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RNA structure and regulation of innate immunity through protein kinase PKR

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Abstract

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 the context of biological RNAs plays a key role in regulation of PKR kinase. Strategies for forming such elements in biology include RNA dimerization, formation of symmetrical helical defects, A-form dsRNA mimicry, and coaxial stacking of helices.

Introduction

Numerous remarkable 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 play key roles in regulating genes. Many of these discoveries have been transformative to our understanding of life processes [2].

A central reason why RNA plays crucial roles in biology is that it embodies both 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

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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 activation of PKR and how RNAs can serve as PAMPs. The last 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 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 (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 (described below) in which the latent protein is locked into 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 region between the dsRBD and the

[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.

kinase domain leads to heterogeneous states. However, a few structures of other dsRBMs bound to dsRNA have been solved; see for 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 be modeled similarly.

The function of PKR in biology is 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/threonine phosphorylating sites, is required for full-scale activation of the kinase [24].

In addition to the antiviral functions, PKR has been implicated in modulating cell-signalling pathways to alter numerous cellular responses [19]. In addition, 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 recent 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 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 additional 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, -monophosphte, -hydroxyl and 7mG cap-containing ssRNAs do not activate PKR [28]. Most endogenous cytoplasmic RNAs contain 5’-monophophate 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. In contrast, activation of PKR by dsRNA does not require a 5’-triphosphate, indicating that PKR uses different strategies for recognition of ssRNA and dsRNA. 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 these may also serve in distinguishing self from non-self.

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. 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 [35-37]. A strategy by which dsRNAs with imperfections can activate PKR is through structural mimicry of perfect 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 structural 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 [36-38]. 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 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. Interestingly, 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 also 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 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 idea 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 RNA structures capable of activating PKR.

Finally, a role for RNA tertiary structure in PKR activation 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

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