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Regulation of innate immunity through RNA structure and the protein kinase PKR

Subba Rao Nallagatla[1,a], Rebecca Toroney[1,a] and Philip C Bevilacqua[1]

Molecular recognition of RNA structure is key to innate immunity. The protein kinase PKR differentiates self from non-self by recognition of molecular patterns in RNA. Certain biological RNAs induce autophosphorylation of PKR, activating it to phosphorylate eukaryotic initiation factor 2α (eIF2α), which leads to inhibition of translation. Additional biological RNAs inhibit PKR, while still others have no effect. The aim of this article is to develop a cohesive framework for understanding and predicting PKR function in the context of diverse RNA structure. We present effects of recently characterized viral and cellular RNAs on regulation of PKR, as well as siRNAs. A central conclusion is that assembly of accessible long double-stranded RNA (dsRNA) elements within biological RNAs plays a key role in regulation of PKR kinase. Strategies for forming such elements include RNA dimerization, formation of symmetrical helical defects, A-form dsRNA mimicry, and coaxial stacking of helices.

Address
[1] Department of Chemistry, The Pennsylvania State University, 104 Chemistry Bldg, University Park, PA 16802, USA

Corresponding author: Bevilacqua, Philip C (pcb5@psu.edu)
[a] These authors contributed equally to this work.

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Introduction

Numerous roles for RNA in biology have been uncovered [1]. RNA is central to translation; it can function as an enzyme (ribozyme) and genetic switch (riboswitch); and small RNAs (siRNAs and miRNAs) play key roles in regulating genes. Many of these discoveries have been transformative to our understanding of life processes and the development of therapeutics [2].

A central reason why RNA plays crucial roles in biology is that it embodies diverse structural and decodable sequence information. The folding of RNA has been described as hierarchical [3], in which primary structure forms as the RNA is being transcribed, followed by folding of secondary structure, and then tertiary structure, as the nascent secondary structural elements assemble (Figure 1a).

There is great diversity present in each element of the hierarchy: Primary structure embodies different sequence and length, as well as modifications at the ends and internally (Figure 1b). Secondary structure has as its basis the A-form helix, but is highly diverse owing to assorted imperfections (defects) present in most helices such as bulges, hairpin loops, and internal loops (Figure 1c). Tertiary structures are compact and often (but not always) globular forms of RNA that bring together helices and are highly diverse (Figure 1d). Adding even further to this complexity, the fold and interactions of RNA are dynamic as well: RNA folds as it is being transcribed, and it interacts with ions, metabolites, proteins, and other RNAs (Figure 1e) [4].

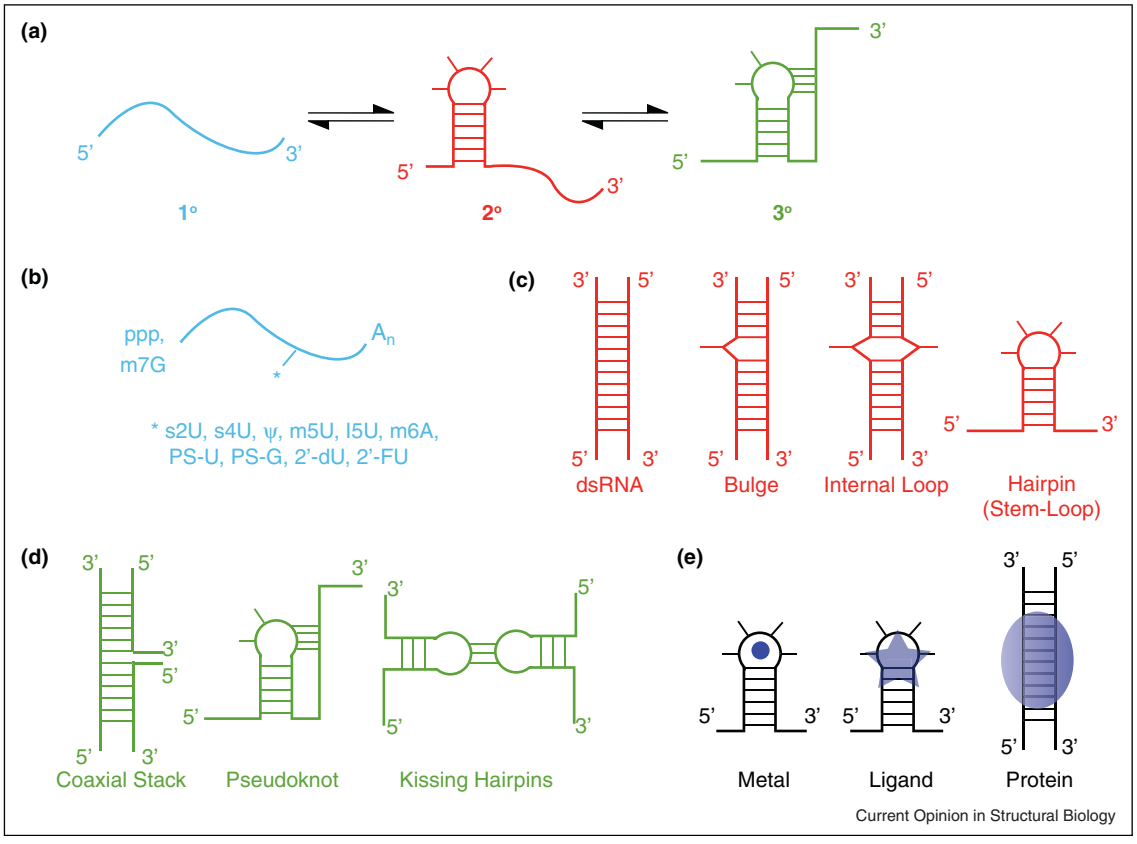
Innate immunity is the initial immune response to invasion by pathogens [5]. Many proteins are involved in this process, including toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I), and the RNA-activated protein kinase (PKR). One key function of these proteins is distinguishing self from non-self through so-called pathogen-associated molecular patterns, or 'PAMPs' [6]. Given RNA's diversity in sequence and structure, it comes as no surprise to find that nature has chosen RNA for many key PAMPs. Specific sequences and structures present in pathogenic RNA allow the innate immune system to distinguish between cellular RNAs and RNAs from viruses and foreign organisms [7].

This review focuses on the RNA-based regulation of PKR and how RNAs can serve as PAMPs. The past few years have witnessed increased understanding of PKR interaction with RNAs of diverse structure. We begin with an overview of PKR structure and its well-known interaction with dsRNA. We then describe recent contributions within the context of the RNA folding hierarchy, proceeding from primary to tertiary structure and ending with siRNAs and a brief comparison to other RNA-based regulating proteins of innate immunity. Our central goal is to develop a cohesive framework for understanding and predicting PKR function in the context of RNA structure.

Structure and function of PKR

The structural biology of PKR is best viewed as a work in progress. PKR is a 551 amino acid protein that consists of two functional domains: an N-terminal dsRNA binding domain (dsRBD) that comprises two dsRNA binding

Figure 1



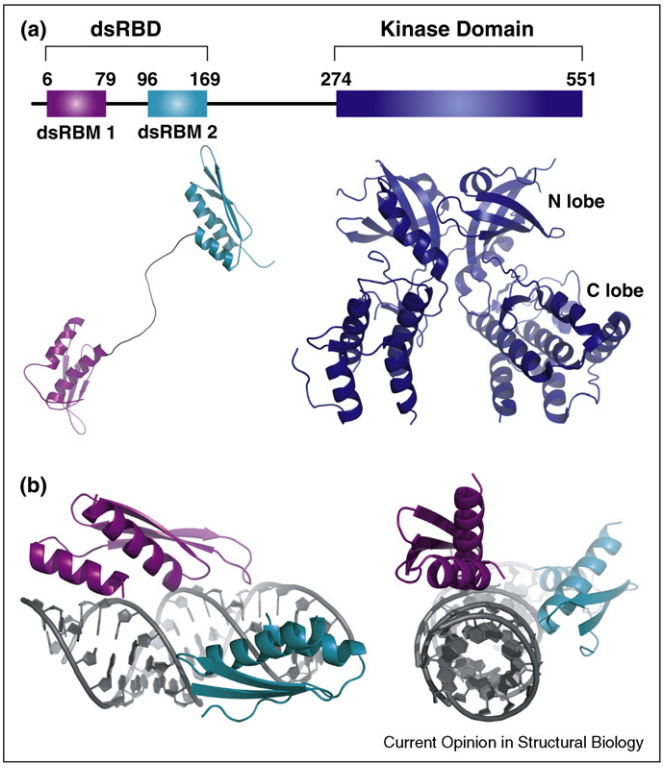
Hierarchy of RNA folding. **(a)** Two-step folding pathway of a pseudoknot RNA, involving primary structure (blue) forming secondary structure (red), here a 5′-proximal hairpin, followed by tertiary structure (green), here interaction of the 3′-tail with the hairpin loop. **(b)** Primary structural elements of RNA (blue), with certain 5′-end, 3′-end, and internal modifications provided. **(c)** Secondary structural elements of RNA (red), with perfect dsRNA, imperfections on one strand to give a bulge, on both strands to give an internal loop, or a stem-loop provided. **(d)** Tertiary structure elements of RNA (green), with coaxial stacking of helices, pseudoknot, and kissing hairpin loops depicted. **(e)** Binding of various species to RNA, with metal ion, ligand, and protein shown.

motifs (dsRBMs) spaced by a flexible 20 amino acid linker,[1] and a C-terminal kinase domain that contains the major sites for phosphorylation (Figure 2a) [8,9]. The dsRBM is a common motif that occurs in all kingdoms of life and is present in a number of notable proteins beyond PKR, including dicer, drosha, and adenosine deaminases that act on RNA (ADARs) [10]. The dsRBM typically recognizes dsRNA non-sequence specifically via minor groove interactions, and several reports indicate interactions with the bases [11,12]. Available structural biology of PKR includes an NMR structure of the dsRBD solved without RNA present [13], and a crystal structure for the kinase domain complexed with eIF2α substrate [14]. The NMR structure reveals the typical αββββα architecture for each dsRBM [13], while the X-ray structure indicates a smaller, mostly β-sheet N-terminal lobe (N-lobe) with a larger, stable, largely helical C-terminal lobe (C-lobe) (Figure 2a). The N-lobe of the kinase domain is involved in dimerization of PKR, whereas the αG helix from the C-lobe acts as a substrate-docking motif [14]. Low-resolution structural models of full length latent (*i.e.* inactive) PKR have been constructed by small angle X-ray scattering (SAXS) and reveal that PKR has intrinsically disordered regions, which may become ordered upon RNA binding; interestingly, data from this method are not fully consistent with the autoinhibition model previously proposed for PKR in which the latent protein is locked into a closed conformation, as described below [15•].

At present there are no RNA-bound structures of PKR, probably because the non-sequence specific nature of RNA binding and the disordered regions lead to heterogeneous states. However, a few structures of other dsRBMs bound to dsRNA have been solved; see for

[1] This nomenclature is the convention used in the PKR field. However, more generally speaking, 'dsRBM' refers to the sequence motif, while 'dsRBD' refers to an independently folding domain.

Figure 2



Structural biology of PKR. **(a)** Structures of the two domains of PKR. PKR is a 551 amino acid protein that contains an N-terminal dsRNA binding domain (dsRBD) that is comprised of two dsRNA binding motifs (dsRBMs) spaced by a flexible 20 amino acid linker, and a C-terminal kinase domain with small and large N-terminal and C-terminal domains. Available are an NMR structure of the dsRBD (pdbid 1QU6) and a crystal structure of the kinase domain complexed with eIF2α substrate (eIF2α omitted here). The kinase crystallizes as a dimer (pdbid 2A1A). **(b)** Structure of a dsRBM from *X. laevis* rbpa bound to dsRNA (pdbid 1DI2) [16]. The dsRNA is 10 bp in length, and two helices are shown stacked end-to-end. Each dsRBM occupies one face of the dsRNA, and packing occurs along different faces of the dsRNA. Shown are side-on and end-on views.

example [16,17]. In general, the dsRBM binds into the wide, accessible minor groove of dsRNA, and multiple dsRBMs can pack along the length of the helix. Shown in Figure 2b is packing of two dsRBMs on ~20 bp of dsRNA. Packing of four dsRBMs on 33 bp of dsRNA, which is the minimum activating length, can also be modeled.

The functions of PKR in biology are quite diverse. A number of excellent reviews of PKR function are available [8,18–20], and only a very brief overview is presented here. In general, activation of latent PKR requires dimerization and autophosphorylation, which occurs upon recognition of sufficiently long dsRNA, such as from intermediates generated during viral replication. In general, 33 bp are needed for minimal activation, with longer dsRNAs activating to a greater extent. Shorter dsRNA, 15–30 bp, inhibits PKR through competitive binding [21,22]. The protein activator PACT and the polyanion heparin can also activate PKR [20], and PKR can even autophosphorylate in the absence of activator if its concentration is high enough [23]. The activated dimer of PKR goes on to phosphorylate its cellular substrate eIF2α on Ser51 leading to translational arrest [8,19]. This process provides essential antiviral and antiproliferative capabilities for the host cell. More recently it was found that phosphorylation of three tyrosine residues on PKR, in addition to multiple serine/threonines is required for full-scale activation of the kinase [24].

In addition to antiviral functions, PKR has been implicated in modulating cell-signalling pathways to alter numerous cellular responses [19]. Moreover, several diseases, such as Huntington, Parkinson and Alzheimer's, have been linked to PKR regulation [20]. A recent report suggested that p53-mediated tumor suppression can be attributed to p53's induction of PKR under genotoxic conditions [25], while another study indicated that PKR regulates insulin action and metabolism in response to nutrient signals and endoplasmic reticulum stress [26].

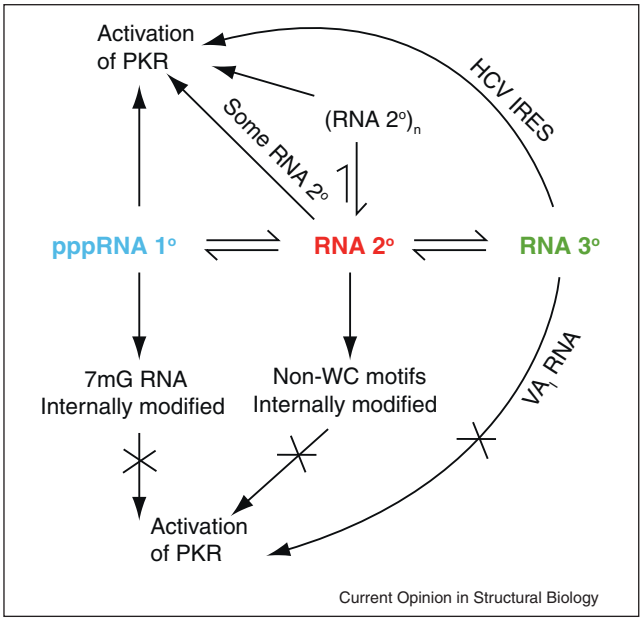
RNA primary structure-based regulation of PKR

As presented in the Introduction, the folding of RNA is largely hierarchical (Figure 1), and high information content exists at each level in the folding hierarchy. The next

three sections consider each of the three levels of RNA folding. The interplay of these RNA elements with regulation of PKR function is summarized in Figure 3.

Early studies used perfect dsRNAs such as poly I:C and T7 transcribed dsRNAs of various lengths to characterize PKR activation [19]. More recent studies reveal activation by RNAs that are non-perfectly double stranded [27,28]. At the primary RNA sequence level, ssRNAs with a small stem-loop and an imperfect 16 base-paired dsRNA with 10–15 nt single-strand tails (so-called 'ss-dsRNA') have been shown to activate PKR in a 5′-triphosphate dependent manner [27,28]. This 5′-triphosphate functional group of ssRNA is key in PKR activation, as 5′-diphosphate, 5′-monophosphate, 5′-hydroxyl, and 7mG cap-containing ssRNAs do not activate PKR [28]. Most endogenous cytoplasmic RNAs contain 5′-monophosphate or 7mG cap, generated through RNA processing, whereas bacterial and some viral RNAs contain 5′-triphosphate; the 5′-triphosphate functionality thus constitutes a PAMP for PKR. By contrast, activation of PKR by dsRNA does not require a 5′-triphosphate, indicating that PKR uses different strategies for recognition of ssRNA and dsRNA [28]. The 5′-triphosphate serves as a PAMP for PKR in recognition of the viral RNA from influenza B virus as well [29••]. Additional experiments have demonstrated that internal nucleoside modifications in 5′-triphosphate ssRNA abrogate PKR activation [30•], indicating that such internal RNA modifications may also serve in distinguishing self from non-self.

Figure 3



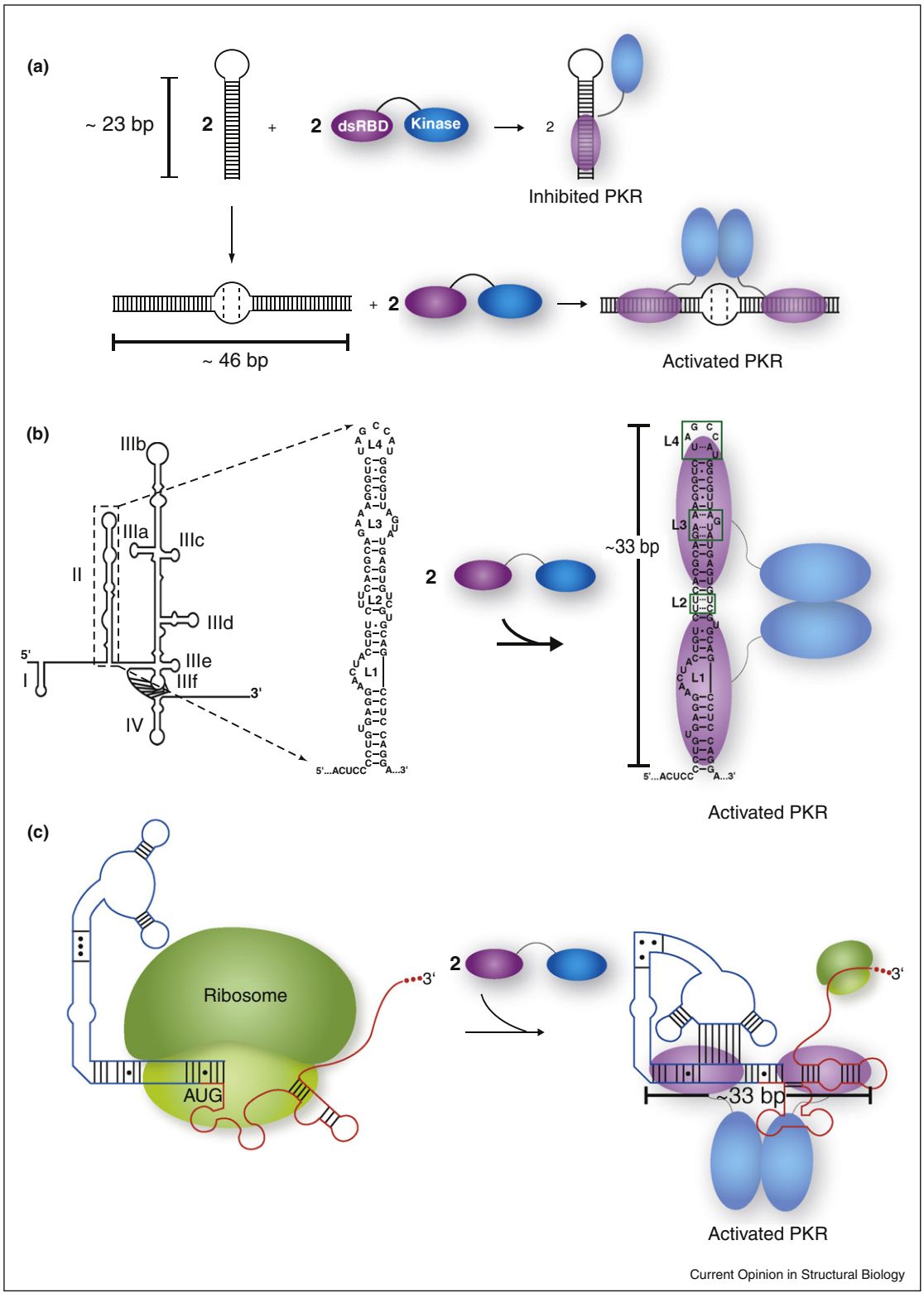
Interplay between the hierarchy of RNA folding and the activation of PKR. The same overall two-step RNA folding pathway presented in Figure 1 is shown, with coloring maintained. At each of the three states along the pathway, the potential for regulation of PKR by RNA exists. At the primary structural level, a 5′-triphosphate helps largely single-stranded RNA activate PKR, whereas a native 7mG cap and internal chemical modifications are incompatible with activation. At the secondary structural level, aggregates of RNA, depicted as '$(RNA\ 2^{\circ})_n$', and certain RNA secondary structures activate PKR, while internal chemically modified RNAs and certain non-Watson–Crick motifs do not. Additionally, certain RNA tertiary structures, such as that of IFN-γ mRNA (see Figure 4c), appear to activate PKR, while others, such as that of the VA_I RNA, do not. See text for details and References.

RNA secondary structure-based regulation of PKR

Long stretches of double-stranded RNA (≥33 bp) activate PKR potently, and have been proposed as the major activators of PKR *in vivo*. The molecular mechanism behind dsRNA-based activation of PKR has been studied extensively. Several models have been advanced, including an autoinhibition model, in which dsRNA binding to the dsRBD releases PKR from an inactive conformation, and a dimerization model, in which dsRNA binding serves to promote kinase dimerization [8]. Recently, analytical ultracentrifugation (AUC) has been employed to investigate the length dependence and stoichiometry of PKR binding to dsRNA [22,31]. These studies have demonstrated that dsRBM1 functions primarily in recognition of shorter dsRNA sequences (<20 bp), while both dsRBMs participate in recognition of longer dsRNAs, which are capable of activating PKR. Additionally, AUC studies have shown that the minimum requirement of ~33 bp for activation of PKR correlates with the ability to bind two PKR monomers. These data are consistent with a model in which long dsRNA functions to bring two PKR monomers into close proximity, which promotes dimerization and thus activation of the kinase domains.

In addition to dsRNA, PKR has been found to be activated by a variety of viral and cellular RNAs, which typically contain various secondary structure imperfections. One such viral RNA is the human immunodeficiency virus transactivation-response region (HIV TAR), a 23 bp hairpin RNA interrupted by three bulges that can exist as a dimer (Figure 4a) [32]. There has been longstanding discrepancy about the role of HIV TAR RNA in regulation of PKR; recent evidence, however, strongly supports that a dimeric form of TAR activates PKR [33••,34•]. In this study, monomers and dimers of TAR were isolated by native gel electrophoresis and studied both structurally and functionally. In particular, it was found that two TAR hairpin monomers refold to form an extended duplex with two asymmetric bulges, which effectively doubles the number of base pairs from ~23 bp in TAR monomer to ~46 bp in TAR dimer. It was found that monomer inhibited PKR, while dimer activated it, consistent with the known dependence of PKR function on dsRNA length. Thus, in this case, RNA dimerization promoted PKR dimerization and activation.

Figure 4



Activation of PKR by complex RNAs. **(a)** HIV TAR RNA undergoes dimerization, which effectively doubles its length from 23 to 46 bp. The 23 bp TAR monomer inhibits PKR, whereas the 46 bp dimer activates PKR. The former binds PKR as a monomer, whereas the latter binds it as a dimer. **(b)** HCV IRES containing four domains, with domain II highlighted. Domain II contains base paired and loop segments, both of which contribute to activation and provide the equivalent of ~33 bp of dsRNA. This element has been shown to mimic A-form dsRNA, including the loop interactions boxed in green [36•]. **(c)** Pseudoknot from the IFN-γ mRNA. The 124 nt 5′-untranslated region is blue, and the coding sequence is red. This domain forms an extended structure with several base pairing elements that also leads to the equivalent of ~33 bp of dsRNA [50••]. In both panels b and c, a dimer of PKR appears to assemble onto the ~33 bp region, leading to an activated state. Panel **(c)** is adapted with permission from [64].

In addition, this study showed that RNA dimers with fewer asymmetrical secondary structure defects were more potent activators of PKR, suggesting that such defects function as antideterminants of PKR binding.

The IRES of HCV has been reported to regulate PKR [35,36•,37•]. One strategy by which dsRNAs with imperfections can activate PKR is through structural mimicry of A-form dsRNA, as recently demonstrated in activation of PKR by domain II of hepatitis C virus internal ribosome entry site (HCV IRES) RNA [36•]. The IRES element of HCV has a complex secondary structure with four distinct structural domains containing multiple symmetric and asymmetric bulges, internal loops, and a pseudoknot (Figure 4b). Despite these complicated elements, several domains of HCV IRES RNA have been reported as activators of PKR, including domains III-IV, which contains several multi-helix junctions and a pseudoknot, and domain II, a shorter hairpin with several internal loops and bulges [36•,37•,38]. Given both the presence of imperfections and the limited number of canonical base pairs (<33 bp), activation of PKR by domain II in particular is surprising. Footprinting and mutational analysis suggest that PKR binds and is potently activated by domain II RNA because the overall topology of its symmetrical loop regions is primarily A-form [36•]. Non-Watson–Crick interactions in the loops of domain II maintain an overall A-form helical backbone geometry and contribute to an activating total of ~33 bp. Mimicry of A-form dsRNA by symmetrical loops may serve as a general mechanism for PKR activation by RNAs with multiple helical imperfections.

Regulation of PKR by RNA secondary structure is also typified by abrogation of PKR dimerization and activation through binding of inhibitory RNAs, such as those encoded by adenovirus (VA_I) and Epstein-Barr virus ($EBER_I$). Both RNAs bind PKR with similar affinity as activating RNAs, but prevent PKR dimerization and subsequent autophosphorylation [39]. VA_I and $EBER_I$ have roughly similar structures with three distinct domains: an apical stem-loop, a central domain, and a terminal stem. In the case of VA_I, the apical stem-loop has been identified as the PKR dsRBD binding site, and the three-way junction within the central domain is the determinant for PKR inhibition [40•]; this domain includes elements of tertiary structure, which will be discussed in the next section. The terminal stem, on the other hand, is completely dispensable for inhibition [41•]. The VA_I apical stem-loop consists of ~18 canonical and non-canonical base pairs, which is sufficient for binding one PKR monomer but not long enough to promote PKR dimerization. Additionally, the apical stem-loop domain of VA_I exists as a population of two conformations, one of which potently inhibits PKR, and the other of which displays markedly decreased inhibition activity [42•]. Possible benefits of these functionally distinct structures, for either the virus or the host, have yet to be determined.

Although the function of PKR is typically to serve as a sensor of non-self RNA, certain cellular RNAs activate PKR. Previous work by Davis *et al.* and Nussbaum *et al.* identified the 3′-UTRs (untranslated region) of several highly structured cytoskeletal mRNAs as activators of PKR [43,44]. Interestingly, PKR activation by cytoskeletal 3′-UTRs is predicted to play a role in the tumor-related activities of these sequences. Similar to previously discussed viral RNAs, these cellular RNAs contain long helical stretches interrupted by bulges, internal loops, and branch points. Also, an element of the 3′-UTR of tumor necrosis factor α mRNA (TNF-α) has been shown to activate PKR [45]. Control of exogenous gene expression by PKR is attenuated by full-length ADAR1 as well as its dsRBMs alone, suggesting that PKR and ADAR1 may compete for binding to the same RNAs [46•,47•]. Whether this effect carries over to cellular RNAs is unclear at present [48].

RNA tertiary structure-based regulation of PKR

Tertiary structure has the potential to activate or inhibit PKR. Given PKR's penchant for dsRNA, one simple model is that if the tertiary structure is globular, activation is unlikely, but that if it is extended, activation is possible. The 5′-UTR of the cellular mRNA for interferon-gamma (IFN-γ) fits this model (Figure 4c). As part of the interferon-mediated antiviral response, PKR participates in a negative feedback loop whereby IFN-γ regulates its own translation via competition between the ribosome and PKR for binding to IFN-γ mRNA [49,50••]. If the level of PKR in the cell is low, the ribosome binds to IFN-γ mRNA to promote interferon synthesis. Upon clearing the ribosome, the 5′-UTR refolds to generate an RNA structure containing a pseudoknot, which is capable of activating PKR. Four adjoining short helices within IFN-γ mRNA coaxially stack within the pseudoknot to cumulate to an activating total of ~33 bp. Thus, in addition to RNA oligomerization by HIV TAR and A-form structural mimicry by HCV domain II, the amalgamation of secondary and tertiary features in IFN-γ mRNA demonstrates another means by which the hierarchical nature of RNA folding can generate structures capable of activating PKR.

Finally, a role for RNA tertiary structure in PKR inhibition lies in the VA_I viral RNA. It was recently determined that Mg^{2+}, which is often required for stabilization of RNA tertiary structure, is required for correct folding of the VA_I central domain and leads to binding of just one PKR. This helps explain the well-established inhibitory role of this RNA [51•]. Melting profiles and compensatory base pair modifications suggested a possible role of RNA tertiary structure in PKR inhibition by VA_I RNA [40•]. It

has been suggested that, while the terminal stem of VA_I may function to stabilize this tertiary structure, in the absence of the terminal stem, PKR binding to VA_I may stabilize tertiary structure [41•], although the exact nature of this tertiary structure has not been fully characterized [52].

siRNA-based regulation of PKR

There exist conflicting reports on the activation of PKR by small interfering RNAs (siRNA). siRNA are short, 19–27 bp, dsRNAs that mediate RNA interference. Several groups have reported that siRNAs of 19–21 bp do not activate PKR, supporting the aforementioned requirement of 33 bp dsRNA for activation [28,53]. In particular, Kim *et al.* [53] designed long siRNAs of 27 bp to enhance RNAi potency and efficiency and showed that they do not activate PKR, while we found that 21 bp double-stranded siRNAs also do not activate PKR [27,28].

In contrast to these observations, activation of PKR by siRNA containing just 19–21 bp has been reported [54,55]. A proposed model for PKR activation by these shorter dsRNAs suggested that a PKR dimer assembled on one siRNA phosphorylates a PKR dimer bound to a different siRNA [55].

Activation of PKR has a strong dependence on ionic strength, and lower salt conditions favor binding of short RNA [22]. Also blunt end siRNAs activate PKR less potently than sticky ends [55]. Thus, experimental conditions, sequence, and helix termini may play crucial roles in determining which siRNAs activate PKR [55]. Lastly, other studies indicate that activation of PKR by siRNAs is more efficient *in vitro* than *in vivo* [56], while others indicated that *in vivo* effects may be indirect [57]. More work is needed to sort out these differences.

Comparison of PKR to other RNA-regulated proteins in innate immunity

RIG-I and Toll-like receptors (TLR 3, 7 and 8) are sensors in innate immunity that also recognize patterns associated with non-self RNAs. Indeed, dsRNA and 5′-triphosphate groups, which PKR recognizes as mentioned, can also be recognized by RIG-I [28,58,59]. Moreover, several natural nucleoside modifications can negate the 5′-triphosphate and dsRNA dependent activation of PKR and RIG-I [30•,58,60]. Indeed, *in vitro* transcribed pseudouridine-containing mRNA translates better than unmodified mRNA owing to diminishing activation of PKR [61•], while modified RNAs provide more efficient reprogramming of cells to pluripotency and directed differentiation, which has been attributed in part to reduced activation of PKR [62]. Regarding TLRs, they are similarly affected: TLR3 is regulated by similar nucleoside modifications in dsRNA [63], while TLR 7 and 8 are regulated by such modifications in ssRNA. Remarkably, PKR, RIG-I, and TLRs are not sequence homologues, supporting unique molecular recognition strategies by each and suggesting convergent evolution.

Conclusions and outlook

The RNA-activated protein kinase PKR is activated by much more than long perfect RNA helices. Recent studies indicate that biological RNAs activate PKR by diverse strategies and to varying extents: dimerization of RNA, inclusion of symmetrical defects, mimicry of A-form dsRNA, and coaxial stacking of helices all contribute to activation of PKR. A common theme is assembly of accessible double-stranded elements that reach the minimum activating length of ~33 bp. Covalent modifications to the 5′-end and internal regions of RNA can either activate or inhibit the kinase. Much remains to be understood about the link between RNA structure and extent of PKR activation, including roles of RNA tertiary structure, RNA aggregation, and co-transcriptional folding. Additional cellular and viral RNAs that regulate PKR surely await discovery, and high-resolution structures of PKR bound to dsRNA and complex biological RNAs are needed. Moreover, mechanisms by which various RNAs alter the fraction and extent of PKR phosphorylation are unknown. Such future advances will help further define the RNA features that allow PKR to perform its essential functions in innate immunity.

Acknowledgements

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