Breaking the Central Dogma by RNA Editing

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1. Introduction

RNA editing is a term associated with structural changes in an RNA strand that alter its coding properties. These enzyme-catalyzed reactions include nucleotide and oligonucleotide insertions and deletions as well as base modifications. The diversity of coding strands created by these reactions contributes to the protein diversity present in cells of higher organisms. In this review, we highlight advances in our understanding of the structure, mechanism, regulation, and biological functions of the ADAR enzymes published in the last five years. The ADARs (adenosine deaminases that act on RNA) are multidomain enzymes capable of converting adenosine to inosine at specific locations in certain RNA substrates. These reactions can change codon meaning in mRNA and lead to changes in the structures of proteins, including ligand-gated and voltage-gated ion channels and G-protein coupled receptors expressed in the central nervous system.

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2. RNA Editing by Adenosine Deamination and Its Consequences in Vivo

The term RNA editing refers to a variety of modification reactions that change the coding properties of RNA molecules from that encoded in their corresponding gene sequences.[1] In this way, editing is similar to splicing, since both are post-transcriptional modifications of an RNA molecule that alter its information content. However, in contrast to splicing reactions, which involve the removal of large fragments from a message and potentially entire domains from an encoded protein, the editing reactions are more subtle, with only one (or a few) nucleotides changed. This can happen by insertion or deletion reactions or by base modifications. Deamination at C6 of adenosine (A) and C4 of cytidine (C) in RNA are examples of base modification RNA editing. Deamination of adenosine generates inosine (I) at the corresponding nucleotide position in RNA. Since inosine is decoded as guanosine (G) during translation, this reaction can lead to codon changes and the introduction of amino acids into a gene product not encoded in the gene (Figure 1).[2,3]

This process is unique to organisms with developed tissue systems (metazoa) and appears to be important in generating the protein structural diversity necessary in these complex life forms, particularly in their central nervous systems. Many of the targets of the A to I modification reaction are mRNAs that encode proteins important for nervous system function, such as ligand-gated and voltage-gated ion channels and G-protein coupled neurotransmitter receptors.[2−5] In several cases, editing of the message has a clear effect on the function of the encoded protein. Editing of the message for the 5-HT_{2C} subtype of serotonin receptor is an example. The editing occurs at five adenosines (A−E) clustered in exon 3 of the 5-HT_{2C}R pre-mRNA in a duplex structure predicted to form with nucleotides present in the adjacent intron 3 (Table 1).[2,6] The A to I conversions change codons for three different amino acids present in the second intracellular loop of the receptor. These structural changes affect the ability of the receptor to transmit signal to the intracellular signal transduction machinery by changing the nature of the surface that interacts with G proteins.[2]

In another example, editing of the pre-mRNA for the B subunit of the glutamate-gated ion channel (GluR-B) causes

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Peter Beal was born in Oxnard, California, and moved to Bismarck, North Dakota, at a young age. There he graduated high school and attended the University of North Dakota. As an undergraduate at UND, he worked with Prof. Donald Bergstrom developing new methods for the synthesis of nucleic acid components. After graduation in 1989, Beal began graduate research in Peter Dervan's lab at the California Institute of Technology where he studied triple helix formation with purine-rich oligonucleotides. He carried out postdoctoral research on the mammalian target of rapamycin (mTOR) with Stuart Schreiber at Harvard University. In 1996, Beal began his independent research career in the Department of Chemistry at the University of Utah where he now holds the rank of Professor. The Beal lab in Utah has an continuing interest in molecules that bind RNA, including proteins involved in RNA editing, RNA interference, and the innate immune response, as well as chemically synthesized small molecules capable of structure-specific binding to RNA. Beal enjoys hiking, skiing, and fly fishing in the mountains of Utah and time spent with his wife Sheila David and two children, Surina and Maya.

codon changes at two different sites (Table 1).[3,7] The Q/R site is so named because a glutamine codon is altered at that site 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 permeability of the channel. Glutamate receptors assembled with GluR-B subunits translated from edited mRNA have low Ca^{2+} ion permeability because the Q/R editing reaction causes the charged arginine residue to be positioned

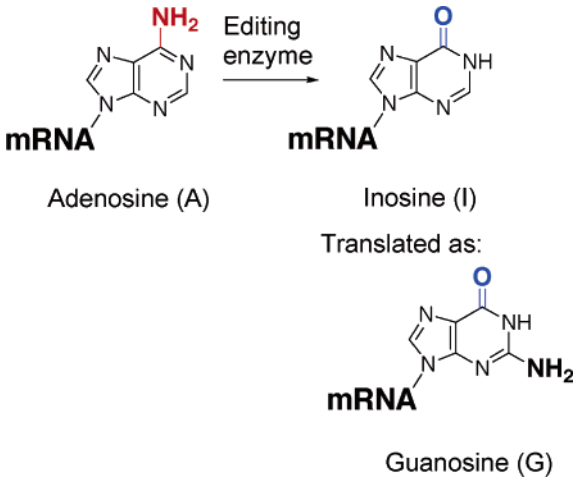


Figure 1. RNA editing by adenosine deamination changes the information encoded in mRNA.

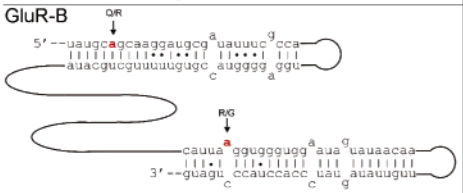
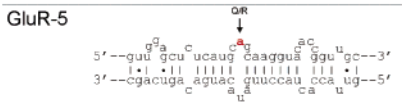
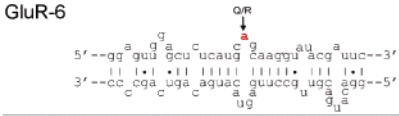
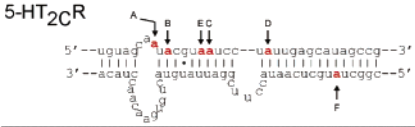
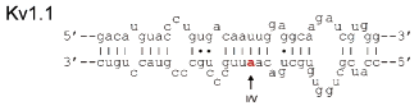
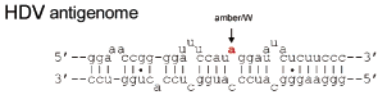
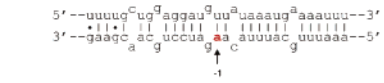
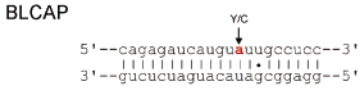
in a critical transmembrane location.[8] Editing at the R/G site affects the rate at which the receptor recovers from ligand desensitization.[7] Other glutamate receptor subunit mRNAs also undergo editing reactions that change codons. For instance, both GluR-5 and GluR-6 pre-mRNAs form duplex structures that guide Q/R codon changes that affect ion permeability of the resulting channel (Table 1).[9,10]

Additional nervous system targets of A to I RNA editing have been identified.[4] In a recent report, Reenan and colleagues showed that A to I editing of the mRNA encoding the human potassium channel Kv1.1 results in an isoleucine to valine mutation that changes channel inactivation kinetics.[5] Interestingly, the observed effect on channel function is similar to those that arise from certain mutations in the human Kv1.1 gene associated with episodic ataxia type 1 (EA1), a neurological disorder characterized by attacks of generalized ataxia brought on by physical or emotional stress.[11] Indeed, two mutations that lead to EA1 are found in the critical hairpin loop structure present in the hKv1.1 mRNA required for A to I editing, suggesting a possible link between mutation at these sites and RNA editing.[5,11]

Viruses generate RNAs that are targets for A to I RNA editing as well. For instance, the hepatitis delta virus antigenomic RNA is edited at an amber stop codon (UAG), and the resulting sequence (UIG) is read through as a tryptophan codon.[12] The virus uses this process to generate two forms of its protein antigen, the shorter form having a role in replication and the longer form involved in packaging of the viral particle.[13,14] Recently, the Tuschel group reported a microRNA from Kaposi sarcoma-associated virus that is edited at the pre-miRNA stage when the RNA has significant duplex secondary structure.[15] Other viral targets of A to I RNA editing have been reported.[16−18] In addition, several other editing sites have been described in mammalian mRNAs that change the structure of the encoded proteins, including in the mRNA for an editing enzyme (ADAR2) (Table 1).[19,20]

RNA editing by adenosine deamination also occurs readily in noncoding introns and 3′ untranslated regions of mRNAs. Indeed, recent studies have shown that the vast majority of A to I conversion reactions occur in these noncoding regions and, therefore, do not cause codon changes.[21−24] Although the function of "editing" in noncoding regions is not fully understood, it may be important for modulating duplex RNA structure, which could affect subsequent RNA processing events such as those involving microRNAs (see section 4).

Table 1. Predicted Secondary Structures of Substrates for Mammalian ADARs Where Codon Changes Occur[a]

Editing substrate	Protein product	Editing enzyme	Effect
GluR-B 	glutatmate receptor B subunit	*ADAR1* R/G *ADAR2* Q/R + R/G	Q/R ion permeability of channel R/G ligand desensitization recovery rate
GluR-5 	glutatmate receptor subunit 5	*ADAR2*	ion permeability of channel
GluR-6 	glutatmate receptor subunit 6	*ADAR2*	ion permeability of channel
5-HT$_{2C}$R 	serotonin receptor 2C subtype	*ADAR1* A,B,C *ADAR2* D,E,F	G protein interactions
Kv1.1 	Kv1.1 potassium ion channel	*ADAR2*	channel inactivation kinetics
HDV antigenome 	hepatitis delta virus antigen	*ADAR1*	expression of large or small antigen
ADAR2 	ADAR2	*ADAR2*	expression of inactive splice variant
BLCAP 	bladder cancer associated protein	unknown	unknown

[a] Data for GluR-B taken from refs 3 and 7, for GluR-5 and GluR-6 from refs 9 and 10, for 5-HT$_{2C}$R from refs 2 and 6, for Kv1.1 from ref 5, for HDV antigenome from refs 1 and 12, for ADAR2 from ref 19, and for BLCAP from ref 20.

Consistent with the observation of nervous system targets for RNA editing, this process appears to be required for a properly functioning nervous system in metazoa.[25−28] For instance, deletion of the gene encoding an enzyme responsible for A to I editing in *Drosophila melanogaster* leads to a morphologically normal fly with dramatic behavioral defects, such as tremors, uncoordinated locomotion, and an inability to fly or jump.[27] Likewise, *Caenorhabditis elegans* containing similar gene deletions shows defects in behaviors such as chemotaxis.[28] Thus, it appears the protein structural diversity that arises through editing of mRNA by adenosine deamination is used in the nervous system to produce complex behavior. Deletions of the A to I editing enzymes in mice are lethal, indicating that this process has become essential for mammals.[25,26] The study of the effect that changes in levels of RNA editing have on humans has only recently been initiated and appears to link abnormal editing to psychiatric disorders.[29−31] Indeed, altered editing levels in nervous system target mRNAs may be related to severe depression side effects experienced by some patients undergoing interferon treatment, since one of the enzymes responsible for these editing reactions is interferon-induced (see below).[6]

3. ADARs: Enzymes Responsible for Adenosine to Inosine RNA Editing

Two different mammalian enzymes have been shown to carry out A to I editing in vitro in the absence of additional proteins or RNAs. These enzymes have been given the name ADAR for adenosine deaminase that acts on RNA (Figure 2).[32] A related family of RNA-modifying enzymes that catalyze adenosine deamination in tRNAs is referred to as ADATs (adenosine deaminase that acts on tRNA).[33] The first ADAR to be discovered (ADAR1) was originally identified as a duplex RNA unwinding enzyme in *Xenopus* embryos.[34] It was later shown that the unwinding activity of this enzyme arose from its ability to deaminate adenosine at C6 in base-paired RNAs.[35] This reaction creates an I:U mismatch, which

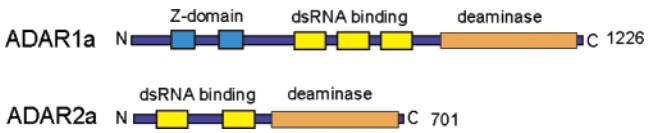


Figure 2. Domain maps of ADAR1a and ADAR2a.[36,39] Yellow boxes indicate approximate locations of dsRBMs in each protein. Blue indicates Z-alpha and Z-beta domains in ADAR1. Orange boxes refer to the CDA-like deaminase domains.

destabilizes the double helical structure and, thus, "unwinds" the duplex. The gene sequence for ADAR1 has been cloned, and the enzyme has been overexpressed.[36] We now know that ADAR1 is expressed in two forms in human cells, a long form (p150) that is interferon-induced and found in the nucleus and cytoplasm and a short form (p110) that is found exclusively in the cell's nucleus.[37] In addition to these two ADAR1 forms, several splice variants have been identified with the pattern of the original ADAR1 cDNA clone referred to as ADAR1a (Figure 2).[36,38] ADAR1 has been shown to efficiently modify the 5-HT_{2C}R A, B, and C sites, the GluR-B R/G site, and the hepatitis delta virus amber/W site, among others (Table 1).[6,39−41] Interestingly, the long form of ADAR1 is believed to play an antiviral role in the cell by nonselectively deaminating viral duplex RNAs found in the cytoplasm.[37] ADAR1 also has an essential function in mammals beyond the nervous system.[26] This function was demonstrated by death of the *ADAR1* null mouse between embryonic days 11 and 12.[26] This phenotype included a rapidly disintegrating liver structure, severe defects in hematopoiesis, and widespread apoptosis, suggesting that editing of some unknown RNA substrate by ADAR1 protects embryos from apoptosis during development.[26]

The failure of in vitro deamination assays with ADAR1 and editing site RNAs that were known to be processed in vivo prompted the search for new RNA-editing adenosine deaminases.[39] This led to the discovery of ADAR2, which deaminates the GluR Q/R and R/G sites, the 5-HT_{2C}R C, D, and E sites, the Kv1.1 I/V site, and sites in its own mRNA, including one that changes the splicing pattern.[5,6,19,39,40] ADAR2 is a smaller protein than ADAR1 (∼80 kDa) with a different N-terminal domain structure.[39] Like ADAR1, multiple splice variants of ADAR2 have been described with the original ADAR2 cDNA clone referred to as ADAR2a.[19,39] Deletion of the *ADAR2* gene in mice is lethal with homozygotes dying between postnatal day 0 and 20.[25] Consistent with an important role for ADAR2 in the nervous system, *ADAR2* null mice become progressively seizure prone after postnatal day 12.[25]

ADAR1 and -2 are expressed in several different tissues, whereas a related protein referred to as ADAR3 is expressed exclusively in the brain.[42] To date, no editing substrate has been identified for ADAR3. Interestingly, unlike ADAR1 and -2, ADAR3 can bind single-stranded RNA in addition to dsRNA, which suggests an ability to target a unique, yet unknown, class of RNA substrates.[43]

3.1. Structure

Cloning and sequencing of ADAR genes allowed for the identification of likely functional domains within the expressed proteins.[36,39] Indeed, the ADARs are modular in their makeup with multiple independently folded domains that work in concert to achieve efficient and selective RNA editing (Figure 2). RNA binding is controlled by sequence motifs (double-stranded RNA-binding motifs, dsRBMs) present in multiple copies in both ADAR1 and ADAR2.[44] The C-terminal domain of each protein harbors the deaminase domain with the catalytic machinery necessary to convert adenosine to inosine. In addition, ADAR1 has an N-terminal Z-domain similar to other known Z-DNA binding domains.[45] Indeed, the Z-alpha subdomain of ADAR1 has Z-DNA and Z-RNA binding activity.[46] Below we describe in more detail the ADAR functional domains.

3.1.1. Double-Stranded RNA-Binding Motifs (dsRBMs)

Found in the primary structure of the ADARs are multiple copies of double-stranded RNA-binding motifs (dsRBMs), also referred to as double-stranded RNA-binding domains (dsRBDs).[44] ADAR1 has three copies of this motif, whereas ADAR2 and ADAR3 have two copies. The dsRBM is a ∼65 amino acid sequence motif found in many double-stranded RNA-binding proteins, such as PKR, the RNA-dependent protein kinase, and Dicer, a ribonuclease involved in the RNA interference pathway.[44] dsRBMs have been structurally characterized in a number of cases, typically revealing a characteristic $\alpha-\beta-\beta-\beta-\alpha$ fold for the motif.[47−50] There are several high-resolution structures of dsRBMs bound to RNA targets (Figure 3).[51−54] The most striking feature of these structures is their similarity, despite the fact that the dsRBMs were derived from different proteins and the RNAs differ in their sequence and structure. In a crystal structure of the second dsRBM of *Xenopus laevis* RNA-binding protein A (Xlrbpa), the dsRBM spans two minor grooves and the intervening major groove at a binding site made up of 16 base pairs (Figure 3 (left)).[51] Protein contacts are primarily at RNA 2′-hydroxyls and phosphodiesters, with only a few base contacts observed in the minor grooves. This explains the known length requirement and lack of a strict sequence requirement for binding for members of the dsRBM protein family. The amino acids that make contact with the RNA are located in three distinct locations in the dsRBM (Figure 3 (bottom)). Residues found in α1 and residues in loop 2 between β1 and β2 contact different minor grooves on one face of the RNA duplex. Residues found in loop 4 between β3 and α2 and some α2 residues are involved in phosphodiester contacts at the opening of the major groove between the two minor groove contact points on the same helical face. The second example is a structure of the dsRBM from yeast RNase III (Rnt1p) in complex with an RNA hairpin determined by NMR (Figure 3 (right)).[54] In this structure, the dsRBM is positioned across one minor and one major groove in the same manner seen in the Xlrbpa−RNA structure; the third region of contact occurs in the loop region of the RNA by α helix 1 (α1) of the protein. This structure also revealed a third C-terminal helix in this motif. The highly resolved structures available are valuable because the binding modes observed can be used to create models for protein−RNA complexes formed by other members of the dsRBM superfamily, including ADARs. In support of this notion, the recent report of the structures of the two dsRBMs from ADAR2 indicate that the canonical $\alpha-\beta-\beta-\beta-\alpha$ topology is maintained for both motifs.[50] Furthermore, chemical shift perturbation studies with added RNA showed that the RNA recognition surfaces of ADAR2's dsRBMs are similar to those observed for other dsRBM proteins.[50]

The presence of dsRBMs in the structures of the ADARs is consistent with the requirement for double-stranded RNA secondary structure of a defined length in the RNA editing substrates.[34] In addition, as would be predicted by the properties of dsRBMs, ADARs bind duplex RNA in a largely sequence-independent fashion, although they deaminate certain substrates with high selectivity for specific adenosines. Our laboratory and others have shown that, although dsRBMs do not require specific sequences of duplex RNA for binding, certain sites on an RNA ligand can be selectively occupied.[50,55−57] The importance of these binding site preferences in determining editing site selectivity has only recently

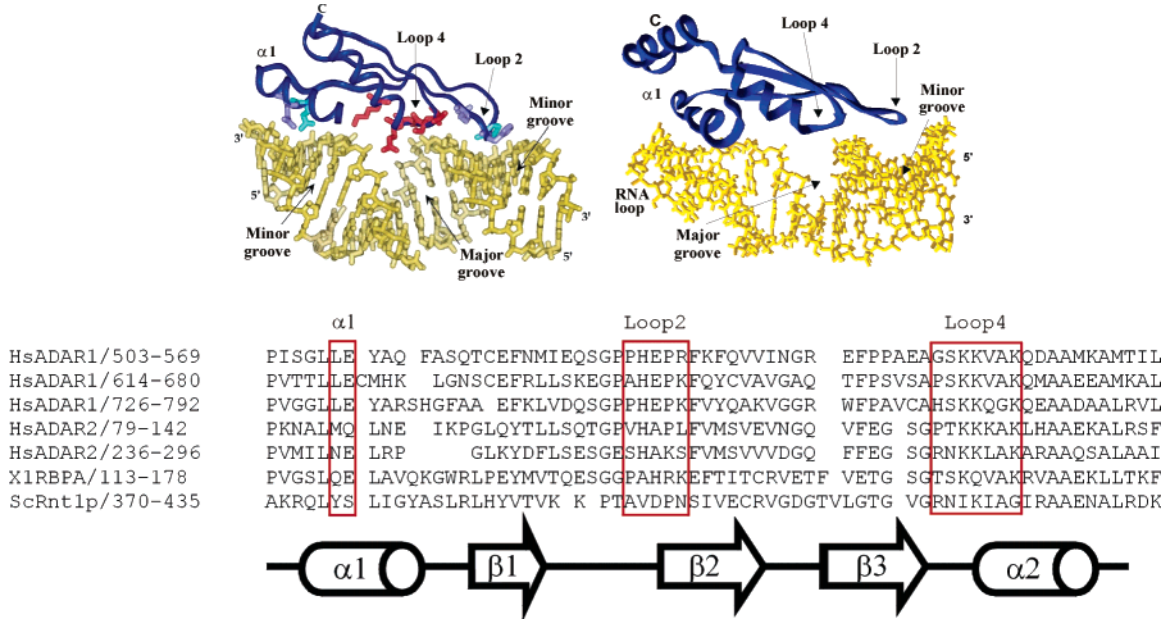


Figure 3. dsRBM−RNA interactions in (top left) the structure of dsRBM III from Xlrbpa bound to duplex RNA[51] and (top right) the structure of dsRBM from yeast RNase III bound to a hairpin stem RNA[54] and (bottom) the sequence of ADAR dsRBMs along with Xlrbpa dsRBM III and yeast RNAse III (Rnt1p) dsRBM with secondary structures indicated.

been explored (see section 3.2.1). These studies indicate that changes in RNA structure at preferred dsRBM binding sites change editing efficiency. Thus, the dsRBMs influence editing selectivity by requiring duplex secondary structure of a certain length and selectively occupying binding sites on the duplex, undoubtedly affecting positioning of deaminase domain over the edited adenosine. ADAR1a has three closely spaced dsRBMs each separated from the other by only ~20 amino acids. ADAR2a has two dsRBMs separated by a significantly larger linker region (~70 amino acids). Interestingly, this region has a proline-rich sequence reminiscent of SH3 domain ligands and multiple sites predicted to be substrates for serine/threonine protein kinases leading us to speculate that this part of ADAR2 may be important in regulation of editing activity.[58−60] How the dsRBMs work together in an ADAR to control editing site selectivity and the role, if any, of the inter-dsRBM linker structures remains to be fully defined.

3.1.2. Z-Domain (ADAR1)

A structural feature that distinguishes ADAR1 from ADAR2 and ADAR3 is a unique nucleic acid binding domain at the N-terminus of the long form (p150). This domain has the ability to bind Z-DNA and Z-RNA both in vitro and in vivo.[46] Two subdomains (Z-α and Z-β) comprise the Z-binding domain of ADAR1 with Z-α having demonstrated Z-DNA and Z-RNA binding activity on its own.[45,61,62] The structures of both of these domains have been solved by crystallography, including Z-α in complex with a short Z-DNA duplex (Figure 4).[45,61] Recent studies indicate that the presence of the Z domain influences editing site selectivity of the ADAR1 long form with preferential editing of duplex RNA substrates containing a 5′-$(CG)_6$-3′ repeat capable of existing in the Z-conformation.[46] How the presence or absence of RNA sequence capable of existing in the Z-conformation influences editing by ADAR1 on natural substrates has not yet been reported. Given the fact that the ADAR1 long form is interferon-induced and cytoplasmic, it is tempting to speculate that the Z domain directs the enzyme to deaminate viral RNA substrates that can adopt a Z-like conformation.

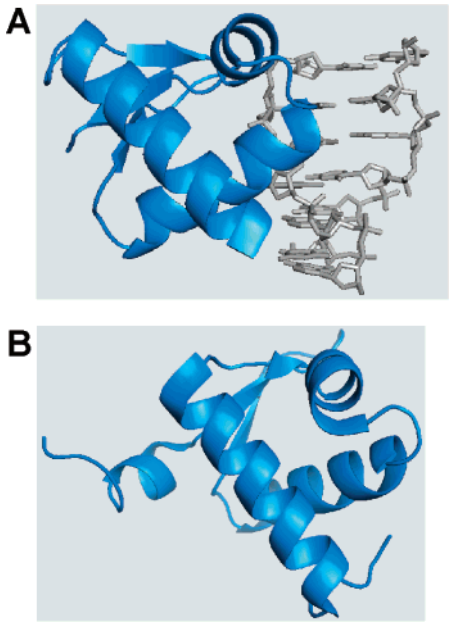


Figure 4. Z-domain of ADAR1: (A) Z-alpha domain (residues 134−198) bound to a 6 bp Z-DNA duplex;[45] (B) Z-beta domain (residues 295−368).[61]

3.1.3. Deaminase Domain

Each ADAR has a region near the C-terminus with sequence similarity to the cytidine deaminases (CDA).[33] Conserved residues in the cytidine deaminase family involved in formation of the active site are also found in ADARs.[33] CDAs are zinc enzymes that catalyze hydrolytic deamination of the pyrimidine cytidine.[63] Two cysteines and one histidine form three of the four ligands to the active site zinc, with the fourth site occupied by a reactive water molecule. In addition, a conserved glutamic acid residue is found in the CDA active site and is believed to be critical for proton

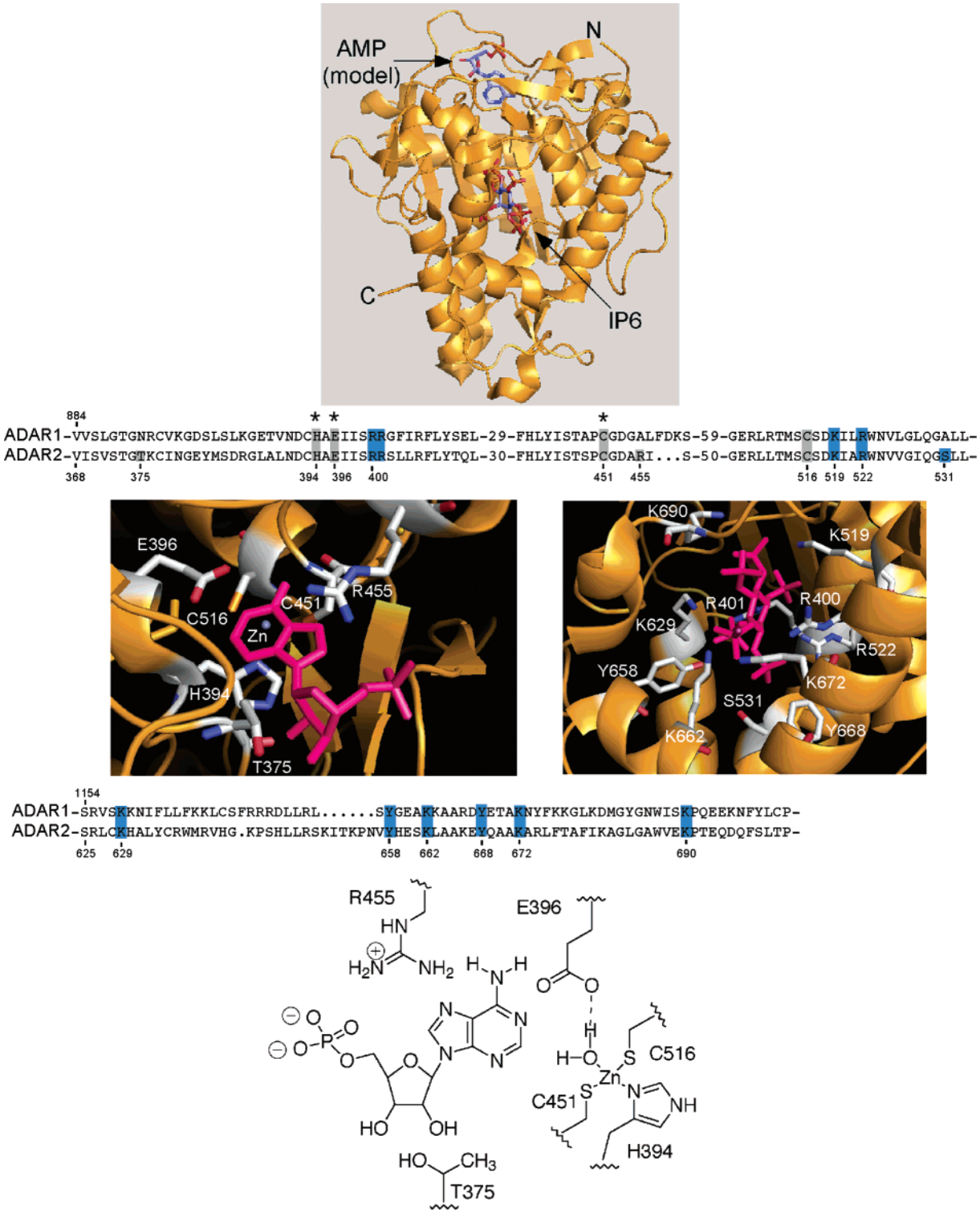


Figure 5. The ADAR2 deaminase domain:[70] (top) fold of ADAR2 deaminase domain showing location of active site and IP6 binding site; (center) residues involved in forming the deaminase active site and IP6 binding site with active site residues (grey boxes) and residues that make direct contacts to IP6 (blue boxes) highlighted on sequence comparison of ADAR1 and ADAR2; (bottom) ADAR2 active site residues. Note that AMP was modeled into the ADAR2 active site based on the location of nucleoside inhibitors in structures of the related cytidine deaminases.

transfers during the deamination reaction.[63] These four active site residues are also conserved in the ADARs (H394, E396, C451, and C516 in ADAR2).[36,39] Indeed, they likely play similar roles in the ADAR2 active site (Figure 5). However, ADARs do not deaminate cytidine to any measurable extent.[64] In addition, a family of enzymes exists that deaminate adenosine and related mononucleotides.[63,65−67] This adenosine deaminase family, although more closely related to the ADARs in the reaction catalyzed, shows no discernible structural similarity to the ADARs. Thus an interesting evolutionary question that exists in this field is why did the RNA editing adenosine deaminases evolve from the CDA family as apposed to from the ADAs. Furthermore, how has the structure of the CDA active site evolved in the ADARs to preferentially catalyze adenosine deamination? These questions are beginning to be addressed with a

combination of structural studies and analysis of site-directed mutants and substrate analogues (see section 3.2).

Until recently, the only information available on the deaminase domain came from its sequence similarity to the cytidine deaminases, site-directed mutagenesis, and the reactivity of substrate analogues. High-resolution structural work with ADARs has been challenging. This type of RNA editing only occurs in organisms with defined tissue systems (metazoa). Thus, the common approach of structural studies using a homologue from a thermophilic bacterium is not possible with ADARs. Active ADARs and deletion mutants can be expressed in yeast including both *Saccharomyces cerevisiae* and *Pichia pastoris*.[68,69] Using this strategy, the Bass lab was able to overexpress, purify, and crystallize the C-terminal deaminase domain of human ADAR2 (amino acids 306−700) (Figure 5).[70] As expected from the sequence similarities, ADAR2 ligates zinc with residues conserved in the CDA protein family (H394, C451, and C516). The fourth ligand to zinc is a water, which is also hydrogen bonded to E396, another conserved catalytic residue in the CDA family. The major differences between the active sites of ADAR2 and CDA are two ADAR2 residues that project into the active site of this enzyme. T375 appears to be in position to interact with the 2′-hydroxyl substituent of a ribonucleotide in the active site. This was discerned from modeling AMP into the structure of the ADAR2 catalytic domain positioning the purine ring to overlay C6 with the known position of the pyrimidine C4 in structures of complexes of CDA and pyrimidine-containing inhibitors (Figure 5).[71] Interestingly, the location of the T375 side chain appears to prevent cytidines from binding productively into the ADAR2 active site. Arginine A455 is also not conserved in CDAs (Figure 5). The arginine side chain is in a position consistent with interaction with the purine ring at N7. However, ADAR2 readily deaminates a 7-deazaadenosine-containing substrate, calling into question the existence of an important interaction with N7 (Figure 8).[64] Understanding of the precise role of the active site residues not also found in CDAs will require additional mutagenesis/modification experiments to determine the importance of potential contacts and solution of high-resolution structures of ADAR−RNA complexes.

The ADAR2 catalytic domain showed an unusual structural feature not seen before. An inositol hexakisphosphate (IP6) molecule was found buried in the core of the protein, hydrogen bonded to numerous polar residues conserved in the ADARs and some ADATs (Figure 5).[70] IP6 was not added to purification buffers or during crystallization, so the protein must have sequestered it during expression in yeast. An important role for IP6 in ADAR function is implied by the fact that active ADAR2 is isolated from overexpression in *S. cerevisiae* only when the biosynthetic pathway for IP6 formation is intact.[70] It seems likely that IP6 is required for ADAR2 folding, since it is difficult to imagine ADAR2 maintaining the fold identified in the crystal structure without IP6 present.

Intracellular localization sequences have been identified in the primary structure of both ADAR1 and ADAR2.[72,73] ADAR2 and the ADAR1 short form (p110) are localized to the nucleus and found to be dynamically associated with the nucleolus.[73−75] The ADAR1 long form has a nuclear export sequence near the N-terminus and is also found in the cytoplasm.[72] Nucleolar association of ADAR2 is dependent on the RNA-binding activity of the dsRBMs and is believed to be due to interaction with ribosomal RNA found in the nucleolus.[73] When editing-competent RNAs are expressed, ADARs delocalize from the nucleolus and accumulate in the nucleoplasm, suggesting that shuttling of ADARs between different subcellular compartments may be an important regulatory mechanism for RNA editing.[74,75]

3.2. Substrate Recognition

3.2.1. RNA Binding Selectivity

An important issue in the ADAR-catalyzed RNA editing reaction is defining the extent to which RNA-binding selectivity controls the editing site selectivity. For the long form of ADAR1, recent work indicates that the Z-conformation binding preferences of the N-terminal Z-domain influence the editing site selectivity for this enzyme.[46] However, much of the RNA-binding affinity for the ADARs comes from the dsRBM-containing RNA-binding domains.[44] How selective are these domains in their binding and what role does that selectivity play in controlling selection of editing sites? ADARs require duplex secondary structure of sufficient length (15−20 bp) for deamination to be observed.[35] This duplex length requirement is clearly related to the known length requirement of dsRBMs.[44] Interestingly, for longer duplex substrates, the presence of internal loops influences the selectivity of the editing reaction.[76] Long perfectly matched duplex RNA substrates are deaminated indiscriminately, having a reaction end point with ∼50% of the adenosines converted to inosines.[76] However, when internal loops or mismatches interrupt the helix, the ADAR reaction is more selective.[76] Thus, editing selectivity is controlled, at least in part, by the combination of the duplex length requirement for the dsRBMs and bulges and mismatches found in the editing substrates that limit the length of perfect duplex available for binding. This effect alone, however, cannot explain the selectivity observed. For instance, Ohman and colleagues have shown that mutation of the nucleotide paired with the R/G site adenosine dramatically decreases editing efficiency at this site, whereas this change has little measurable effect on binding.[77]

Our laboratory has also carried out experiments to determine whether the ADAR2 RNA-binding domain containing dsRBM I and dsRBM II binds duplex RNAs at specific locations and whether any selective binding sites observed are important for editing.[78] We have had success in identifying dsRBM binding sites using directed hydroxyl radical cleavage experiments involving EDTA·Fe-modified proteins.[56] Furthermore, binding to these sites can be blocked by incorporating N^2-benzylguanosine at positions in the RNA predicted to be in contact with the dsRBMs based on the directed hydroxyl radical cleavage data and the available dsRBM−RNA structures.[78,79] Introducing a benzyl group at N2 of guanosine in duplex RNA projects steric bulk into the minor groove and prevents the binding of dsRBMs to that site on the substrate. Since dsRBMs are not highly sequence specific, simple nucleotide substitutions are often ineffective at disrupting dsRBM−RNA binding. This approach was used to identify binding sites for both dsRBM I and dsRBM II on the GluR-B Q/R site duplex.[78] Benzylated RNAs were then used to determine whether these sites were important for editing at the Q/R site by ADAR2. Indeed, locations on the duplex RNA predicted to be in contact with dsRBMs were shown to be important for ADAR2 editing at the Q/R site. Furthermore, a deletion mutant of ADAR2 lacking dsRBM I was not inhibited by a specific benzyl

modification, suggesting that the modification blocked dsRBM I binding. These studies showed that ADAR2's dsRBMs can bind editing substrates selectively and that disruption of the binding to these sites inhibits the editing reaction. However, a full understanding of the basis for the binding selectivity observed will require additional structural studies on the relevant protein−RNA complexes. Importantly, the binding sites for ADAR2 dsRBMs identified in this study were shown to be distinct from that of PKR's dsRBM I, illustrating the importance of the amino acid sequence of the dsRBM in dictating preferred binding sites.[78]

Structural studies of an ADAR dsRBM/RNA complex have been recently reported.[50] The Allain lab used NMR spectroscopy to solve the structures of the individual ADAR2 dsRBMs.[50] In addition, chemical shift perturbation studies identified nucleotides in the GluR-B R/G site duplex that bound the isolated motifs. The results suggested that dsRBM I of ADAR2 contacted nucleotides in the duplex and the loop of the RNA, in analogy to the Rnt1p−RNA complex (Figure 3). Furthermore, mutagenesis of the loop caused a diminished editing efficiency at the R/G site, consistent with a role for dsRBM I−loop interactions in R/G site-specific editing.

3.2.2. Nearest Neighbor Effects

The ADARs have been shown to preferentially deaminate adjacent to certain base pairs in matched dsRNA substrates.[80] These "nearest neighbor" preferences differ for ADAR1 and ADAR2. ADAR1 preferentially deaminates adenosines that are 3′ to U = A > C > G. These are ADAR1's preferred 5′ nearest neighbors. ADAR1 has no apparent 3′ nearest neighbor preferences. In contrast, ADAR2 has both 5′ and 3′ nearest neighbor preferences. The preferred 5′ nearest neighbors for ADAR2-catalyzed adenosine deamination are U = A > C = G. Its preferred 3′ nearest neighbors to the edited adenosine are U = G > C = A. Thus, ADAR2 has preferred triplets that are optimal editing sites including UAU, AAG, UAG, and AAU. Although the basis for these nearest neighbor preferences is not known, they may arise from favorable interactions between the nucleotides adjacent to the edited adenosine and structure found in the ADAR catalytic domain. It is also possible that flipping of the adenosine into the ADAR active site is facilitated with certain flanking base pairs (see section 3.3.1).

The studies carried out to determine these preferred nearest neighbors used long perfectly matched dsRNAs as substrates and assumed no preferred binding registers for the RNA-binding domains of the enzymes. In addition, the extent to which these preferences can be overridden by other factors is revealed by the fact that the Q/R editing site on the GluR-B duplex resides in a sequence (CAG) that would not be expected to be an optimal site for ADAR2, whereas this enzyme deaminates the Q/R site quantatively in vivo.[3] Thus, in some cases, factors such as preferred binding sites on the substrate for the RNA binding domain are likely crucial in determining editing site selectivity.

3.2.3. Dimerization

Several groups have addressed the requirement for ADARs to self-associate for substrate binding and editing activity.[81−83] This was suggested as a possibility since other dsRBM proteins, including the RNA-dependent protein kinase PKR, are known to require dimerization for activity.[84] Early studies were consistent with dimerization as a necessity for editing activity. For instance, MacMillan argued based on kinetic data that the active editing complex for ADAR2 includes two molecules of enzyme and one of substrate with the rate-limiting step of the reaction being the formation of this E·S·E complex.[82] In addition, both ADAR1 and ADAR2 were shown to self-associate during purification.[81] It has also been demonstrated that the N-terminus of *Drosophila* ADAR is required for protein self-association and an enzyme lacking this sequence is not active in an editing assay.[83] However, there are conflicting reports regarding the migration of ADAR1 and ADAR2 on gel filtration columns that could resolve monomer from dimer.[81,85] Furthermore, a deletion mutant of human ADAR2 active in a site-specific editing assay exists as a monomer in solution.[69] Thus, while it is clear that ADARs have the ability to oligomerize, both in an RNA-dependent and in an RNA-independent manner, it is less certain that this oligomerization will always be required for enzymatic activity.

3.3. Deamination Mechanism

3.3.1. Conformational Changes in Substrate and Enzyme

One of the consequences of the double helical nature of the ADAR substrate is the requirement for conformational changes in the RNA prior to deamination. It is clear from the structure of the catalytic domain of ADAR2 that the reactive nucleotide must adopt a conformation that removes the edited base from the helical stack before it can access the zinc-containing active site (Figure 5).[70] Our laboratory addressed the issue of conformational changes in the ADAR substrate early on using RNAs bearing the fluorescent base 2-aminopurine (2-AP) at different positions, including at a known editing site.[86,87] Stacking into a duplex quenches the fluorescence of 2-AP. Thus, 2-AP can be used as a probe of the stacking environment of a nucleotide under different experimental conditions.[88−92] Our studies demonstrated 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 active site (Figure 6).[86,87] This effect was not observed with 2-AP in single-stranded RNA. The ADAR2-induced increase in fluorescence also required the presence of the deaminase domain, suggesting an active role for this part of the enzyme. Furthermore, the change in 2-AP fluorescence was significantly faster than deamination indicating that this step was not rate limiting for the substrate studied. More recently, molecular dynamics simulations have been used to study base flipping processes for adenosines in different duplex RNA sequence environments.[93] These efforts demonstrated that an adenosine at a known editing site (R/G of GluR-B) is more prone to move out of the helical stack than other adenosines present in the simulated duplex.[93] Thus, the local structure of the RNA may facilitate the base-flipping step in the editing reaction. The extent to which an increased propensity to flip affects the rate of the deaminase reaction at different editing sites remains to be determined.

Protein conformational changes have been studied by monitoring differences in the tryptophan fluorescence of ADAR2 when RNA binds.[87] Interestingly, even though all the tryptophans of ADAR2 are found in the C-terminal deaminase domain, RNA binding induces an increase in the observed protein fluorescence. This result points to a coupling of RNA substrate binding and conformational rearrangements in the ADAR2 catalytic domain. These

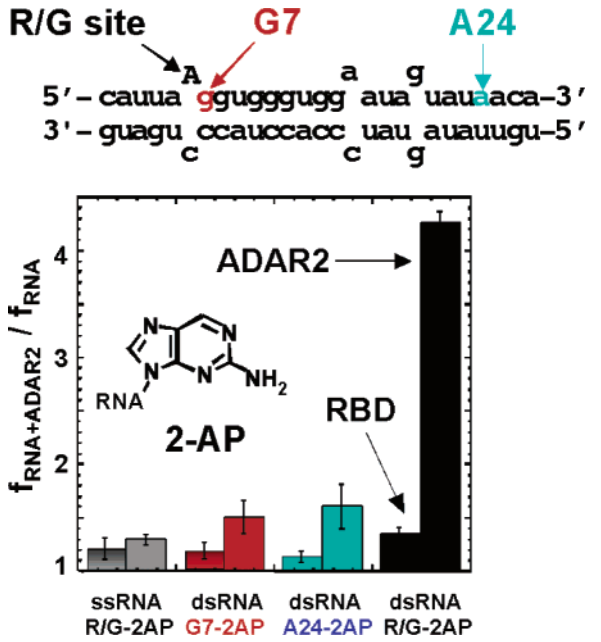


Figure 6. ADAR2 induces an increase in the fluorescence of 2-aminopurine (2-AP) when 2-AP is substituted for the R/G editing site adenosine in a duplex substrate but not in single-stranded RNA or at two control sites in duplex RNA (G7, A24).[87] The RNA binding domain (RBD) alone does not show this effect.

observations are consistent with a recent report suggesting that ADAR2 exists in an autoinhibited conformation until it binds an RNA substrate capable of engaging both of its dsRBMs.[69] Macbeth and Bass deleted the ADAR2 N-terminal domain including dsRBM I.[69] The resulting enzyme retained the ability to efficiently deaminate at the R/G site of GluR-B. Importantly, this truncation mutant was also able to deaminate short substrates that the full-length enzyme was unable to process. This could be rationalized by invoking autoinhibition by ADAR2's N-terminal domain that is relieved upon dsRBM I binding to RNA (Figure 7). This idea was further substantiated by inhibition of the deletion mutant's reaction with a short substrate by addition of the N-terminal domain in trans. Thus, it appears that ADAR2 has a duplex length requirement for its substrates arising from the requirement to bind both dsRBM I and dsRBM II, with dsRBM I binding causing a conformational change in the protein that relieves autoinhibition. This requirement would clearly limit unwanted reaction with other cellular RNA substrates containing short (<15 bp) duplex segments. Unfortunately, at this time, a detailed understanding of ADAR2 autoinhibition is lacking. For instance, the specific residues in the N-terminal domain important for this effect are not known nor is it known how they interfere with editing. It will also be important to determine whether RNA binding to dsRBM I is the only way autoinhibition is relieved and whether ADAR1 is autoinhibited in a similar manner.

3.3.2. Catalysis

The structure of the ADAR active site implies a deamination mechanism similar to that of CDAs. CDAs use a zinc-bound water molecule to carry out hydrolytic deamination with a conserved glutamic acid (E104 in *Escherichia coli* CDA) available for proton transfers. Mutation of the ADAR active site residues involved in zinc binding (H394, E396, C451, and C516 in ADAR2) causes a loss of activity, as expected.[81,94] In addition, the reactivity of substrate analogues supports the proposed hydrolytic deamination mechanism. Our group has synthesized several adenosine analogues, incorporated them into an ADAR2 substrate via protected phosphoramidites, and compared their reactivity to that of adenosine (Figure 8).[64,95,96] These studies were also useful in comparing the reaction of ADAR2 with that of adenosine deaminase (ADA), since many of the nucleosides incorporated by us into the ADAR2 substrate had also been studied in the ADA reaction.[97−99] We found that ADAR2 does not absolutely require the 2′-hydroxyl group at the editing site (2′-H and 2′-F, k_{rel} = 0.3 and 0.5, respectively) but deaminates 2′-*O*-methyladenosine very slowly (k_{rel} = 0.004). It appears that the side chain of T375 in the ADAR2 active site is not important for hydrogen bonding to the 2′-hydroxyl but blocks access by 2′-*O*-methyladenosine (Figure 5). Interestingly, snoRNA-directed 2′-O-methylation at an editing site in a serotonin receptor pre-mRNA is apparently used naturally for regulating editing at that site (see section 3.4.3).[75] ADAR2 can also displace small C6 substituents other than $-NH_2$ with a rate that is independent of leaving group pK_a (k_{rel} for $-OCH_3$ and $-NHCH_3$ = 0.02).[64,96] In addition, unlike the reactions of these nucleosides with ADA, 7-deazaadenosine in RNA is readily converted to product by ADAR2, whereas 2,6-diaminopurine in RNA is not an ADAR2 substrate.[64,96] These latter observations indicated that ADAR2 and ADA use different base recognition strategies as suggested by their different active site geometries.[65,70]

Interestingly, a large rate acceleration was realized for the ADAR2 reaction when 8-azaadenosine replaced adenosine.[96] 8-Aza substitution of adenosine decreases its pK_a from 3.5 to 2.2.[100] Protonation at N1 is a necessary step in the formation of the tetrahedral intermediate in a hydrolytic adenosine deamination reaction (Figure 9). If this protonation were rate limiting, then a substitution that makes the purine less basic might be expected to slow the reaction. However, the 8-aza substitution also facilitates hydration across the N1−C6 double bond.[101] The intrinsic difference in hydration free energies of purine vs 8-azapurine has been estimated to be as much as 7 kcal/mol.[101] This is largely a result of the difference in resonance energy, the purine ring system being significantly more stabilized by resonance. Thus, 8-aza substitution makes hydration of the purine ring a more favorable process. Since this substitution accelerated the ADAR reaction rate, it appeared likely that attack on the purine was rate limiting for the substrates tested in our

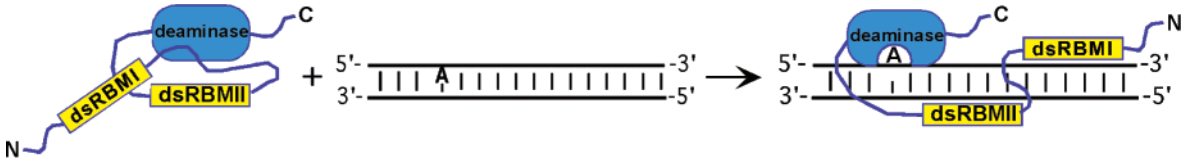


Figure 7. Model for conformational changes that occur during the ADAR2 reaction.[69] ADAR2 is thought to exist in an autoinhibited conformation until it binds a duplex RNA long enough to engage both its dsRBMs. When this happens, autoinhibition is relieved, placing the deaminase domain over the editing site. The enzyme extracts the adenosine from the helical stack and into the zinc-containing active site. In some cases, formation of an ADAR dimer may occur on the RNA during the editing reaction.[81,83]

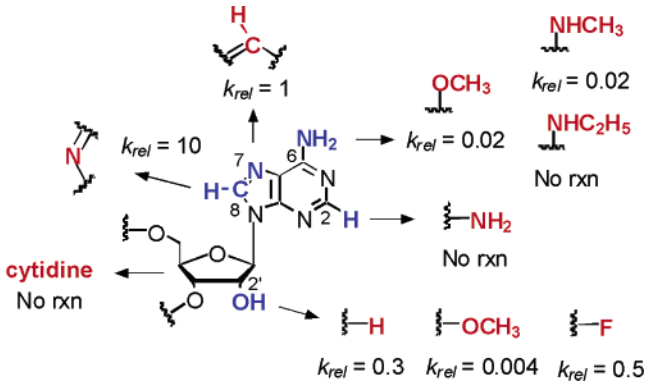


Figure 8. Comparison of rates of product formation for different nucleoside analogues under single turnover conditions at saturating ADAR2 concentration.[64,95,96] k_{rel} = (k_{obs} for analogue)/(k_{obs} for adenosine) in a model duplex RNA substrate.

studies. These results also indicated that the 8-azapurine ring system was well-suited for interaction with the ADAR2 active site, which has been confirmed by the inhibition of ADAR2 by 8-azanebularine nucleoside and high-affinity binding of the enzyme to 8-azanebularine-containing RNA (see section 3.3.3).[96,102]

Given the observations discussed above, a mechanism for the ADAR2 reaction has been proposed.[102] Initially, the dsRBMs bind selectively to certain sites on a duplex RNA substrate. If enough recognition surface is present allowing both dsRBMs to bind simultaneously, the deaminase domain is relieved of autoinhibition and contacts the RNA. The reactive adenosine is flipped out of the helix and occupies 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 (Figure 9). This is followed by protonation at N1, generating a high-energy tetrahedral intermediate. Proton transfer to N6 and from N1 with departure of ammonia from this intermediate would follow, yielding the inosine product.

Important questions about the ADAR mechanism remain unanswered, for instance, is the rate-determining step the same for all editing substrates? What role do active site residues not found in the CDAs play (e.g., T375, R455, etc.)? In addition, to what extent do flanking nucleotides control the rate of deamination and enzyme affinity? Answers to these questions will require additional kinetic analyses with different RNA substrates and various ADAR mutants, along with structural studies of ADAR−RNA complexes.

3.3.3. Inhibitors

After the initial purification of ADAR1 from *Xenopus* eggs, its sensitivity to various added inhibitors was evaluated.[103] Reagents known to react with cysteine residues (iodoacetic acid, *N*-ethylmaleimide, *p*-hydroxymercuriphenylsulfonic acid, and *p*-tosyl-L-lysine chloromethyl ketone) all inhibited the enzyme at low millimolar concentrations. These results are consistent with the role of cysteine residues in ligating zinc in the ADAR active site.[70] The presence of basic proteins, intercalators, and high salt, conditions that inhibit protein−RNA binding, also inhibited editing by ADAR1.[103,104] No inhibition was seen with acidic proteins, ATP, GTP, Mg^{2+}, Ca^{2+}, EDTA, or EGTA. The lack of an effect with added chelators at millimolar concentrations shows the high affinity with which ADARs bind zinc, a value that has not been directly measured. Early studies also showed ADARs to be inhibited by high concentrations of RNA substrate.[103] The substrate inhibition most likely arises from substrate binding to the dsRBMs at high concentrations and interfering with formation of the active ADAR−RNA complex. Substrate inhibition has been explained further by invoking a 2:1 complex as the active species with an ADAR dimer bound to RNA substrate.[82] High concentrations of RNA substrate could lead to a nonproductive 1:1 ADAR−RNA complex. These observations suggest that RNAs with affinity for ADAR's dsRBMs will function as inhibitors. Indeed, VA_1 RNA from adenovirus, a known inhibitor of PKR, also inhibits the activity of ADAR1.[105]

More recent studies have focused on the development of ADAR inhibitors containing nucleoside analogues.[96] 8-Azanebularine (8-azaN) is a derivative of 8-azaadenosine with hydrogen substituted for the C6 amino group (Figure 10). This compound is a potent inhibitor of ADA with a K_i = 40 nM, a value 400-times lower than that for purine ribonucleoside (nebularine).[66] It has been suggested that this increase in potency is due to the greater propensity of the 8-aza analogue to undergo covalent hydration of the C6−N1 double bond.[101] Because the covalent hydrate is an excellent mimic of the transition state for adenosine deamination, structural changes of the purine that facilitate hydration increase the potency of the inhibitor.[101] Since 8-azaadenosine substitution caused a significant increase in the rate of the ADAR2 reaction, it followed that 8-azaN could function as an ADAR2 inhibitor.[96] The inhibition observed required high concentrations of the nucleoside (IC_{50} = 15 ± 3 mM).[96] However, this was undoubtedly because much of ADAR2's substrate binding affinity comes from contacts made between the RNA and the RNA-binding domain, which are not possible for the nucleoside inhibitor. High-affinity binding would require incorporation of the 8-azaN into an RNA ligand of ADAR2.[102] It remains to be seen whether a tight-binding small molecule inhibitor can be developed for the ADARs. Such a compound would be a useful reagent for

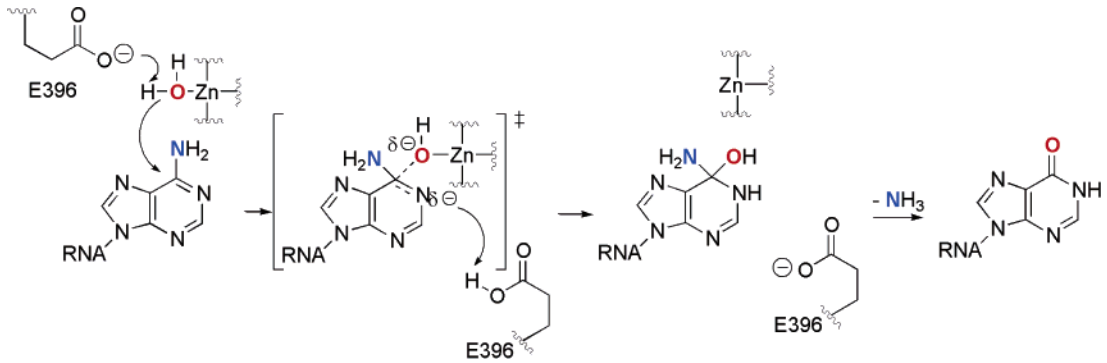


Figure 9. Proposed mechanism for adenosine deamination in RNA catalyzed by the ADAR2.

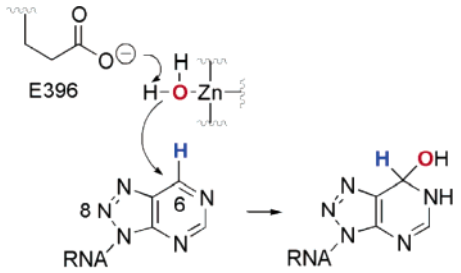


Figure 10. Proposed hydration of the 8-azanebularine base in the ADAR2 active site.

studying RNA editing and could have therapeutic value, for instance, by blocking ADAR1 activity stimulated during interferon treatment for viral infection.[6]

To increase the affinity of 8-azaN for ADARs, our laboratory prepared a phosphoramidite of this nucleoside and used it to make modified RNA for binding studies with ADAR2.[102] Given that full length ADAR2 binds duplex RNA of any sequence with high affinity, the initial studies of 8-azaN-containing RNA involved a deletion mutant of the enzyme lacking dsRBM I.[69] This deletion mutant was shown to maintain high activity on the R/G site duplex.[69] By deleting dsRBM I from the protein, nonselective dsRNA binding was decreased and the effects on substrate binding arising from interactions with the deaminase domain were maximized. The R/G site duplex RNA with 8-azaN at the editing site bound the deletion mutant with a dissociation constant of 2 nM. Duplex RNA of the same sequence with adenosine at the editing site bound in the micromolar concentration range. Similar weak binding was observed when the 8-azaN was positioned at a site other than the natural editing site or when a mutation was made in a key active site residue (E396A). Interestingly, full length ADAR2 bound the 8-azaN containing RNA with nearly the same affinity as the deletion mutant, indicating that tight binding does not require dsRBM I. However, if dsRBM II is deleted, leaving only the deaminase domain ($ADAR2_{300-701}$), weak binding was again observed ($K_D > 1\ \mu M$). Thus, these studies indicated that a tight binding ligand for an ADAR can be generated by incorporation of 8-azaN into a duplex RNA. Importantly, the tight binding observed was dependent on a functional active site. Mutation of the critical conserved glutamate residue in the ADAR active site had been shown by others to inhibit enzyme activity but have minimal effect on RNA binding to unmodified RNA.[94] However, this mutation dramatically reduces the affinity with which the ADAR2 deletion mutant binds the duplex modified at the R/G site with 8-azaN.[102] This is most likely because tight binding requires formation of the covalent hydrate, as observed in the crystal structure of ADA bound to nebularine, and hydration requires the catalytic residues of the enzyme (Figure 10).[65] Formation of the covalent hydrate of 5-fluorozebularine in the active site of *E. coli* CDA is prevented by the E104A mutation in that enzyme as well.[106]

The generation of substrates for mechanism-based trapping of ADARs, like 8-azaN-modified RNAs, should prove useful in structural studies of ADAR−RNA complex. Additional trapping strategies to access different points along the reaction coordinate will also be valuable.[107] Results of these studies will be necessary for advancing our understanding of substrate recognition and catalysis as well as editing site selectivity.

3.4. Regulation

As one might expect for a process that can change the identity of critical amino acid residues in proteins, RNA editing by adenosine deamination is highly regulated. This regulation manifests itself in different ways with new regulatory mechanisms continuing to be discovered. For instance, ADAR1 is regulated at the transcriptional level with the long form under the control of an inducible promoter.[108] ADAR mRNAs are also modified post-transcriptionally by splicing and editing.[19] The ADAR1 protein can undergo post-translational modification that affects its activity.[109] Tissue distribution and intracellular localization of ADARs are also used to regulate RNA editing.[38] Finally, modulation of the structure of editing substrate RNAs controls editing efficiency.[75] Below we describe these regulatory mechanisms in more detail.

3.4.1. Transcriptional Control and Intracellular Localization

As discussed above, the long form of ADAR1 is induced by interferon, whereas the short form is constitutively expressed.[37] This difference arises from the use of alternate promoters that lead to different 5′ end structures in ADAR1 mRNAs. The short form is translated from an mRNA whose 5′ exon structure excludes methionine 1 of ADAR1a, and thus, initiation starts at M246 leading to the N-terminal truncation. This difference in structure includes the nuclear export sequence present in the N-terminus of the long form not found in the shorter form. Thus, in addition to being induced by interferon, the long form also shuttles between the nucleus and the cytoplasm.[110] Since interferon-stimulated genes are known to play an important role in the antiviral response, it is likely that one function of the long form of ADAR1 is to deaminate viral duplex RNAs found in the cytoplasm as a defense strategy.[111] This proposed protective antiviral effect of ADARs is not unlike that observed for other RNA/DNA deaminases. Indeed, the DNA cytidine deaminase APOBEC 3G, which has been shown recently to also be induced by interferon-α, inhibits retroviral infection and is the target of the Vif protein of HIV-1.[112−114] The presence of the ADAR1 long form in the cell's cytoplasm also shows that ADARs can access RNAs found in either the nucleus or the cytoplasm, which has important ramifications for other cellular processes involving duplex RNAs, like microRNA processing and RNA interference (see section 4).

A recent study indicates that the localization of ADAR1 and ADAR2 activity differs even within the nucleus.[75] By targeting exogenous substrates to either the nucleolus or nucleoplasm, Huttenhofer and colleagues demonstrated that ADAR1-mediated and ADAR2-mediated RNA editing can occur in the nucleoplasm but only ADAR2-mediated editing occurs in the nucleolus.[75] These studies were necessary to evaluate the possibility that small nucleolar RNAs (snoRNAs) could control editing at specific sites within pre-mRNAs (see section 3.4.3).

3.4.2. Post-Transcriptional and Post-Translational Modification of ADAR Structure

Another mechanism by which RNA editing activity can be modulated is through control of ADAR structure. This occurs both at the level of the mRNA encoding ADARs and at the ADAR protein level. As mentioned above, ADAR1 and ADAR2 pre-mRNAs are alternatively spliced to give

multiple splice forms that vary not only in their intrinsic enzymatic activity but in other important aspects as well.[19,38] Indeed, a recent study describing ADAR1 expression in a mouse model for acute inflammation described the generation of three different forms of ADAR1 that arose from alternative splicing and differed in the functional domains present (Z-domain, dsRBMs, localization sequences, etc.), their tissue distribution, and the extent to which they were upregulated in response to inflammation.[38]

Editing of ADAR2 pre-mRNA by the ADAR2 protein has also been observed.[19] A specific adenosine in intron 4 of the ADAR2 pre-mRNA generates a new 3′-splice site acceptor (Table 1). Splicing at this new site results in change in reading frame and loss of expression of the catalytically active ADAR2 protein. *Drosophila* ADAR also edits its own mRNA at a codon for a conserved residue in the catalytic domain.[115] This editing reaction causes a serine to glycine codon change resulting in loss of deaminase activity. Although not fully understood, these autoediting reactions may function as negative feedback to prevent excess levels of ADAR activity, which could lead to unwanted, nonselective adenosine deamination in various RNAs.

ADAR regulation can occur post-translationally.[109] Covalent attachment of the ubiquitin-like modifier SUMO to a target protein is a post-translational regulatory mechanism in eukaryotes that can alter the protein's localization, activity, or stability.[116] Human ADAR1 is modified with SUMO-1 on lysine 418, which lies between the Z-domain and the first dsRBM.[109] Sumoylation of ADAR1 reduces its RNA editing activity in vitro and in vivo, suggesting a role for SUMO in regulating cellular RNA editing by ADAR1. How SUMO-modification of ADAR1 is itself controlled and the precise role for SUMO-mediated inhibition of ADAR1 have not been defined.

The crystal structure of the ADAR2 catalytic domain suggests another mechanism by which RNA-editing activity may be regulated post-translationally. Inositol hexakisphosphate (IP6) was found buried in the core of this protein bound by multiple conserved polar residues (Figure 5).[70] The observed fold of the protein leads to the convergence of several positively charged side chains onto the IP6 binding site. This conformation is not likely to be stable in the absence of the IP6 molecule. Therefore, it is reasonable to propose that IP6 binding is required for proper folding of the catalytic domain and ADAR activity.[70] In support of this, the *S. cerevisiae* kinase (Ipk1p) that converts IP5 to IP6 is required for expression of active human ADAR2 in this yeast.[70] It remains to be seen whether this is true in mammalian cells. It is possible that ADAR2's structure (and its editing activity) fluctuates with IP6 levels in human cells, linking RNA editing by adenosine deamination to inositol signaling pathways.

Interestingly, although protein kinase substrate algorithms predict numerous phosphorylation sites in the ADARs, regulation of ADAR activity by phosphorylation has yet to be reported.[60] Advances in mass spectrometry and other methods for analyzing post-translational modifications of proteins make it likely that additional regulatory modifications, including phosphorylations, will be identified for the ADARs.

3.4.3. Editing Site Modification

One way to control efficiency of editing at a specific site is to control the structure of the RNA at that site. Since

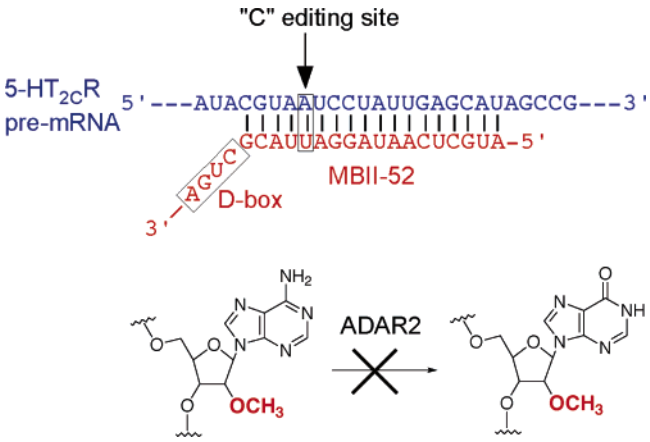


Figure 11. The brain-specific C/D box snoRNA MBII-52 is predicted to direct 2′-O-methylation of the "C" editing site adenosine in the 5-HT$_{2C}$R pre-mRNA and block editing at that site by ADAR2:[75] (top) duplex formed by MBII-52 binding to the 5-HT$_{2C}$R pre-mRNA with D-box and site of methylation indicated; (bottom) methylation at the 2′-position of an editing site adenosine inhibits ADAR2.

ADARs require duplex secondary structure in the editing site RNA, any alteration that inhibits duplex formation will decrease editing efficiency. Furthermore, most of the editing sites identified in exons are found in duplexes formed with sequence distal to the editing site in a downstream intron (the editing site complementary sequence or ECS).[117] Therefore, splicing and editing are often intrinsically linked, with the editing reaction only taking place on the pre-mRNA before splicing, since the splice product RNA lacks the ECS necessary to support editing.[117]

A more subtle modification of the editing site RNA that affects deamination efficiency is 2′-O-methylation of the edited adenosine. In our studies of the reactivity of various adenosine analogues, we showed that 2′-O-methylation severely inhibited the ADAR2 reaction (Figure 8).[95] Since that time, a snoRNA has been discovered that is predicted to inhibit editing of the 5-HT$_{2C}$R pre-mRNA by directing 2′-O-methylation to an edited adenosine (Figure 11).[75] The 2′-O-methylation of ribosomal and spliceosomal RNAs is directed by short guide RNAs that contain conserved sequence elements and accumulate in the nucleolus (small nucleolar RNAs or snoRNAs).[118] These guide RNAs act by forming a Watson−Crick duplex with the RNA target and directing 2′-O-methylation of the nucleotide in the target paired with the fifth position upstream from the conserved D box sequence motif in the C/D box snoRNAs.[75,119] The brain-specific C/D snoRNA MBII-52 is predicted to hybridize with the 5-HT$_{2C}$R pre-mRNA and direct 2′-O-methylation of the "C" editing site adenosine (Figure 11).[75] Since editing by ADAR2 can occur in the nucleolus, it followed that this snoRNA may reduce editing efficiency by ADAR2 at the C-site. Indeed, MBII-52 specifically decreases efficiency of editing at the C-site by ADAR2 with no measurable effect on the nearby D-site, which is also an ADAR2 substrate.[75] Furthermore, artificial snoRNAs could be generated that blocked editing at other sites in a sequence-specific manner. Thus, snoRNA-mediated 2′-O-methylation can be used to inhibit ADAR2 editing at specific sites in RNAs localized to the nucleolus.

4. Relationship between RNA Editing and the microRNA/RNA Interference Pathways

Since ADARs are capable of binding and deaminating numerous different double helical RNA structures found both in the nucleus and in the cytoplasm, it would be expected that they would have effects on other pathways that involve duplex RNAs, such as microRNA biogenesis and RNA interference. This is indeed the case.[120,121]

microRNAs (miRNAs) are small noncoding RNAs that lead to translation suppression of specific messenger RNAs containing partially complementary sequence in their 3′ untranslated regions.[122] miRNAs can also function to silence mRNA much like short interfering RNAs (siRNAs) by integrating into the RNA-induced silencing complex (RISC) and guiding the degradation of target message.[122] Although the function of most miRNAs is not known, strong links have been made between miRNAs and the regulation of development.[123] These 20−22 nt single-stranded RNAs are generated through the action of two RNAse III-like endoribonucleases.[123] Drosha intercepts the primary transcript encoding a miRNA (the primary miRNA or pri-miRNA) in the nucleus and cleaves it into ∼70 nt hairpin−stem structure referred to as the pre-miRNA. The pre-miRNA is exported from the nucleus into the cytoplasm where Dicer cleaves it into the miRNA that ultimately binds target message and causes translation suppression. Thus, duplex RNA structures found both in the nucleus and in the cytoplasm are important for miRNA biosynthesis. Furthermore, specific miRNA precursors have been shown to be substrates for adenosine deamination by ADARs.[15,121,124] For instance, Maas showed that the stem−loop precursor to human miRNA22 is edited at multiple adenosines by the ADAR1 long form, although the functional consequence of these editing events was not established.[124] More recently, Nishikura showed that several pre-miRNAs were substrates for both ADAR1 short form and ADAR2 in the nucleus.[121] In addition, processing of a specific pri-miRNA by Drosha was suppressed by the editing reactions on that RNA, leading to lower levels of the mature miRNA. Thus, RNA editing by the ADARs can directly effect miRNA biosynthesis and regulate the levels of mature miRNA formed. It is also true that editing can change the sequence of the mature miRNA. The Kaposi sarcoma-associated virus miRNA-K12-10b has an editing site within the sequence of the mature miRNA.[15] Therefore the possibility exists for RNA editing to generate multiple miRNAs from a single precursor to control expression of different target genes. This has yet to be shown, however. An intriguing possibility is that the severe developmental defect observed in the *ADAR1* null mouse is related to the lack of editing of some critical miRNA.

ADARs also interact with components of the RNA interference pathway. ADAR knockouts in *C.* elegans have chemotaxis defects.[125] These defects can be reverted to normal in RNA interference defective strains, indicating a dependence of the *ADAR* null phenotype on RNAi. In addition, recent reports show that ADARs can antagonize the desired effect of transfected siRNA duplexes.[120,126] RNAi is suppressed in cells that overproduce ADAR1 and is more effective in mouse fibroblasts homozygous for an *ADAR1* null mutation than in wild-type cells.[121] ADAR1 was shown to bind tightly to siRNA duplexes, but no deamination could be observed for these RNAs. Thus, the inhibitory effect of ADAR1 likely arises from binding and sequestering siRNA duplexes by the long form in the cytoplasm, preventing them from entering the RNAi pathway. Therefore, it is likely that cell lines that naturally express high levels of ADAR1 will require higher concentrations of added siRNA to achieve the desired knockdown in RNA interference experiments with those cells. Furthermore, chemical modifications of siRNAs that prevent interactions with ADARs could lead to more efficient gene silencing.

5. Summary and Prospectus

Our understanding of the biological role, structure, mechanism, and regulation of the ADARs has advanced significantly in the last five years. However, intriguing questions that must be addressed remain in the each of these areas. For instance, what is the function of editing in noncoding sequence? What are the targets of ADAR1 that lead to such profound developmental defects in the *ADAR1* null mouse? How widespread is editing of microRNA precursors? What is the role of editing in modulating behavior? Are there specific behaviors that can be related to specific editing sites? Can potent small molecule inhibitors be developed to target RNA editing enzymes or editing sites? What effect will these compounds have in adult mammals? How would an animal respond to inhibition of editing at different points in development? In addition to these issues related to the biological function of ADARs, important gaps in our understanding of ADAR structure, mechanism, and regulation remain. High-resolution structures of ADARs bound to RNAs at different points along the reaction coordinate are needed to fully define the molecular recognition and the extent to which editing selectivity arises from contacts with the RNA-binding domains and deaminase domains. Additional studies with different model substrates are necessary to determine the role of RNA structure in the ADAR reaction. Also, is IP6 binding used to control ADAR activity? What does this tell us about the relationship between inositol signaling and RNA editing? Are other post-translational modifications of ADARs used for regulation? What is the basis for autoinhibition of ADAR2? Is this a point for further regulation of ADARs? Experiments designed to answer many of these questions are underway in laboratories worldwide. We expect the next five years of research in RNA editing by ADARs to be even more exciting than the last!

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7. Note Added in Proof

Support for ADAR dimerization in vivo was recently presented in Chilibeck, K. A. et al. *J. Biol. Chem.* **2006**, *281*, 16350.

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