the gel with purple lines. Comparison between RNA+CDG and RNA-only lanes in G83C at nucleotides C46, A48, C59, C64, and C65 support ligand binding to G83C. Positions A48 and C59, which are at the docked interface, have enhanced cleavages in G83C. Cleavage comparisons at these nucleotides (and others) reveal footprinting of PKR in WT but not G83C. This suggests that PKR has many weak binding sites on the undocked Vc2 G83C structure, allowing RNase V1 to cleave double-stranded regions. (B) Secondary structure of Vc2 with G83C labeled and tertiary interactions that involved this nucleotide removed. (Same as Figure B2, provided here for convenience.)
Figure B4: Structure mapping of the Vc2FL riboswitch and prediction of the terminator and antiterminator. (A) RNA was 5’-end labeled, incubated alone or in the presence of cdiGMP, subjected to limited RNase T1 digestion, and fractionated by 12% denaturing PAGE. Lanes are as follows: RNA-only, hydrolysis ladder (OH−), denaturing RNase T1 ladder (‘Den T1’), and Native T1 mapping. The right side of the gel displays RNase T1 digestion under native conditions at 0.5 or 4 mM Mg2+ in the absence or presence of cdiGMP. Vc2FL is a 350 nt 5’-UTR containing the Vc2 aptamer and expression domains. The numbering scheme includes the aptamer as 1-110 and nucleotides in the UTR flanking this domain are labeled as numbers below or above this range. Cleavages of interest are labeled with the corresponding nucleotides and labels at the right of the gel denote the aptamer domain and predicted antiterminator and terminator stem-loops. (B) Model for switching between the antiterminator and terminator forms. Formation of the antiterminator prevents formation of the overlapping terminator (red box), which gives transcription readthrough. This model is supported by gain of RNase T1 cleavage at G152 in the presence of c-diGMP (panel A) and is consistent with a model proposed for the cdiGMP riboswitch in Clostridium difficile.1
Appendix B References

Table B1: Summary of activation assay data for WT Vc2 and Vc2FL in 0.5 and 4 mM Mg$_{2+}$ conditions.

<table>
<thead>
<tr>
<th>Vc2 0.5 mM Mg$_{2+}$</th>
<th>Vc2FL 0.5 mM Mg$_{2+}$</th>
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<td>--- PKR Activation ---</td>
<td>--- PKR Activation ---</td>
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<tr>
<td>[RNA], µM</td>
<td>Trial 1</td>
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<tr>
<td>0.01</td>
<td>5.8</td>
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<th>Vc2FL 4.0 mM Mg$_{2+}$</th>
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<td>--- PKR Activation ---</td>
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Table B2: Summary of activation assay data for WT Vc2 and Vc2FL in 0.5 and 4 mM Mg\(^{2+}\) conditions in the presence of cdiGMP.

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<table>
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<table>
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<td>0.0375</td>
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<tr>
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Table B3: Summary of activation assay data for G83C mutants Vc2 and Vc2FL in 0.5 and 4 mM Mg$^{2+}$ conditions.

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<th>% Range</th>
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Vc2 FL 0.5 mM Mg$^{2+}$

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<th>% Range</th>
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Vc2 4.0 mM Mg$^{2+}$

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Vc2FL 4.0 mM Mg$^{2+}$

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Table B4: Summary of activation assay data for G83C mutants Vc2 and Vc2FL in 0.5 and 4 mM Mg\(^{2+}\) conditions in the presence of cdiGMP.

### Vc2/ cdiGMP 0.5 mM Mg\(^{2+}\)

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### Vc2/ cdiGMP 4.0 mM Mg\(^{2+}\)

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<td>0.72</td>
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### Vc2FL/ cdiGMP 0.5 mM Mg\(^{2+}\)

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### Vc2FL/ cdiGMP 4.0 mM Mg\(^{2+}\)

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Table B5: Summary of activation assay data for cleaved glmS in 0.5 and 4 mM Mg$^{2+}$ conditions.

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Table B6: Summary of activation assay data for varying length twister RNAs in 4 mM Mg\textsuperscript{2+} conditions and T+15 in 0.5 and 4 mM Mg\textsuperscript{2+} conditions.

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<td>Trial 3</td>
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Chapter 4

Mechanistic characterization of the 5’-triphosphate- dependent activation of PKR: Lack of 5’-end nucleobase specificity, evidence for a distinct triphosphate binding site and a critical role for the dsRBD

[Published as a paper entitled “Mechanistic characterization of the 5’-triphosphate-dependent activation of PKR: Lack of 5’-end nucleobase specificity, evidence for a distinct triphosphate binding site and a critical role for the dsRBD.” By Rebecca Toroney, Chelsea M. Hull, Joshua E. Sokoloski & Philip C. Bevilacqua in RNA 2012, 18, 1862-74.]

Contributions: RT designed and performed activation assays, competition assays, ITC and EMSAs, CH designed and performed crosslinking experiments and cloned and prepared the dmPKR. JS helped with ITC experiments and analysis.

4.1 Abstract

The protein kinase PKR is activated by RNA to phosphorylate eIF-2a, inhibiting translation initiation. Long dsRNA activates PKR via interactions with the dsRNA-binding domain (dsRBD). Weakly structured RNA also activates PKR and does so in a 5’-triphosphate (ppp)-dependent fashion, however relatively little is known about this pathway. We used a mutant T7 RNA polymerase to incorporate all four triphosphate-containing nucleotides into the first position of a largely single-stranded RNA and found absence of selectivity, in that all four transcripts activate PKR. Recognition of 5’-triphosphate, but not the nucleobase at the 5’-most position, makes this RNA-mediated innate immune response sensitive to a broad array of viruses. PKR was neither activated in the presence of γ-GTP, nor recognized by NTPs other than ATP in activation
competition and ITC binding assays. This indicates that the binding site for ATP is selective, which contrasts with the site for the 5’-end of ppp-ssRNA. Activation experiments reveal that short dsRNAs compete with 5’-triphosphate RNAs and heparin for activation, and likewise gel-shift assays reveal that activating 5’-triphosphate RNAs and heparin compete with short dsRNAs for binding to PKR’s dsRBD. The dsRBD thus plays a critical role in the activation of PKR by ppp-ssRNA and even heparin. At the same time, crosslinking experiments indicate that ppp-ssRNA interacts with PKR outside of the dsRBD as well. Overall, 5’-triphosphate-containing, weakly structured RNAs activate PKR via interactions with both the dsRBD and a distinct triphosphate binding site that lacks 5’-nucleobase specificity, allowing the innate immune response to provide broad-spectrum protection from pathogens.

4.2 Introduction

Host recognition of molecular patterns in RNA serves as an integral component of the innate immune response in humans. A number of RNA sensors have been identified as part of this response, including Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I), and the protein kinase PKR. These sensors respond to specific pathogen-associated molecular patterns (PAMPs) that distinguish certain RNAs as non-self, such as long double-stranded stretches and internal nucleoside modifications. The 5’-triphosphate (ppp) has been identified as a PAMP for both RIG-I and PKR. The structural determinants of this activation are different for both proteins: RIG-I recognizes the 5’-triphosphate of short blunt-end dsRNA, while PKR recognizes the 5’-triphosphate of primarily ssRNA with short stem-loops. Because cellular RNA is typically capped by
7-methylguanosine at its 5'-end, the 5’-triphosphate serves as molecular pattern to identify an RNA as potentially pathogenic.

PKR is typically activated by long stretches of dsRNA that accumulate as intermediates of viral transcription and replication, which allows PKR to phosphorylate its substrate, eIF2α, thereby disrupting GTP-GDP exchange and ultimately inhibiting translation initiation. PKR activation, or autophosphorylation, is mediated by the two tandem N-terminal dsRNA binding motifs (dsRBMs) that comprise PKR’s dsRBD (Fig. 4.1A). PKR autophosphorylation requires binding of two PKR monomers to dsRNA of at least 33 bp, which results in dimerization of the C-terminal kinase domains and subsequent phosphorylation at Thr446. Given the requirement of 33 bp for activation of PKR by dsRNA, activation by ppp-ssRNA with stem-loops as short as 5 bp is surprising and suggests a distinct mechanism of activation.

We previously identified and characterized the primary RNA structural requirements for PKR activation by ppp-ssRNA in vitro and in vivo, and found that all three phosphates in the triphosphate moiety are required for activation, as ssRNA with 5’-end signatures of pp and p did not activate PKR. Moreover, we showed that dsRNA constructs have no requirement for this 5’-triphosphate for PKR activation, which is unlike RIG-I. Additionally, we demonstrated that PKR activation by ppp-ssRNA requires ~47 nt and is optimal when the short stem-loops are placed near the middle of the RNA. The mechanistic nature of the above RNA structural requirements for ppp-ssRNA-mediated PKR activation has remained unclear however.

One parsimonious model states that the 5’-triphosphate of ssRNA uses the known catalytic ATP binding site. This model assumes that there is only one triphosphate-
binding site within PKR, which would then serve two functions. Given the dependence of PKR activation on protein dimerization for both dsRNA and heparin activators \(^{11,13}\) these functions would be: RNA 5'-triphosphate recognition in one protomer and ATP binding for catalysis in the other. Supporting this economical model, inspection of the crystal structure of the PKR kinase domain with the ATP analog AMP-PNP bound in the catalytic cleft reveals steric accessibility for extension of an RNA chain from the 3'-hydroxyl of AMP-PNP.\(^{14,15}\) A second model assumes that two functionally distinct triphosphate-binding sites reside within PKR.

In the current study, we seek to provide insight into the mechanism of 5'-triphosphate-dependent activation of PKR. Our study supports the second model, with distinct triphosphate binding sites, wherein the catalytic ATP binding site has high specificity and binding affinity, while the 5'-triphosphate binding site has lower specificity and affinity. We see that ppp-ssRNA interacts with regions of PKR outside of the dsRBD, but that this RNA and also heparin require PKR’s dsRBD for strong activation. These findings suggest that the dsRBD aids in recognition of diverse PKR activators and reinforce the importance of the dsRBD as a universal target for anti-viral therapeutics that function by upregulating PKR.
4.3 Materials and Methods

4.3.1 Sequences of RNA and protein

**ssRNA:**

**ssRNA-47:** Different versions of ssRNA-47 were prepared, starting with various 5’-end nucleotides, which are indicated in parenthesis.

5’-(pppG, pppA, pppC, pppU, pppA, OH-G, OH-A)GGCACCAACUCAAGUAUACC
UUUAUACAACCGUUCUACACUCAACG

**ssRNA-20:** 5’-GGCACCAACUCAAGUAUACC

**dsRNA:** Top strands (TS) are provided below; the bottom strand is the Watson-Crick complement to the top strand. Double-stranded RNAs were prepared by annealing equimolar concentrations of the two strands.

**dsRNA-79 TS:** 16
5’GGGUUUUUCCCAGUCACGACGUUGUAAAACGACGCGGCGAUAUUCCGAGC
UCGUAACCCGGGAUCCUCUAGAGUCGACC

**dsRNA-20:** 10
5’-GGGUUUCCCUGGUUUCGGUCU

**dsRNA-40:** 17 5’GGACCUGUGCGUGAUCCUGGAGCAUCCUCUAGGUACGUCC

**ss-dsRNA:** 16

**ss-dsRNA(9,11):**

GGGAGAGAGGUCACUGACUAAGUGGUAAUCUUGA
AUCAGUGACAAAGGAAGG (Single-stranded tails are underlined.)
**Protein:**

In order to generate K60A and K150A point mutations in PKR, which are known to abrogate dsRNA binding, site-directed mutagenesis (QuikChange, Agilent Tech) was performed on pET28a-wtPKR using the following primers: K60A, 5’-CCAGAAGGTGAAGGTAGATCA CAGGAAAGC-3’ and K150A, 5’-CAGGTTCTACTGCACAGGAAGCCAAAACAATTTG-3’, where the mutated codon is in bold. To generate the double mutant (dmPKR), point mutations were made sequentially, starting with K60A.

### 4.3.2 RNA preparation

As described previously, dsRNA-79 was prepared by transcribing the two strands of pUC19 separately and later annealing. Wild-type (pppG-) ssRNA-47 (first nucleotide is G) was prepared *in vitro* by a standard T7 run-off transcription from a linearized pUC19 plasmid (BstU1 digested) containing a T7 promoter, as previously described. However, because WT T7 polymerase very strongly favors RNAs that start with a G, a different strategy had to be employed to get transcripts to begin with the other 5’-triphosphate nucleotides. The QuikChange site-directed mutagenesis kit was utilized to generate plasmids with mutations at the first templating position. In an effort to obtain pppA-, pppC, and pppU-ssRNA-47, plasmid sequences were verified by dideoxy sequencing following maxipreps, and were digested as per the pppG-ssRNA-47 template. For each RNA, 0.2 µg/µL of linearized plasmid was combined with 40 mM Tris (pH 8), 25 mM MgCl₂, 2 mM DTT, 1 mM spermidine, 3 mM of each NTP, and 1 µM T7 polymerase containing a P266L mutation.
Transcription reactions were incubated for 4 h at 37 ºC and quenched by addition of one volume 95% (v/v) formamide loading buffer. RNA was purified by fractionating on a polyacrylamide denature gel (7 M urea, 1X TBE). The transcript was identified by UV shadowing, excised from the gel, and eluted overnight at 4ºC in 1X TEN250. The RNA was ethanol precipitated, resuspended in 1X TE buffer (pH 7.5), and stored at –20 ºC. RNA concentration was determined spectrophotometrically.

To verify the identity of the starting nucleotide as pppG-, pppA-, pppC-, or pppU- in ssRNA-47, these RNAs were also transcribed in the presence of trace amounts of [γ-32P]ATP or [γ-32P]GTP. Because the radiolabel is on the gamma phosphate, a band should only be observed when the radioactive nucleotide is incorporated in the first position of the transcript.

RNAs starting with a bona fide 5’-OH (A-ssRNA-47 and G-ssRNA-47) were chemically synthesized by Dharmacon.

4.3.3 Protein preparation

Full-length PKR, K296R (catalytically inactive mutant), and P20 (dsRBD of PKR) containing N-terminal His6 tags were purified from Escherichia coli BL21(DE3) Rosetta cells (Novagen) as described.10,16,22 Briefly, cells were lysed by sonication, and protein was purified by FPLC with a Ni2+-NTA column (Invitrogen) followed by dialysis in a protein storage buffer (PSB) consisting of 10 mM Tris pH 7.6, 50 mM KCl, 2 mM MgCl2, 10% glycerol, and 7 mM β-mercaptoethanol.

A dsRNA-binding deficient double mutant of PKR (dmPKR) was also purified in a similar fashion, with the following modifications to ensure removal of nucleic acids
from the prep. The mutations in dmPKR are K60A in dsRBM1 and K150A in dsRBM2, which have been shown previously to interfere with binding of dsRNA even as single mutations. After sonication of cells containing overexpressed dmPKR, 5% polyethyleneimine (PEI) was added dropwise to the cleared cell lysate for a final concentration of 0.025% PEI. The lysate was then spun down at 20,000 rpm (Beckman) for 30 minutes and the supernatant was collected. The protein was ammonium sulfate precipitated by slowly adding solid ammonium sulfate to the supernatant to a final percentage of 60% (wt/vol) followed by stirring at 4ºC for 30 minutes. After centrifuging at 11,500 rpm for 30 minutes, the supernatant was decanted and the pellet was redissolved in 50 mM sodium phosphate pH 8.0, 700 mM NaCl, 5 mM imidizole, 7mM BME, and 0.1 mM PMSF. Purification of dmPKR via FPLC and Ni\(^{2+}\)-NTA then proceeded as described above.

The P266L mutant T7 polymerase was also prepared from *Escherichia coli* ENS0134T BL21 cells (provided by Dr. Marc Dreyfus of Laboratoire de Genetique Moleculair) and purified on a Ni-NTA (Invitrogen) column, as described previously. Protein concentrations were determined spectrophotometrically.

### 4.3.4 PKR activation assays

The ssRNA-47 RNAs containing each of the four starting nucleotide triphosphates were tested for their ability to activate PKR autophosphorylation. Activation assays were identical to those conducted with canonical dsRNA activators. PKR was first dephosphorylated by treatment with λ-PPase (NEB) for 1 h at 30 ºC, followed by addition of freshly made phosphatase inhibitor, sodium orthovanadate.
Next, 15 μCi [γ-32P]ATP (Perkin-Elmer), 0.8 μM dephosphorylated PKR, and RNA at various concentrations were incubated in 20 mM HEPES (pH 7.5), 4 mM MgCl₂, 50 mM KCl, and 100 μM ATP (Ambion) for 10 min at 30 °C. In order to determine specificity for the catalytic ATP binding site, 15 μCi [γ-32P]GTP and 100 μM GTP were added instead of [γ-32P]ATP and ATP. All reactions were quenched by addition of SDS loading buffer. Samples were heated at 95 °C for 5 min and loaded on 10% SDS-PAGE gels (Pierce). After electrophoresis, gels were exposed to a storage PhosphorImager screen and labeled band intensities were quantified on a PhosphorImager (Molecular Dynamics). A background value averaged from different portions of the gel was subtracted from each band prior to normalization. The 5’-triphosphate dependence for activation was calculated by first subtracting the amount of phosphorylation in the absence of added RNA from the bands in the presence of a 5’-triphosphate-containing RNA and the presence of a non-5’-triphosphate-containing RNA, and then dividing the resultant values.⁶

4.3.5 PKR activation competition assays

Two PKR activation competition assays were conducted: NTP-competition assays and RNA-competition assays. In the NTP-competition assays, each of the four NTPs was tested for its ability to compete for triphosphate binding sites during PKR activation by dsRNA or ppp-ssRNA. Activation assays were conducted as described above, with the following exceptions: various concentrations of ATP, CTP, GTP, and UTP (all complexed with equimolar Mg²⁺) were incubated along with other reaction components (see above). In the RNA-competition assays, the PKR activator, dsRNA, ssRNA, or
heparin was competed by short dsRNA or ssRNA. In the RNA-competition assays, the activating RNA or heparin was held constant while competitors were added in a range of concentrations. All gels were run and analyzed as above.

4.3.6 Isothermal titration calorimetry

ITC was utilized to obtain thermodynamic parameters for binding of NTPs to K296R. Data were collected using a MicroCal Auto-iTC200 (GE Healthcare). The sample cell contained 40 µM K296R in PSB while the syringe contained 720 µM ATP, GTP, CTP, or UTP dissolved in PSB as 100 mM stocks and diluted with further PSB. Titrations were performed at 30 ºC and involved nineteen 2 µL injections into the 200 µL sample cell. ATP titration curve was fitted to a model for two binding sites using MicroCal Origin software (Version 7.0) with the average value of the last three points of the saturated portion of the curve used to baseline correct the integrated data.

4.3.7 UV crosslinking

To induce crosslinks between RNA and protein, the photoreactive nucleotide analog 4-thio-UTP was fully incorporated into ppp-ssRNA-47 at all uridine positions. This transcript was then 5'-32P-end-labeled and 1 nM of RNA was incubated with 4 µM wtPKR, P20 or dmPKR in 10 mM Tris (pH 7.6), 50 mM KCl, and 2 mM Mg(OAc)2·4H2O in a 20 µL reaction volume. Crosslinking reactions were immediately placed directly on the surface of a handheld UV lamp (UVP model UVGL-25), which had been turned upside-down and covered with Saran Wrap. Reaction samples were irradiated with 365 nm light (Mineralight lamp multiband UV-254/365). At various time points, 3 µL aliquots were quenched with 2X FLB. Samples were then fractionated on a
7%, 1X TBE, 7 M urea denaturing gel for 45 min, and the gel was dried and exposed to a PhosphorImager screen overnight.

**4.3.8 Electrophoretic mobility shift assays (EMSAs)**

To analyze binding to P20, excess protein (3 µM) and trace amounts of 5'-32P-end-labeled dsRNA-20 (~ 2 nM) were incubated along with various unlabeled competitor RNAs or heparin and 1 mg/mL herring sperm DNA, 10 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.1 mg/mL bovine serum albumin, and 0.01 % NP-40 for 30 min at 22 ºC. Samples were then fractionated on 10% 0.5X TBE native gels (29:1 crosslink) at 16 ºC for 2.5 h. Gels were dried and exposed to storage PhosphorImager screens overnight.

**4.4 Results**

In this work, the designation “ssRNA” refers to an RNA that is comprised of one strand; *i.e.* it does not contain a complementarly strand. A ssRNA may contain some secondary structure and is numbered according to length in nucleotides (See Fig. 4.1, B and C).
Figure 4.1: Protein and RNA constructs used in this study. (A) Schematic of PKR primary sequence. The N-terminal dsRNA binding domain (dsRBD, also referred to as P20), comprised of two tandem dsRNA-binding motifs (dsRBM 1 and 2), and the catalytic C-terminal kinase domain are indicated. Positions of point mutations used in this study are indicated. The double-mutant PKR (dmPKR) contains both K60A and K150A mutations, and the K296R mutation in the kinase domain renders PKR catalytically inactive. (B) Experimentally determined secondary structure of ssRNA-47. Similarly, “dsRNA” refers to RNA comprised of two strands and is numbered according to length in basepairs. In one specific instance, “ss-dsRNA” refers to single-stranded RNA with a helical stem and unstructured 5’- and 3’-end tails (Fig. 4.1D). Starting nucleotide is indicated in boldface; G is shown, which is typical of transcripts made from WT T7 polymerase, but RNAs were also transcribed containing pppA, -pppC, and -pppU using mutant T7 polymerase. (C) Sequence of ssRNA-20. Secondary structure prediction via free energy minimization (mFold) indicates that this RNA is essentially completely unstructured. (D) Experimentally determined secondary structure of ss-dsRNA.
4.4.1 Recognition of 5'-triphosphate-containing RNA lacks specificity for the 5'-nucleobase.

Previously, we established that PKR activation by 5'-triphosphate-dependent ssRNAs has high selectivity for the triphosphate moiety. For example, we showed that transcripts beginning with 5'-OH and 7-methyl-guanosine abrogate PKR activation, and transcripts beginning with 5'-p and 5'-pp were also very poor activators relative to the same RNA beginning with 5'-ppp. Specificity of PKR activation for the identity of the base at position 1 of the transcript has not been explored, however, with all studies to date involving RNAs that begin pppG. Although RNAs transcribed \textit{in vitro} by T7 polymerase typically require G in the first position for transcription initiation, bacterial and viral RNAs are initiated by G or A, and even occasionally by C. Base specificity of PKR activation has implications for both specificity regarding the structural nature of the triphosphate binding site in PKR and for the functional permissiveness of PKR activation by non-self ppp-RNAs in innate immunity.

We utilized a mutant T7 polymerase containing a P266L change, which reduces abortive cycling during transcription owing to decreased affinity of the promoter binding site. This variant should be more tolerant to transcripts that start with nucleotides other than pppG, typically required by wild-type T7 polymerase. We transcribed four versions of ssRNA-47 (Fig. 4.2A), which we have previously characterized as a strong 5'-triphosphate-dependent activator of PKR, with pppG-, pppA-, pppC-, and pppU-starting nucleotides. We generated linearized plasmid templates for each of the four starting nucleotides and, in an effort to verify insertion of the correct starting nucleotide, transcribed each RNAs in the presence of [\(\gamma^32\text{P}\)]ATP or [\(\gamma^32\text{P}\)]GTP (Fig. 4.2A).
Figure 4.2: All ssRNA-47 5’-triphosphate-starting nucleotides activate PKR.  (A) Verification of starting nucleotide identity.  RNA was transcribed from DNA templates coding for starting the transcript with pppG, -pppA, -pppC, and -pppU, in the presence of [γ-32P]-ATP or [γ-32P]-GTP.  Expected starting RNA nucleotide based on template (nt. 1: pppX) is indicated.  Percent incorporation was calculated by normalizing the counts at the indicated mobility “ssRNA-47” to the counts from the pppA lane for [γ-32P]-ATP incorporation, or the pppG lane for [γ-32P]-GTP incorporation.  7 M urea gel is shown.  (B) Activation of PKR by ssRNA-47 with pppG, -pppA, -pppU, and -pppC.  RNA concentrations were: 0.31, 0.63, 1.3, 2.5, 5.0, and 10 µM for pppG- and pppA-ssRNA-47; 0.15, 0.31, 0.63, 1.3, 2.5, 5.0, and 10 µM for pppU-ssRNA-47; and 0.15, 0.31, 0.63, 1.3, 2.5, and 5.0 µM for pppC-ssRNA-47.  Phosphorylation activities are provided under the gels and were normalized to the dsRNA-79 lane in the top gel.  (C) Graphical representation of phosphorylation activities from panel (B) as a function of RNA concentration.  (D) Activation of PKR by A-ssRNA-47 and G-ssRNA-47 are both dependent on a 5’-triphosphate.  RNAs starting with 5’-triphosphate were generated by in vitro T7 transcription, while RNAs starting with 5’-OH-G and 5’-OH-A were chemically synthesized.  Phosphorylation activities are provided under the gel and were normalized to the dsRNA-79 lane.  For both panels (B) and (D), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated.
Because the radiolabel is located on the γ-phosphate, a band should only be visible when the labeled nucleotide is inserted at the first position. We observed preferential incorporation of [γ-32P]ATP and [γ-32P]GTP at position 1 where expected (Fig. 4.2A, lanes 2 and 5), and limited misincorporation of these nucleotides in transcripts starting with the other three nucleotides.

These RNAs were then tested for activation of PKR. We observed PKR activation of similarly high potency for ssRNA-47 with all four 5’-NTPs (Figs. 4.2B, C). For example, pppG-ssRNA-47 activated maximally (88% the level of activation by the long 79 bp activator, ‘dsRNA-79’) at 5 µM, with a bell-shaped dependence on RNA concentration, as observed previously for this RNA. Maximal activation intensities for RNAs starting with A and U were similar as for G, but their bell-shaped activation profiles were shifted to even lower RNA concentrations (~1.25 µM RNA), implying higher affinity (Fig. 4.2C). Potent activation of PKR by pppA-ssRNA-47 and pppG-ssRNA-47 is consistent with the known prevalence of viral transcripts containing 5’-G or 5’-A (see Discussion).

We also used a synthetic versions of 5’-A-ssRNA-47 and 5’-G-ssRNA-47, which contain bona fide 5’-OHs, to test whether ssRNA-47 starting with a nucleotide other than G still activates PKR in a 5’-triphosphate-dependent fashion. As shown in Figure 4.2D, both 5’-A-ssRNA-47 and 5’-G-ssRNA-47 have similar 41- and 54-fold triphosphate dependence for activation, with the value for 5’-G-ssRNA-47 in agreement with an earlier study. Thus, the specificity of PKR activation for 5’-triphosphate does not depend on 5’-nucleobase identity.

Activation by pppC-ssRNA-47 was within at least 2/3 the maximum value of the
other three RNAs, although its profile was shifted to higher RNA concentration. In summary, potent activation of PKR by ppp-ssRNA containing different bases at the 5’-position suggests that the binding site for the 5’-triphosphate of ssRNA is permissive and that PKR-mediated contacts are primarily to the triphosphate moiety of the 5’-nucleotide rather than the nucleobase.

4.4.2 Recognition of ATP has specificity for the nucleobase: Activation competition experiments.

The parsimonious model for the 5’-triphosphate-dependent activation of PKR is that the 5’-triphosphate binds in the ATP active site. This model is supported by steric accessibility at the active site for chain extension off the 3’-hydroxyl of ATP and by the observation that the major contacts between ATP and amino acids at the active site that are necessary to position the phosphates for catalysis occur to the ribose sugar and triphosphate rather than the base. Moreover, more potent activation by pppA-ssRNA-47 would seem to suggest potential binding of this RNA into the ATP binding site.

The results presented here on PKR activation by ss-RNA-47s starting with any of the four triphosphate nucleotides, and the reported 5’-triphosphate-dependent activation of PKR by a number of different ssRNAs with G as the starting nucleotide suggest that if the ATP active site also serves as the 5’-triphosphate binding site for ssRNA, then the active site NTP may also be permissive to GTP. To test this notion, we performed a PKR activation assay in which all of the ATP (unlabeled and [$\gamma^{32}$P]ATP) was replaced with GTP and [$\gamma^{32}$P]GTP. In contrast to the parsimonious model, we observed complete
abrogation of activation in the presence of GTP only (Fig. 4.3A, first two sets of lanes), suggesting that the ATP binding site has high nucleotide specificity for chemistry. Furthermore, we showed that a radiolabeled \( \gamma \)-phosphate of a 5’-triphosphate activator is not transferred to PKR (Nallagatla and Bevilacqua, unpublished results), suggesting that the 5’-triphosphate end of ssRNA cannot fill the ATP binding site. It thus appears that PKR has separate triphosphate binding sites—one with base specificity and one without.

In order to probe further these two models, activation assays were performed in which activation by dsRNA-79, which does not require a 5’-triphosphate, and by pppG-ssRNA-47 were challenged with each of the four NTPs. As shown in Fig 4.3B, C, for each RNA activator, only ATP was able to effectively compete with PKR activation (up to ~10-fold inhibition). (Note that these plots, which are in the background of 100 mM ATP, are consistent with the \( K_d \) value for ATP of ~19 mM obtained by ITC, see below.) Because dsRNA-79 has no triphosphate dependence for activation, the observed inhibition is reflective of unlabeled ATP competing out radiolabeled ATP from the active site. Thus, the catalytic site is specific for ATP not only for chemistry, but also for binding. Given the apparent tolerance of PKR for different activating 5’-triphosphate groups on RNA (Fig. 4.2B), the lack of competition for NTPs other than ATP provides further evidence that PKR contains two separate triphosphate binding sites.

A similar specificity of strong inhibition by ATP but not the other NTPs was also seen for activation by ssRNA-47 (Fig. 4.3B, C). This observation suggests that the same, specific ATP binding site is used for this activator. We do note slight (~40%) inhibition of activation by GTP and CTP at their highest concentrations of 2 mM; this inhibition was not present in the presence of dsRNA-79 (Fig. 4.3C). Weak inhibition by GTP and
CTP in the presence of pppG-ssRNA-47 but not dsRNA-79 suggests that these other NTPs can compete very weakly for pppG-ssRNA-47’s triphosphate binding site and that ssRNA-47 binds rather weakly to PKR. Failure of GTP, CTP, and UTP to potently inhibit activation by pppG-ssRNA-47 suggests that 5’-triphosphate binding at this putative second triphosphate binding site is strengthened relative to free NTPs, probably through tethering of the triphosphate to PKR via the remainder of the RNA. Interactions of the remainder of the RNA with PKR are explored below in more depth.

4.4.3 Recognition of ATP has specificity for the nucleobase: ITC experiments.

In this section, we further explore the specificity of ATP recognition by turning from activation competition experiments to isothermal titration calorimetry (ITC) experiments. To investigate further the nature of triphosphate binding, ITC was employed to determine the thermodynamics of NTP binding to PKR. In order to prevent coupling of binding with enzymatic activity, a catalytically inactive mutant of PKR, K296R, was used (Fig. 4.1A). Binding was to K296R in the absence of RNA, with injections of each of the four individual NTPs in 18-fold excess over protein. As shown in Fig. 4.4A, the titration curve for GTP revealed a very weak interaction that was barely noticeable in comparison to titration against buffer, and UTP and CTP titration curves were essentially equivalent to the buffer titration. In contrast, titration of ATP revealed a strongly exothermic interaction.
Figure 4.3: The ATP binding site has high specificity: Activation assays. (A) Only ATP supports PKR phosphorylation. Activation of PKR by dsRNA-79 using [γ-32P]-GTP or [γ-32P]-ATP as the phosphate source. RNA concentrations are provided. In ‘γ-GTP’ lanes, activation assays were performed as per standard assay conditions (see Materials and Methods) with the following exceptions: 100 µM GTP was used instead of ATP, and 0.1 or 1.5 µCi/µL of [γ-32P]GTP were added instead of 1.5 µCi/µL [γ-32P]ATP. In ‘γ-ATP’ lanes, standard assay conditions were used. (B) NTP-competition assays reveal that only free ATP competes with ATP for activation. PKR activation by dsRNA-79 or pppG-ssRNA-47 was assayed in the presence of increasing concentrations of unlabeled ATP, GTP, CTP, and UTP. Concentrations of dsRNA-79 and pppG-ssRNA-47 were 0.1 and 2.5 µM, respectively. Concentrations of each NTP were 0.1, 0.5, 1, and 2 mM. (All of these are added concentrations and are in the background of 100 µM ATP.) No-RNA and no-competitor-NTP lanes are included as negative and positive controls, respectively. Phosphorylation activities were normalized to the no-competitor-NTP lane in the middle gel. For both (A) and (B), 10% SDS-PAGE gel is shown, with position of phosphorylated PKR (p-PKR) indicated. (C) Graphical representation of phosphorylation activities from panel (B).
The ATP titration curve presented in Figure 4.4B using initial concentrations of 40 mM K296R and 720 mM ATP was best fit to a two-site binding model. (Fitting these data to a one-site model (not shown) in which all the parameters floated gave \(n=0.55\) and a poor fit to injections where \([\text{ATP}]/[\text{K296R}] \geq 1.5\), while fitting to a one-site model with \(n\) forced to 1, gave a poor fit to all injections.) The first site had a \(K_d\) of \(19 \pm 1.8\) µM and \(n = 1.32 \pm 0.30\), suggesting a 1:1 stoichiometry of K296R to ATP (Fig. 4.4B), as expected based on the crystal structure of PKR.\(^{14}\) This \(K_d\) is similar to that seen for ATP binding to other kinases such as PKA and MAPK,\(^{30,31}\) and in agreement with a value from Cole and co-workers of \(20 \pm 2\) µM, measured for ATP binding to K296R using competition fluorescence anisotropy experiments.\(^{32}\) The second site was tighter in affinity (\(K_d = 3.9 \pm 1.4\) µM) but with a small binding stoichiometry of \(n= 0.11 \pm 0.09\) (Fig. 4.4B).

Given the high affinity at both sites as determined by ITC and the lack of binding with the other three NTPs, it seems unlikely that one of these two sites is representative of the proposed non-specific triphosphate binding site, which the preceding data suggest is much weaker binding than the catalytic site (Fig. 4.3C, bottom panel). One possible explanation for the two-site fit involves the different conformers of Mg\(^{2+}\)-coordinated ATP, namely the “open” conformer, in which the metal ion only coordinates the phosphate, and the “macrochelated” conformer, in which the metal ion is coordinated to the phosphate and the base.\(^{33}\) Interestingly, the macrochelated form represents \(\sim 10\%\) of the ATP population at physiological salt conditions and could thus represent the “site 2” observed by ITC titration (Fig. 4.4B).\(^{33}\) (Specificity of the ATP binding site was also probed by a series of fluorescence competition and pulse-chase stopped-flow experiments involving the fluorophore mant-ATP and the competitors ATP, GTP, and UTP, and
confirmed specificity for ATP\textsuperscript{15}.)

Figure 4.4: The ATP binding site has high affinity: ITC experiments. ITC titration curves for NTP binding to K296R. (A) GTP (green trace), UTP (blue trace), and CTP (orange trace) binding to K296R. Titration of each NTP into buffer is included (black traces). NTP traces are offset from buffer traces by ~0.1 µcal/sec in raw data (upper panels) for clarity. Legends are provided in plots. (B) ATP binding to K296R (red trace). Titration of ATP into buffer is included (black trace). ATP titration curve is fitted to a two-site binding model (see Materials and Methods), and thermodynamic parameters are provided to the right of the plot. The major contribution is with site 1 (n=1.32 ± 0.09) and a $K_d$ of 19 ± 1.8 µM. Note that uncertainty in ΔH is due to the lack of a good lower baseline, which is common with µM $K_d$ experiments. However, this does not affect the $K_d$ value as that is mainly based on the slope of the transition region.
In sum, the results from PKR competition assays and ITC suggest that two triphosphate binding sites exit on PKR and that they are quite different: a catalytic triphosphate binding site that binds ATP with both high specificity and high affinity, and a 5’-nucleotide triphosphate binding site that is non-specific to 5’-nucleobase identity and low affinity.

4.4.4 Binding of 5’-triphosphate RNA and heparin require the dsRBD

We previously demonstrated that the magnitude of PKR activation by ppp-ssRNA is dependent upon the presence and positioning of a short ~5 bp stem-loop, thus implicating a role for the dsRBD in PKR activation. The optimal placement of this stem-loop was ~20-45 nt from the 5’-end of ppp-ssRNA. Additionally, ppp-ssRNA activators displayed certain similarities to dsRNA activators, such as a requirement for length (~47 nt. of single-stranded RNA versus ~33 bp of double-stranded RNA) and a bell-shaped dependence on RNA concentration. Despite these similarities, distinct differences in the 5’-end requirements of ppp-ssRNA and dsRNA exist—requiring and not requiring a 5’-triphosphate, respectively—suggesting potential mechanistic disparities between these two classes of activators. Indeed, ssRNA displays coarse structural similarity to a non-RNA activator of PKR, the polyanion heparin, which has been shown to bind basic residues near the active site in the kinase domain. In order to elucidate the mechanism of activation of PKR by ppp-ssRNA, we sought to elucidate the role, if any, of the dsRBD in such activation.

We first performed PKR competition assays, in which three activators—canonical dsRNA (dsRNA-79), pppG-ssRNA-47, and heparin—were competed by either short, 20
bp dsRNA “dsRNA-20” or short unstructured 20 nt ssRNA with a 5’-triphosphate “ppp-ssRNA-20” (Fig. 4.1B, Fig. 4.5). Both competitor RNAs have been demonstrated to bind PKR but not activate.$^6,10$ As shown in Figure 4.5A and B, dsRNA-20 effectively competed with both RNA activators (6-fold inhibition for dsRNA-79 and 26-fold for ppp-ssRNA-47), while ssRNA-20 had no effect on dsRNA-mediated activation and only a small ~20% effect on ppp-ssRNA-47-mediated activation. For dsRNA-79, these results are consistent with the well-established model of long dsRNA interacting with the dsRBD, which drives binding of multiple PKR molecules (Fig. 4.5A). Short dsRNA competes with this interaction hence leading to inhibition, while ssRNA either does not bind to the same part of PKR as dsRNA, or cannot effectively compete with longer dsRNA.

Competition of ppp-ssRNA-47 by dsRNA-20, on the other hand, was surprising. In fact, dsRNA-20 competes more effectively with ssRNA activator than dsRNA activator, 6- and 26-fold, respectively. This finding suggests that ppp-ssRNA-47 makes critical contacts with the dsRBD, most likely through its short stem-loop. The fact that ppp-ssRNA-20 is at best a poor competitor for ppp-ssRNA-47, despite the fact it contains a 5’-triphosphate, may be due to absence of the short stem-loop in ssRNA-20. This notion is also consistent with the above inability of NTPs to compete for ppp-ssRNA-47.
Figure 4.5. PKR activation by ppp-ssRNA and heparin is inhibited by dsRNA, but not by short ppp-ssRNA. RNA-competition assays. (A) Competition for dsRNA-79-mediated PKR activation by short dsRNA and ssRNA. PKR activation by dsRNA-79 was assayed in the presence of increasing concentrations of dsRNA-20 and ssRNA-20. Concentration of dsRNA-79 was held constant at 0.1 µM, and concentrations of dsRNA-20 and ssRNA-20 were 0.1, 1, 2.5 and 5 µM. (B) Competition for ppp-ssRNA-47-mediated PKR activation by short dsRNA and ssRNA. PKR activation was assayed as in (A), with the concentration of ssRNA-47 held constant at 2.5 µM. (C) Competition for heparin-mediated PKR activation by short dsRNA and ssRNA. PKR activation was assayed as in (A), with the concentration of heparin (average molecular weight of 6 kDa) held constant at 75 µg/mL, and concentrations of competitor RNAs at 0.1, 1, 2.5, 5, and 10 µM. For all panels, 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. No-RNA and no-competitor lanes are included as negative and positive controls, respectively. Phosphorylation activities are provided under the gels and were normalized to the 0.1 µM competitor lane for each set of competitors; activities were normalized in this manner because in some cases the presence of competitor at low concentrations appeared to stimulate the reaction.
In an effort to provide further insight into the role of the dsRBD, we considered the mechanism of PKR activation by the known activator heparin, a sulfated glycosaminoglycan. Because ssRNA and heparin are both non-dsRNA polyanion activators, we thought that they might have similar mechanisms of activation. Heparin has been shown to bind clusters of basic amino acids at the entrance to the kinase active site that are non-overlapping with the dsRBD, suggesting a primarily electrostatic as opposed to structure-based mechanism of activation. Furthermore, it has been shown that heparin can activate a truncated version of PKR that does not contain the dsRBD, although the extent of activation was strongly diminished relative to full-length PKR. We thus anticipated that ppp-ssRNA activators might compete for activation by heparin while short dsRNA would not compete; indeed, such a result would suggest that the 5’-triphosphate binding site overlaps with the cluster of basic residues that serve as the binding site for heparin.

We thus performed competition assays in which PKR activation by a constant concentration of heparin (average MW 6 kD) was challenged with increasing concentrations of dsRNA-20 or ppp-ssRNA-20. In contrast to the above expectations, ppp-ssRNA-20 was unable to compete with heparin for activation, while dsRNA-20 was a potent inhibitor of heparin-mediated activation of PKR (Fig. 4.5C). Lack of inhibition by ppp-ssRNA-20 indicates that either the 5’-triphosphate does not share a binding site with heparin, or that heparin binds more tightly than this particular RNA. Even more surprising, heparin-mediated activation of PKR is strongly inhibited by dsRNA-20 (up to 7.7-fold inhibition). This observation suggests an important role for the dsRBD in activation of PKR by both heparin and ppp-ssRNA.
4.4.5 The 5’-triphosphate-binding site is located outside the dsRBD

Results so far indicate that 5’-triphosphate-containing ssRNA do not interact with the ATP binding site, but do interact, at least in part, with the dsRBD. Our next step in defining the mechanism of 5’-triphosphate-dependent PKR activation was to investigate where the ppp-ssRNA physically associates with PKR. We transcribed ppp-ssRNA-47 (Fig. 4.1B) in which all uridines were replaced with 4-thiouridine, termed “ppp-4thioU-ssRNA-47”. This RNA was radiolabeled at the 5’-end, and crosslinked to PKR via exposure to 365 nm light (see Materials and Methods). Several different forms of PKR were investigated: full-length (wtPKR), P20 (dsRBD only), and a mutant PKR with two point mutations in the dsRBD, K60A and K150A (dmPKR) (Fig. 4.1A). It has been shown previously that these two changes abrogate binding to dsRNA.\textsuperscript{9,18}

As shown in Figure 4.6 and Figure C1, ppp-4thioU-ssRNA-47 crosslinks to wtPKR in a protein-, 4-thioU-, and UV-dependent fashion, confirming interaction of this activator with PKR. Surprisingly, there is at most very weak crosslinking between 4thioU-ssRNA-47 and P20. To confirm that the crosslinks between this RNA and PKR were outside of the dsRBD, we conducted crosslinking on dmPKR.
Figure 4.6. Photochemical crosslinking reveals interaction of ppp-ssRNA-47 with PKR regions outside the dsRBD. 4thioU-substituted ppp-ssRNA-47 was incubated with wild-type PKR, P20, or dmPKR and exposed to 365 nm light for 0, 10, 20, or 30 min and analyzed by 7% denaturing (7M urea) PAGE. Positions of free RNA and crosslinked products are indicated. Protein-dependence of crosslinked products was confirmed by absence of products upon irradiating 4-thioU-ppp-ssRNA-47 in buffer alone (first three lanes).

As shown in Figure 4.6, we observe strong, UV-dependent interaction of ppp-4thioU-ssRNA-47 with dmPKR, which supports the notion that ppp-ssRNA-47 interacts with a region of PKR outside the dsRBD. Given the above support for interaction of ssRNA-47 with the dsRBD, poor crosslinking to the dsRBD is most likely because the quantum yield of crosslinks between PKR and 4thioU-ssRNA-47 are low: there are several AU base pairs in the short stem region of ssRNA-47, which are likely broken due the thio group on the Watson-Crick face.

To further evaluate the role of the dsRBD in interacting with various PKR activators, we performed EMSA competitions, with P20 pre-bound to radiolabeled 20 bp dsRNA (Fig. 4.7). These binding-competition experiments complement the activation-competition experiments described above (Fig. 4.5), but also extend them because it is
possible to use actual activators as competitors in the EMSAs. As expected based on previous studies characterizing interaction with short dsRNAs of various length, P20 binds to trace amounts of 5'-$^{32}$P-radiolabeled dsRNA-20 in two distinct complexes (Fig. 4.7, lane 3). A long classical dsRNA activator, dsRNA-40, competes completely for binding to both complexes, as revealed by loss of complexes 1 and 2, and by gain in free p*dsRNA-20 (Fig. 4.7, lanes 4-5). Moreover, in agreement with our activation assays (Fig. 4.5), we find that ssRNA-20 is unable to displace dsRNA-20 from P20, as revealed by absence of gain of free p*dsRNA-20. We do note, however, that ssRNA-20 appears to induce conversion of complex 1 into complex 2, indicating some type of rearrangement (Fig. 4.7, compare lane 3, where complex 2 represents only ~10% of bound product, to lanes 6-9, where complex 2 represents ~50-60% of products). This suggests that short, purely single-stranded RNA is capable of making contacts with the dsRBD, albeit contacts non-productive for activation. This effect is 5’-triphosphate independent (Fig. 4.7, compare lanes 6-7 to 8-9), further supporting that the 5’-triphosphate binding site is not located within the dsRBD.
Figure 4.7. Competition for P20 binding by dsRNA, ssRNA, and heparin by EMSAs. Trace amount of p*dsRNA-20 was incubated with P20 in the presence of various unlabeled competitors and analyzed by 10% native PAGE. Top strand (TS) dsRNA-20 was 5'-32P-labeled and annealed to excess unlabeled bottom strand (BS). Formation of annealed p*dsRNA-20 duplex was confirmed by the microshift of p*TS in the presence of BS (lane 2). A no-competitor mobility shift was detected upon addition of 3 µM P20, with slight formation of a second complex (lane 3). In remaining lanes, 3 µM P20 bound to trace p*dsRNA-20 was challenged with either unlabeled RNA or heparin competitor. Competitor RNA concentrations were 5 and 10 µM, and heparin concentrations were 10, 100, 1000, and 2000 µg/mL. Mobility of p*dsRNA-20, free and bound to P20 complexes, is indicated. Quantitation of fraction p*dsRNA-20 bound in each complex is provided under each lane and was determined by drawing boxes around the free RNA and two complexes.
Next, we find that two different 5’-triphosphate-dependent activators, ssRNA-47 and ss-dsRNA (9,11), compete with dsRNA-20 for binding to both P20 complexes. This is revealed by loss of complex 1 and especially complex 2, and by gain in free p*dsRNA-20 (Fig. 4.7, lanes 10-13). These observations are consistent with the dsRNA-20 competition experiments in Figure 4.5B, which suggested that the dsRBD plays a critical role in PKR activation by ppp-ssRNA. This binding competition, in contrast to the rearrangement of complexes induced by non-activating ssRNAs, is in agreement with a role for the small stem-loops of 5’-triphosphate-dependent RNAs in binding the dsRBD during activation. Consistent with this interpretation, ss-dsRNA (9,11), a previously characterized activator containing a 16 bp stem-loop flanked by 9 and 11 nucleotide single-stranded tails (Fig. 4.1D), competes more effectively for p20 than ssRNA-47, which has less double-stranded character (5 and 4 bp helices) (compare gain of ~50% free p*dsRNA-20 in lane 11 to ~75% free p*dsRNA-20 in lane 13). It is worth noting that the effects here are not simply due to ionic strength, as ssRNA-20 offers no competition at its highest concentration, which is effectively equal to the concentration of phosphate in the lower concentration lanes for ssRNA-47 and ss-dsRNA (9,11).

Finally, we observe that heparin can also compete with dsRNA for binding (Fig. 4.7, lanes 14-17). This result corroborates our activation competition assays, wherein activation by heparin was effectively competed by dsRNA-20 (Fig. 4.5c), and further supports a key role of the dsRBD in activation of PKR by heparin. This interaction with the dsRBD appears to be dependent on the heparin concentration: at very low heparin concentrations (10 µg/mL), heparin behaves like short, non-activating ssRNAs, promoting conversion of complex 1 to complex 2 (Fig. 4.7, lane 14), while at higher
concentrations, heparin competes out dsRNA-20 for P20 binding, resulting in free p*dsRNA-20. Overall, the EMSA competition studies provide strong support for a general role of the dsRBD in PKR activation by both classical dsRNA as well as non-dsRNA activators of ppp-ssRNA and heparin.

4.5 Discussion

Surveillance of foreign RNA by PKR and other components of the innate immune response is a critical first line of defense against viral infection. While long stretches of double-stranded RNA have been recognized as the primary molecular signature for PKR activation, recent studies indicate that PKR can be activated by a multitude of additional RNA structural elements, including short coaxially stacked helices, RNA helices interrupted by bulges and internal loops, and internal or 5’-end modifications, such as the 5’-triphosphate. Given this expanding landscape of RNA elements that regulate PKR, it is critical to understand the mechanisms by which PKR is specifically activated by diverse RNA structures.

We previously identified the 5’-triphosphate of weakly structured ssRNA as a PAMP for PKR activation, which allows for discrimination between self and non-self RNA. This PAMP is also recognized by another innate immune sensor, RIG-I, suggesting that the 5’-triphosphate is a more widespread signal for the innate immune system. We sought to better define the interaction of PKR and ppp-ssRNA by characterizing the 5’-triphosphate binding pocket via a variety of functional and biophysical assays.
The simple “one-triphosphate site” model is one in which the catalytic binding cleft in the kinase domain serves as a common site for binding both ATP for catalysis and the 5’-triphosphate of ssRNA for activation. This model is suggested by the fact that PKR and RIG-I, which also contains an ATP binding cleft, are both able to bind a common RNA ligand despite lacking sequence and structural homology. An alternative is the “two-triphosphate site” model, in which the 5’-triphosphate binding site is located either elsewhere in the kinase domain or in the dsRBD and is separate from the catalytic ATP site. The data provided herein support the two-triphosphate site model. There is low specificity for the 5’-nucleobase in 5’-ppp-ssRNA-mediated activation of RNA (Fig. 4.2); but high specificity for ATP, as neither GTP, CTP, nor UTP can compete efficiently for ATP in activation (Fig. 4.3). Moreover, ATP is the only NTP that gives detectable heat of binding to PKR (Fig. 4.4). An early study observed ATP and GTP competition for photoaffinity labeling of PKR at a site proposed to represent either the catalytic site or a secondary allosteric regulatory site.39 The ATP specificity at the catalytic site presented herein supports the possibility that these investigators may have probed the secondary triphosphate site. Indeed, selectivity at the PKR catalytic site for ATP is consistent with the behavior of most kinases.40,41 This binding specificity likely serves as a selective pressure to avoid catalysis by GTP, which could have detrimental consequences for PKR regulation in the cell. Conversely, lack of nucleobase selectivity for the proposed 5’-triphosphate site allows for activation of PKR by 5’-triphosphate RNA activators that start with any of the four nucleotides. Indeed, bacterial and viral RNAs can begin with A, C, or G,25-27 suggesting that lack of specificity at this nucleobase position allows a broad immune response. In particular potent activation of PKR by pppA-ssRNA-47 supports
recognition of the highly conserved pppA present in influenza A, B, and C viruses,\textsuperscript{42,43} consistent with the 5’-triphosphate-dependence of PKR activation by influenza B virus.\textsuperscript{44}

We have previously shown that 5’-triphosphate-mediated activation of PKR is dependent upon the characteristics of the downstream RNA structure, such as length and presence and positioning of short stem-loops.\textsuperscript{6} Activating 5’-triphosphate RNAs interact with PKR outside the dsRBD (Fig. 4.6), raising the question of whether the dsRBD still plays a role in recognition of 5’-triphosphate activators. Surprisingly, we find that the dsRBD plays important roles in binding and activation of PKR by both ppp/ssRNA and heparin (Figs. 4.5 and 4.7). Despite extensive structural differences between canonical dsRNA, 5’-ppp/ssRNA, and heparin, all three require interactions with the dsRBD for optimal activation. Given that PKR activation has a bell-shaped dependence on concentration of ppp/ssRNA (Fig. 4.2) and heparin\textsuperscript{13} (and data not shown), the dsRBD may drive dimerization of PKR on ppp/ssRNA and heparin as well, although these non-dsRNA activators also take advantage of additional contacts outside the dsRBD. Indeed, a clear mechanistic basis for the bell-shaped dependence of PKR activation on heparin concentration has been elusive.\textsuperscript{13} We are continuing work to define the specific PKR residues involved in these non-dsRBD-mediated contacts.

It has been demonstrated that PKR recognizes the 5’-triphosphate of influenza B viral RNA in human cells,\textsuperscript{44} and other studies have shown that PKR mediates the antiproliferative action of heparin treatment for atherosclerosis.\textsuperscript{45} As such, defining regions of PKR that contact disparate activators, such as key regions of PKR’s dsRBD, may facilitate construction of therapeutic RNAs capable of activating PKR during viral infection or may provide insight into the mechanism of heparin therapeutics.
4.6 Acknowledgments

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4.7 References


Figure C1. Photochemical crosslinking of ppp-ssRNA-47 with wtPKR is protein-, 4-thioU-, and UV-dependent. 4-thioU-substituted ppp-ssRNA-47 (4SU) or unsubstituted ppp-ssRNA-47 (UTP) was incubated with wtPKR and exposed to 365 nm light for 0, 2, 5, or 10 min and analyzed by 7% denaturing (7M urea) PAGE. Positions of free RNA and crosslinked products are indicated. Protein-dependence of crosslinked products was confirmed by comparing the first and third set of lanes; 4thioUTP-dependence was confirmed by comparing third and fourth set of lanes; and UV-dependence was confirmed by comparing lanes 1 and lanes 2-4 of the third set of lanes.
Chapter 5

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

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Contributions: LH designed and performed all experiments for the first trial. SRN and CH repeated all duplicate experiments upon request of reviewers of the RNA Journal.

5.1 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 RNA structure, prediction of whether a given RNA 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 towards 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. Lastly, 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.

5.2 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 2α (eIF2α), and consequently translation is inhibited.\textsuperscript{1,2} Activators of PKR include viral dsRNA replication intermediates from both DNA and RNA viruses, non-viral RNA aptamers, and a pseudoknot IFN-γ mRNA promoter.\textsuperscript{3-8} PKR interacts with these RNAs in a non-sequence specific fashion, and is regulated by RNAs containing helical defects such as bulges, internal loops, stem-loops, multi-stem junctions, and even more complex tertiary interaction such as pseudoknots and kissing hairpins.\textsuperscript{9-12} 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.\textsuperscript{12} 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 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, transient electric birefringence (TEB), and ligase-catalyzed cyclization. Electrophoretic mobility and TEB analyses indicate that bulges are not points of flexibility, but rather are rigid. 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<sub>n</sub>- than U<sub>n</sub>-bulges. TEB analyses provide quantitative measurements for bend angles. Consistent with mobility measurements, A<sub>n</sub> bulges induce more acute bend angles than U<sub>n</sub> bulges. Moreover, addition of bases opposite a bulge, to make an internal loop, reduces bending.

The effect of salt on bulge angle has also been examined. Addition of magnesium ions, which can compact the folds of certain RNA motifs, has little effect on A<sub>n</sub> bulge angles. 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.

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. 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.

5.3 Materials and Methods

5.3.1 Protein expression and purification

Full-length PKR, containing an N-terminal (His)$_6$, was cloned into pET-28α (Novagen, Inc.) and transformed into E. coli BL21 (DE3) Rosetta cells (Novagen, Inc.), as previously described. Briefly, cells were sonicated and the protein was purified by a Ni$^{2+}$-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 b-mercaptoethanol (βME). Isolated protein was treated with λ-PPase (NEB) before PKR activation assays as described below.

5.3.2 RNA preparation and purification

RNAs were prepared by transcription from a hemiduplex template by T7 RNA polymerase, except for dsRNA-79, which was prepared from pUC19 as previously described. 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 TEN$_{250}$ [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 (CIP), followed by polynucleotide kinase (PNK) treatment in the presence of [γ-32P]ATP.

5.3.3 Native gel mobility analysis

Native gels containing 10 or 15% of 29:1 (acrylamide: bis) crosslinking 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 µM unlabeled top and 11 µM 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.26 Duplex RNAs were prepared as above, except unlabeled top and bottom strand RNA concentrations were 2 and 3 mM, 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 and 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.

5.3.4 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 µM) 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 µM ATP (Ambion), and 15 µCi [γ-³²P]-ATP. RNA duplexes were renatured at 10 µM 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.
5.4 Results

5.4.1 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 5.1 for shorthand and a list of RNAs studied). Representative cis and trans RNAs containing two [2x0] A bulges in the context of a 51 bp helix, termed ‘cis 2’ and ‘trans 2’, are provided in Figure 5.1A.\textsuperscript{15,21} Cis RNAs have a geometry in which 20 bp flanking helices are on the same side of a central 11 bp helix, while 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\textsuperscript{26,29} but \( \geq 30 \) bp for dimerization and activation.\textsuperscript{8,29,30} 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 1 turn of A-form dsRNA.
Table 5.1. Summary of RNA duplex constructs and nomenclature

<table>
<thead>
<tr>
<th>Construct name</th>
<th>LH-(Center)-RH (bp)</th>
<th>Total bp</th>
<th>Total nt</th>
<th>Bulge size and strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRNA-40a</td>
<td>20-20</td>
<td>40</td>
<td>80</td>
<td>None</td>
</tr>
<tr>
<td>bent [2x0]a</td>
<td>20-20</td>
<td>40</td>
<td>82</td>
<td>[2x0] TS</td>
</tr>
<tr>
<td>flex [2x2]a</td>
<td>20-20</td>
<td>40</td>
<td>84</td>
<td>[2x2]</td>
</tr>
<tr>
<td>dsRNA-51b</td>
<td>20-11-20</td>
<td>51</td>
<td>102</td>
<td>None</td>
</tr>
<tr>
<td>dsRNA-51-chi b c</td>
<td>20-11(D)-20</td>
<td>51</td>
<td>102</td>
<td>None</td>
</tr>
<tr>
<td>dsRNA-52b</td>
<td>20-12-20</td>
<td>52</td>
<td>104</td>
<td>None</td>
</tr>
<tr>
<td>dsRNA-53b</td>
<td>20-13-20</td>
<td>53</td>
<td>106</td>
<td>None</td>
</tr>
<tr>
<td>cis 1d</td>
<td>20-11-20</td>
<td>51</td>
<td>104</td>
<td>2[1x0] TS</td>
</tr>
<tr>
<td>trans 1d</td>
<td>20-11-20</td>
<td>51</td>
<td>104</td>
<td>[1x0] TS, [1x0] BS</td>
</tr>
<tr>
<td>cis 1s de</td>
<td>20-5-20</td>
<td>45</td>
<td>92</td>
<td>[1x0] TS, [1x0] BS</td>
</tr>
<tr>
<td>trans 1s de</td>
<td>20-5-20</td>
<td>45</td>
<td>92</td>
<td>2[1x0] TS</td>
</tr>
<tr>
<td>cis 2d</td>
<td>20-11-20</td>
<td>51</td>
<td>106</td>
<td>2[2x0] TS</td>
</tr>
<tr>
<td>cis 2-chi d e</td>
<td>20-11(D)-20</td>
<td>51</td>
<td>106</td>
<td>2[2x0] TS</td>
</tr>
<tr>
<td>trans 2d</td>
<td>20-11-20</td>
<td>51</td>
<td>106</td>
<td>[2x0] TS, [2x0] BS</td>
</tr>
<tr>
<td>bent Ad</td>
<td>46-5</td>
<td>51</td>
<td>104</td>
<td>[2x0] BS</td>
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<td>[2x0] BS</td>
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<td>36-15</td>
<td>51</td>
<td>104</td>
<td>[2x0] BS</td>
</tr>
<tr>
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<td>31-20</td>
<td>51</td>
<td>104</td>
<td>[2x0] BS</td>
</tr>
<tr>
<td>bent Ed</td>
<td>26-25</td>
<td>51</td>
<td>104</td>
<td>[2x0] BS</td>
</tr>
<tr>
<td>bent E [1x0]d</td>
<td>26-25</td>
<td>51</td>
<td>103</td>
<td>[1x0] BS</td>
</tr>
<tr>
<td>bent E [2x0]d</td>
<td>26-25</td>
<td>51</td>
<td>104</td>
<td>[2x0] BS</td>
</tr>
<tr>
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<td>26-25</td>
<td>51</td>
<td>106</td>
<td>[4x0] BS</td>
</tr>
<tr>
<td>bent E [6x0]d</td>
<td>26-25</td>
<td>51</td>
<td>108</td>
<td>[6x0] BS</td>
</tr>
</tbody>
</table>
In these dsRNAs 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. All bulged nucleotides are A’s. The dash depicts positions of A substitutions. See Figure 5.1 for diagram of cis and trans orientations. ‘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.
Figure 5.1 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 5.1 footnote) were constructed with two [2x0] A-bulges on the top strand and a bulge-free bottom strand. Trans RNAs containing a dsRNA-51 core were constructed with one [2x0] A-bulge on the top strand and one on the bottom strand. Nomenclature for all duplexes is detailed in Table 5.1. Number of A-bulges is represented by the number of concentric circles; duplex length and bend angle in the absence of Mg\textsuperscript{2+} 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.
In general, the mobility of RNA on native PAGE is controlled by global geometry, with the following migration order: linear > trans > cis.\textsuperscript{15} 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. 5.1B). Since all three duplexes are 106 nt, the observed differences in mobility can be attributed to differences in global geometry rather than mass. Addition of KCl to native gels did not improve resolution of these RNAs, and cis and trans duplexes containing [1x0] bulges were found to have less distinct electrophoretic mobilities (data not shown).

5.4.2 Effect of bulge position on PKR activation

We next investigated how position of 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, while 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 a one [2x0] A-bulge positioned 5, 10, 15, 20, or 25 bp from the end of a 51 bp duplex (Fig. 5.2A). Mobility of these RNAs on native PAGE is progressively more retarded as the [2x0] bulge is moved towards the center of the duplex, as expected (Fig. 5.2B).
A

RNA: dsRNA-51

B

RNA: dsRNA-52

C

dsRNA-79

D

Fold decrease (dsRNA-51/bulge)
Figure 5.2: Effects of bulge position on PKR activation by bent RNAs. (A) Global geometry of dsRNA-51 core bent RNAs containing one [2x0] bulge located 5, 10, 15, 20, or 25 nt from the right helical end. RNAs are named ‘bent A’ through ‘bent E’ (see panel and Table 5.1). (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.

Next, activation assays were performed to assess differences in PKR phosphorylation upon changing the position of the single A2 bulge (Fig. 5.2C, D). Overall, activation decreases as the bulge is moved towards the center of the duplex. At 0.02 µM 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 µM 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 4-fold, 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,

5.4.3 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 5.3). Bent E RNA had the slowest native gel mobility, and was the weakest activator of PKR (Fig. 5.2).
compared mobility of and PKR activation by four duplexes: bent E [1x0], bent E [2x0], bent E [4x0], and bent E [6x0] (Fig. 5.3A). Approximate bulge angles, based on the studies of Zacharias and Hagerman\textsuperscript{21} are presented in Figure 5.3A. As expected, electrophoretic mobility on native PAGE becomes more retarded as bulge size increases, owing to a more acute bend (Fig. 5.3B).\textsuperscript{31}

An activation assay was then performed to assess differences in PKR phosphorylation upon increasing the size of the single bulge (Fig. 5.3C, D). Overall, activation decreases as bulge size increases. In particular, at 0.02 µM RNA, PKR activation by bent E [1x0], [2x0], [4x0], and [6x0] 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 [6x0] with its approximate right angle bend (Fig. 5.3A), only serves to further abrogate activation of PKR.

### 5.4.4 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 \textit{two} bulges, with either cis or trans global geometry. Cis and trans geometries were engineered into 45 or 51 bp RNAs with either [1x0] or [2x0] bulges (Fig. 5.4A-D). In addition, bulge defect flexibility was examined in the context of 40 bp RNAs comparing [2x0] and [2x2] defects (Appendix Fig. D1). Three perfect duplex control RNAs were also included in these assays: dsRNA-40, dsRNA-51, and dsRNA-79. 40 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.

**Figure 5.3 Effect of bulge size on PKR activation.** (A) Global geometry of bent E RNAs containing one [1x0], [2x0], [4x0], and [6x0] A-bulge. Approximate bulge angles, based on the studies of 21 are provided in the panel. (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. (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.
First, we tested PKR activation by 51 bp cis and trans RNAs with [1x0] or [2x0] 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. 5.4A and 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. 5.4A, B and E). Similar effects hold at 0.1 mM RNA (Fig. 4). When compared to the shorter dsRNA-40, overall effects were more modest in magnitude, but general trends were unaffected (Fig. 5.4). In addition, we also tested cis and trans duplexes containing [3x0] A-bulges and found them to activate PKR similarly to [2x0] RNAs (data not shown).

Second, we examined effects of shortening cis 1 and trans 1 RNAs from 51- to 45-bp (Fig. 5.4C). The shortened duplexes, termed ‘cis 1s’ and ‘trans 1s’, have a 5 bp central helix and 20 bp flanking helices (Table 5.1). 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 presence of just a half-turn of dsRNA in the central helix, helices were engineered with bulges positioned on strands opposite those shown in Figure 5.1A for 51 bp cis and trans duplexes—see Table 5.1.) PKR was significantly less activated by the shortened central helices for the cis constructs (Fig. 5.4C). 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. 5.4D), which does not support dsRBM binding.26
Activation of PKR by dsRNA-51-chi decreased on average by 35-fold compared to
dsRNA-51 at 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 [2x2] symmetric internal loop in a 40 bp RNA,
termed ‘flex [2x2]’, on activation of PKR was also tested (Appendix Fig. D1). We
reasoned that if we provided bases opposing the bulged bases, which removes bending,24
stronger activation might be restored; as noted earlier, internal loops are points of
flexibility.23 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 [2x0]’. At 0.02 µM RNA, PKR
activation by bent [2x0] and flex [2x2] are 3.8- and 5.7-fold less than dsRNA-40,
respectively (Appendix Fig. D1). Thus, incorporating a flexible internal loop disfavors
PKR activation and has a larger effect than an asymmetric bulge.
Figure 5.4: 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 [1x0] 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.

Lastly, 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 C2). 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, and 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 non-activating modified RNAs gave similar binding activity as activating unmodified RNAs. 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 subtle issues.
5.4.5  **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. In an effort to gain insight into how helical flexibility might affect PKR activation, we investigated effects of lowering salt concentration from the standard value of 100 mM 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. 5.5 and gels in Appendix Fig. D3).

PKR activation assays were first performed in 50 mM KCl, which is below biological ionic strength, and revealed only small differences between the three duplexes relative to dsRNA-51, with cis 2 and bent D being slightly better activators than trans 2 (Fig. 5.5, open symbols). For instance, at 10 min and an RNA concentration of 0.02 µM, 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 (Appendix Fig. D3A). Moreover, initial rates of phosphorylation were similar for all RNAs, with bulged RNAs differing from perfect dsRNA by just 1.5-2.3 fold (Table 5.2).
Table 5.2. Ionic strength dependence of initial rates of PKR phosphorylation

<table>
<thead>
<tr>
<th>RNA construct</th>
<th>50 mM KCl(^a)</th>
<th>100 mM KCl(^b)</th>
<th>Fold-effect of ionic strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRNA-51</td>
<td>13.3</td>
<td>5.3</td>
<td>2.5</td>
</tr>
<tr>
<td>bent D</td>
<td>9.0</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>cis 2</td>
<td>8.3</td>
<td>0.3</td>
<td>28</td>
</tr>
<tr>
<td>trans 2</td>
<td>5.8</td>
<td>0.1</td>
<td>58</td>
</tr>
</tbody>
</table>

Results are from linear fits to data plotted in Figure 5.5. \(^a\)Initial rates were determined using only the first time point for all RNAs. \(^b\)Initial 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.

Next, we considered time courses of PKR activation assays in higher and more biologically relevant concentrations of KCl. Remarkably, assays in 100 mM KCl revealed much greater loss in activity for the three bulge-containing duplexes than perfect dsRNA-51 (Fig. 5.5, closed symbols). For example, at 10 min and an RNA concentration of 0.02 mM, cis 2, trans 2, and bent D were reduced in activity relative to dsRNA-51 by 11-, >20-, and 9.2-fold, respectively (Appendix Fig. D3B). 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. 5.5, closed symbols). Also, initial rates for bulged RNAs were lower by a striking ~30-60 fold in 100 mM KCl (Table 5.2).
Figure 5.5 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 µM dsRNA-51 (○,●) and bent D (△,▲). (B) Plot of PKR activation versus time for 0.02 µM cis 2 (□,■) and trans 2 (◇,◇). Percent PKR activation is normalized to 0.01 µM dsRNA-79 RNA. Arrows depict effect of increasing ionic strength. Gels are provided in Appendix Fig. D3.

Lastly, 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 mM (Appendix Fig. D3). 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.

5.5 Discussion

The protein kinase PKR acts in the innate immunity pathway by sensing RNA. 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. 5.2D). 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. 5.2C, D). Notably, bent C has a bulge-free segment of just 36 bp, which is similar to the minimal length needed for dimerization and to the activating length of ~33 bp. 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 A2-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, we might expect bent B and dsRNA-40 to activate to the same extent. However, at 0.02 μM RNA activation is 6-fold higher for bent B than dsRNA-40 (Fig. 5.2C and Fig. 5.4A and 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. 5.2C, D) (see model below).

Increasing the size of the bulge from [1x0] to [6x0] progressively decreased activation of PKR. Presumably this arises because increasing the bend angle in the RNA from 15 to 93° 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 below).

A minimal model for activation of PKR by bulged RNAs is presented in Figure 5.6. 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.\textsuperscript{1,2} 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.

Figure 5.6: Model for PKR dimerization and activation on bulge-containing RNAs. (A) Activation by perfect dsRNA. (B) Activation by cis 2 dsRNA. (C) Activation by cis 2-chi dsRNA. Positions of equilibrium are denoted with various arrowheads.
Straightening, diagramed in this panel, is supported by independent studies.\textsuperscript{9} Weaker activation of PKR by this RNA as compared to perfect dsRNA, as observed in Figure 5.4, can be understood in the thermodynamic penalty for binding to bent RNAs.\textsuperscript{9} 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. Weakest activation by this RNA, as observed in Figure 5.4, 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.\textsuperscript{9,26} PKR having to contend with this RNA-DNA hybrid region rejects any through-space interaction of PKR monomers.

To address 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.

Lastly, bulged RNAs had a large negative dependence on ionic strength, while perfect dsRNAs did not (Fig. 5.5). Reduced flexibility (\textit{i.e.} increased persistence length) of nucleic acids has been reported in lower ionic strength.\textsuperscript{25,38} 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.\textsuperscript{9} Consistent with this notion, an RNA containing a flexible [2x2] internal loop was a poor activator of PKR (Fig. D1).
Since 100 mM KCl is similar to, or still slightly lower than, physiological ionic (140 mM KCl),\textsuperscript{33} these data suggest that ability of PKR to discriminate between various biological RNA substrates is enhanced under physiological salt conditions. 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.\textsuperscript{39,40}

5.6 Acknowledgements

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5.7 References


Appendix D: Supporting Information for Chapter 5

Figure D1. **Effect of RNA flexibility on PKR activation.** PKR activation by dsRNA-40, bent [2x0], and flex [2x2]. Percent PKR activation is normalized to dsRNA-40, and fold effects relative to dsRNA-40 are provided. Reactions were stopped at 10 min and gel is 10% SDS-PAGE.
Figure D2. Binding of perfect and bulge-containing duplexes to P20 by mobility-shift assays. Trace amounts of radiolabeled dsRNAs were mixed with P20 and analyzed by 10% native PAGE (29:1 crosslink). Top strand of the duplex (p*TS) was 5'-32P labeled and pre-annealed in the presence unlabeled top strand and bottom strand, with bottom strand in excess. Formation of duplex was confirmed by a microshift of the top strand upon addition of bottom strand (Compare first and second lanes in each RNA set.). Protein concentrations used are 0.16, 0.32, 0.63, 1.25, 2.5 and 5 mM.
Figure D3. **Time-dependence of effects of geometry and ionic strength on PKR activation.** Time points were collected at 4, 10, 30, and 60 min. (A) PKR activation by dsRNA-51, cis 2, trans 2, and bent D in **50 mM KCl (low salt)**. (B) PKR activation in standard **100 mM KCl (standard salt)**. PKR activation is normalized to 0.01 µM 79 bp RNA due to the time dependence of activation on the four RNAs tested.
Chapter 6

Toward *in vivo* characterization of PKR interaction with cellular and bacterial RNA

6.1 Abstract

The interferon-induced RNA-activated protein kinase, PKR is an RNA-binding protein and an essential sensor in the innate immune response. PKR is known for its ability to bind long stretches (>33bp) of double-stranded RNA (dsRNA), non-sequence specifically, through its N-terminal dsRNA binding domain (dsRBD), allowing its C-terminal kinase domain to dimerize and undergo autophosphorylation. This allows PKR to phosphorylate the eukaryotic initiation factor 2α (eIF2α), which leads to inhibition of translation initiation. PKR has a well-characterized role in recognizing viral dsRNA and initiating an immune response in order to clear the virus, but recently has been shown to be more permissive, binding other functional, biological and non-conventional RNAs, as described in Chapters 2-5 of this thesis. While this RNA-mediated process is how PKR is regulated *in vitro*, there are not sufficient data about what really occurs *in vivo*. In this chapter, I present advancements toward understanding how PKR functions *in vivo* including optimization of the crosslinking and immunoprecipitation (CLIP) method to identify all RNAs that PKR interacts within the cell and testing for PKR activity by transfections and infections of bacteria. Additionally, conditions bridging the gap between *in vitro* and *in vivo* were tested by looking at PKR activity *in vitro* under macromolecular crowding conditions as well as testing how total RNA extracted from *E.*
coli activates PKR. These methods were challenging but I will present what was learned from each, as well as some future directions in how to improve these methods.

6.2 Introduction

As described in previous chapters, the innate immune response is the first line of defense for humans and the RNA-activated protein kinase, PKR is an essential sensor in this system. PKR is regulated by RNA and is classically known to bind long stretches of viral dsRNA. Over the last decade PKR has been shown to be activated by multiple levels of RNA in vitro and I have continued to expand this in the previous four chapters of my thesis. My goal in this chapter was to learn more about how PKR interacts with RNA in vivo.

It has recently been found that PKR is activated by total bacterial RNA in vivo. This activity was confirmed at the RNA level by RNase treatment of these samples, but nothing is known about the identity of the RNAs that lead to this activation.\(^1\)\(^2\) It has also been shown recently that another innate immune protein, RIG-I is activated by bacterial RNA from the RNA-secreting bacterium *Listeria*,\(^3\) suggesting a general role for the innate immune system in protecting from bacteria. Work from chapters 2 and 3 showed that leader RNA, and riboswitches and ribozymes activated PKR, and when we purify PKR from overexpression in bacterial cells it is already activated, perhaps by bacterial RNA. In this study, it was not only found that the RNA from *Listeria* led to regulation of RIG-I and the innate immune system, but that *Listeria* had to be alive and functioning for RIG-I detection. In this case, RIG-I was shown to detect RNAs secreted from *Listeria* in the cytoplasm of the host cell.\(^3\)
In an effort to understand how PKR detects bacterial RNA, we collaborated with Prof. Eric Harvill’s lab at PSU, who studies bacterial secretion systems of the bacteria *Bordatella bronchiseptica* (referred to for now on by the strain they study which is RB50).\(^4\)\(^5\) We hypothesized that RNA secreted from RB50, which has similar secretion pathways as *Listeria*, could activate PKR *in vivo* and we could use this as our system of interest to study how PKR interacts with bacterial RNA.

It would be very interesting to know how PKR recognizes the difference between self and non-self at the RNA level *in vivo*, because it is latent in humans even though cellular RNAs present. Overall there are not sufficient data regarding PKR’s interaction with any types of RNA *in vivo*. This chapter contains multiple methods that I applied in order to learn more about this. I will present four studies that I used to acquire information on this interaction including crosslinking and immunoprecipitation (CLIP) of PKR to cellular RNA in human cells, *in vivo* PKR activity assays on human cells infected with a series of multiplicities of infection (MOI) from RB50, *in vitro* PKR activity assays on total RNA extracted from bacteria, and lastly simulating *in vivo* conditions by adding a molecular crowding reagent to an *in vitro* PKR activity assay.

### 6.3 Materials and Methods

#### 6.3.1 Protein Preparation

Full-length wtPKR containing an N-terminal (His)\(_6\) tag was cloned into pET-28a vector and transformed into *E. coli* BL21 (DE3) Rosetta cells (Novagen) as described in earlier chapters. Cells were sonicated and purified on a Ni\(^{2+}\) -NTA agarose column (Qiagen) run over a high-salt imidazole gradient on a Bio-Rad FPLC. Purified protein
was dialyzed into storage buffer [10 mM Tris (pH 7.6), 50 mM KCl, 2 mM Mg(OAc)$_2$, 10% glycerol and 7 mM β-mercaptoethanol].

6.3.2 RNA Preparation, purification and treatment

The 79 bp control RNA, dsRNA-79, was prepared by transcribing opposing strands of a portion of the pUC19 vector using two separate hemi-duplex templates as described in earlier chapters. The transcripts were purified by denaturing PAGE and annealed to yield a perfectly double-stranded 79 bp dsRNA, which we previously used as a positive control for PKR activation.

Bacterial mRNA was prepared as follows. Wild-type MG1655 *E. coli* cells (a gift from Prof. Paul Babitzke’s lab) were grown overnight in LB broth and pelleted. Cells were treated with the RNAprotect bacterial reagent (Qiagen) to help prevent RNA degradation and then purified by the RNEasy Qiagen kit. A Ribozero kit (Epicentre) was used to greatly diminish rRNAs, which was confirmed by a Bioanalyzer run (Agilent). Ribosomal RNA was depleted to avoid introducing naked rRNA, which is non-physiological. In addition an aliquot of this purified total *E. coli* RNA was separately digested with 0.03 U/µL RNase T1 (Ambion) at 50˚C for 10 min then again purified before preparation for PKR activation assays.

6.3.3 Crosslinking and immunoprecipitation (CLIP)

The general method for crosslinking and immunoprecipitation was adapted from Professor Robert Darnell’s lab$^6$ and is summarized in Figure 6.1. Huh7.5 cells were grown in 100-150 mm plates (to about 9-18 x10$^6$ cells) and crosslinked in a Stratalinker
(Prof. Joe Reese Lab) with 400 mJ/cm² of 254 nm light. Cells were pelleted and frozen at -80°C until use. Cells were lysed and washed in the same conditions as previously published⁶ and partially digested with a cocktail of RNase A & T1 (Ambion) and purified. PKR and any associated RNAs were then immunoprecipitated with anti-PKR [Y117] (Abcam) antibody attached to protein A magnetic Dynabeads (Thermo Scientific). RNAs were then prepared for ligation to 3’- and 5’-RNA linkers (RL) of known sequence. Sequences of RNA linkers can be seen in Figure 6.2. First, the 3’-end was treated with T4 PNK to remove the 3’ phosphate followed by ligation with a 3’-puromycin protected RNA-linker and T4 RNA ligase (Fermentas). Next the 5’-end was prepared for a separate ligation by first dephosphorylating the 5’-end with Calf intestinal phosphatase (NEB) then 5’-end labeling with γ-³²P-ATP.

The RNA/protein complexes were purified by SDS-PAGE and transferred to a nitrocellulose membrane then selected for RNA size according to appropriate RNA size markers. Samples were extracted from the gel, treated with proteinase K (Roche) to remove any remaining PKR protein and the 5’-RNA linker was ligated. Next, RNAs were prepared for sequencing by RT-PCR, purified and then amplified with Illumina sequencing primers having barcodes (See Figure 6.2). Lastly, DNAs were quantified by qPCR, bioanalyzed for quality, and a batch of this DNA was TOPO cloned and sequenced on a 96 well tray before high-throughput sequencing. Lastly, the libraries are sent to the PSU genomics facility for high-throughput sequencing. Optimization of RNase treatment, preparation of ends for ligation, and ligation steps were tested and are explained in section 6.4.1.
6.3.4 Transfections and Infections

The transfection procedure was similar to previously reported. Hek293 or HeLa cells were grown in 100 mm plates and transferred to 96 well trays. Approximately 1.0 x 10^6 cells were transferred to each well in a 96 well tray and primed with 1 U/mL IFN α or γ and incubated at 37°C for 24h prior to transfection. DOTAP (Roche) and DharmaFect siRNA (Thermo Scientific) transfection reagents were both tested to transfec dsRNA-79 into cells and their corresponding protocols were followed. RNAs were allowed to transfec for 8 h prior to trypsin treatment and pelleting of cells. Further details on methodology are explained in in section 6.4.2.

6.3.5 Western Blotting

The western blotting procedure was similar to previously reported. Cells were lysed in 4 µL SDS loading dye by boiling for 5 minutes and loading directly onto a pre-cast 10% Bis-Tris SDS-PAGE gel (Life Tech). Protein was transferred to 0.2 µm Nitrocellulose membrane (GE Protran BA83) using a Transblot SD semi-dry electrophoretic transfer apparatus (BioRad) in 1x Bis-Tris transfer buffer/ 10% methanol (25 mM Bicine, 25 mM Bis-Tris, 0.01% SDS, 10% methanol) for 45 min at 10 V (other variations tested include, 2x transfer buffer, 2 sets blotting paper, PVDF membrane, 30 min/15V transfer). Membranes were blocked in 5% non-fat dry milk/ 1x TBS-T ( 20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h. Membranes were incubated in primary antibody [PKR, p-PKR or p-eIF2a (Abcam)] at a 1:1000 dilution in 1xTBS-T with 0.1% milk added 60 min at room temperature or 4°C overnight. Membranes were washed three times in 1x TBS-T for 10 min. Next, incubation in anti-rabbit secondary
antibody conjugated with HRP to detect ECL (chemiluminescence) at 1:10,000 dilution in 1x TBS-T with 0.1% milk added for 30-60 min at room temperature. Membranes were washed again 3 times then dried and exposed to Pierce pico-chemiluminescent substrate (or Pierce femto-chemiluminescent substrate for higher sensitivity) for 5 min. Exposure to film for varying times were tested (1 min, 10 min, up to 30 min) and were developed in developer/fixer system. Other variations tested include: lysing technique: lysis buffer, sonnicator, fluorescent secondary antibody from Prof. Craig Cameron’s lab, staining gels/membranes after transfer to see if protein transferred well with Ponceau dye.

6.3.6  PKR activation assays under standard and molecular crowding conditions

RNAs were tested for their ability to mediate PKR autophosphorylation. PKR purified from E. coli is in a phosphorylated form so it was first dephosphorylated by treatment with lambda protein phosphatase λPP (NEB). PKR and λPP were incubated at 30°C for 1 h followed by inhibition of the λPP by freshly made 2 mM sodium orthovanadate8–9. Subsequently, the dephosphorylated PKR was incubated at a final concentration of 0.8 μM with various concentrations of RNA. Reactions were in PKR activation buffer [20 mM HEPES (pH 7.5), 50 mM KCl] and 1.5 mM DTT, 100 μM ATP (Ambion) plus 15 μCi [γ-32P]-ATP. For varying magnesium concentrations, the appropriate concentration of free Mg2+ was added to the RNA alone and incubated at 30°C for 5 min prior to the addition of PKR activation buffer. Standard incubation conditions were 30 °C for 10 or 20 min (indicated in each experiment), followed by quenching with SDS loading buffer. Samples were fractionated on a 10% Bis-Tris (Novex) SDS PAGE gel, dried, and exposed to a storage PhosphorImager screen.
(Molecular Dynamics). Phosphorylated PKR bands were detected using a Typhoon PhosphorImager and quantified with ImageQuant. Each activation assay gel contains one negative control, which is a sample containing 1x TEN instead of RNA, as well as a positive control, which is a sample containing 0.1 µM dsRNA-79 that was used to normalize all phosphorylation activities. For PKR activation assays under molecular crowding conditions, PEG 8000 was added to the same buffer mixture as above with the water volume adjusted accordingly so the final percentage of PEG in each 10 µL reaction was 20%.

6.4 Results and Discussion

6.4.1 Steps toward optimizing crosslinking and immunoprecipitation (CLIP)

In an effort to determine the RNAs that PKR interacts with in human cells, the standard CLIP procedure was followed according to the protocol published by Professor Robert Darnell’s lab. The end goal was to obtain CLIP data for both healthy human cells and bacterially infected human cells in order to compare the RNAs that PKR interacts with in self vs. non-self systems. I performed the entire method on healthy human cells following all steps in Figures 6.1 and 6.2, and TOPO cloning was done on the resulting DNA library. Colonies were chosen and sent for sequencing in a 96-well tray. Unfortunately, it was determined that all sequences were only parts of PCR primers with no detectable human RNA sequences of interest. On one occasion before I took over the CLIP project in the lab, one set of samples prepared by a previous postdoc, Dr. Saikat Ghosh, were sent for high-throughput sequencing and the results were also of bad quality,
only having regions of sequence that mapped to the human genome of less than 12 nucleotides.

Figure 6.1: Steps in CLIP protocol. Briefly, cells are treated with IFN-α and crosslinked at 254 nM in a stratalinker. Cells are lysed, RNAs are digested with ribonucleases to produce smaller fragments and PKR is immunoprecipitated with an antibody attached to magnetic beads. Ends of the RNAs were treated to prepare for ligation to known RNA sequences, first the 3' end was dephosphorylated with T4 PNK and the 3' RNA linker was ligated, then the 5' end was dephosphorylated with CIP and radiolabeled with T4 PNK. Products were run on SDS PAGE gels, transferred to nitrocellulose and bands corresponding to approximately 200-400 nt RNA were extracted. Remaining protein was digested with proteinase K and finally the 5' RNA linker was ligated.
**Figure 6.2: CLIP linker ligation and amplification steps.** Steps involved ligating known RNA sequences to the 3′- and 5′- ends of your RNA pool of interest, reverse transcribing the RNA and PCR amplifying it. Then another PCR step must be performed to barcode RNA libraries and add on known Illumina sequencing adaptors. Lastly, amount and quality of your DNA libraries must be analyzed by qPCR and bioanalyzer before sending for high-throughput sequencing. Note: adding three adenosines nearest the ligating end of the RNA linkers depicted on the first line of this figure greatly enhances ligation efficiency (as shown in Figure 6.3).
I next went even more carefully through the protocol and checked the products step by step (not normally done because of the already low yields). I realized through this process that the RNA linkers were not ligating; thus upon washing, all of the RNA linkers would get washed away and by the time I got to the RT-PCR step, there were no corresponding linkers to RT so the PCR primers ended up amplifying themselves. I decided to focus on optimizing the RNA linker ligation steps. The first step was to look at how these RNAs fold using mFold and determine if they were optimal for ligation as I had not been involved in the design process. It was determined that both the 5' and 3' RL’s had too much structure nearest their ligating ends and according to literature did not have the most ideal ending nucleotides (A’s) for ligation, thus new RNA linkers were designed with three adenosines at the acceptor/donor end of the linker. Model RNAs were used in order to optimize these ligation steps including the bottom strand of dsRNA-79, and single-stranded structured RNAs of similar size to RNAs we were looking to purify from cells including HCV IRES domain II (80 nt) and cleaved glmS RNA (145 nt). In the process, I identified the important steps as RNase digestion, removal of 2',3'-cyclic phosphate (2',3'-cP) post digestion and ligation of the linker. It was not trivial to remove this 2',3'-cP and a simple Calf Intestinal Phosphatase (CIP) treatment was not sufficient. According to literature, addition of T4 PNK in the absence of ATP is a similar but more efficient way of removing the 2',3'-cP. Upon using this PNK treatment on my digested RNAs, I immediately had ligations working. I found that T4 PNK treatment in the absence of ATP, so as to avoid unwanted phosphorylation of the 5'-end of the RNA, prepared the 3'-end for ligation and in a later step T4 PNK treatment in the presence of ATP prepared the 5'-end for ligation. The next step was to optimize the RNase conditions.
to achieve limited digestion. The results from these experiments can be seen in Figure 6.3. RNase reactions were at 37°C for 10 min, quenched on ice and immediately phenol-chloroform extracted. Post-purification, RNAs were treated with PNK in absence or presence of 0.2 mM cold ATP at 37°C for 1h and immediately phenol-chloroform extracted. Products were split in half and tested for ligation to either the 5’- or 3’- RNA linker (both linkers labeled). Previously optimized conditions for ligation with T4 RNA Ligase (NEB) were used, which were: 16°C overnight, followed by heating to 70°C for 10 min then quench with 2x FLB and run on a 10% denaturing PAGE gel (Figure 6.3). Results indicate a broad range of ligated products for most RNAs when ligated to the 3’-RNA linker using library RNAs that were T4 PNK treated in the absence of ATP and when ligated to the 5’ RNA linker using library RNAs that were T4 PNK treated in the presence of ATP.
Figure 6.3: CLIP ligation trials between the 5’- and 3’- RNA linkers and various model RNAs. Left side of gel are labeled RNAs for size and marker purposes. Right of gel are RNase treated cold RNAs (dsRNA-79_bottom strand, HCV IRES domain II, or glmS cleaved). Right side of gel are RNase and ligation experiments under noted RNase conditions (in all 4 underlined lanes in each set) that were T4 PNK treated in presence or absence of ATP and ligated to 5’- or 3’- radiolabeled RNA linker (label denoted with asterisk).

6.4.2 Bacterial infections and transfections

In order to determine if live bacteria had an effect on activation of PKR *in vivo* when they were in different stages of bacterial infection (with intact secretion systems) and when they were lysed to release all of their molecular information (heat-killed), infections of human cells with *Bordetella bronchiseptica* (RB50) bacteria in competition with transfected dsRNA-79 (in order to determine if bacterial RNA was a PKR inhibitor), were performed. First, HEK293 cells were used and were primed with IFN-α to increase production of PKR. As shown in Figure 6.4, cells were either transfected with no RNA (just reagents as a negative control), with dsRNA-79 and no bacteria, or with dsRNA and
varying multiplicities of infection (MOI’s) of RB50 or heat killed RB50 (HK bacteria).

Some samples were only infected with bacteria and not transfected with RNA. Figure 6.4 shows western blots to p-PKR for each of these described experiments in triplicate. The results indicate that background was high in non-transfected cells and among transfected and infected cells, levels of p-PKR were inconsistent (within the three same experiments), leading us to believe that the cells were not healthy enough for p-PKR detection.

Figure 6.4: Western blots to phospho-PKR from transfections and infections of dsRNA-79 and RB50 bacteria at multiple MOI’s in HEK293 cells. (a-c) Transfections were incubated for 8 hours prior to cell pelleting and lysis. Each experiment was performed in triplicate. The first two lanes on each western blot are a negative control (transfected with reagents and no dsRNA-79) and a positive control (dsRNA79 only transfected). (a) Cells were infected with MOI’s of RB50 at 0.1, 1 and 100 with no dsRNA79 transfected. (b) Cells were infected with MOI of 100 with no RNA transfected and MOI’s of 0.1 and 1 with dsRNA79 transfected. (c) Cells were infected with MOI’s of 1, 100 and transfected with dsRNA79 and cells were infected with lysed [or heat-killed (HK)] bacteria in absence and presence of transfected dsRNA79.
Many attempts were made at solving this issue including changing the cell
types (HEK, HeLa, Huh), very gentle handling of cells to avoid stress, changing
transfection reagent (DOTAP, Dharmafect-I), and varying transfection reagent and RNA
concentrations among others, but background was never eliminated. We thought there
were possibilities of contamination of antibodies or a problem with my western blotting
procedure so western blots were also attempted in both Prof. Craig Cameron and Prof.
Eric Harvill’s labs. We found similar results with high pPKR background in non-
transfected or infected cells.

The possibility that IFN-α was not producing enough PKR for the antibodies to
detect was also investigated by looking at PKR levels in cells with no IFN, IFN-α and
IFN-γ as shown in Figure 6.5. Again, experiments were performed in triplicate and this
time were tested for presence of non-phosphorylated PKR (first), then the membranes
were stripped of antibody and reprobed, for p-PKR using corresponding antibodies. At
this point we decided to only test RNA transfection and did not add bacteria for
infections. Data seemed more promising than previously seen where IFN-α yielded the
best levels of PKR, and p-PKR levels were enhanced upon transfecting dsRNA-79
(Figure 6.5). However, some problems were still present including high levels of p-PKR
in cells where only the transfection reagents were added (-RNA), indicating that the
transfection reagents could be leading to increased levels of p-PKR. Also, levels of p-
PKR were high and non-reproducible in cells that were not primed with interferon and
not transfected with RNA or transfection reagents at all, indicating cells were not
consistently healthy.
Figure 6.5: Western blots to PKR and pPKR to test new cell type (HeLa) and different types of interferon to attempt to get background down and increase amounts of PKR present in cells. (a-d) Cells were primed with interferon for 24 h prior to transfections, which were incubated for 8 h followed by cell pelleting and lysis. Each experiment was performed in triplicate. (a) Antibody to PKR from cells that were primed with either IFN-γ or no IFN and transfected with dsRNA-7, transfection reagents only (-dsRNA79) or nothing at all (no tfn). (b) Antibody to PKR from cells that were primed with either IFN-α or no IFN and transfected with dsRNA-79, transfection reagents only (-dsRNA79) or nothing at all (no tfn). (c) Antibody to pPKR from cells that were primed with either IFN-γ or no IFN and transfected with dsRNA-79, transfection reagents only (-dsRNA79) or nothing at all (no tfn). (d) Antibody to p-PKR from cells that were primed with either IFN-α or no IFN and transfected with dsRNA-79, transfection reagents onl7 (-dsRNA79) or nothing at all (no tfn).

Some final measures were taken to try to solve these issues, including one person (myself) doing the entire experiment (previously my collaborator Dr. Liron Bendor would prep cells and I would take over and do transfections, lysis and western blots), and I added in a control for protein loading levels in the western blots, which was to probe for the housekeeping gene GAPDH. Results did not improve and it seemed as though protein was loaded equally in each lane according to GAPDH levels.
6.4.3 Total *E. coli* RNA findings

The ability of a mixture of *E. coli* RNAs to activate PKR was tested under both 0.5 and 4 mM Mg$^{2+}$ concentrations. We isolated total RNA from MG1655 *E. coli* and depleted rRNAs using standard procedures described in section 6.2. As shown in Figure 6.6a, total *E. coli* RNA activated PKR under both 0.5 and 4 mM Mg$^{2+}$ conditions and did so to a similar extent, with ~30% activation (relative to 0.1 µM dsRNA-79) at a total bacterial RNA concentration of 22 ng/mL. This activation is within ~10-fold that of dsRNA-79 considering RNA concentration and percent PKR activation. The effect of partially digesting these total *E. coli* RNAs with RNase T1 was a slight increase in PKR activation at a lower concentration of RNA (Figure 6.6b-c). Although this effect was modest and unexpected, it could be of potential interest, where digesting potentially very large bacterial RNAs down to sizes that may allow PKR to dimerize increases PKR’s activity. This could also have a biological significance because a bacterial RNA may be degraded upon infection. However, more experiments must be done to confirm these hypotheses (see section 6.4.5).
Figure 6.6: Total RNA extracted from *E. coli* activates PKR and partial RNAse digestion of this RNA modestly increases potency of activation. (a) PKR activation assays by *E. coli* total RNA in low (0.5 mM) and normal (4 mM) Mg\(^{2+}\) concentrations. The *E. coli* total RNA was rRNA depleted and incubated in 0.5 mM or 4 mM Mg\(^{2+}\) at 30˚C for 5 min and then incubated with PKR for 20 min. RNA concentrations used were 4- fold dilutions: 21.7, 5.4, 1.4, 0.34, 0.08 and 0.02 (ng/µL). Experiments were performed twice and a representative gel is provided here (b-c) PKR activation assays by *E. coli* total RNA partially digested with RNase T1 in low (0.5 mM) and normal (4 mM) Mg\(^{2+}\) concentrations. The *E. coli* total RNA was rRNA depleted and incubated in (b) 0.5 mM or (c) 4 mM Mg\(^{2+}\) at 30˚C for 5 min and then incubated with PKR for 20 min. RNA concentrations used were 14.4, 4.0, 1.0, 0.25, 0.063 and 0.02 (ng/µL). For all experiments a buffer-only negative control is included and PKR activation is normalized to dsRNA-79 under indicated Mg\(^{2+}\) conditions. The position of phosphorylated PKR is indicated as ‘p*PKR’.

### 6.4.4 Molecular crowding experiments on PKR activation

In an effort to test for PKR activation under more cellular-like conditions, the molecular crowding reagent, 20% w/v PEG 8000 was added to *in vitro* PKR activation assays. PEG and other molecular crowders are known to stabilize RNA structure and at the same time stimulate ribozyme catalysis.\(^{12,13}\) Addition of molecular crowders to ribozyme reactions
also displayed a lower Mg$^{2+}$ concentration necessary for catalysis. Additionally, thermostability of nucleic acids in molecular crowding conditions are enhanced. The hypothesis was that molecular crowder would lower the concentrations of RNA needed for PKR activation, and the first step was to try this experiment on the standard PKR activator dsRNA-79. The results from this experiment are shown in Figure 6.7 where dsRNA-79 was titrated into a standard activation assay from 0.0007 to 7 µM in 10-fold dilutions at both 0.5 and 4 mM Mg$^{2+}$ and assays were performed for either 10 min (Fig. 6.7a and c) or 20 min (Fig. 6.7b and d) and in the presence (Fig. 6.7a and b) or absence (Fig. 6.7c and d) of PEG-8000. Each gel contains a negative control with no PEG or RNA, two positive controls with 0.1 µM dsRNA-79 at 0.5 and 4 mM Mg$^{2+}$ and no PEG, and one negative control with no RNA but in the presence of PEG. As seen in Figure 6.6a and b, it seems as though PEG alone leads to an increase in phosphorylated PKR, but there was already substantial background in complete negative controls with no RNA or PEG in this particular gel, something that has been observed as a random event in our lab. This could be due to the fact that because I had to accommodate for the volume of PEG, I added γ-$^{32}$P-ATP to the tube with PKR/λPP before adding to the RNA/master mix rather than vice versa (standard conditions- adding γ-$^{32}$P-ATP to the master mix). This is confirmed by high background in Figure 6.7c and d where PEG was not present. Another observation is that bell-shaped dependence on activation of PKR by dsRNA-79 was seen in Fig. 6.7c and d where PEG was not present, but no apparent trend was seen in Fig. 6.7a and b in the presence of PEG. One apparent effect seen in these experiments was a large difference between PKR activation in 20% PEG at the two different Mg$^{2+}$ concentrations for the 20 min assays where activity was almost lost in 4 mM Mg$^{2+}$, which is a non-
physiological condition. Lastly, it can be seen in Fig 6.7a and b that PKR seems to run faster on the gels where PEG was added.

Figure 6.7: SDS PAGE gels showing phosphorylated-PKR in PKR activity assays under molecular crowding conditions. (a-d) PKR activation assays by dsRNA-79 in low (0.5 mM) and normal (4 mM) Mg\(^{2+}\) concentrations. The dsRNA-79 was incubated in 0.5 mM or 4 mM Mg\(^{2+}\) at 30°C for 5 min and then incubated with PKR for 10 or 20 min. RNA concentrations used were 10-fold dilutions from 7 to 0.0007 µM. For all experiments a buffer-only negative control, RNA-only positive controls at both Mg\(^{2+}\) concentrations and a PEG-only negative control are included, and PKR activation is normalized to dsRNA-79 under 4 mM Mg\(^{2+}\) conditions. The position of phosphorylated PKR is indicated as ‘p*PKR’.
Because of the difference in how PKR ran on the gels in the presence of PEG-8000, I decided to re-run the samples from Fig.6.7a and b on new SDS-PAGE gels after diluting the samples 2-fold with SDS loading dye and keeping the wells intact before drying the gels (to see if any p*PKR was getting stuck). The result of this experiment is shown in Fig. 6.8; thus Fig. 6.8a is the same as Fig. 6.7a and Fig. 6.8b is the same as Fig. 6.7b. The results indicate that samples were getting stuck in the wells when PEG was added to the experiment, but the amount getting stuck in the wells was not consistent so no useable data can be acquired from this experiment. In the future if one were to conduct PKR activation assays under molecular crowding conditions, these conditions would need to be resolved or another crowder other then PEG-8000 should be tried.
Figure 6.8: SDS PAGE full gels from same experiments performed in Fig. 6.7 showing phosphorylated-PKR in PKR activity assays under molecular crowding conditions. Gels were re-ran after diluting in half with SDS loading dye and entire gels were scanned to see if samples were sticking in wells.
6.4.5 Future Directions

A few of the experiments attempted in this chapter have promising directions including CLIP of PKR. I did end up getting to a point where the RNA linkers were ligating efficiently to known RNAs, however we have come to realize that there is a completely easier method to pursue CLIP on PKR. The best direction to take this is to follow a simplified protocol as described in Figure 6.9. The attempt of ligating our own RNA adaptors and preparing RNAs for high-throughput sequencing proved to be so problematic that the best way to move forward would be to follow a more standard RNA-seq method. The steps below were suggested by Dr. Craig Praul, Director of the Penn State Genomics Core Facility.

![Modified CLIP schematic](image)

**Figure 6.9: Modified CLIP schematic.** The quickest proposed way to get HT-sequencing data on PKR-binding RNAs would be to follow the pictured steps including crosslinking cells, RNase-treating RNAs, extracting PKR via immunoprecipitation on proteinA DynaBeads, removing protein via protease, isolating and purifying the RNA and sending off to the sequencing center for Illumina sequencing (RNA-seq).
As for the *in vivo* PKR activation assays with transfections and infections, I felt like I learned a lot and was eager to gain information from these exciting experiments, however I was never able to deplete background levels of pPKR in negative controls. Thus to move forward, someone with more expertise in working with these cells under gentle conditions would be necessary. PKR is very sensitive to cell stress including nutrition stress in cells, thus it is very important to have very healthy cells in order to study PKR *in vivo*.

It was also interesting that upon partial digestion of the pool of RNA from *E. coli* that the activity of PKR was somewhat enhanced, suggesting that smaller pieces of this total RNA pool may be better capable of activating PKR. Some future studies to test this would be to control for size and to test if certain sizes of this bacterial RNA have more of an effect on PKR activity. Finding different RNAse conditions that would allow for specific sizes of RNA product and radiolabeling an aliquot of them to look on denaturing PAGE gels for confirmation of size could support this idea.

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6.6 References


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Department of Chemistry Eugene and Jane Apple Fellowship Continuing Graduate Student Award, The Pennsylvania State University, 2015-2016

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