Duplex RNA-Binding Enzymes: Headliners from Neurobiology, Virology, and Development

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1. Introduction

The discoveries of gene silencing by RNA interference (RNAi) and microRNA regulation of development have focused much attention recently on the properties of double-stranded RNA (dsRNA) and dsRNA-binding proteins. However, the importance of dsRNA to the fields of virology and neurobiology has been recognized for some time. In all these cases, key proteins have been identified that bind dsRNA through one or more copies of a small domain called the double-stranded RNA-binding motif or (dsRBM; sometimes referred to as the dsRBD).[1–3] In this minireview, I will discuss five human enzymes implicated in neurobiology, virology, and development that bind dsRNA through dsRBMs and highlight opportunities for advancing our understanding of these fascinating enzymes by chemical biology.

1.1. The dsRBM: A versatile dsRNA-recognition module

The dsRBM is a ~65 amino acid motif found in many, but not all, dsRNA-binding proteins (Figure 1).[3] First identified in *Drosophila melanogaster* Staufen protein and *Xenopus laevis* RNA-binding protein A (Xlrbpa), it is now known to be present in over 100 different gene products from a variety of organisms.[1,2] dsRBMs have been structurally characterized in a number of cases, revealing a characteristic α–β–β–β–α fold for the motif.[4–6] There are also two high-resolution structures of dsRBMs bound to RNA targets.[7,8] In a crystal structure of the second dsRBM of Xlrbpa, the dsRBM spans two minor grooves and the intervening major groove at a binding site made up of 16 base pairs (Figure 1 A).[7] These three locations on the RNA are contacted by three different parts of the motif: α1 binds one minor groove, loop 2 between β1 and β2 binds a second minor groove, and loop 4 between β3 and α2 contacts phosphodiesters that frame the opening of the major groove between the two minor groove sites (Figure 1 A). Contacts are primarily due to RNA 2′-hydroxy groups and phosphodiesters, with only a few base contacts observed in the minor-groove-recognition sites. This explains the RNA specificity and lack of a strict sequence requirement for binding for members of the dsRBM protein family. The NMR structure of Staufen dsRBM III bound to a hairpin-stem revealed a similar protein–RNA interface.[8]

Although dsRBMs do not require specific sequences of duplex RNA for binding, our laboratory has shown that certain sites on an RNA ligand can be selectively occupied by a dsRBM.[9–12] We demonstrated this by using dsRBM proteins tethered with EDTA·Fe. The EDTA·Fe modification converts the dsRNA-binding protein into a dsRNA-cleaving protein and analysis of the sites of cleavage allowed us to conclude that selective binding does occur. This binding selectivity is due to both structural features in the RNA ligand and the structure of the dsRBM involved and can be important to the function of the protein containing the motif (see Section 2).[12] Selective binding has also been demonstrated by others for dsRBM proteins by using footprinting techniques and scanning force microscopy.[13,14]

Proteins that have a dsRBM more often than not have multiple copies.[2] Multiple dsRBMs can increase RNA binding affinity and/or selectivity.[8,15] Furthermore, the dsRBMs carry out functions other than recognition of dsRNA. For instance, dsRBM II of the RNA-dependent protein kinase (PKR) acts as an autoinhibitory domain through interactions with the catalytic domain in that enzyme.[16] Other examples of protein–protein interactions mediated by dsRBMs have also been reported.[2]

dsRBMs are present in several enzymes of varying activity including deaminases, kinases, and nucleases. In the following sections, I will describe properties of several of these dsRNA-binding enzymes (Figure 1 B). ADAR1 and ADAR2 are adenosine deaminases that act on dsRNA. ADARs deaminate adenosines in duplex structures found in pre-mRNA and are essential for a properly functioning central nervous system in metazoa. Dicer and Drosha are dsRNA-specific ribonucleases implicated in the RNA interference pathway and in the generation of microRNA regulators of translation. PKR is a protein kinase regulated by dsRNA and a member of a family of eIF2α kinases that control translation initiation rates in response to stress. PKR is involved in the interferon antiviral response and is also an intracellular signal transducer.

ADARs: RNA Editing and the Nervous System

2.1. RNA editing by adenosine deamination

RNA editing refers to a wide variety of modification reactions that change the sequence of an RNA molecule from that encoded by the gene sequence.[17] Deamination at C6 of adenosine (A) in RNA generates inosine (I) at the corresponding nucleotide position. Since inosine is decoded as guanosine

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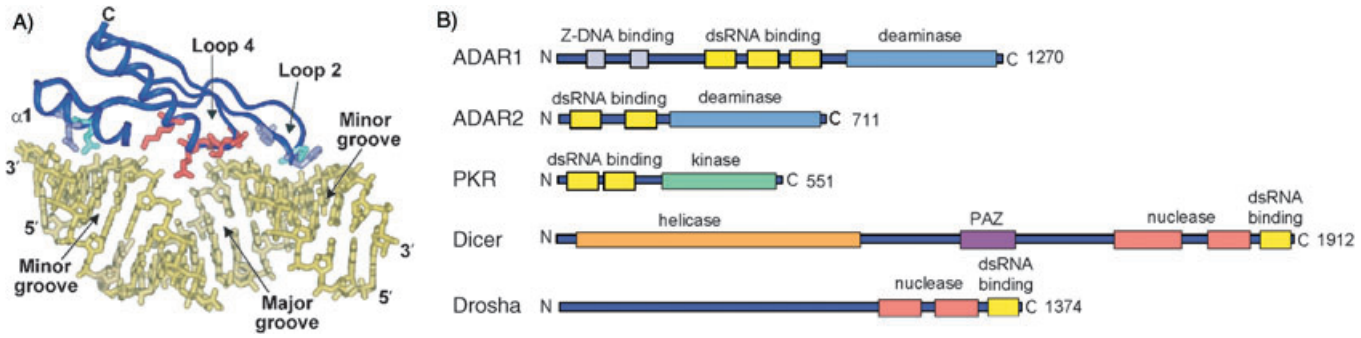


Figure 1. *The double-stranded RNA-binding motif and dsRBM-containing proteins. A) The structure of a single dsRBM from* X. laevis *RNA-binding protein A bound to dsRNA.*[7] *Three regions of the dsRBM (loop 2, loop 4, and α1) bind two minor grooves and contact phosphodiesters that frame the intervening major groove in a 16 bp binding site. B) Domain maps for dsRBM proteins discussed in this minireview.*

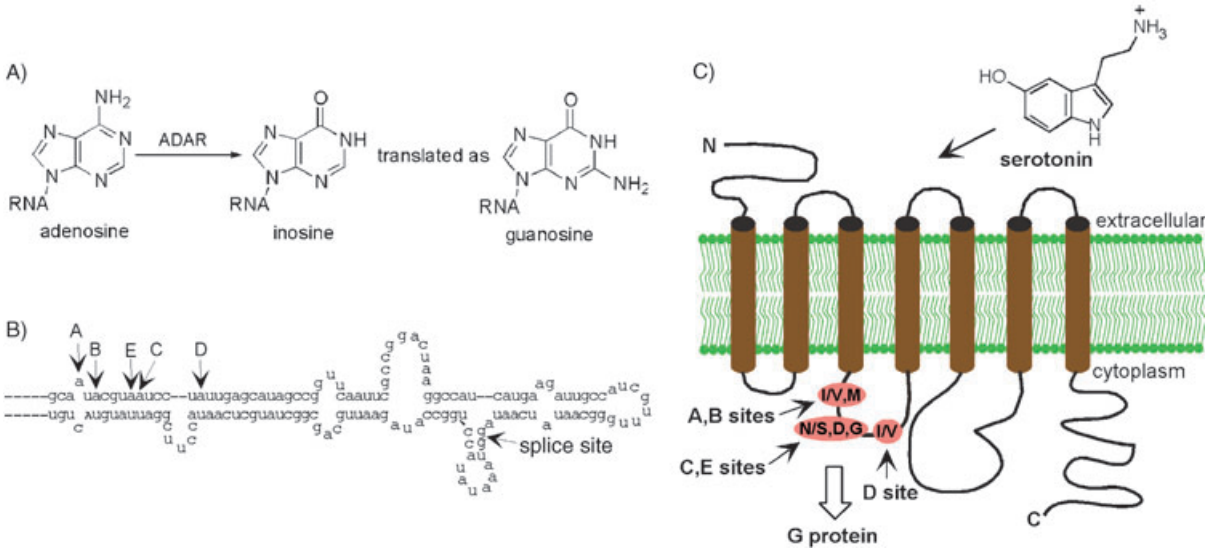


Figure 2. *RNA editing by adenosine deamination leads to neurotransmitter receptor diversity. A) ADARs catalyze the deamination of adenosine generating inosine at that site in the RNA. Since inosine is translated as guanosine, the deamination can change codon meaning. B) Editing sites A–D in the serotonin receptor pre-mRNA (5-HT$_{2C}$ subtype. C) Model of a serotonin receptor and location of amino acids that are altered by editing of its pre-mRNA.*[125]

during translation, this RNA modification can lead to codon changes and the introduction of amino acids into a gene product not encoded in the gene (Figure 2A).[18,19]

Interestingly, many targets of the A-to-I editing reaction are the pre-mRNAs that encode receptors for neurotransmitters.[18–20] It is clear now that complex behavioral patterns are made possible by the dizzying array of neurotransmitter receptors on the surface of neurons that differ very subtly in structure and in function. One way mammals create these different receptors is through editing of the messenger RNAs that encode them. Thus, a single receptor gene can produce different forms of the receptor protein through mRNAs edited at different sites. Indeed, the many combinations of editing sites lead to dozens of different forms of the receptor, each subtly different from the next. In several cases, editing of the message has been shown to have a clear effect on the function of the encoded receptor. For instance, editing at sites A–D in the message for the serotonin receptor 5-HT$_{2C}$ subtype alters the ability of the receptor to transmit a signal to the intracellular transduction machinery by changing the nature of the intracellular surface of the receptor that interacts with G proteins (Figure 2B, C).[18] In another example, editing of the pre-mRNA for the B subunit of the glutamate receptor (GluR-B) causes codon changes at two different sites.[19,21] The Q/R site is so named because at that site a glutamine codon is altered such that the new sequence encodes arginine. The R/G site is located in an arginine codon that is converted to a sequence that encodes glycine. Editing at the Q/R site affects the ion permeability of the channel, whereas editing at the R/G site affects the rate at which the receptor recovers from ligand desensitization.[21,22] Other nervous system targets of A-to-I RNA editing have also been identified.[20]

Consistent with the observation that the editing targets include messages for neurotransmitter receptors, A-to-I RNA editing is necessary for a properly functioning central nervous system in metazoa.[23–26] For instance, deletion of the gene encoding an enzyme responsible for A-to-I editing in *D. melanogaster* leads to a morphologically normal fly with dramatic

behavioral defects, such as tremors, uncoordinated locomotion, and an inability to fly or jump.[25] Likewise, *Caenorhabditis elegans* containing similar gene deletions show defects in behaviors such as chemotaxis.[26] The study of the effect that changes in levels of RNA editing have on human behavior has only recently been initiated and appears to link abnormal editing to psychiatric disorders.[27–30] Altered RNA-editing levels might also be responsible for severe depression side effects experienced by some patients undergoing interferon treatment, since one of the enzymes that carries out this reaction (ADAR1) is interferon-induced.[30]

Interestingly, ADAR1 also has been shown to have an essential function in mammals beyond the nervous system. This function was demonstrated by the death of the ADAR1-knockout mouse because of a failure in erythropoesis.[24] Furthermore, the ability of ADARs to extensively deaminate messages in noncoding regions suggests the possibility of a more widespread role for this process in controlling RNA structure and function.[31]

2.2. ADARs: Enzymes responsible for A-to-I RNA editing

Two different enzymes edit GluR-B and 5-HT_{2C}R pre-mRNA in vitro in the absence of additional proteins or RNAs. These enzymes have been given the name ADAR for *a*denosine *dea*minase that acts on *R*NA (Figure 1 B).[32] ADAR1 is purified from tissue sources as a ~120 kDa protein[33,34] and efficiently modifies the 5-HT_{2C}R A–C sites and the GluR-B R/G site.[30,35,36] Interestingly, ADAR1 is interferon-stimulated and is believed also to play an antiviral role in the cell by nonselectively deaminating viral duplex RNAs.[37] In addition to three dsRBMs in its RNA-binding domain, ADAR1 also has a unique Z-DNA-binding domain near its N terminus.[38] ADAR2 is a ~80 kDa protein that deaminates the 5-HT_{2C}R D and E sites and the GluR-B Q/R and R/G sites.[30,35,36] ADAR2 has two dsRBMs for RNA recognition. ADAR1 and ADAR2 are expressed in most tissues.[39] Since the phenotypes of ADAR knockouts are related to nervous-system function, and known substrates for ADARs are primarily nervous system targets, the function of ADAR1 and ADAR2 in non-neural tissues is not firmly established at this time.

Deamination of adenosine occurs in an active site made up of amino acids located C-terminal to the dsRBMs.[40,41] This region of the protein harbors conserved sequences similar to those found in nucleoside deaminases. Cytidine deaminases (CDAs) and adenosine deaminases (ADAs) have been extensively characterized.[42] These metalloenzymes use a zinc-activated water molecule to carry out hydrolytic deamination of their nucleoside substrates. The active sites of CDA and ADA are composed of highly conserved amino acids that provide the ligands to the active-site zinc and the acidic/basic groups for the necessary proton transfers. The ADARs and a family of adenosine deaminases that act on tRNA (ADATs) have conserved sequences similar to consensus sequences for the CDAs.[43] Putative active-site residues identified in this analysis have been altered by site-directed mutagenesis with a corresponding loss of editing activity.[41] Furthermore, Bass and colleagues have shown that the source of the oxygen atom present in the inosine of the ADAR product is water.[44] These observations indicate that the deamination steps in the ADAR reaction are likely similar to those of the CDA reaction. In addition, although there is no sequence conservation between the ADARs and ADAs, the mechanistic similarity between the CDA and ADA reactions makes it likely that ADARs and ADAs also share common mechanistic features. However, our work with substrate analogues and inhibitors has shown that structure–activity relationships are not entirely overlapping for the ADA and ADAR reactions.[45,46] Thus, ADA is an imperfect model for adenosine deamination catalyzed by ADARs.

2.3. The ADAR reaction as we currently understand it

More detailed biochemical experiments have been carried out for ADAR2, and its reaction mechanism is more clearly defined than that of ADAR1. Given the necessary trajectory of the attacking hydroxide for hydrolytic deamination of adenosine, it seemed highly unlikely that the adenosine would be buried in the core of the RNA double helix throughout the ADAR reaction. Indeed, by using fluorescence spectroscopy with 2-aminopurine-containing RNAs, our laboratory showed that ADAR2 causes a nucleotide position-specific conformational change in an RNA substrate consistent with flipping the reactive base from the helix into the enzyme's active site.[47,48] We have also shown that the dsRBMs of ADAR2 bind selectively on a model substrate for the Q/R editing site in the GluR-B pre-mRNA, and occupation of the selective binding sites is important for deamination at the Q/R site.[12] It has also recently been shown that dsRBM I can be deleted from ADAR2 and that the resulting enzyme retains the ability to efficiently deaminate at an editing site in a model substrate.[49] Furthermore, this truncation mutant can deaminate short substrates that the full-length enzyme is unable to process. These observations have been rationalized by invoking an autoinhibitory role for ADAR2's dsRBM I (or the linker between the dsRBMs). Also, we have shown that 8-azaadenosine in RNA is a better substrate for ADAR2 than adenosine.[46] Since nucleophilic aromatic substitution reactions are more facile for the 8-azapurine ring system (compared to purine), these results suggest that attack of the hydroxide on the heterocycle is rate limiting in the substrates tested.[50] Evidence has also been presented from several laboratories that supports dimerization of ADARs during the editing reaction,[51–53] although it remains to be seen if this is important for all ADAR reactions.[49]

Given the observations discussed above, a mechanism for the ADAR2 reaction can be proposed (Figure 3). Initially, the dsRBMs recognize dsRNA of sufficient length and selectively occupy sites on this duplex substrate. If enough recognition surface is present to allow both dsRBMs to bind simultaneously, the deaminase domain is relieved of the autoinhibition and contacts the RNA. The reactive adenosine is flipped out of the helix and binds into the active site. A metal-bound water is deprotonated by E396, and the resulting hydroxide attacks the C6 position of the purine ring in the rate-determining step of the reaction. This is followed by protonation at N1 to generate a tetrahedral intermediate. Proton transfer to N6 and from N1

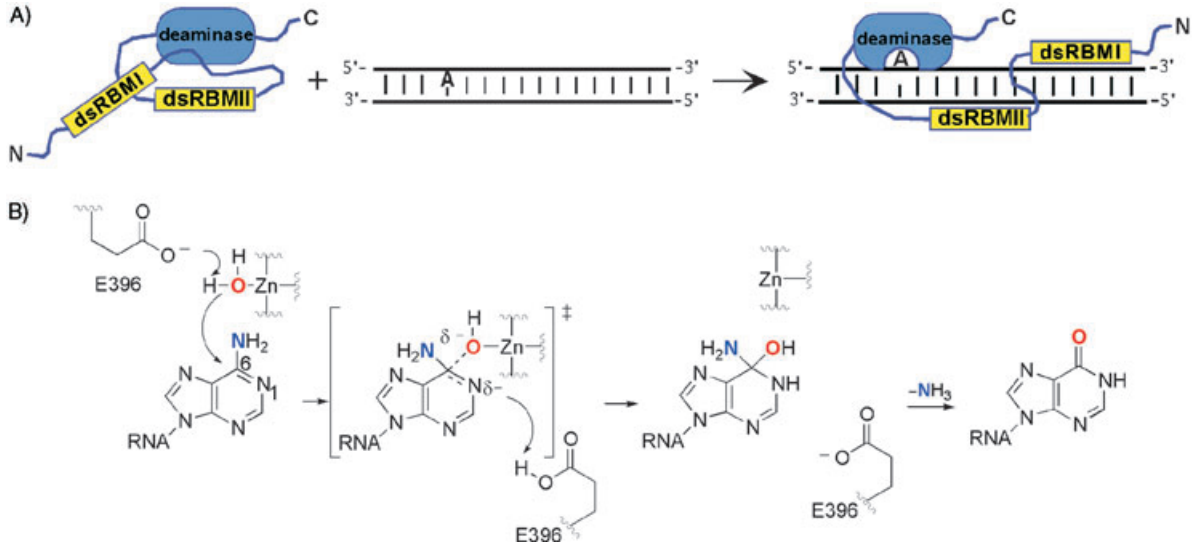
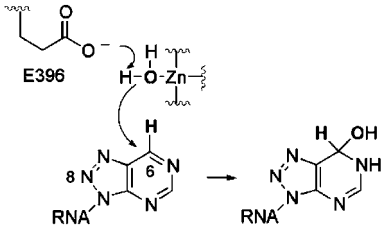


Figure 3. *Proposed mechanism for the reaction of ADAR2. A) Binding of ADAR2's dsRBMs to a dsRNA substrate relieves the deaminase domain of autoinhibition and allows the adenosine to flip into the active site. B) Deprotonation of a zinc-bound water is followed by attack of the hydroxide on the purine ring in the rate-limiting step of the reaction. Protonation at N1 leads to tetrahedral intermediate formation. Loss of ammonia from this intermediate generates the inosine product.*

with departure of ammonia from this intermediate would follow, yielding the inosine product.

In support of this mechanism, we have shown that 8-azanebularine (8-azapurine ribonucleoside), when incorporated at a known editing site in a model RNA substrate, binds tightly to ADAR2 and the deletion mutant lacking dsRBM I.[54] This binding requires a functional active site, consistent with the hypothesis that the high-affinity binding occurs to the covalent hydrate, which is an excellent mimic of the proposed reaction transition state (Scheme 1). The development of a tight-binding nucleotide analogue for mechanism-based trapping of an ADAR bound to RNA is distinctly advantageous. As stated earlier, dsRBM binding occurs without strict sequence requirements. Thus, dsRBM proteins typically bind multiple sites on a dsRNA ligand, and the complexes formed have similar stabilities; this complicates structure studies. Trapping the protein–RNA complex via a transition state analogue in the RNA reduces heterogeneity in solution and facilitates analysis of the structure. This general approach has seen application in the study of a number of other nucleic acid modifying enzymes.[55]



Scheme 1. *Mechanism-based trapping of an ADAR. 8-Azanebularine, which differs from substrate by the replacement of the amine leaving group with hydrogen and insertion of a nitrogen atom for C8, binds tightly to ADAR2 when present in a duplex RNA at a known editing site.[54] This binding requires E396 and is most likely in the form of the covalent hydrate.*

While the importance of ADAR RNA editing to nervous-system function is firmly established, important basic questions remain about these enzymes. For instance, how certain adenosines in an editing substrate are selectively deaminated continues to be an important and challenging question. Also, without a high-resolution structure, it remains unclear precisely how ADARs recognize the adenosine nucleotide and catalyze its deamination in an active site evolutionarily related to that of the cytidine deaminases. Furthermore, little information is available on regulation of ADAR editing activity or on their role in non-neural tissues.[56]

Dicer, Drosha, and Development

3.1. The RNase III family: Duplex-specific endoribonucleases

dsRBMs are also found in the RNase III family of ribonucleases.[3,57] Class I RNases III are the structurally simplest members of this group containing one dsRBM and one nuclease domain.[58] As expected from the presence of the dsRBM, all RNase III proteins are specific for duplex-RNA secondary structures. Class I RNases III, such as *E. coli* RNase III, are involved in the generation of mature, functional RNAs through the cleavage of duplex structures found in the initial transcripts.[59] Two other RNase III family members, Dicer and Drosha, have been implicated in duplex RNA-mediated regulation of gene expression (Figure 1 B).[60,61] Each of these multidomain enzymes has a single dsRBM at its C terminus. Both Dicer and Drosha also have two nuclease domains in the C-terminal third of their sequences. In addition to these functional domains, Dicer has a DExH helicase domain and a PAZ domain, the latter is believed to be important in the binding of short overhangs at the ends of RNA duplexes.[62] Drosha-like proteins are referred to as class II enzymes, whereas the more complex Dicers are class III RNases III.

Duplex RNA has been shown to regulate gene expression by inhibiting translation in a message-specific manner via two mechanistically distinct pathways (reviewed in ref. [63]). In the RNA interference (RNAi) pathway, long, perfectly matched duplex RNAs are cleaved into short interfering RNAs (siRNAs) approximately 21–24 bp in length that are incorporated into a multiprotein complex referred to as the RNA-induced silencing complex (RISC). Sequences found in these "triggers" guides RISC to selectively cleave mRNA with Watson–Crick complementarity, leading to destruction of the message. In a related pathway, short oligoribonucleotides (~22 nt) referred to as microRNAs (miRNAs) bind to sites of imperfect complementarity in mRNAs. The formation of such complexes leads to translation inhibition from that message, likely through the assembly of a noncleaving RISC at this site. A key distinction between the two pathways appears to be the extent of complementarity between either the siRNA antisense strand or miRNA and the target site in the mRNA. The RNase III family members Dicer and Drosha are involved in different steps in these pathways. Drosha intercepts the primary transcript encoding a miRNA (the primary miRNA or pri-miRNA) in the nucleus and cleaves it into a ~70 nt hairpin–stem structure referred to as the pre-miRNA (Figure 4).[61] The pre-miRNA is exported from the nucleus into the cytoplasm where Dicer cleaves it into the miRNA that ultimately causes translation suppression.[64] Dicer also cleaves exogenous, long, perfectly matched dsRNA in the cytoplasm into siRNAs that trigger degradation of specific messages via RISC (Figure 4).[65]

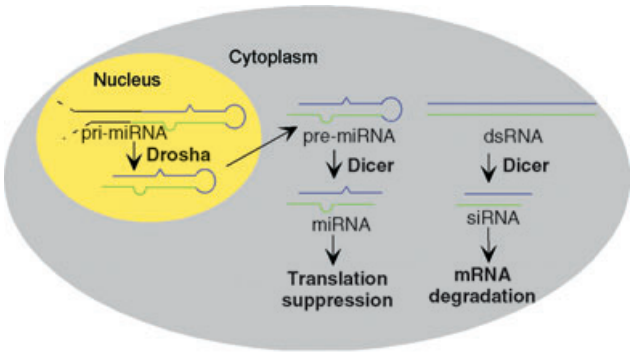


Figure 4. *The role of Dicer and Drosha in duplex RNA-mediated regulation of gene expression . Drosha intercepts the primary miRNA transcript (pri-miRNA) in the nucleus and cleaves it into a ~70 nt hairpin-stem. The Drosha product is exported into the cytoplasm where Dicer cleaves it into the miRNA. The miRNA causes suppression of the translation of mRNAs with imperfect Watson–Crick complementarity. Dicer also cleaves exogenous long dsRNA molecules into 21–24 nt siRNA triggers for the RNA interference pathway where complementary mRNAs are degraded in the RISC complex.*

3.2. The miRNA/siRNA pathway and development

The RNAi machinery in eukaryotic cells is routinely usurped by biologists to silence genes through the addition of synthetic siRNAs that mimic the Dicer products. These siRNAs are incorporated into the RISC and lead to the degradation of the target message. However, a natural function for this pathway is most likely the protection of the genome against invading nucleic acids, such as RNA viruses or transposons. In addition, there are clear links between the RNAi/miRNA regulation of gene expression and development. For instance, Dicer knockouts in both mice and zebrafish lead to severe developmental defects.[66,67] Indeed, developmental arrest in the zebrafish knockout correlated with a loss of production of miRNAs, consistent with the notion that a primary function for Dicer is in the production of miRNAs necessary in different organs at different stages of development.[66] This mirrors results obtained in *C. elegans* where specific miRNAs have been shown to be involved in the transition between specific developmental stages.[68] In addition, the *bantam* gene encodes a miRNA with a role in the control of cell proliferation and apoptosis during development in *D. melanogaster*.[69] Hundreds of different miRNA sequences have been identified and the vast majority have no known biological function. Thus, an important challenge for future work in this area is identifying targets of specific miRNAs and the effect miRNA regulation has on the function of those targets.

3.3. The RNase III reaction mechanism

Although no structures of RNase III enzymes have been reported with dsRNA bound, our understanding of the RNase III reaction mechanism is aided by the availability of a structure of the catalytic domain of *Aquifex aeolicus* RNase III, a class I enzyme.[70] The class I RNases III are homodimers with active sites at the dimer interface made up of conserved acidic residues.[70] As with all RNase III proteins, class I enzymes generate products with characteristic 2 nt 3′ overhangs with 5′-phosphate and 3′-hydroxy termini. Recent studies by Filipowicz and colleagues have shed additional light on the reaction mechanism of Dicer, a class III RNase III.[71] This enzyme appears to function as a monomer with a single dsRNA-processing center made from residues found in each of the two nuclease domains of the protein. Thus, although the active enzyme is a monomer, one can consider the active site forming through the association of the two nuclease domains in a pseudodimer. Each nuclease domain has a catalytic site that is responsible for the cleavage of one strand of the duplex. The relative positions of the two catalytic sites in the pseudodimer are thought to generate products with 2 nt 3′ overhangs. Interestingly, Dicer preferentially cleaves duplexes with 3′ protruding ends in a stepwise fashion releasing ~20 bp fragments from the end of a dsRNA substrate. It appears the Dicer PAZ domain recognizes the 3′ overhang at the duplex end and the single dsRBM gives the enzyme general dsRNA-binding affinity.[71] The preference Dicer has for substrates bearing the 3′ overhang is consistent with its role in the processing of pre-miRNAs generated by the cleavage of pri-mRNAs by Drosha, another RNase III.[61]

Dicer is reported to be an inefficient catalyst.[72] Part of the reason for this is the enzyme's high affinity for the ~20 bp duplex product of the reaction. Recent studies suggest that Dicer accompanies an siRNA trigger it generates into the RISC complex.[73] It is tempting to speculate that tight binding to its

reaction product prevents the duplex RNAs generated by Dicer from interacting with other dsRNA-binding proteins in the cell (see Section 4).

Our understanding of the function and mechanism of the RNase III family members Dicer and Drosha has proceeded rapidly in the last few years. It will be important for these studies to continue to include analysis of the structures of these proteins and their complexes with RNA. Furthermore, defining the effect RNA structure has on the efficiency and selectivity of each enzyme will be critical, particularly as it relates to miRNA biogenesis. In addition, methods for controlling the function of specific endogenous miRNAs will be necessary to fully define the function of the Drosha/Dicer miRNA products in development. It is interesting to note in this regard that 2′-*O*-methyl oligoribonucleotides injected into *C. elegans* larvae have recently been shown to inhibit miRNA function sequence specifically.[74] Small molecules capable of binding to specific pre-miRNAs should also prove valuable in this studies.[75]

PKR: An Antiviral Protein Kinase

4.1. PKR is an RNA-regulated eIF2α kinase

The RNA-dependent protein kinase (PKR) was originally identified as a protein that causes translation inhibition in reticulocyte lysates treated with RNA.[76] It is now known that this effect comes from the PKR's ability to phosphorylate and inhibit a key translation-initiation factor (eIF2) in response to double-stranded RNA (dsRNA).[77] Indeed, phosphorylation of eIF2 on its alpha subunit is a general mechanism eukaryotic cells use to control translation in response to various environmental stresses.[78] eIF2 normally binds GTP, and the eIF2·GTP complex mediates the association of Met-tRNA with ribosomal 40S subunits. eIF2 is released from the 40S subunit when GTP is hydrolyzed to GDP. Phosphorylation of eIF2α blocks binding of the guanine nucleotide–exchange factor necessary for the GTP-for-GDP exchange, locking eIF2 in the GDP-bound form. Thus, the phosphorylation event prohibits turnover of eIF2 and blocks further initiation of protein synthesis. Four different eIF2α kinases have been discovered; each activated by a different cellular stress (PKR: viral infection;[79] PERK: unfolded proteins;[80] GCN2: amino acid deprivation;[78] HRI: heme deficiency).[81] All of these protein kinases have structurally similar kinase domains with regulatory domains corresponding to the unique activation mechanism of each. PKR has an N-terminal RNA-binding domain selective for dsRNA, which is an indicator of viral infection in higher eukaryotes.[82]

4.2. PKR is a component of the interferon antiviral response and is an intracellular signal transducer

Viral infection induces transcription of interferons α, β, and γ. These interferons, in turn, induce the transcription of a number of genes including PKR.[82] However, PKR is synthesized in a latent, inactive form that requires association with RNA to be activated. In vitro, PKR is activated by binding to dsRNA molecules of greater than approximately 20 bp.[83,84] In vivo, PKR is believed to be activated by viral replicative intermediates that build up in the cell during infection. This activation manifests itself initially by autophosphorylation. Once autophosphorylated, the enzyme can phosphorylate eIF2α.[85] Viruses pathogenic to eukaryotic cells have evolved mechanisms by which they circumvent the activity of PKR.[86,82,87] For instance, adenovirus and Epstein–Barr virus synthesize highly structured RNAs designated VA and EBER, respectively.[88,89] These RNAs bind PKR and block its activation, allowing continued translation in the cell. Other viruses, such as influenza and vaccinia, synthesize protein inhibitors of PKR.[82]

Several studies suggest that PKR plays a role in the normal maintenance of cell-growth control in the absence of viral infection.[90–92] PKR activity also induces differentiation in myogenic cells, and expression of a dominant negative PKR mutant interferes with the differentiation program.[93] Thus, PKR plays a role in the exit of muscle cells from the cell cycle during differentiation. Furthermore, overexpression of PKR induces apoptosis in several systems.[94,95] Interestingly, overexpression of Bcl-2 protects cells from PKR-induced apoptosis, suggesting that Bcl-2 is downstream of PKR in these apoptotic pathways.[96] PKR is involved in other signal-transduction pathways that control transcription. For instance, PKR activates the transcription factors NFκB and IRF-1, whereas it leads to an inhibition of the activity of STAT1.[97–99] Currently it is not known whether PKR's effects on cell growth, differentiation, apoptosis, and transcription are all mediated by phosphorylation of eIF2α or if PKR can directly affect these pathways through phosphorylation of other substrates. Evidence for PKR's direct phosphorylation of the NFκB inhibitor, IκB, has been presented.[97] PKR can also phosphorylate the transcription regulator NF-90.[100]

4.3. The structure of PKR and its RNA-binding properties

The 68 kDa human PKR consists of a 20 kDa N-terminal RNA-binding domain (RBD) and a C-terminal kinase domain (Figure 1 B).[101] Currently, no high-resolution structure of the full-length PKR molecule exists neither are any high-resolution structural data available on a PKR·RNA complex. The PKR RBD is composed of two dsRBMs linked by a stretch of approximately 20 amino acids. The lack of structural data on PKR·RNA complexes is undoubtedly related to the ability of the dsRBM-containing RNA-binding domain to bind most dsRNA ligands at multiple sites. This creates a heterogeneous mixture of complexes in solution when PKR binds RNA. Covalent trapping of a PKR·RNA complex might be necessary to achieve the homogeneity necessary for high resolution structure studies.[55] The solution structure of the PKR RBD was solved by NMR spectroscopy and shows that both dsRBMs have the characteristic fold of the dsRBM family.[6]

The binding of PKR has been shown to be selective on certain dsRNAs.[13,10,11] Indeed, our laboratory has demonstrated that the RBD of PKR binds the 167 nt Epstein–Barr virus-encoded RNA 1 (EBER1) at two places in its stem-loop IV.[11] Furthermore, we found that a 37 nt RNA could be generated that recapitulated the binding observed on the full-length transcript. We generated a molecular model of the complex formed be-

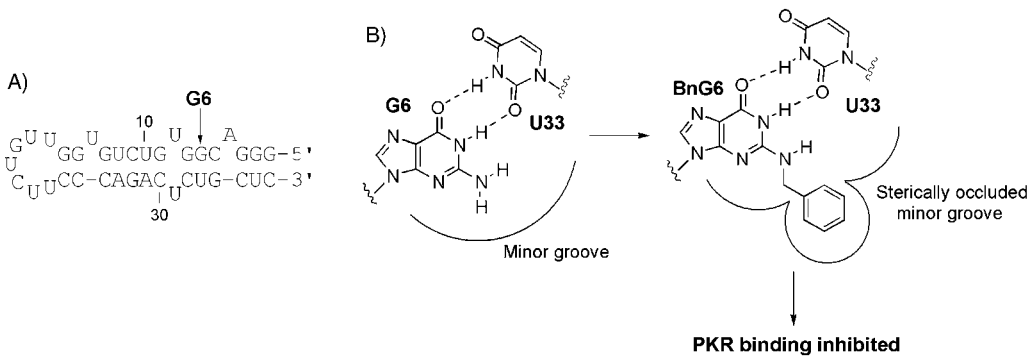


Figure 5. *Controlling dsRBM binding by steric occlusion of the dsRNA minor groove. A) Stem-loop IV from Epstein–Barr virus-encoded RNA 1 (EBER1) is a binding site for PKRs dsRBM I.*[11] *B) Introducing N^2-benzylguanosine at specific locations in this RNA blocks PKR binding.*[102]

tween PKR and EBER1 which predicted important contact surfaces on the RNA for interaction with dsRBM I. This model was then tested by substituting specific nucleotides in the RNA predicted to be near the protein contact site with N^2-benzylguanosine.[102] Introducing a benzyl group at specific purine N^2-positions blocks access to the minor groove at these locations (Figure 5). This experimental approach allowed us to confirm the importance of minor-groove contact sites for PKR binding on the EBER1 RNA.

4.4. The mechanism of RNA-regulation of PKR kinase activity

The dsRBMs play a key role in PKR regulation as both kinase-activating and -inhibiting RNAs bind PKR through these motifs. Previous studies of PKR's RBD have provided some insight into how the two dsRBMs interact with RNA ligands. It has been shown that dsRBM I has a higher affinity for dsRNA than does dsRBM II.[103] However, both motifs are required for maximal dsRNA binding by PKR's RBD, suggesting cooperativity between the two motifs.[15,104] Furthermore, binding of RNA to PKR's RBD induces an activating conformational change in the kinase domain of the enzyme.[83,85,105] Structural studies led to the suggestion that dsRBM II of PKR is an autoinhibitory domain, masking the kinase domain in the inactive conformation.[16,106] It was suggested that an RNA ligand capable of kinase activation could bind cooperatively to both dsRBMs, relieving the kinase domain of its interaction with dsRBM II. Therefore, an inhibiting RNA ligand might interact with PKR in a distinct manner that does not allow for relief of autoinhibition by dsRBM II. Interestingly, although only ~16 bp of duplex RNA is required for binding to PKR, activation of the enzyme requires a longer duplex region, consistent with the idea that an activation event requires the simultaneous binding of both dsRBMs.[107] A requirement for simultaneous contact to both dsRBMs could ensure that PKR is only activated by long stretches of dsRNA, which would be relatively rare in the absence of viral infection. Our results with EDTA·Fe-modified PKR proteins, and various kinase-activating and inhibiting RNAs are consistent with this activation mechanism.[10,11]

It has also been clearly established that dimerization is required for PKR activation.[108] Indeed, heterologous dimerization domains have been shown to functionally replace the first 258 amino acids of PKR, a region that includes the RBD.[108] The importance of dimerization in the PKR activation mechanism was further substantiated in this study by using chemical inducers

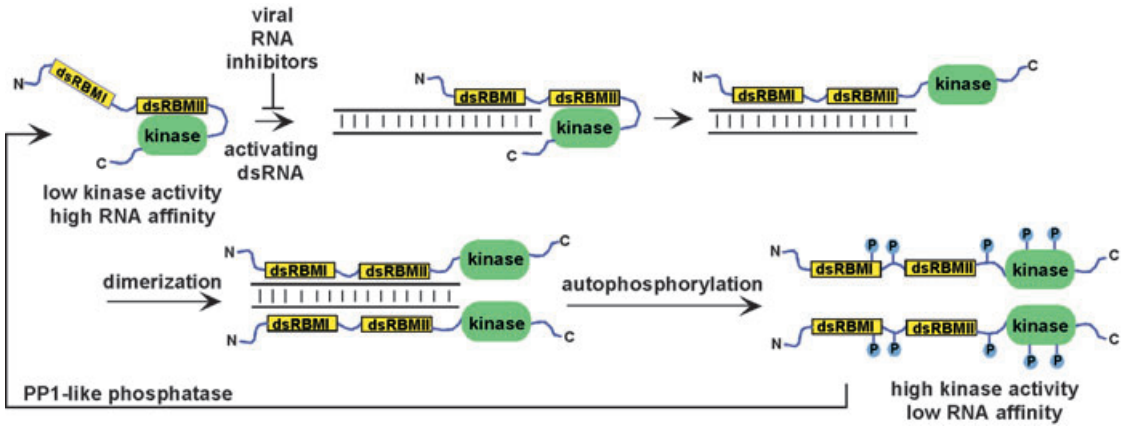


Figure 6. *Model for the activation of PKR by dsRNA. Simultaneous binding of both of PKR's dsRBMs to an RNA activator relieves the kinase domain of autoinhibition and reveals sites for dimerization. Viral RNAs that bind tightly only to PKR dsRBM I can prevent this step. Autophosphorylation generates a form of the protein with high protein-kinase activity and low RNA affinity. Dephosphorylation with a PP1-like phosphatase inactivates the kinase and restores RNA affinity.*

of dimerization (CIDs) to bring PKR kinase domain monomers together.[108,109] For native PKR, the binding of an activating RNA ligand in the RBD is thought to unmask dimerization sites leading to PKR self association and intermolecular autophosphorylation between active monomers in the dimer.[16] Thus, an additional mechanism by which a viral inhibitor of PKR might function is to prevent productive dimerization.

Autophosphorylation occurs throughout the PKR molecule, including sites in the kinase domain, the RBD, and in the linker between the two.[110,111] We have shown that autophosphorylated human PKR isolated from an *E. coli* expression system is fully activated and binds RNA poorly.[112] However, treatment with the catalytic subunit of protein phosphatase 1 efficiently dephosphorylates the enzyme, generating a form that has low kinase activity, binds tightly to RNA, and can be activated by RNA in vitro. A summary of the current model for PKR activation by RNA is shown in Figure 6.

4.5. PKR and RNA interference

Interestingly, the failure to induce a specific RNAi effect in eukaryotic cells with long dsRNA molecules can be attributed, at least in part, to the activation of PKR and the resulting nonspecific inhibition in translation.[113] To overcome this obstacle, siRNAs have been used that mimic the Dicer products of the natural pathway.[114] These 19 bp duplex RNAs with two nucleotide 3′ overhangs were thought at the time to be too short to activate PKR. However, recent studies indicate that siRNAs can activate PKR both in vitro and in vivo (our unpublished results).[84] Indeed, there are several conflicting reports in the current literature on the extent to which activation of PKR specifically, or other components of the dsRNA-induced antiviral pathways in general, are the cause of off-target effects observed in RNAi experiments with mammalian cells.[84,115,116] It is interesting to note in this regard that Invitrogen, Inc. (Carlsbad, California, USA) offers a line of "Stealth" siRNA reagents that are claimed to eliminate the nonspecific stress response of the PKR/interferon pathways that can be induced by traditional siRNA. Defining the effect siRNAs have on dsRNA-binding proteins other than those involved in the RNAi/miRNA pathways and developing ways to avoid these effects will clearly be important for the future development of RNAi as a research tool and therapeutic approach.

4.6. Reagents available for studying PKR signaling

To test the importance of PKR in antiviral, antitumor, and apoptotic pathways at the whole-organism level and to generate PKR-null cell lines for further investigation of PKR's role in cell signaling, two PKR-null mice have been generated.[117,118] However, analysis of the antiviral response, susceptibility to tumors, and tumor necrosis factor X α-induced apoptosis in cells from these mice gave conflicting results regarding the role of PKR in these pathways in vivo. Interestingly, in neither case is the mouse truly a PKR-null organism since each expresses fragments of the protein displaying different activities.[119] These results highlight the need for additional approaches to modulate PKR activity in intact cells or animal models for the purpose of studying PKR signaling.

Small molecule inhibitors of protein kinases are powerful tools for studying their role in cell signaling pathways.[120] Indeed, 2-aminopurine (2-AP) has been used at millimolar concentrations to inhibit PKR's kinase activity in intact cells (Figure 7 A).[121] Although 2-AP does show some selectivity in its

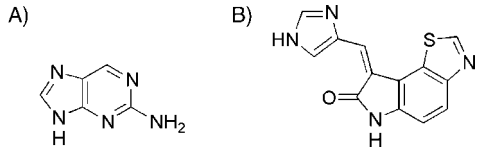


Figure 7. *Small molecule inhibitors of PKR activity. A) 2-Aminopurine.[121] B) An oxoindole PKR inhibitor discovered in a library screen.[124]*

ability to inhibit protein kinases, it is not completely specific to PKR.[122] MAP kinases, for instance, are also inhibited by 2-AP at these high concentrations.[123] In an effort to discover new small molecule inhibitors of PKR, our laboratory screened a library of ligands for the ATP-binding site of protein kinases generously provided to us by GlaxoSmithKline.[124] We observed that various oxoindoles inhibit PKR at submicromolar concentrations and, in certain cases, are selective enough to rescue translation in rabbit reticulocyte lysates from the block imposed by PKR activation (Figure 7 B). Thus, in addition to 2-aminopurine, the oxoindole scaffold might provide the basis for the development of new, selective small-molecule inhibitors of PKR. These will be useful reagents for the study of PKR signaling and could find application in blocking PKR activity induced by siRNAs.[84]

Prospects

The significance of dsRNA-binding enzymes is highlighted by the role PKR plays in the interferon antiviral response and signal transduction, the effect ADARs have on the function of the nervous system, and the activity of Dicer and Drosha in miRNA biogenesis and development. Chemical biologists will continue to contribute to our understanding of these important proteins with new ways to study their structures and mechanisms, as well as provide methods to control their activity both in vitro and in vivo.

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Keywords: deaminases · protein kinases · proteins · ribonucleases · RNA

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