MOLECULAR MECHANISMS OF ADAR2 LOCALIZATION AND SUBSTRATE SPECIFICITY

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I can still smell the excitement and nervousness when the airplane was landing at Detroit Metro Airport on June, 28th 2001. Since then I have devoted myself to the hard work for this degree, Doctor of Philosophy, in the United States. I think the naming of this degree makes perfect sense because after a few successes and many failures during these five years, I need to move on and work on a brand new scientific field and the only thing left for me is this document and better understanding of philosophy.

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I am the only child of my family so I consider myself selfish to leave my parents on the other side of the planet and only visit them twice during the five years. I remember seeing them crying every time I leave China. My parents support every decision I made and care about me in any conditions. I can not thank my parents enough and I wish them health and happiness. I would also like to thank my girlfriend for the happy time she brings to my life during the last two years. I want to be with her forever.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT_{2C}R	2C subtype serotonin receptor
A	adenosine
aa	amino acid
ACF	APOBEC-1 complementation factor
ADAR	vertebrate adenosine deaminase that act on RNA
ADAT	adenosine deaminase that acts on RNA
ADR	*C. elegans* adenosine deaminase acts on RNA
AMP	adenosine 5'-monophosphate
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoB	apolipoprotein B
APOBEC-1	ApoB editing catalytic subunit 1
A-to-I	adenosine to inosine
bp	base pairs
C	cytidine
CDA	cytidine deaminase
cDNA	complementary DNA
CNS	central nervous system
CTD	C-terminal domain
C-6	carbon number 6 of the purine ring
C-terminal	carboxyl-terminal
C-to-U	citidine to uridine
dADAR	*Drosophila* ADAR
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dsRBM	double-stranded RNA binding motif
dsRBP	dsRNA binding protein
dsRNA	double-stranded RNA
eIF	eukaryotic initiation factor
Fn	average nucleolar fluorescence intensity
Fo	average nucleoplasmic fluorescence intensity
G	guarnosine
GluR	glutamate receptor subunit
GPCR	G-protein coupled receptor
HDV	hepatitis delta virus
IFN	interferon
IMP	inosine 5'-monophosphate
IP_6	inositol hexaphosphate
kD	kilo Dalton
LDL	low density lipoprotein
mRNA	messenger RNA
NMR	nuclear magnetic resonance
NES	nuclear export signal

NFAR	nuclear factor associated with RNA
NLS	nuclear localization signal
NMDA	N-methyl-D-asparate
nt	nucleotide
N-terminal	amino-terminal
OH	hydroxyl group
PCR	polymerase chain reaction
PKR	RNA-dependent protein kinase
PACT	protein activator
pre-mRNA	precursor messenger RNA
pre-rRNA	precursor ribosomal RNA
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
Q/R site	glutamine/arginine editing site
RDE-4	RNAi-deficient 4
RHA	RNA helicase A
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
RT	reverse transcription
R/G site	arginine/glycine editing site
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SUMO	small ubiquitinlike modifier
TENR	testis nuclear RNA binding protein
TRBP	TAR-RNA binding protein
tRNA	transfer RNA
Xlrbpa	*Xenopus laevis* RNA binding protein A
U	uridine
UTR	untranslated region

CHAPTER I

INTRODUCTION

dsRNA binding motif and dsRNA binding protein

RNA (mRNA, tRNA and rRNA) represents the messenger and a component of the machinery necessary to convey the genetic information from DNA into variety of functional proteins in the cells (Crick, 1958). However, many viruses use RNA as their genomes and perform RNA-dependent RNA synthesis for replication or transcription (Lawton *et al.*, 2000; Patton and Spencer, 2000) and the presence of double-stranded (ds) RNA used to be considered as a sign of viral invasion (Jacobs and Langland, 1996). Recent discoveries of RNA interference (RNAi) and microRNA signaling pathways have illustrated the importance of cellular dsRNA in regulating gene expression (Lee et al., 1993; Napoli et al., 1990; van der Krol et al., 1990). Therefore, the comprehensive understanding of dsRNA biology is of great interest.

Double-stranded RNAs (dsRNAs) interact with dsRNA-binding proteins (dsRBPs), the great majority of which contain a sequence called the dsRNA-binding motif (dsRBM). The interaction between dsRNA with different dsRBPs may cause diverse biological outcomes, including cleavage or covalent modification of dsRNA, the phosphorylation of translational machinery and the transcriptional activation of specific genes (Fierro-Monti and Mathews, 2000).

Double-stranded RNA binding motif (dsRBM)

All dsRBMs are ~70 amino acids in length and the alignment of their primary sequences revealed a high level of sequence homology (Fierro-Monti and Mathews, 2000) (Figure 1). The structures of the dsRBMs present in several proteins from different organisms have been solved by nuclear magnetic resonance (NMR) and X-ray crystallography, with a conserved fold comprising an overall α-helix 1, β-strand 1, β-strand 2, β-strand 3 and α-helix 2 topology. The two α-helices are packed along the face of a three-stranded antiparallel β sheet (Bycroft *et al.*, 1995; Kharrat *et al.*, 1995; Nanduri *et al.*, 1998) (Figure 2). There are three regions that are highly conserved, α1, loop2 (between β1 and β2) and loop 4 (between β3 and α2); all three regions are exposed on one side of the motif and form the potential RNA-binding surface.

Recognition of an A-form RNA helix by the dsRBM

The dsRBM was initially named for its ability to recognize dsRNA, yet this ability is not universal to all dsRBMs. For instance, dsRBM1, dsRBM3 and dsRBM4, but not dsRBM2 and dsRBM5, of *Drosophila* Staufen bind to dsRNA in vitro (Micklem *et al.*, 2000). To date, no good correlation between the dsRNA binding affinity and the sequence of dsRBM has been established (Chang and Ramos, 2005).

The structure of dsRBM2 from *Xenopus laevis* RNA-binding protein A (XlrbpA) bound to a non-physiological dsRNA molecule provided the first molecular insights into dsRBM-dsRNA recognition (Ryter and Schultz, 1998) (Figure 2A). The dsRNA forms a nearly ideal A-form helix, whose major groove is deep and

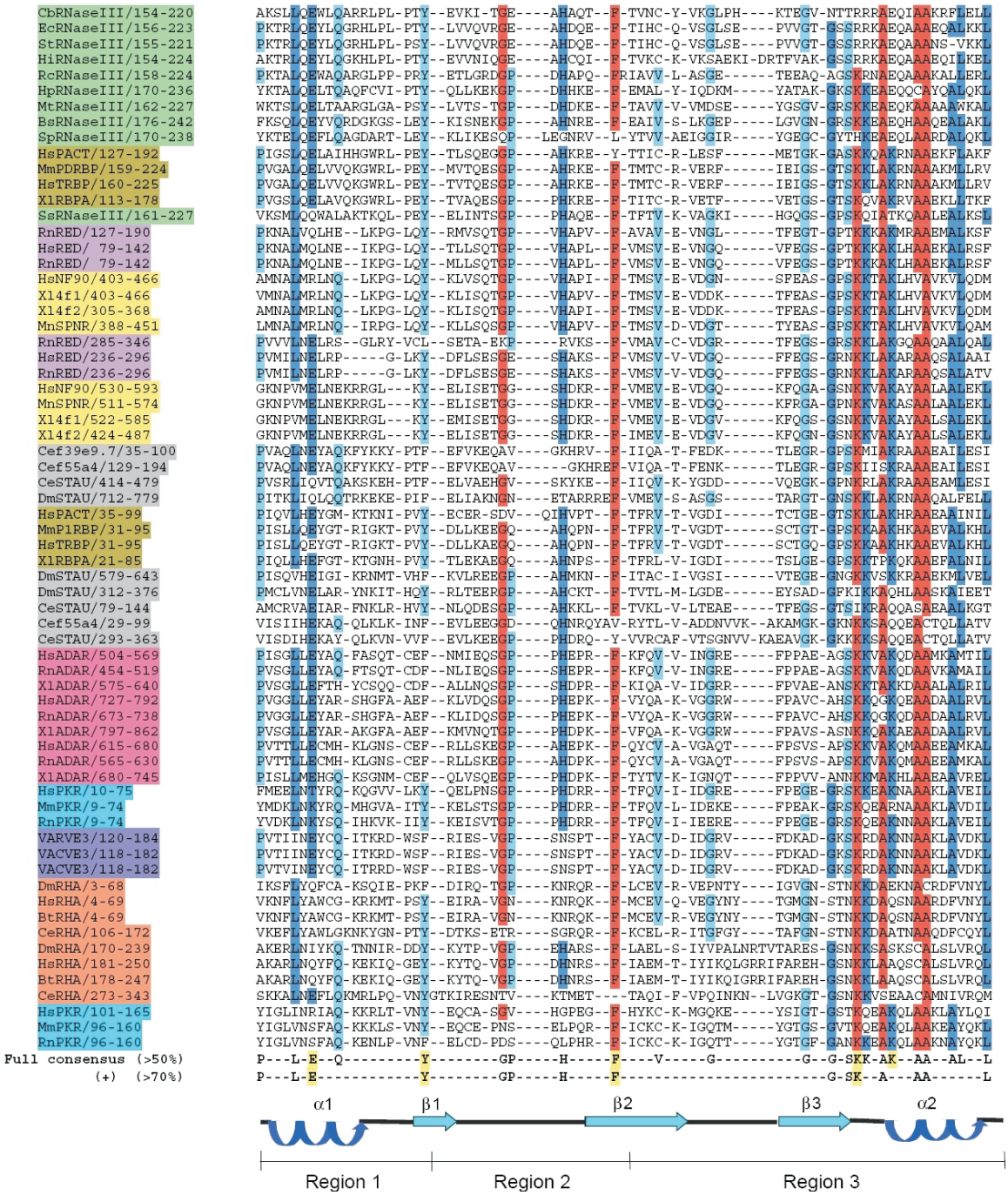

Figure 1. Sequence alignment of double-stranded RNA-binding motifs (dsRBM). The panel displays a multiple alignment of 70 dsRBM sequences clustered into protein sequence families using the PFAM domain alignment database. Residues that are >50%, >70% and >80% conserved are shown in light blue, dark blue and red, respectively. The full consensus is defined as residues that are >50% conserved among all dsRBM sequences; the (+) consensus consists of residues that are >70% conserved among those sequences that are known to bind dsRNA strongly. A schematic representation of the dsRBM sequence fold, its component structural elements and the three regions involved in RNA interactions are shown at the bottom of the alignment, (adapted from Fierro-Monti and Mathews, 2000).

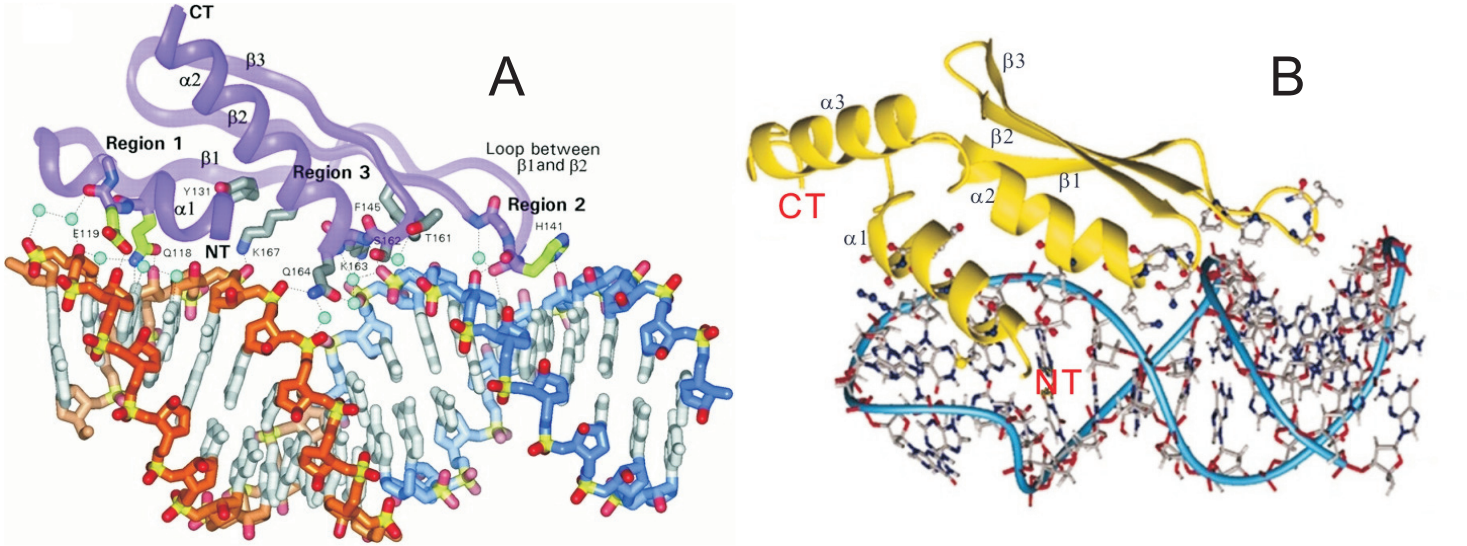

Figure 2. Structures of complexes formed by dsRBM and dsRNA (A) Overview of the A-form dsRNA-Xlrbpa-dsRBM2 interactions. Protein is shown as an carbon trace in purple. Side chain residues interacting with the RNA minor groove in regions 1 and 2 are colored green, and side chains interacting with the RNA major groove in region 3 are colored gray. Also displayed in gray are the side chains of Y131 and F145, which serve to position K167 and K163, respectively. Oxygen atoms are shown in red, nitrogen atoms in blue and phosphorus in yellow. Hydrogen bonds are indicated as black lines. (B) Overview of the lowest energy structure of Rnt1p dsRBM-snR47h RNA. The RNA is shown in lines with the helical backbone indicated by thin blue cylinder and the protein in ribbons with amino acids at the protein–RNA interface shown as ball and sticks,(adapted from Ryter and Schultz, 1998 and Wu et al., 2004).

inaccessible while the minor groove is shallow. The dsRBM binds across 16 RNA base pairs (bp) and interacts with successive minor, major, minor grooves. The α1 and loop2 regions interact with two minor grooves and the loop 4 region binds to the intervening major groove. Almost all the interactions to dsRNA are made by direct or water-mediated contacts to 2'-OH and phosphate group. These interactions explain dsRBM specificity for dsRNA (over ssRNA or dsDNA) and the apparent lack of sequence specificity. Structures of two other dsRBM-dsRNA complexes, one with *Drosophila* Staufen dsRBM3, one with the dsRBM from *E. Coli* RNase III, confirmed this common pattern of dsRBM-dsRNA recognition (Blaszczyk et al., 2004; Ramos et al., 2000).

A very interesting phenomenon was revealed by analysis of *Drosophila* Staufen dsRBM3-dsRNA interaction. Initial NMR analysis detected high frequency motions in loop 2 and loop 4 of the dsRBM, and later this flexibility was rationalized by a molecular dynamics study which demonstrated that the positively charged lysine side chains of loop2 and loop 4 do not make single, well defined interactions with RNA molecule, but rather switch between different polar interactions on a very fast timescale (Castrignano *et al.*, 2002). This special feature gives rise to the tolerance for non-identical positions of the negatively charged acceptor on RNA, and may explain the negligible effect of the small variations in the helix geometry resulted from different primary RNA sequences.

Recognition of specific structured RNA by the dsRBM

Studies with a number of dsRBPs have shown clearly that a single dsRBM is sufficient to provide a dsRBP with the specificity to distinguish between different

dsRNAs (Doyle and Jantsch, 2002; Doyle and Jantsch, 2003; Liu et al., 2000; Nagel and Ares, 2000). Since it is unlikely that the primary sequences of the A-form RNA helix provides such specificity, the recognition of particular sequences or secondary structure elements flanking the helix could indeed be the key determination of dsRNA targeting.

Interactions between the dsRBM and non-helical structural determinants, were observed in the structural studies of the complex between *S. cerevisiae* RNase III 1 protein (Rnt1p) and its physiological target, an AGNN (N: A or C or G or U) RNA hairpin (Leulliot *et al.*, 2004; Wu *et al.*, 2004) (Figure 2B). The α-helix 1 of the dsRBM recognizes the bases in the distorted minor groove of the RNA tetraloop. The recognition is considered structure-specific, rather than sequence-specific, because the α1 helix of dsRBM makes contacts with the non-conserved nucleotides (nt) in the tetraloop only. In contrast to the α1 helix, residues in loop 2 and loop 4 still make contact with the 2'-OH and phosphate groups of sequential minor and major grooves.

A similar binding theme was detected in the complex between Staufen dsRBM3 and a non-physiological UUCG RNA hairpin, where α-helix-1 contributes to the binding specificity (Ramos *et al.*, 2000). The outstanding question raised by these studies is whether the specific recognition patterns described here are common for all dsRBM-RNA complexes.

Interactions of dsRBMs with non-dsRNA binding partners

Although dsRNA-binding is the major defining feature of dsRBMs, other binding partners have been identified. For example, in addition to their

dsRNA-binding activity, the isolated dsRBMs of RNA-dependent protein kinase (PKR) can form a heterodimer with full-length protein mediated by dsRBM-dsRBM interactions (Patel and Sen, 1998). This interaction is thought to inactivate PKR when no dsRNA target is present. Similar dsRBM-dsRBM interactions are observed in protein activator (PACT) and TAR-RNA binding protein (TRBP) whose dsRBMs are capable to form dsRNA-independent heterodimers with the dsRBMs of PKR and affecting PKR kinase activity (Daher *et al.*, 2001; Peters *et al.*, 2001).

Double-stranded RNA binding motifs are not only able to interact with another dsRBM, but also able to bind other functional domains using either intra- or intermolecular interactions. The dsRBM2 of PKR binds to its kinase domain and blocks the enzymatic activity in the absence of dsRNA (Romano *et al.*, 1998). In a similar way, the dsRBM in RNase III has also been shown to interact with the catalytic domain (Blaszczyk *et al.*, 2004). The dsRBM5 of *Drosophila* Staufen does not bind to dsRNA, but it has been shown to interact with protein Miranda, which mediates protein and RNA localization in the developing nervous system (Schuldt *et al.*, 1998).

The amino-terminal 250 amino acids of RNA helicase A (RHA) contain two dsRBMs, yet the dsDNA binding activity of this protein has also been mapped to this region (Hung *et al.*, 2003). It is not clear that how the dsRBMs can accommodate both A-form dsRNA and B-form dsDNA, although some data have suggested that distinct but overlapping sets of amino acids are involved in each binding activity. Highly conserved in primary sequence and tertiary structure, dsRBMs work as a versatile macromolecular docking platform to mediate the

regulation of dsRNA functions.

Double-stranded RNA binding proteins (dsRBPs)

Almost all dsRBPs contains dsRBMs, with only one copy in the RNase III family and five copies in *Drosophila* Staufen (Figure 3). Previously, the dsRBPs were divided into two groups based upon whether they possessed a catalytic domain (Fierro-Monti and Mathews, 2000); the first group included protein families containing a catalytic domain, such as PKR, RHA, RNase III and ADAR (adenosine deaminase that act on RNA), while the second group included protein families with Staufen, TRBP, NFAR (nuclear factors associated with dsRNA) and RDE-4 (RNAi-deficient 4). The functions of identified dsRBPs involve the regulations of dsRNA localization, RNA processing and modification, or to modulate the dsRNA-dependent signal pathways (Saunders and Barber, 2003) (Table 1).

PKR

PKR contains two dsRBMs in the N-terminus and one serine/threonine kinase domain in the C-terminus (Green and Mathews, 1992; Meurs et al., 1990). The expression of PKR is induced by the antiviral cytokine interferon, consistent with its key role in the cellular antiviral response (Clemens and Elia, 1997).

The dsRBMs of PKR function as a molecular switch during PKR activation by dsRNA. In the absence of dsRNA, dsRBM2 interacts with the catalytic domain and negatively regulates the kinase activity of PKR (Vattem et al., 2001; Wu and Kaufman, 1997). Upon viral infection, dsRNA will induce a dsRBM-mediated

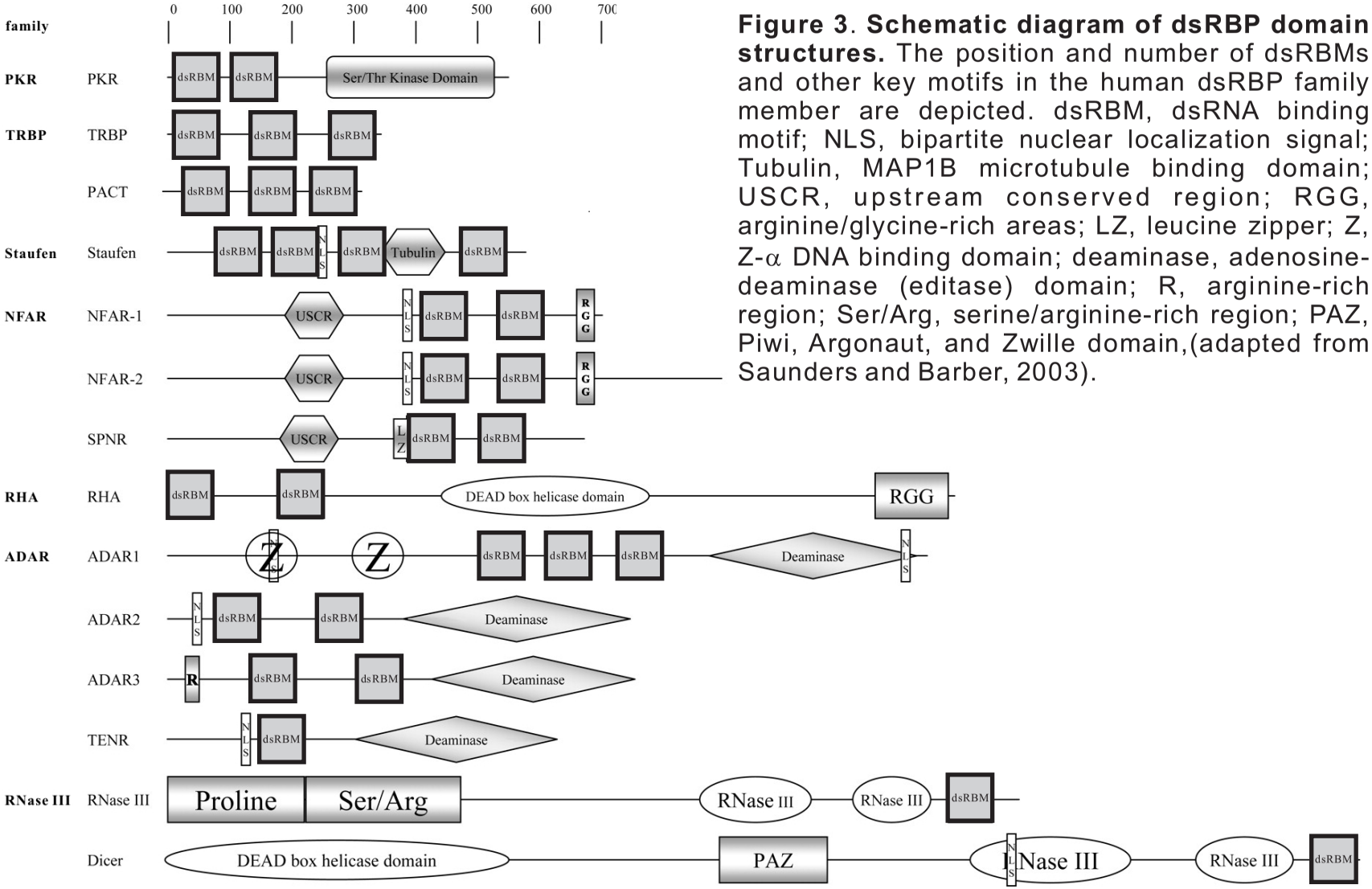

Figure 3. Schematic diagram of dsRBP domain structures. The position and number of dsRBMs and other key motifs in the human dsRBP family member are depicted. dsRBM, dsRNA binding motif; NLS, bipartite nuclear localization signal; Tubulin, MAP1B microtubule binding domain; USCR, upstream conserved region; RGG, arginine/glycine-rich areas; LZ, leucine zipper; Z, Z-α DNA binding domain; deaminase, adenosine-deaminase (editase) domain; R, arginine-rich region; Ser/Arg, serine/arginine-rich region; PAZ, Piwi, Argonaut, and Zwille domain,(adapted from Saunders and Barber, 2003).

Table 1. Features and Functions of dsRBP family members.

dsRBP	Alternative names	Species	MW (kD)	Cellular localization	Cellular role	Genetic knockout
PKR	p68/DAI	Human	68	Cytoplasm	Anti-viral defense	Impaired anti-viral response in
		Rat		~20% nuclear	Cellular stress response	homozygous null mice
	TIK/p65	Mouse	65			
TRBP		Human	40–50	Both	Translation activation	Homozygous null mice are small, die at
	PRBP	Mouse				weaning; oligospermic
PACT		Human	35	Cytoplasm	Activates PKR in response to stress	N/A
	RAX	Mouse				
	Xlrbpa	*Xenopus*	33			
	CG6866	*Drosophila*				
Staufen		Human	60, 65	Cytoplasm nucleus?	Localization and translation activation	N/A
		Rat		(NLS)	of mRNAs	
		Mouse				
		Drosophila				
NFAR1	DRBP76	Human	90	Nuclear	mRNA processing; interacts with PKR;	N/A
NFAR2			110		transcription	
	ILF3	Rat				
	mILF3	Mouse				
	4F.1	*Xenopus*				
	4F.2					
	$CBTF_{122}$					
SPNR	p74	Human	74	Nuclear?	mRNA transportation; interacts with	Homozygous null mice have a high rate
		Rat			PKR	of mortality, slow growth rate, defects
		Mouse	71	Cytoplasm		in spermatogenesis, reduced fertility,
						and neurological defects
RHA		Human	130	Nuclear	Helicase activity; interacts with RNA	Early embryonic lethal phenotype for
		Mouse			pol II-role in mRNA processing	homozygous null mice; apoptosis of
	MLE	*Drosophila*				ectodermal cells during gastrulation
ADAR1		Human	150/110	Nuclear	A to I mRNA editing of viral and brain	Heterozygous embryonic lethal; liver
	DRADA1	Rat	130	150 kDa IFN-inducible	mRNAs as well as unidentified targets	defects in erythropoiesis
		Mouse		form also	in the liver	
	dsRAD-1	*Xenopus*	120	cytoplasmic		
		C. elegans				
ADAR2		Human	90	Nuclear	A-to-I mRNA editing of Q/R site in	Homozygous null mice are seizure
	RED1	Rat			GluR	prone and die by day 20; rescue by
		Mouse				edited GluR transgenes
	DRADA2	*Drosophila*	80			*Drosophila* ADAR2 null no editing in
						3 ion channel transcripts, extreme
						behavioral defects
ADAR3		Human	81		Brain-specific	N/A
	RED2	Rat			No adenosine deaminase activity	
		Mouse			detected	
TENR		Human			Testis-specific posttranscriptional	N/A
		Mouse	72	Nuclear	mRNA processing	
RNaseIII		Human	160	Nuclear	Endoribonuclease activity cleaves	Antisense to human RNase III
	Drosha	*Drosophila*	~153		dsRNA	induces cell death, accumulation of
		C. elegans			Processing of pre-rRNA	pre-rRNA; Microdeletions in
	RNT/PAC	*S. cerevisiae/pombe*	50			*Drosophila* Drosha lethal
	RNC	*E. coli*	26			
Dicer	Helicase MOI	Human	220	Cytoplasmic multiple	Helicase and endoribonuclease activity	Key role in RNAi and development
		Mouse	215	NLS?	Role in RNAi	in *A. thaliana*, *C. elegans*, and
		Drosophila	210			*Drosophila*
		C. elegans				
		A. thaliana				
		S. pombe				
RDE-4		*C. elegans*			Deliver dsRNAs to Dicer to activate RNAi	N/A

Abbreviation : dsRBP, double-stranded RNA binding protein; ADAR, adenosine deaminase that act on RNA; NFAR, nuclear factors associated with dsRNA; PACT, protein activator; PKR, RNA-dependent protein kinase; RDE4, RNAi-deficient 4; RHA, RNA helicase A; TAR, transactivator RNA; TRBP, TAR RNA-binding protein; SPNR, spermatid perinuclear RNA binding protein; TENR, testis nuclear RNA binding protein,(adapted from Saunders and Barber, 2003).

homodimerization and unmask the kinase domain (Patel and Sen, 1998). As a consequence of dimerization, PKR will autophosphorylate, its dimer partner on multiple serine and threonine residues to become activated (Galabru and Hovanessian, 1987; Thomis and Samuel, 1995).

The most well characterized PKR substrate is eukaryotic initiation factor 2α (eIF2α); phosphorylated eIF2α sequesters eIF2B, a rate-limiting component in protein translation, leading to a dramatic inhibition of protein synthesis in the cell and subsequent apoptosis (Choi et al., 1992; Levin and London, 1978). Several less well-established PKR substrates, including IκB (the inhibitory subunit of transcription factor NF-κB), Tat (the HIV-1 transcription factor), p53 (a tumor suppressor gene), NFARs and RHA, may also contribute to the cellular consequences of PKR activation.

Since PKR is a great threat to the replication of dsRNA viruses, some viruses have developed strategies to counteract PKR activation. For example, vaccinia virus encodes two proteins, E3L and K3L; E3L contains one dsRBM and competes dsRNA-binding with PKR in the nucleus (Chang *et al.*, 1992; Kibler *et al.*, 1997), and K3L shares homology to eIF2α, and is thought to competitively inhibit eIF2α phosphorylation (Beattie *et al.*, 1991; Carroll *et al.*, 1993; Davies *et al.*, 1993). PKR appears to be the dsRNA sensor and regulates protein synthesis and cell apoptosis mostly through the phosphorylation of eIF2α.

Staufen

Staufen was originally discovered as a protein necessary for normal oocyte development in *Drosophil*a (St Johnston *et al.*, 1991). Now it is known that this

Staufen anchors *bicoid* mRNA at the anterior pole and *oskar* mRNA at the posterior pole of the oocyte, but at different stages of development (St Johnston et al., 1991; St Johnston et al., 1989). The recognition between Staufen and these maternal mRNAs is mediated by interactions between the dsRBMs of Staufen and the 3'-UTRs of the target RNAs (Ferrandon *et al.*, 1994). *Drosophila* Staufen contains five dsRBMs, but only three of them have demonstrated dsRNA-binding activity (Micklem et al., 2000; St Johnston et al., 1992); dsRBM2 and dsRBM5 cannot bind dsRNA but they are necessary for Staufen function, probably through protein-protein interactions. It was reported that dsRBM2 is necessary for microtubule-dependent localization of *oskar* mRNA, while dsRBM5 is required for the activation of *oskar* mRNA translation after localization. In addition, both motifs are required for correct *bicoid* mRNA anchoring and dsRBM5 is also involved in actin-dependent localization of *prospero* mRNA (Micklem *et al.*, 2000).

Homologues of *Drosophila* Staufen (dStau) have been cloned from humans (hStau), mice (mStau) and rats (rStau). The hStau possesses only four dsRBMs, corresponding to dsRBM2-5 in dStau, and a putative microtubule binding domain which has been reported to bind to tubulin (Wickham *et al.*, 1999). The hStau is ubiquitously expressed and detected on the rough endoplasmic reticulum, microtubules and polysomes, indicating that hStau may transport mRNA to the site of translation in the cell (Marion *et al.*, 1999; Wickham *et al.*, 1999). The mStau and rStau have also been found in RNA-protein complexes and have been suggested to be involved in assembly of large RNP particles and RNA transport (Kiebler *et al.*, 1999; Kohrmann *et al.*, 1999). Staufen, in both *Drosophila* and

mammals, appears to be involved in anchoring specific mRNAs to distinct subcellular locations to achieve their desired distribution.

NFAR

Two protein isoforms of NFAR are generated from a single human gene by alternative splicing. NFAR-2 (110kD) is 20kD larger than NFAR-1, due to an extended C-terminus. Both proteins contain two dsRBMs, as well as UCSR (upstream conserved region) and RGG (arginine/glycine-rich areas) domains. Besides the physical association with PKR, NFARs appear to be substrates of PKR in vitro, indicating that NFAR may function in PKR-mediated antiviral responses in the cell (Saunders *et al.*, 2001). Both NFARs were found to regulate transcription in tissue culture studies, probably at the level of mRNA elongation or splicing. The extended C-terminus, unique to NFAR-2, has been shown to interact with proteins, such as p68, SMN and FUS, which are involved in mRNA splicing. In agreement with these observations, NFAR-2 has been identified as a component of the spliceosome, suggesting that NFARs are involved in mRNA splicing too (Hartmuth et al., 2002; Ogilvie et al., 2003; Saunders et al., 2001; Zhou et al., 2002).

RNase III family

Members of the RNase III family have been found in multiple organisms and are all characterized as endoribonucleases that selectively cleave dsRNAs (Nicholson, 1996; Robertson et al., 1968). *E. coli* RNase III has an N-terminal endonuclease domain and single C-terminal dsRBM. It is reported that the dsRBM is dispensable for the catalytic activity, but required for cleavage

specificity (Conrad *et al.*, 2001; Sun *et al.*, 2001). *E.coli* RNase III has been shown to participate in the processing of pre-rRNA, tRNA and phage mRNA and has been suggested to have a global role in gene regulation (Gitelman and Apirion, 1980). The homologues of RNase III in *E. coli* have been identified in *Saccharomyces cerevisiae* (Rnt1) and *Schizosaccharomyces pombe* (Pac1) and have been shown to be required for the processing of pre-rRNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) (Iino et al., 1991; Rotondo and Frendewey, 1996; Xu et al., 1990). An RNase III family member, Drosha, in *C. elegans* and *Drosophila* has been shown to contain an additional RNase III signature sequence, comparing to *E.coli* homologue. This feature suggests that Drosha in *C. elegans* and *Drosophila* may be able to work as a monomer, whereas RNase III in *E. coli* forms a homodimer and requires a divalent metal ion (Mg^{2+}) to catalyze the cleavage (Dunn, 1976; Filippov *et al.*, 2000; Sun and Nicholson, 2001). The human homologue of Drosha has also been also been identified and characterized. It has an amino-terminal region that is proline-rich, followed by a serine/arginine-rich domain, and two RNase III signature motifs as well as a dsRBM at the C-terminus. It is expressed ubiquitously and has been shown to be required for pre-rRNA processing (Wu *et al.*, 2000). Recently, Drosha has also been shown to be important for microRNA processing. It cleaves pri-miRNA and releases a 60-70 nt pre-miRNA hairpin; this specific cleavage is critical for determining the sequence of the mature microRNA and contributes to the specificity of microRNA (Lee et al., 2003).

Another RNase III-like endoribonuclease, Dicer, has been found in *S. pombe*,

C. elegans, *Drosophila*, mice and humans. Dicer contains an amino-terminal helicase domain, a PAZ (Pinwheel, Argonaut, Zwille) domain, two RNase III motifs and a single dsRBM. Dicer is also involved in the microRNA processing pathway, downstream of Drosha, by cleaving 22 nt from the ends on both strands of the pre-miRNA (Bernstein *et al.*, 2001; Knight and Bass, 2001; Nicholson and Nicholson, 2002; Zhang *et al.*, 2004a). Dicer is also required for the cleavage of long dsRNAs into 21-23 bps small interfering RNAs (siRNAs) that represent a critical component of RNA interference (Bernstein *et al.*, 2001). RNase III family is required for numerous RNA processing events and may serve as a regulatory step for gene expression.

ADAR

ADARs are dsRNA-specific adenosine deaminases that involved in RNA editing of adenosine (A) to inosine (I) in cellular mRNAs and viral RNAs (Chen et al., 2000; O'Connell et al., 1995). ADAR family members have been found in numerous species including *C. elegans*, *Drosophila*, fish, *Xenopus*, rats, mice and humans (Bass, 2002). All ADARs contain one to three copies of the dsRBM and a conserved adenosine deaminase domain at the C-terminus. The functions and regulation of ADARs have been studied extensively and will be discussed later in this chapter.

A-to-I RNA editing and ADARs

Although the central dogma of molecular biology states that genetic information flows from DNA through RNA to the encoded proteins, in most cases

one cannot accurately predict the nucleotide sequences of the mRNA or the amino acid sequence of protein only based on the sequence of genomic DNA (Crick, 1958). This is because eukaryotic mRNAs undergo numerous post-transcriptional modifications including capping, polyadenylation, alternative splicing and RNA editing before they can become mature mRNAs that serve as templates for translation (Emeson *et al.*, 1989; Kable *et al.*, 1996; Keller and Minvielle-Sebastia, 1997; Varani, 1997; Wang and Manley, 1997).

The term “RNA editing” is used to describe a number of mechanistically distinct RNA modifications, including post-transcriptional insertion/deletion (Kable *et al.*, 1996), co-transcriptional insertion (Visomirski-Robic and Gott, 1997), nucleotide exchange (Price and Gray, 1999) and base modification/substitution (Auxilien et al., 1996; Sommer et al., 1991). Notably, all RNA editing events discovered in mammals are base modification/substitution events and within this category, a great majority of the known RNA editing events result from nucleoside deamination [cytidine-to-uridine (C-to-U) or adenosine-to-inosine (A-to-I)] (Backus and Smith, 1991; Rueter *et al.*, 1995).

The first identified RNA editing in mammals was a C-to-U conversion in mRNA transcripts encoding apoplipoprotein B (ApoB), where a glutamate codon (CAA) is converted into a stop codon (UAA) by RNA editing (Chen *et al.*, 1987). In the liver, the non-edited mRNA transcript encodes a full-length protein, ApoB-100, which is secreted as a component of lipoproteins and binds to the LDL receptor, delivering cholesterol to the cells by receptor-mediated endocytosis. In the intestine, the RNA editing-dependent generation of a stop codon terminates translation

prematurely and gives rise to a truncated protein, ApoB-48, which lacks the LDL receptor binding domain and functions in the absorption and transport of dietary lipid (Wang et al., 2003; Yao and McLeod, 1994). Conversion of C-to-U in the intestine is catalyzed by a complex of proteins including a zinc-dependent cytidine deaminase (APOBEC-1) and an accessory protein (ACF), which specifically interacts with an 11 nt mooring sequence downstream of the editing site (Backus and Smith, 1991; Mehta *et al.*, 1996). Overexpression of APOBEC-1 in the livers of mice and rabbits results in liver dysplasia and hepatocellular carcinoma. In this case, aberrant C-to-U editing was found in the transcripts normally not modified, suggesting that uncontrolled RNA editing can aberrantly modify RNAs and lead to disease (Yamanaka *et al.*, 1995; Yamanaka *et al.*, 1997).

The most widespread base-modification type of RNA editing is the conversion of adenosine to inosine (A-to-I) (Auxilien *et al.*, 1996), where the amino group on the C-6 position of the adenine ring is replaced by a ketone, changing the corresponding adenosine to inosine. Since inosine has base-pairing properties similar to guanosine, it is read as guanosine during translation, splicing and reverse transcription. Inosine was first discovered at position 34 and 37 of yeast alanine tRNA ($tRNA^{Ala}$), where inosine 34 in the wobble position of the anticodon loop is essential for base-paring with cytosine, adenosine or uridine to degenerate codons during translation (Holley *et al.*, 1965). A-to-I RNA editing events have also been found to be able to change the coding potentials of mRNAs and viral RNAs, including transcripts encoding glutamate-gated ion channel subunits (GluRs), the 2C-subtype of serotonin receptor (5-HT_{2C}R), the voltage-gated

potassium channel subunit (K_V1.1) and the hepatitis delta virus antigen (HDAg) (Brusa et al., 1995; Burns et al., 1997; Higuchi et al., 2000; Sommer et al., 1991) (Table 2). Despite the identification of these editing events that play a critical role in the function of the encoded protein products, the great majority of identified A-to-I RNA editing events are located in non-coding regions of RNAs and non-translated RNA species, and the functional consequences for most of these editing events have not been examined (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004; Luciano et al., 2004; Morse et al., 2002; Yang et al., 2006). It is speculated that such RNA modifications may affect a variety of RNA functions, including splicing, localization, translation efficiency and stability.

Except for tRNAs, all A-to-I RNA editing events are catalyzed by a family of enzymes known as adenosine deaminases that act on RNA (ADARs) (Bass *et al.*, 1997). ADARs were initially identified as the activity to “unwind” dsRNA when injected into *Xenopus* oocytes (Bass and Weintraub, 1988), resulting from the conversion of stable A-U base-pairs to unstable I-U base-pairs (Wagner *et al.*, 1989). Based on this activity, the first ADAR was purified from *Xenopus* (Hough and Bass, 1994). Later, the mammalian homologue was identified and named ADAR1 (Kim *et al.*, 1994b). Meanwhile, A-to-I conversion was discovered in transcripts encoding the second subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype glutamate receptor (GluR-2) and this modification was shown to alter the ion permeability of heteromeric glutamate receptors (Rueter et al., 1995; Sommer et al., 1991). These studies lead to the

Table 2. A summary of characterized A-to-I RNA editing events.

RNA Substrate	protein function	Functional consequences
Vertebrate		
ADAR2	A-to-I RNA editing enzyme	Alteration in splicing pattern, proteine expression
GluR-2,3,4	Glutamate-gated cation channel subunits (AMPA subtype)	Alteration in calcium permeability and kinetics
GluR-5,6	Glutamate-gated channel subunit (Kainate subtype)	Alteration in calcium permeability
5-HT_{2C}R	G-protein coupled receptor	Reduction in G-protein coupling
Kv1.1	votage-gated potassium channel	Faster recovery from inactivation
ETB	endothelin receptor	unknown
PTPN6	phosphatase	unknown
FLNA	Filamin, alpha	unknown
BLCAP	bladder cander associated protein	unknown
CYFIP2	cytoplasmic FMR2 interacting protein	unknown
IGFBP7	insulin-like growth factor binding protein 7	unknown
Invertebrate		
4f-rnp	unknown	unknown
Para	sodium channel	unknown
Da5	nACh receptor subunit	unknown
ARD	nACh receptor subunit	unknown
SBD	nACh receptor subunit	unknown
Rdl	GABA receptor	unknown
DSCI	sodium channel	unknown
Ca-alpha1T	Calcium channel	unknown
DmCa1D	Calcium channel	unknown
$\alpha 2\delta$	Calcium channel accessory subunit	unknown
Shaker	votage-gated potassium channel	unknown
Eag	votage-gated potassium channel	unknown
Slowpoke	votage-gated potassium channel	unknown
synaptotagmin	synaptic vesicle calcium sensor	unknown
Dunc-13	SNARE protein	unknown
stnB	unknown	unknown
complexin	SNARE protein	unknown
Iap	adapter protein in synaptic release	unknown
Pop-1	C elegans HMG-box protein	unknown
Laminin-γ	unknown	unknown
SSADH	aldehyde dehydrogenase	unknown
sqKv1.1	votage-gated potassium channel	Alteration in channel gating and tetramerization
sqKv2	votage-gated potassium channel	unknown
virus		
Hepatitis delta virus HDAg	virus replication/release	Switch from HDAg-S (replication) to HDAg-L (release)
measle virus hemagglutinin	hemadsorption	generate nonlytic persistant virons

identification of the second ADAR family member, ADAR2 (Melcher *et al.*, 1996b). Subsequent biochemical purification and molecular cloning has allowed the identification and characterization of ADARs from variety of organisms, including three ADAR genes in mammals (Melcher *et al.*, 1996a), two ADR genes in *C. elegans* (Tonkin *et al.*, 2002), one dADAR gene in *Drosophila* (Palladino *et al.*, 2000b; Slavov *et al.*, 2000) (Figure 4).

Functional domains of ADARs

ADAR proteins from different organisms have a common modular domain organization, including a variable N-terminal region followed by one or several copies of dsRBMs and one highly conserved adenosine deaminase domain at the C-terminus.

The N-terminal region

Full-length ADAR1 contains two Z-DNA binding motifs, which specifically binds to the left-handed Z-DNA/Z-RNA with high affinity (Herbert *et al.*, 1998). The exact role of these Z-DNA binding motifs in ADAR1 is not fully understood, although some data have suggested that ADAR1 editing activity can be modulated by Z-RNA in vitro (Koeris *et al.*, 2005). In ADAR3, an arginine-rich single-stranded RNA (ssRNA) binding domain is found in N-terminal region, making ADAR3 unique within the ADAR family by possessing the ability to bind to ssRNA (Chen et al., 2000; Melcher et al., 1996a).

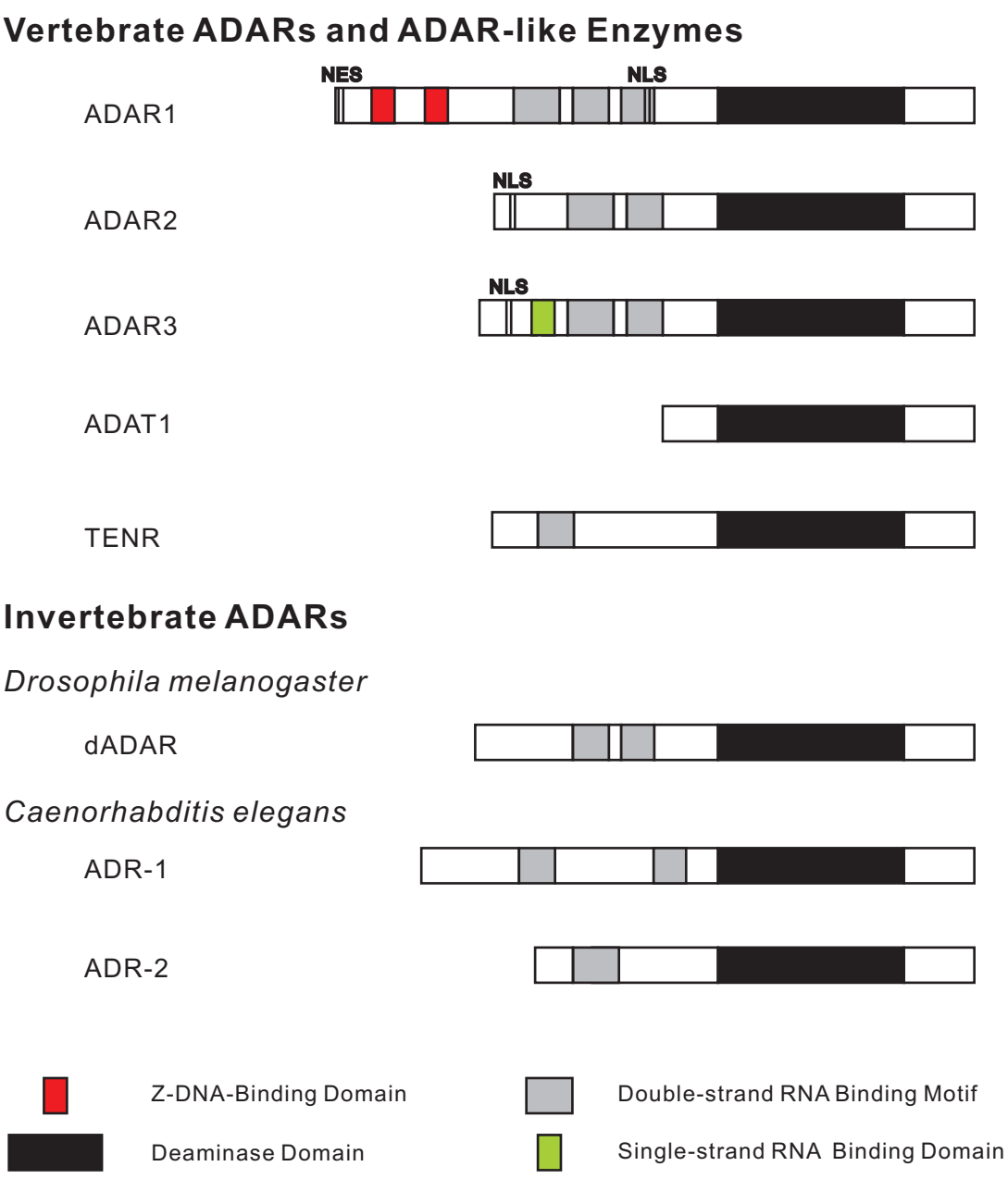

Figure 4. Functional domains of ADARs from different species. Schematic diagram of domain structures for ADAR proteins from vertebrates and invertebrates are presented to depict the organization and phylogenetic conservation of functional domains in the enzymes that catalyze A-to-I conversion in RNA substrates. The positions for nuclear export signal (NES) and nuclear localization signal (NLS) for vertebrate ADARs are indicated.

The dsRBMs

ADAR1 contains three copies of dsRBMs, while ADAR2 and ADAR3 each contain two copies. The dsRBMs provide ADARs with the ability to bind dsRNA over ssRNA, and consistent with this observation, ADAR-mediated editing can be found only within extended RNA duplexes (Bass, 2002; Higuchi et al., 1993; Rueter et al., 1999). The dsRBMs have also been demonstrated to be required to target nuclear ADAR1 or ADAR2 to the nucleolus in mammalian cell lines (Desterro *et al.*, 2003; Sansam *et al.*, 2003).

Although dsRBMs of ADARs are highly conserved in sequence and structure, they seem to play differential roles in regulating ADAR functions. While the deletion/mutation of the first and the third dsRBM from ADAR1 inhibited editing, the second dsRBM seemed to be dispensable (Lai *et al.*, 1995; Liu and Samuel, 1996). In addition, the dsRBMs can target ADAR1 to the chromosome in *Xenopus laevis* but individual dsRBMs are capable of recognizing distinct chromosome sites in an apparently specific manner (Doyle and Jantsch, 2003).

The deaminase domain

Phylogenetic studies have suggested that the catalytic mechanism of ADAR is similar to the ancestral mononucleotide cytidine deaminase (CDA) in *E. coli* (Carter, 1995). A recently structure study of the human ADAR2 deaminase domain confirmed this similarity (Macbeth *et al.*, 2005). The active site has an ordered zinc ion that coordinates a water molecule, presumably displacing ammonia during the deamination reaction. The positions of the zinc ion coordinated by His 394, Cys 451, and Cys 516, and hydrogen bonding of the

water molecule by the proton-shuttling residue, Glu 396, are essentially identical to the geometry seen at the catalytic centers of CDA (Figure 5A and B). The residues that are important to form the active site are also highly conserved throughout the ADAR family and ADAT, which catalyzes A-to-I editing of tRNAs, further supporting the existence of a conserved catalytic mechanism (Gott and Emeson, 2000) (Figure 5C). Presumably, the zinc-bound water molecule will perform nucleophilic attack on the C-6 position of the adenine ring to generate a tetrahedral intermediate, followed by the release of ammonia to produce the inosine (Bass, 2002) (Figure 5D).

Surprisingly, an inositol hexaphosphate (IP_6) molecule is found buried within the enzyme core mostly surrounded by C-terminal residues. This feature is unique and is not presented in CDA. The ADAR2 expressed in an IP_6-deficient yeast strain was not active, suggesting that IP_6 is required for ADAR2 activity, most likely by defining and stabilizing the folding of the deaminase domain (Macbeth *et al.*, 2005).

Substrate specificity of ADARs

Numerous studies have been performed to understand the substrate specificity of ADARs and why they edit specific adenosines over others in RNA targets. Some of the specificity comes from the RNA substrates themselves as all editing sites are found in highly structured imperfect RNA duplexes (Figure 6). RNA duplex structures are required for editing since disruption of the secondary structure ablates the A-to-I conversion (Bhalla et al., 2004; Burns et al., 1997;

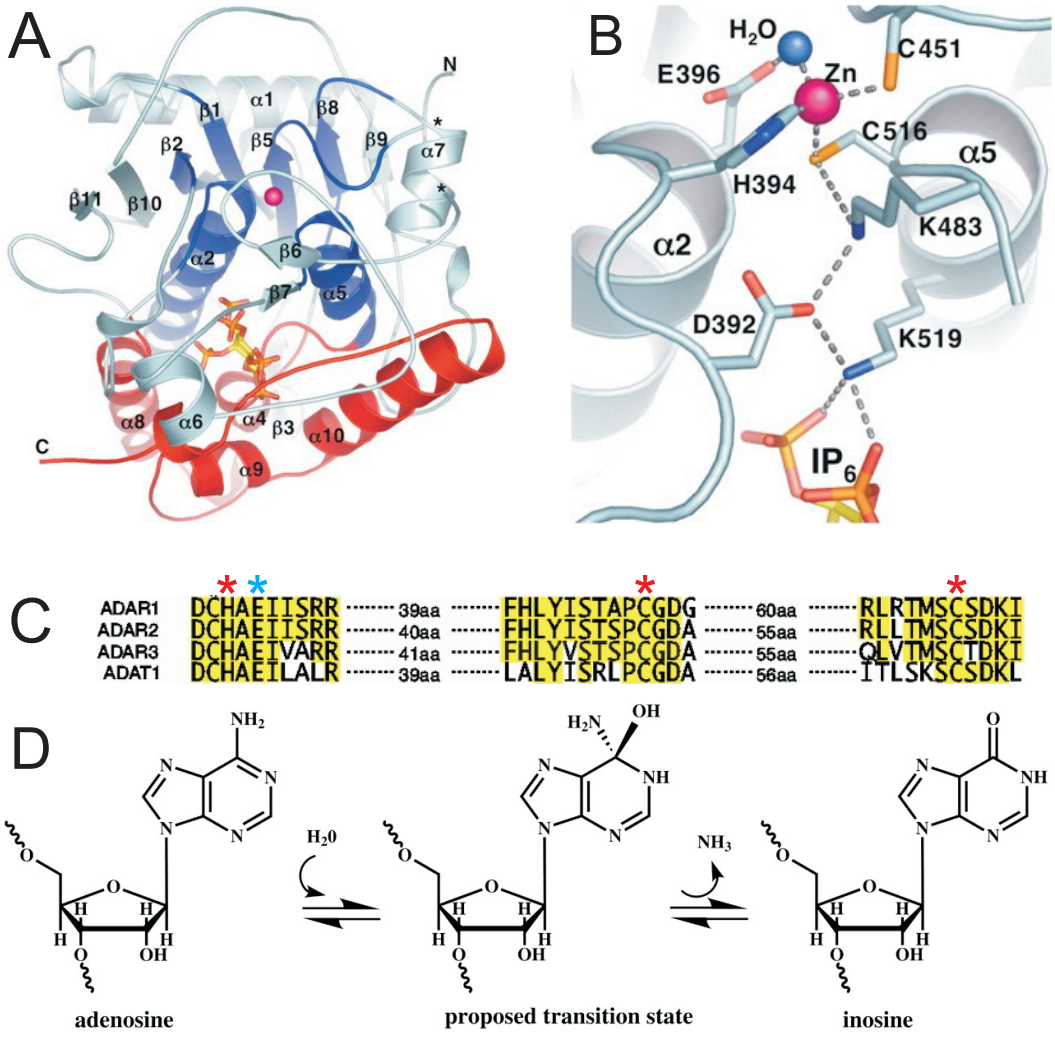

Figure 5. The conserved mechanism for deamination. (A) Ribbon model of human ADAR2 deaminase domain. The zinc atom is represented by a magenta sphere. The N-terminal domain is colored cyan, with the region that shares structural similarity with CDA and TadA colored dark blue. The C-terminal helical domain, which with contributions from the deamination motif makes the major contacts to IP_6, is colored red. (B) Residue interactions at the active site. Shown are the zinc ion, coordinating residues, the nucleophilic water (blue sphere), and the proposed proton-shuttling residue, E396. The hydrogen-bond relay that connects the active site to the IP_6 is also indicated. (C) An amino acid sequence comparison within the catalytic domain of ADARs and ADAT proteins is presented with asterisks indicating the residues thought to be critical for active-site zinc coordination. (D) The stereochemistry of the proposed tetrahedral intermediate. The intermediate is drawn as if water attacks from the same side of the base as observed with the CDA enzymes, (adapted from Bass, 2002; Gott and Emeson, 2000 and Macbeth et al., 2005).

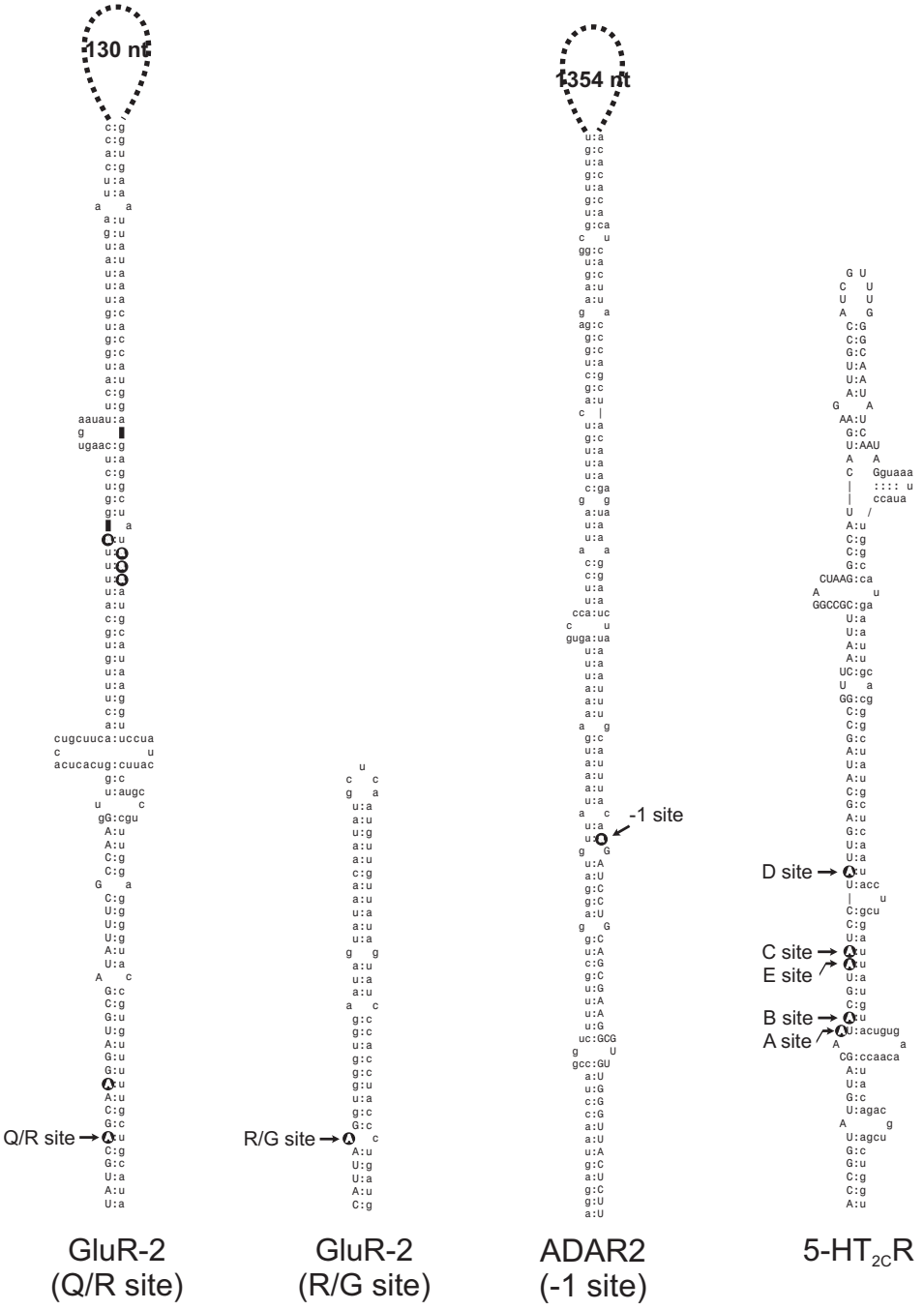

Figure 6. Predicted secondary structures for pre-mRNA substrates modified by A-to-I editing. Double stranded RNA structures are required for ADAR-mediated editing. The predicted secondary structure of pre-mRNA transcripts encoding non-NMDA receptor subunits (GluR-2), ADAR2 and the 2C-subtype of serotonin receptor (5-HT_{2C}R), in the regions of major editing modifications, are presented using an RNA folding algorithm [*mfold*; Science **244**:48-52 (1989)]. The positions of edited adenosine residues are indicated with inverse lettering; exon and intron sequences are represented with uppercase and lowercase lettering, respectively, and the number of nucleotides omitted from the figure are indicated in the loops.

Feng et al., 2006; Higuchi et al., 1993; Rueter et al., 1999). The detailed structure of the ADAR target also influences editing specificity. Adenosines in a long, perfect A-form RNA duplex can be deaminated non-selectively (Nishikura *et al.*, 1991; Polson and Bass, 1994); while naturally-occurring RNAs with mismatches, bulges, or loops within a duplex region undergo more selective deamination. It was suggested that small disruptions within the duplex contribute to ADAR selectivity by limiting the ability of these enzymes to bind short, perfect-matched A-form RNA duplex regions (Lehmann and Bass, 1999). In RNA duplexes, all the bases are surrounded by the sugar-phosphate backbone and are largely inaccessible to proteins. It is speculated that the one adenosine needs to flip out of the duplex to be edited by ADARs (Hough and Bass, 1997). Recent molecular dynamic analysis has suggested that the adenosine at the R/G editing site in GluR-2 transcripts needs less energy to flip out compared to neighboring, editing-incompetent adenosines and this may contribute to determining the specificity of A-to-I conversion too (Hart *et al.*, 2005).

On the other hand, editing specificity also rises from the ADARs themselves as ADAR1 and ADAR2 have distinct but overlapping editing specificity. For example, on the pre-mRNA of GluR-2, the Q/R site is edited only by ADAR2 and the +60 site is edited primarily by ADAR1, although both ADARs can efficiently deaminate the R/G site (Melcher *et al.*, 1996b). Using a long perfect dsRNA as the substrate, ADARs have also been shown to have different preferred neighboring sequence for targeting. ADAR1 and ADAR2 both prefer a uridine as 5'-neighbor to the targeted adenosine. ADAR1 rarely edits adenosine moieties close to either

end of a synthetic dsRNA substrate, whereas ADAR2 can target adenosines as close as 3 nt from either terminus, and ADAR2 also has a unique 3' neighbor preference for uridine (Lehmann and Bass, 2000; Polson and Bass, 1994).

It is not fully understood whether the dsRBMs contribute to defining ADAR substrate specificity. Since a highly conserved deaminase domain is also found in ADAT, which can specifically edit adenosines in tRNA without the presence of any dsRBMs, it is believed that the deaminase domains themselves can confer both substrate specificity and activity. In agreement with this idea, ADAR1, lacking all three dsRBMs, showed editing activity and some specificity in tissue culture cells (Herbert and Rich, 2001). In another study, the dsRBMs of ADAR1 and ADAR2 were swapped, and the authors claimed that the deaminase domain is the major determinant of the specificity, since they observed that a chimeric protein containing the ADAR1 deaminase domain displayed similar specificity to ADAR1 while a chimeric enzyme containing the ADAR2 deaminase domain displayed similar specificity to ADAR2 (Wong *et al.*, 2001). In addition, the dsRBMs of ADARs have also been shown to have good affinity for nonspecific binding to long perfect dsRNA, enabling ADAR1 and ADAR2 to edit over 50% of adenosines on those dsRNAs (Cho et al., 2003; Dawson et al., 2004; Lehmann and Bass, 2000; Liu et al., 1999). Therefore, dsRBMs were thought to contribute to ADAR-mediated editing only by increasing the affinity to the substrates by nonspecific binding to perfect dsRNA regions.

However, recent biochemical work has demonstrated that the dsRBMs of ADAR2 can bind specifically on natural RNA substrates and may contribute to

editing specificity (Stephens *et al.*, 2004). In this study, each amino acid (M84 or T96) in dsRBM1 of rat ADAR2 was replaced by cysteine to direct hydroxyl radical cleavage upon binding to the target RNA. The cleavage sites on RNA therefore indicate the position(s) of these mutated amino acid residues on the RNA duplex (Figure 7A and B). The pattern generated was used to develop a model for the ADAR2 dsRBM1-RNA interaction (Figure 7C). To functionally test this model, benzyl modification at guanosine 2-amino groups was introduced at three sites on RNA, where two of them specifically block the binding of dsRBM1 ($A6G^{N2Bn}$, $A12G^{N2Bn}$) and the other one does not ($G9G^{N2Bn}$) (Figure 7D). The mutation of $A6G^{N2Bn}$, $A12G^{N2Bn}$ dramatically decreased ADAR2 editing efficiency while $G9G^{N2Bn}$ had no effect, supporting their specific binding model and further suggesting the specific binding of dsRBMs may contribute to substrate specificity (Stephens *et al.*, 2004).

Functional consequences of specific A-to-I editing events

After the targeted adenosines are converted to inosines by ADARs in the nucleus, the modified transcripts are transported into the cytoplasm where they serve as templates for translation. Several extensively studied A-to-I modifications result in non-synonymous codon changes in mRNA sequences and the production of proteins with altered functional properties. Examples in this category include the editing of transcripts encoding subunits of ionotropic glutamate receptors (GluR-2 to 6) and the 2C-subtype of serotonin receptor (5-HT_{2C}R) (Burns et al., 1997; Niswender et al., 1999; Sommer et al., 1991) (Table 2).

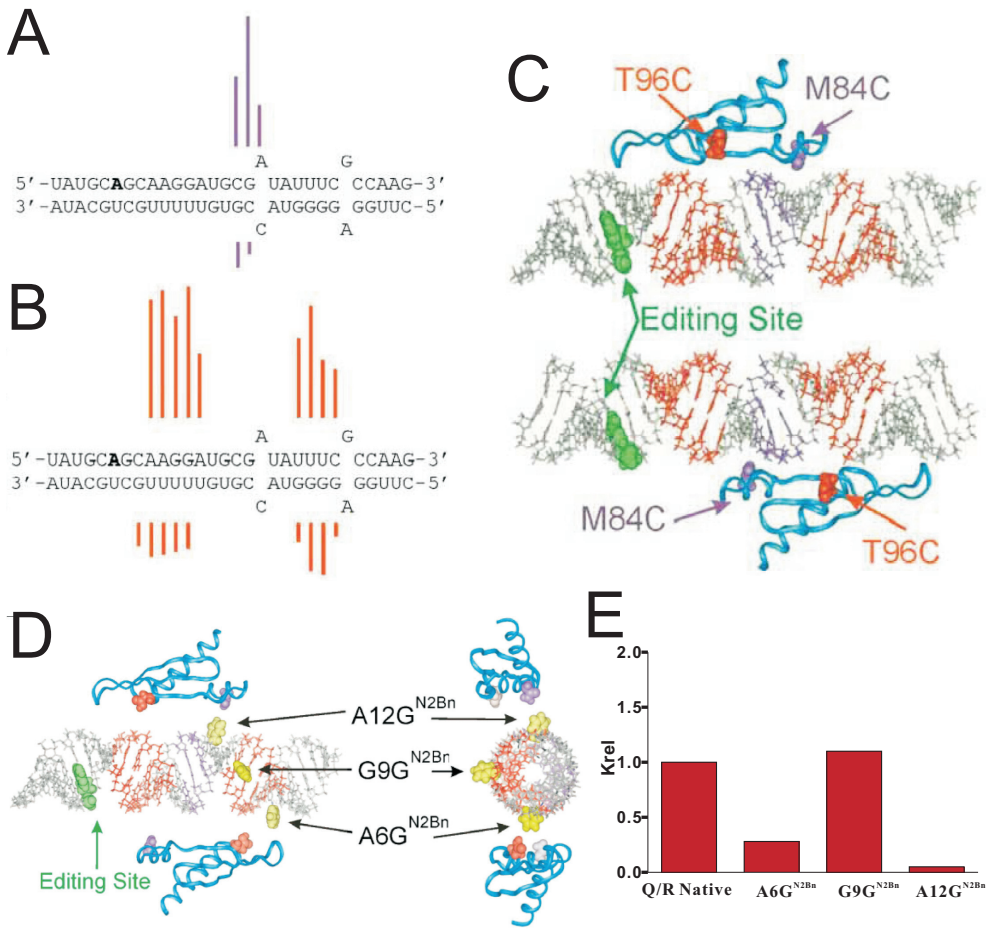

Figure 7. Specific interaction of ADAR2 dsRBM1 and GluR-2 RNA (Q/R site). (A) Directed hydroxyl radical cleavage of the Q/R substrate using the EDTA•Fe modified M84C mutant. The mapping of the major cleavage sites on the Q/R substrate secondary structure is shown. Lines indicate sites of cleavage and line lengths indicate relative cleavage efficiencies. (B) Directed hydroxyl radical cleavage of the Q/R substrate using of T96C-EDTA•Fe. (C) Models generated in Insight II using the α carbon skeleton from the Xlrbpa dsRBM II structure to model potential binding sites identified by the cleavage experiments. Nucleotides cleaved by M84C-EDTA•Fe are highlighted in purple. Nucleotides cleaved by T96C-EDTA•Fe are highlighted in red. The Q/R editing site is depicted in green. (D) The relative location of the benzyl modifications to the two binding sites for ADAR2 dsRBMs are depicted in yellow. (E) A bar graph describing the relative rate constants for deamination assays on the GluR-B Q/R editing site substrate analogs. All rates are normalized to that of the native Q/R substrate,(adapted from Stephens *et al.*, 2004).

Recent studies also demonstrate that A-to-I RNA editing in non-coding regions can affect alternative splicing pattern or the nuclear retention of modified RNA transcripts (Kumar and Carmichael, 1997; Prasanth et al., 2005; Rueter et al., 1999).

RNA editing on Q/R site of GluR transcripts

Glutamate is the major excitatory neurotransmitter in the CNS of the vertebrates and is critical for fast excitatory neurotransmission, synaptic plasticity and has been shown play a role is both chronic and acute neural disorder including stroke, epilepsy, amyotrophic lateral sclerosis and Parkinson's Disease (Wisden and Seeburg, 1993). The impact of glutamate is mediated by either ionotropic (iGluR) or metabotropic receptors (mGluR) (Ozawa *et al.*, 1998). The ionotropic glutamate receptors can be divided to into three subtypes, AMPA, NMDA and kainate, based upon specific agonist for each receptor subtype. They are all tetrameric cation channels and four subunits contribute to the inner channel lining a pore loop structure (Seeburg and Hartner, 2003). A-to-I editing events have been identified in transcripts encoding 5 subunits (GluR2-6) of the GluRs which are involved in the assembly of AMPA or kainate receptors, but not the NMDA receptor.

Editing of the Q/R site in GluR-2 transcripts represents the earliest and the most well characterized example of A-to-I editing in mammals. The glutamine residue, located in the second hydrophobic domain of the GluR-2 subunit, is essential for forming the narrow constriction of the channel and in determining the ion permeation and electrophysiologic properties of heteromeric AMPA receptors.

RNA editing by ADAR2 converts the genomically encoded CAG (glutamine, Q codon) into CIG and translated as CGG (arginine, R codon), which makes the channel impermeable to calcium ions and also helps the channel assemble in the endoplasmic reticulum (Greger et al., 2003; Higuchi et al., 2000; Melcher et al., 1996b; Sommer et al., 1991) (Figure 8A). The Q/R site is edited essentially to completion in whole brain, except subsets of striatal and cortical neurons. Decreased Q/R site editing in these neurons is consistent with their high vulnerability to excitotoxicity due to calcium excess (Kim *et al.*, 2001). Genetically modified mice expressing one copy of an editing-incompetent GluR-2 allele die of epileptic seizure three weeks after birth, further emphasizing the importance of Q/R site editing for normal brain function (Brusa *et al.*, 1995). Interestingly, ADAR2-null mice demonstrated a very similar phenotype to mice lacking Q/R site editing. The adverse effects observed in ADAR2-null mice can be rescued by introducing mutant GluR-2 alleles which encode an arginine residue at the Q/R site, suggesting the seizure and lethal phenotype in ADAR2-deficient mice is primarily due to the lack of editing at the GluR-2 Q/R site (Higuchi *et al.*, 2000). It is still puzzling as to why evolution has developed editing at the Q/R site rather than encoding an arginine-codon in the genomic DNA, since mice expressing GluR-2 with a genomically encoded arginine at this site demonstrated no discernable phenotype (Kask *et al.*, 1998).

At the analogous position to the GluR-2 Q/R site, Q/R site editing has also been discovered in transcripts encoding GluR-5 and GluR-6, the subunits of heteromeric kainate receptors. Unlike GluR-2 Q/R site, which is edited almost

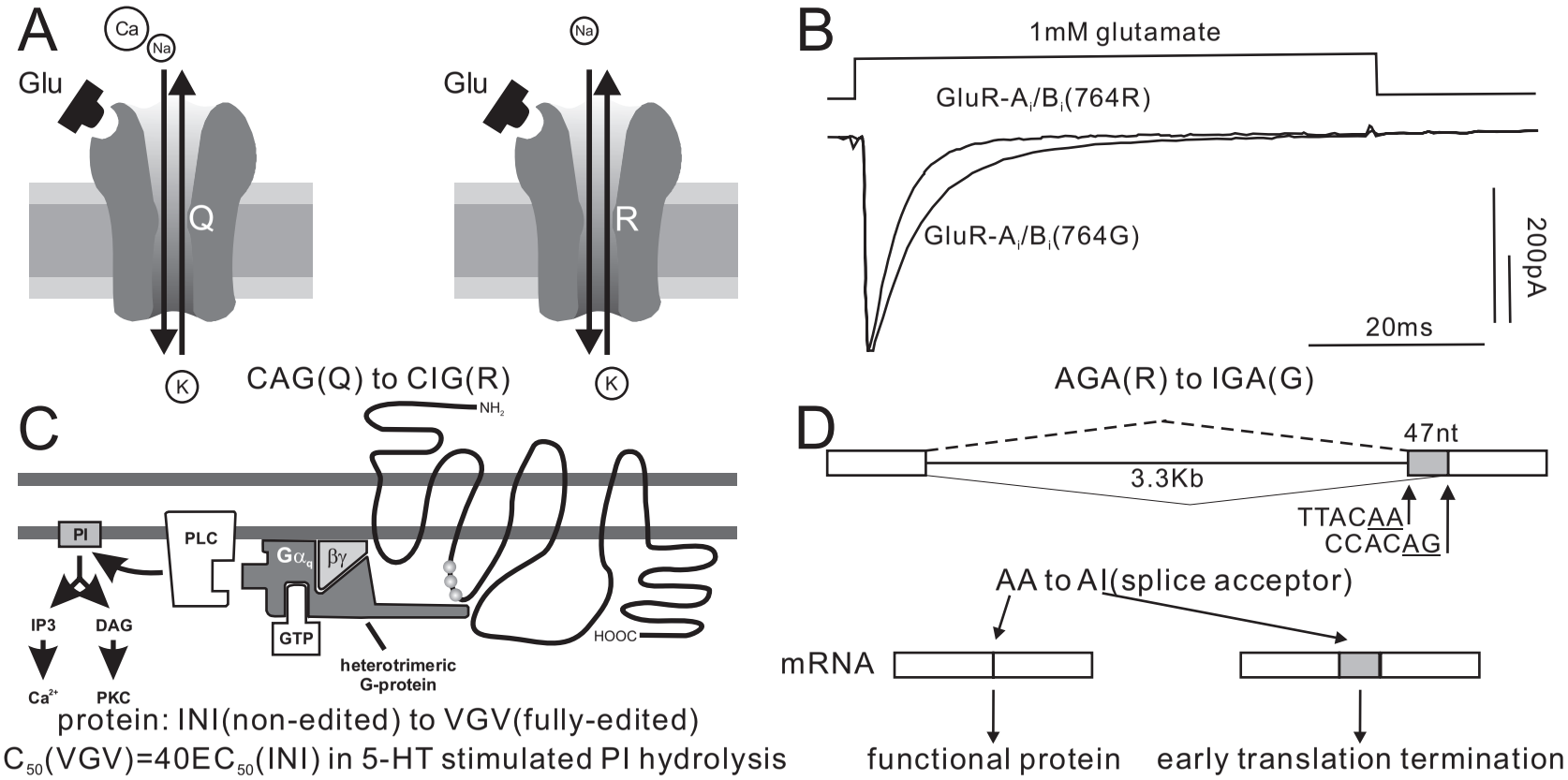

Figure 8. The functional consequences of A-to-I RNA editing. (A) A cartoon diagram of the ion permeation properties of heteromeric AMPA receptor containing GluR-2 subunits encoded by the nonedited (Q) and edited (R) transcripts, respectively. (B) A current trace indicating the recovery of AMPA receptors containing GluR-2 subunit encode by nonedited (R) or edited (G) transcripts at the 764 amino acid position. (C) A schematic diagram of the predicted topology of the 5-HT_{2C} receptor and signaling pathway. The approximate positions of amino acid alterations within the second intracellular loop of the receptor, resulting from RNA editing, are indicated. (D) A schematic diagram showing an alteration in splicing pattern and protein expression due to the editing at -1 site, (B, adapted from Lomeli *et al*., 1994).

completely, both nonedited and edited isoforms of the GluR-5 and GluR-6 RNAs are expressed and the editing at these sites is developmentally regulated. Mutant mice in which the GluR-6 Q/R site cannot be edited display NMDA receptor-independent long-term potentiation (LTP) in the medial perforant path-dentate gyrus synapse. Both heterozygous and homozygous mutant animal were more vulnerable to kainate-induced seizures, indicating a role for GluR-6 Q/R site editing in synaptic plasticity and circuit excitability (Vissel *et al.*, 2001).

RNA editing of the R/G site in GluR transcripts

A-to-I RNA editing was also been found in another RNA duplex structure of the GluR-2 transcript, referred as R/G site, since the adenosine to inosine conversion replaced the genomically encoded AGA (arginine, R codon) with IGA (glycine, G codon). The AMPA receptor containing the edited GluR-2 subunit recovers more rapidly from receptor desensitization than receptors harboring the non-edited isoform, demonstrating that RNA editing at R/G site plays a role in regulating receptor kinetics (Lomeli *et al.*, 1994) (Figure 8B). Recent in vitro studies also have suggested that R/G site editing affects the splicing of GluR-2 transcripts since the editing site is next to a splice junction (Bratt and Ohman, 2003). The R/G site of GluR-2 pre-mRNA is often used as a model system to study A-to-I editing as it forms a small and conserved 70 nt stem-loop with three mismatches (Aruscavage and Bass, 2000; Macbeth et al., 2004; Ohman et al., 2000; Stefl et al., 2006; Stephens et al., 2000) A similar R/G site editing was identified on GluR-3 and GluR-4, yet the functional consequences of these modifications remain unknown.

RNA editing of 5-HT_{2C}R transcripts

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that modulates numerous sensory and motor processes as well as a wide variety of behavior including locomotion, thermoregulation, pain perception, sleep, appetite and sexual behavior (Sanders-Bush *et al.*, 2003). 5-HT exerts its biological functions by activating at least 14 distinct receptor subtypes that differ in their tissue distribution, binding affinity and coupling to intracellular signaling pathways (Hoyer *et al.*, 1994). The 5-HT_2 family of receptors contains three members (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R), all of which mediate Gq-coupled phospholipase C (PLC) activation, which leads to the increasing intracellular inositol phosphates (IPs) and diacylglycerol (DAG) (Figure 8C). In addition, it is reported that both 5-HT_{2A}R and 5-HT_{2C}R can activate phospholipase D (PLD) and phospholipase A2 (PLA2) through interactions with other G proteins (Sanders-Bush *et al.*, 2003).

5-HT_{2C}R is the only known GPCR that undergoes A-to-I RNA editing. In its pre-mRNA five genomically encoded adenosines can be converted into inosines (termed sites A-E), generating up to 32 mRNA species, encoding as many as 24 different protein isoforms (Burns *et al.*, 1997; Niswender *et al.*, 1999). Sequence analysis of cDNAs from rat and human brains showed the region-specific expression of editing-generated 5-HT_{2C}R mRNA species, suggesting differentially edited 5-HT_{2C}R may serve distinct biological functions in those regions where they are expressed (Burns *et al.*, 1997; Niswender *et al.*, 1999). The five editing sites can affect the identify of three amino acids in the second intracellular loop of the receptor, a region interacting with heterotrimeric G-protein (Pin et al., 1994; Wong

et al., 1990). The functional consequences of RNA editing in the 5-HT_{2C}R transcript were revealed by comparing the potency of 5-HT to induce PLC activation in a heterologous tissue culture system, where the nonedited 5-HT_{2C}R couples to the G-protein 40-fold greater than the fully edited isoform. It has also been reported that the fully edited receptor isoform has reduced constitutive activity, compared to the non-edited receptor (Niswender *et al.*, 1999; Price *et al.*, 2001).

RNA editing on -1 site of ADAR2 pre-mRNA transcript

ADAR2 is a dsRNA specific adenosine deaminase that catalyzes numerous A-to-I editing events in mammals (Higuchi et al., 2000; Melcher et al., 1996b). Interestingly, A-to-I RNA editing sites were also discovered within intron 4 of the ADAR2 pre-mRNA, one of which (-1 site) has been shown to regulate alternative splicing of the ADAR2 transcript itself (Dawson et al., 2004; Rueter et al., 1999).

In the absence of RNA editing, exon 3 will be spliced to exon 5 using the genomically-encoded 3'-splice acceptor (AG) immediately 5' to the exon, subsequently producing a mature mRNA that encodes a functional ADAR2 protein (Figure 8D, solid line). Forty-seven nucleotides upstream of the normal 3'-splice junction, an AA dinucleotide can be converted to an AI by RNA editing and serve as a new 3'-splice acceptor. Editing at the -1 site allows the use of this proximal 3'-splice site and results in the addition of 47 nt to the ADAR2 open reading frame (Figure 8D, dotted line). The 47nt inclusion results in early translation termination and generates a 9kD protein without any functional domains required for A-to-I conversion (Rueter *et al.*, 1999). Subsequent studies

revealed that editing of the -1 site is catalyzed by ADAR2 itself (Dawson *et al.*, 2004), suggesting an autoregulatory negative-feedback mechanism by which ADAR2 regulates its own level of expression (Feng et al., 2006).

Physiological significance of ADARs

The conversion of A-to-I by RNA editing has been shown to modulate the functions of specific proteins by sequestering mRNA in the nucleus, altering codons, or changing alternative splicing patterns. However, the functions of ADARs may have been underestimated since most of the newly identified adenosine to inosine editing events have been located in intronic or untranslated regions of RNA transcripts and the relevance of such modifications has not been examined (Levanon et al., 2004; Morse et al., 2002). In addition, ADARs can potentially modulate the functions of other dsRBP functions by competing for interactions with other targeted dsRNAs (Chen *et al.*, 2000). Furthermore, ADARs may also play a role in the processing and targeting of siRNAs and microRNAs (Luciano et al., 2004; Yang et al., 2006; Yang et al., 2005). Therefore, genetically modified animals, in which ADARs are deleted or overexpressed, can provide more insights into the physiological roles of these editing enzymes.

Mammalian ADARs

In mammals three ADAR genes have been identified. ADAR1 and ADAR2 are ubiquitously expressed, while ADAR3 is exclusively detected in the brain (Chen *et al.*, 2000). The greatest level of ADAR2 expression is found in the brain, consistent with the observation that brain mRNA contains the highest inosine

content compared to other tissues (Paul and Bass, 1998). All three ADARs contain nuclear localization signals (NLS) and are localized to the nucleus, with the exception of the interferon(IFN)-inducible isoform of ADAR1, which also harbors a nuclear export signal (NES) in the N-terminus and shuttles between the nucleus and cytoplasm (George and Samuel, 1999; Kawakubo and Samuel, 2000; Strehblow *et al.*, 2002). All three ADARs contain a conserved adenosine deaminase domain, but only ADAR1 and ADAR2 demonstrate editing activity. ADAR3 is enzymatically inactive on both synthetic RNA duplexes or naturally-occurring A-to-I editing substrates, although it can efficiently bind to both ssRNA and dsRNA (Chen et al., 2000; Melcher et al., 1996a). ADAR3 can compete with ADAR1 or ADAR2 on natural substrates in vitro, suggesting that it may work as a modulator of A-to-I editing in the brain (Chen *et al.*, 2000).

Systematic screening for novel A-to-I editing events in the human transcripsome have revealed that the vast majority of editing sites occur in non-translated RNA species and UTRs of mRNA transcripts, especially in Alu repetitive elements, which comprising more than 10% of human genome (Athanasiadis *et al.*, 2004; Blow *et al.*, 2004; Levanon *et al.*, 2004). Since inverted Alu elements in the same transcript can form a stable, extended RNA duplex, the only known requirement for ADAR editing, it is not surprising to find extensive RNA editing events in these regions. It has been speculated that editing in the UTR of RNA transcripts may affect RNA stability, localization and translation efficiency (Kumar and Carmichael, 1997; Prasanth et al., 2005; Scadden, 2005).

ADAR1- and ADAR2-deficient mice have been generated by gene-targeted

homologous recombination, and both die during development. As early as embryonic day 11.5 (E11.5), mouse embryos lacking ADAR1 expression show a rapid disintegration of liver structure, severe defects in embryonic erythropoiesis and stress-induced apoptosis, suggesting ADAR1 is critically important for the development of non-nervous tissues, although the exact mechanism and RNA targets remain unknown (Hartner *et al.*, 2004; Wang *et al.*, 2004). In contrast, the knockout of ADAR2 in mice does not cause embryonic lethality, but rather, these mice develop progressive seizures after birth and die before postnatal day 21 (P21). ADAR2-null mice, demonstrated a significant decrease in the extent of editing at numerous sites including the R/G site of GluR-2, the Q/R site of GluR-5 and the D site of 5-HT_{2C}R transcripts, it has been shown the lethal phenotype primarily results from a lack of editing at the GluR-2 Q/R site (Higuchi *et al.*, 2000).

Drosophila dADAR

dADAR, the only ADAR gene identified in *Drosophila*, contains two dsRBMs and one deaminase domain, sharing 42% amino acid homology with the human ADAR2 protein (Palladino *et al.*, 2000a).

Mutant flies lack dADAR expression have been generated by transposon-mediated P-element excision (Palladino *et al.*, 2000b). These flies show a slight decrease in viability under ideal growth condition, and the only morphological defect observed in young animals was a structural defect in the retina. However, adult mutant flies demonstrated uncoordination and paralysis with severe motion defects (Palladino *et al.*, 2000b). Furthermore, vacuolated lesions developed in the brain of the mutant flies by day 30, and the lesions, as

well as the behavioral defects, become more severe during aging (Chen *et al.*, 2004). The behavioral similarity between dADAR null flies and flies containing mutations in specific ion channels, together with the later discovery of A-to-I editing within RNAs encoding these ion channels, suggested that the primary role of dADAR is to modulate neurotransmission in the CNS (Hoopengardner et al., 2003; Littleton et al., 1999). Recent studies also found that dADAR null flies have a prolonged recovery from anoxia stupor and changes in gene expression for reactive oxygen species (ROS) scavengers, indicating that dADAR may have additional functions in *Drosophila* (Chen *et al.*, 2004). An A-to-I editing event catalyzed by dADAR has also been found on dADAR mRNA transcript itself, where editing results in the conversion of a highly conserved serine residue within the deaminase domain into a glycine, with decreased enzymatic activity (Keegan *et al.*, 2005).

C. elegans ADRs

Two ADAR genes have been identified in the *C. elegans* genome, ADR-1 and ADR-2. The ADR-1 protein contains two dsRBMs and one atypical deaminase domain lacking the zinc-coordination and proton-shuttling residues. In contrast, the ADR-2 protein has one dsRBM and a highly conserved catalytic domain. ADR-1 deficient strains of *C. elegans* have severely reduced editing activity while the deletion of ADR-2 totally eliminates A-to-I conversion, suggesting that ADR-2 can function alone, but that ADR-1 may need ADR-2 for activity and they may edit the targeted adenosines as a heterodimer (Tonkin *et al.*, 2002). *C. elegans,* which are deficient in either one or both ADR genes, displayed severe chemotaxis

defects (Tonkin *et al.*, 2002). Interestingly, this defect can be rescued by loss of function mutations in genes involved in RNA interference pathway, suggesting the chemotaxis defect was caused by the aberrant RNAi activity normally inhibited by ADRs (Tonkin and Bass, 2003).

Regulation of ADAR function

ADAR-mediated RNA editing is under spatiotemporal regulation through a variety of mechanisms to satisfy variations in demand from the cellular environment (Gurevich *et al.*, 2002; Yang *et al.*, 2004). The inaccuracy of tight control over this process could result in a number of physiological alterations including epilepsy and lethality.

Transcriptional regulation

Two ADAR1 isoforms, different in their expression patterns, domain composition, subcellular localization and function, are the result of alternative promoter use. Initially, two alternative exon 1 structures were identified in the human ADAR1 gene using 5'-rapid amplification of cDNA ends (RACE) analysis (George and Samuel, 1999). Using the start codon in exon 1A, the exon1A-exon2-containing mRNA encodes an interferon-inducible 150kD protein (p150), which contains two Z-DNA binding domains at the N-terminus, three dsRBMs and one C-terminal deaminase domain. The NLS overlapping with the third dsRBM and the NES at the N-terminus gives p150 the capability to shuttle between the cytoplasm and nucleus. The exon1B-exon2-containing mRNA, using

the start codon in exon2, is translated into a constitutively expressed 110kD protein (p110), which contains an N-terminal truncation compared to p150. The region missing in p110 contains the first Z-DNA binding domain and NES, which subsequently restricts p110 to the nucleus (Patterson and Samuel, 1995; Strehblow *et al.*, 2002). Recently, a very similar alternative transcription initiation mechanism has been reported for the mouse ADAR1 gene (George *et al.*, 2005).

Based on the genomic location of exon1A and exon1B, together with the transiently transfection analysis using reporter constructs, two functional human promoters were identified. Pc is the promoter for the constitutional expression of exon1B-exon2 mRNA, which generates ADAR1-p110; Pi is the promoter for the IFN-inducible expression of exon1A-exon2 mRNA, which generates ADAR1-p150 (George and Samuel, 1999). The Pi promoter possesses an IFN-stimulated response element (ISRE) responsible for IFN-inducibility, as well as an upstream kinase conserved sequence-like (KCS-I) element, which has previously been found as an important IFN-responsive element in the promoter of PKR (Markle *et al.*, 2003).

Alternative splicing

Alternative splicing is an important mechanism to produce functionally different protein isoforms from a single RNA transcript. Both ADAR1 and ADAR2 are subject to this regulation. Three naturally occurring spliced isoforms of human ADAR1 have been identified by comparisons of genomic sequence and cDNA sequences (Liu *et al.*, 1997). The full-length ADAR1 protein was designated as

ADAR1-a; the isoform containing a 26 amino acid deletion between the third dsRBM and the deaminase domain is designated as ADAR1-b, while ADAR1-c refers to the isoform possessing an additional 19 amino acid deletion between dsRBM2 and dsRBM3 compared to the ADAR1-b isoform. All three isoforms are expressed in human kidney, while only ADAR1-a and ADAR1-b were detected in a human placenta library. On a synthetic long RNA duplex, all three ADAR1 isoforms displayed similar enzymatic activity. However, site-directed mutagenesis of the dsRBMs revealed that the shortening of the spacers between functional domains altered the relative importance of the dsRBMs in ADAR1 (Liu *et al.*, 1997). Analyses of the editing activity for these splicing variants on natural substrates demonstrated that the ADAR1-b and c isoforms had a consistently higher activity than the ADAR1-a isoform on the 5-HT_{2C}R A site (Liu *et al.*, 1999), suggesting that the alternative splicing could contribute to the regulation of ADAR1 cellular activity.

Multiple alternative splicing events have also been found in transcripts encoding ADAR2. The first such splicing event affects the deaminase domain, where alternative splicing results in a 40 (human) or 10 (rat, mouse) amino acid insertion between the second and the third zinc-coordination motifs. This insertion in the catalytic domain has been shown to produce a 2-fold increase in ADAR2 editing activity (Gerber *et al.*, 1997; Rueter *et al.*, 1999). A second alternative splicing event changes the C-terminus of the human ADAR2 protein, where the last 29 amino acids of the functional protein are replaced by a 2 amino acid tail, making the resultant ADAR2 protein inactive. It is speculated this inactive splicing

variant may play a regulatory role in ADAR2 cellular function (Lai *et al.*, 1997), yet studies to address this issue have not been performed.

ADAR autoediting

Exon 7 of the *Drosophila* dADAR mRNA can form an imperfect RNA duplex, and within this duplex one adenosine can be specifically converted into inosine by dADAR itself (Keegan et al., 2005; Palladino et al., 2000a). This A-to-I editing event modifies an AGU codon into an IGU codon, changing the encoded serine residue (S) into a glycine (G). The serine is highly conserved across species and near the second zinc-coordination motif of the deaminase domain. The substitution of the serine with glycine has been shown to dramatically decrease enzymatic activity in vitro and in vivo on multiple substrates (Keegan *et al.*, 2005). More importantly, this autoediting is developmentally regulated, where the editing is low in embryonic and pupal mRNAs and increases by more than 40-fold from embryo to adult (Palladino *et al.*, 2000a). Ubiquitous expression in embryos and larvae of a dADAR transcript that is resistant to autoediting was lethal, suggesting the autoediting is critical for the normal development of *Drosophila* (Keegan *et al.*, 2005).

While autoediting in *Drosophila* changes the amino acid identity, the autoediting in vertebrates affects alternative splicing. ADAR2 can edit one adenosine (-1 site) within intron 4 of its own pre-mRNA and convert an AA to an AI dinucleotide, a noncanonical 3'-splice acceptor. The alternative splicing product using the ADAR2-dependent splice acceptor adds 47 nt of intronic

sequence into the mature ADAR2 mRNA, resulting in the translation of a 9kD protein lacking all of the functional domains required for A-to-I conversion (Dawson et al., 2004; Rueter et al., 1999) (Figure 9). Similar alternative splicing strategies are evolutionarily conserved in all vertebrates examined incluiding humans, rats, mice, chickens, and fish, indicating the biological importance of this process (Slavov *et al.*, 2000). In genetically-modified mice whose autoediting was ablated by disrupting the required RNA duplex structure, ADAR2 protein level increased up to 4-fold in a tissue-specific manner. Moreover, all known ADAR2 substrates displayed an elevated editing level, suggesting autoediting is a key regulator of ADAR2 expression (Feng et al., 2006).

Observations that both hyperediting and hypoediting are both lethal to an organism demonstrate the necessity to maintain homeostatic control of ADAR expression. Although different in details, dADAR and ADAR2 independently developed autoediting strategies to modulate their own level of activity. When ADAR protein is overexpressed, it will edit on its own transcript, producing a less active protein (dADAR) or a nonfunctional protein (ADAR2), and return the total ADAR activity back to normal.

Dimerization

The catalytic domain of ADAR is very similar to that of the *E. coli* CDA and APOBEC-1. Homodimerization is essential for the enzymatic activity of cytidine deaminases, prompting investigations into ADAR dimerization. Employing sequential affinity chromatography and size exclusion column chromatography,

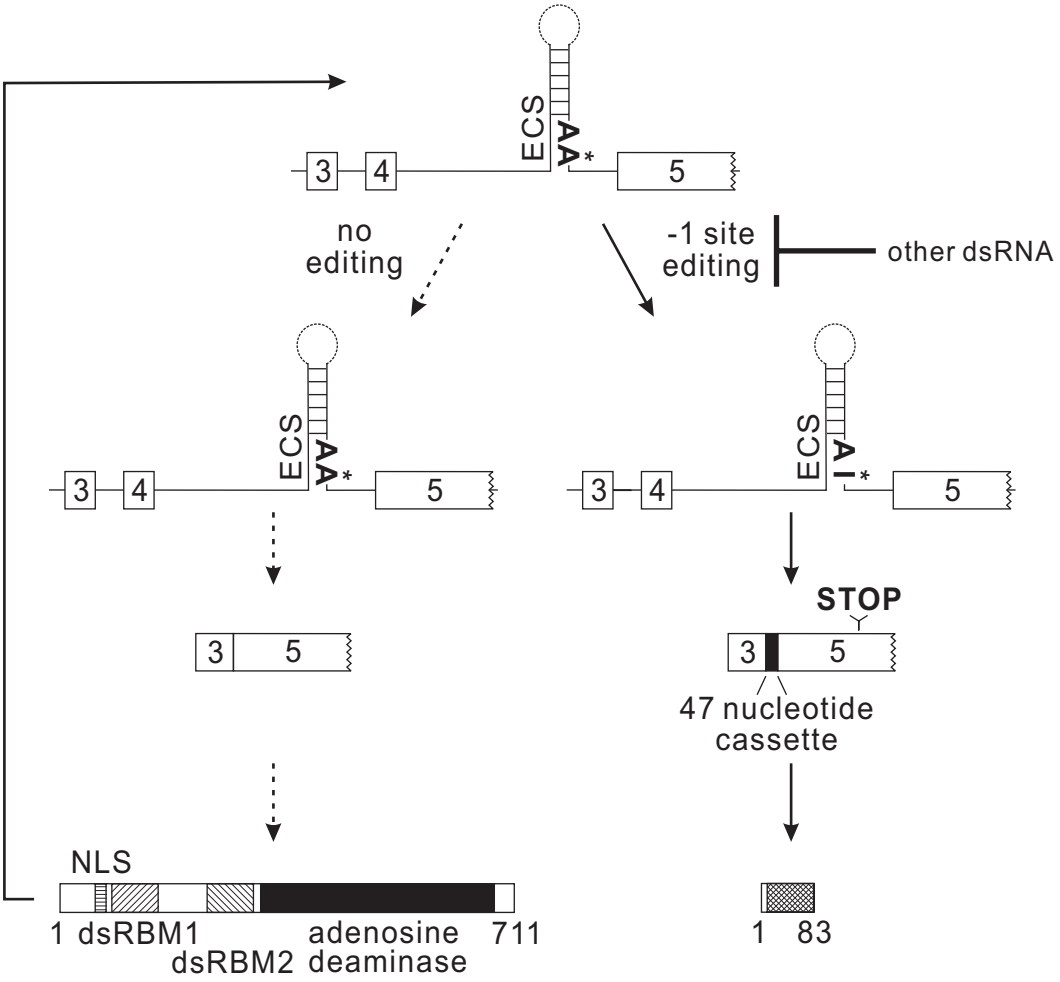

Figure 9. Model of ADAR2 autoregulation. A schematic diagram of the biosynthetic processes involved in the production of ADAR2 is presented, showing a region of the ADAR2 pre-mRNA between exon 3 and 5, the predicted RNA duplex required for A-to-I editing, and the position of the -1 site (*). The RNA processing pathway lacking -1 site editing, leading to the production of full-length (711aa) ADAR2 is indicated with dashed arrows; the functional domains in ADAR2 (NLS, nuclear localization signal; dsRBM, double-stranded RNA binding motifs and adenosine deaminase domain) are indicated. The biosynthetic pathway involving A-to-I conversion at the -1 site (*), leading to the production of a predicted 9 kD (83aa) protein, is indicated with solid arrows; the hatched box represents the amino acid sequences encoded by an altered reading frame resulting from proximal 3'-splice acceptor use and inclusion of an additional 47nt in the mature ADAR2 transcript,(adapted from Feng *et al.*, 2006).

the formation of complexes between differentially epitope-tagged ADAR monomers expressed in insect cells was tested (Cho *et al.*, 2003). It was found that both ADAR1 and ADAR2 form stable enzymatically active homodimers, while ADAR3 remains in a monomeric, enzymatically inactive form. In addition, no heterodimerization among different ADAR family members was detected. Interestingly, endogenous ADAR3 in brain extracts was found to form a homodimer, indicating the existence of a brain-specific mechanism for ADAR3 dimerization (Cho et al., 2003).

The kinetics of ADAR2 editing for the GluR-2 R/G site has been shown to be consistent with a reaction scheme in which the formation of an ADAR2-RNA ternary complex is required for efficient RNA editing and gel-shift analyses have revealed two complexes are formed on the RNA as protein concentration is increased. In addition, ADAR2 molecules have been cross-linked to one another in an RNA-dependent fashion, suggesting that ADAR2 functions as homodimer on their RNA substrates (Jaikaran *et al.*, 2002).

Taking advantage of yeast two hybrid system, dADAR has also been shown to form homodimers (Gallo *et al.*, 2003). The minimum region required for dimerization is the N-terminal region and the first dsRBM. Although dsRBM1 is required for both dsRNA binding and dimerization, these two functions can be uncoupled by deleting the N-terminus. The mutant protein retains the ability to bind dsRNA, but is monomeric and enzymatically inactive, indicating the dimerization is essential for editing but not dsRNA binding.

Although several studies have suggested ADAR may form homodimers, the

conclusions are not always consistent. While one study demonstrated that ADAR2 homodimerization was RNA-independent (Cho et al., 2003), a second study suggested it was RNA-dependent (Jaikaran et al., 2002). The first study showed that the ADAR homodimer is very stable and almost never dissociates, while the second study indicated that the homodimer only forms on RNA substrate and formation is transient. Therefore, the nature of the ADAR dimerization in vivo needs further validation.

Sumoylation

Sumoylation is a reversible and highly dynamic posttranslational modification in eukaryotes, where the targeted protein is conjugated with the small ubiquitin-like modifier, SUMO. It can affect targeted protein function by altering its subcellular localization, activity or stability. The modification process involves formation of an isopeptide bond between the C-terminus of SUMO and the amino group of a lysine side-chain of the targeted protein; while the removal of SUMO from proteins is carried out by specific cysteine proteases that have both hydrolase and isopeptidase activity. Three SUMO proteins have been identified as SUMO-1, -2 and -3 in vertebrates and sumoylation has been found in proteins such as RanGAP1, PCNA, IkBa, p53, c-jun, topoisomerases, promyelocytic leukemia protein, Sp100 and the MEKK1, most of which are nuclear proteins or proteins shuttling to the nucleus (Hay, 2005). In addition, since most enzymes involved in the SUMO pathway are also localized in the nucleus, it is believed that sumoylation is predominantly a nuclear process (Seeler and Dejean, 2003; Zhang

et al., 2002).

Subnuclear localization of SUMO-1 was found to overlap with that of the ADAR1, raising questions regarding whether ADAR1 is a target for SUMO-1 (Desterro *et al.*, 2005). Further analyses demonstrated that ADAR1 can be modified by SUMO-1 at lysine residue 418. Substitution of this lysine residue with an arginine abolished sumoylation without affecting the nucleolar localization of ADAR1. However, this substitution stimulated ADAR1 editing activity both in vitro and in vivo. Moreover, modification of ADAR1 by SUMO-1 in vitro reduced RNA editing activity, indicating that sumoylation is a novel posttranslational mechanism to regulate ADAR1 activity.

Nucleolar sequestration

While ADAR1 p150 can shuttle between the nucleus and cytoplasm (Strehblow *et al.*, 2002), ADAR1 p110 and ADAR2 are restricted to the nucleus. The nucleus is highly compartmentalized and the compartmentalization may play an important role in regulating protein function. ADAR1 p110 and ADAR2 have been demonstrated to highly concentrate in the nucleolus of mammalian cells, which is different from the proposed nucleoplasmic site where A-to-I editing is though to occur. The ADAR proteins dynamically shuttle between the nucleolus and nucleoplasm, suggesting that the nucleolar localization may represent a regulatory process to control ADAR cellular activity (Desterro *et al.*, 2003; Sansam *et al.*, 2003).

The compartmentalization of the nucleus

The separation of the cytoplasm and nucleus is achieved by a nuclear envelope, but such membrane barriers do not exist in the nucleus. Based on their structure and function, the nucleus can be largely divided into nucleolus and nucleoplasm.

Ribosomal RNA (rRNA) synthesis and ribosomal subunit assembly occur in the nucleolus of eukaryotic cells. The 45S pre-rRNA, transcribed by RNA polymerase I from the ribosomal DNA (rDNA), contains the 18S, 5.8S and 28S rRNAs with external and internal spacer sequences. After transcription, the pre-rRNA undergoes a series of cleavage events to release the 18S, 5.8S and 28S rRNAs. The fully processed rRNAs are then assembled with 5S rRNA, which is transcribed by RNA polymerase III in the nucleoplasm, and at least 80 proteins to form complete ribosomal subunits that get exported to the cytoplasm. Besides their well-known role in the synthesis and assembly of ribosomes, the nucleolus has also been suggested to be a place for functional sequestration of proteins. The human telomerase protein is concentrated in the nucleolus, but functions on the DNA ends in the nucleoplasm. In normal cells, the release of telomerase to the nucleoplasm from the nucleolus is enhanced at the expected time of telomere replication in a cell-cycle dependent manner. However, in transformed cells, there is an almost complete dissociation of telomerase from nucleoli at all stages of the cell cycle, indicating that the telomerase activity is released from the nucleolus when needed and sequestrated by the nucleolus when not necessary (Wong *et al.*, 2002).

The nucleoplasm is the compartment for mRNA transcription and processing and can be further divided into the chromatin and interchromatin space. Actively transcribed DNA has a consistent topology on chromatin, with mRNA transcription occurring at, or near, the surface of the compact chromatin domains (the perichromatin region), depositing newly synthesized RNA directly into the interchromatin space (Verschure *et al.*, 1999). Before being exported from the nucleus to the cytoplasm for translation, the pre-mRNAs undergo extensive RNA processing events, including capping, splicing and polyadenylation. Abundant cytological and biochemical evidence supports that pre-mRNA processing occurs co-transcriptionally in the perichromatin region, which is mediated by the interaction between various RNA processing machineries and the C-terminal domain (CTD) of the RNA polymerase II (Cramer et al., 2001; Fong and Bentley, 2001; Herbert et al., 1997; Hirose and Manley, 1998; Ho et al., 1999; McCracken et al., 1997). The co-transcriptional splicing of pre-mRNA has important implications for where ADAR-mediated RNA editing actually takes place. Since editing of many pre-mRNAs requires intronic sequence to form the RNA duplex necessary for A-to-I conversion (Burns et al., 1997; Higuchi et al., 1993; Rueter et al., 1999), editing must occur prior to intron removal by splicing. Since splicing occurs co-transcriptionally, it is believed that ADARs also edit their targets co-transcriptionally in the nucleoplasm, although direct evidence for this hypothesis is lacking.

Functional sequestration of ADARs by the nucleolus

Since ADARs are thought to edit RNA transcripts in the nucleoplasm, it was surprising to find out that both ADAR1 (p110) and ADAR2 are highly concentrated in the nucleolus of human and mouse cells (Desterro *et al.*, 2003; Sansam *et al.*, 2003) (Figure 10A).

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analyses suggested that ADARs shuttle rapidly between the nucleolus and nucleoplasm (Desterro *et al.*, 2003; Sansam *et al.*, 2003). When one selected nucleolus was photobleached in a cell stably expressing eGFP-ADAR2, the recovery of fluorescence was essentially complete within 1min and the fluorescence intensity of other nucleoli in the same cell decreased and reached a steady-state level within a similar time-scale to the recovery, indicating that ADAR2 is constantly moving between nucleoli through the nucleoplasm at a speed approaching the rate of diffusion (Sansam *et al.*, 2003).

Although the nucleolus contains high concentration of ADAR proteins, it does not seem to be the subcellular location in which editing occurs. A transiently expressed editing-competent RNA in the nucleus is excluded from the nucleolus, and overexpression of this nucleoplasmic ADAR substrate causes endogenous ADAR proteins to be translocated to the nucleoplasm. This translocation depends on whether the RNA can be edited by ADAR since a control RNA did not cause any change in ADAR subnuclear localization (Desterro *et al.*, 2003) (Figure 10B). Furthermore, translocation of ADAR2 to the nucleoplasm, by inhibition of rRNA synthesis, resulted in elevated A-to-I RNA editing on multiple substrates (Sansam

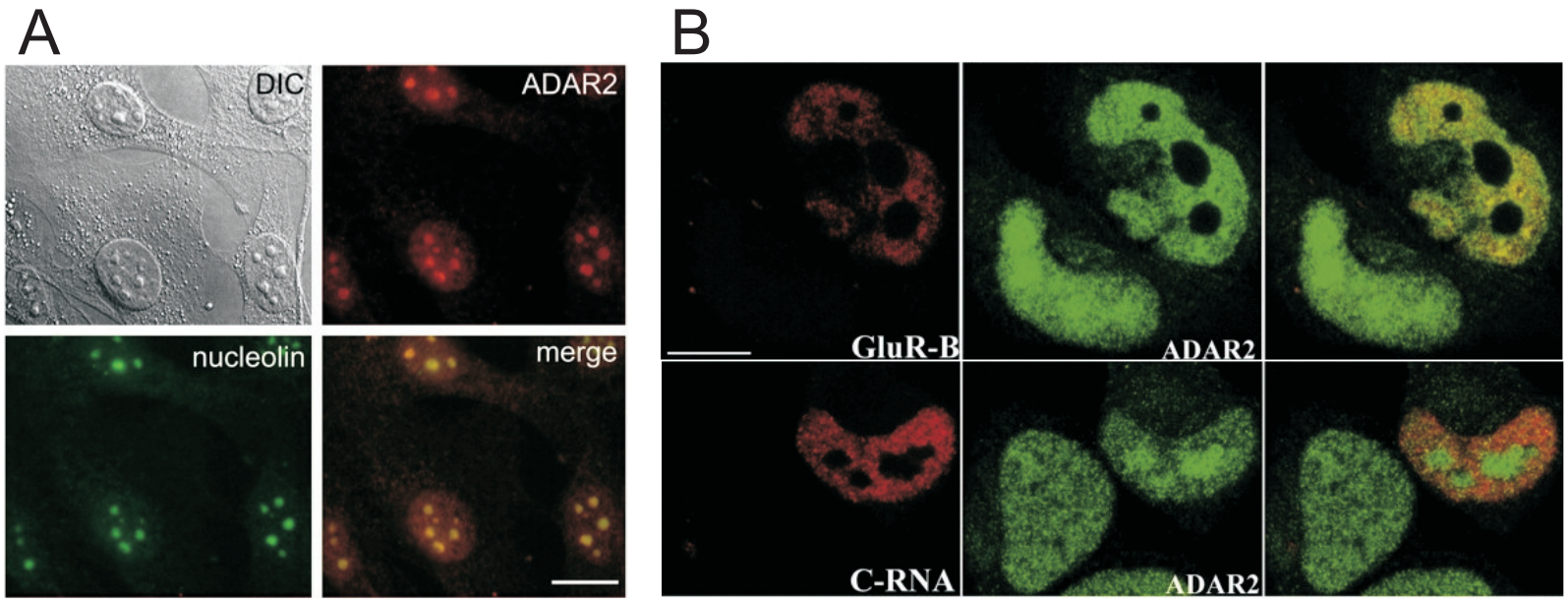

Figure 10. Dynamic association of ADAR2 with the nucleolus. (A) The nucleolar localization of endogenous ADAR2. The subcellular localization of endogenous mouse ADAR2 in NIH/3T3 cells was determined by using affinity-purified ADAR2 antiserum with a Cy3-conjugated secondary antibody. Nucleoli were identified by using differential interference contrast or antinucleolin immunofluorescence microscopy. (B) Endogenous hADAR2 is excluded from the nucleolus of cells expressing an editing substrate. HeLa cells were transiently transfected with a plasmid containing either the editing-competent murine GluR-B (GluR-2) gene portion or a control gene portion from the Friends virus genome (C-RNA). Endogenous localization of ADAR2 was monitored by indirect immunofluorescence microscopy with antibodies directed against ADAR2. GluR-B- and C-RNA-transcribing cells were visualized by fluorescent in situ hybridization with a GluR-B or C-RNA probe labeled with digoxigenin. The hybridization sites were detected using a Cy3 anti-digoxigenin secondary antibody. The GluR-B and C-RNA transcripts were detected ~40 hours after transfection. A superimposition of the corresponding double-labeled images are shown on the right panels. Bar, 10 μm, (adapted from Sansam *et al.*, 2003 and Desterro *et al.*, 2003).

et al., 2003). Taken together, these data indicated that A-to-I RNA editing takes place in the nucleoplasm and ADARs are functionally sequestered in the nucleolus.

Molecular mechanisms for ADARs nucleolar localization

Mutation of critical residues for dsRNA binding in the dsRBMs makes the resultant protein diffuse through the nucleus, indicating the double-stranded RNA binding activity is required for ADAR2 nucleolar localization (Sansam *et al.*, 2003). The pre-rRNA and rRNA in the nucleolus contain extensive RNA duplexes, and rRNA is also essential for ADAR2 nucleolar localization, suggesting ADAR2 may bind to the duplex regions contained within rRNA transcripts.

Summary and specific aims

ADAR2-mediated RNA editing is able to change the ion permeability and the kinetics of ion channels, the coupling efficiency between receptor and G protein and the alternative splicing pattern of its own pre-mRNA. With over 12,000 editing events in Alu elements recently identified, ADAR2 may also modulate a number of additional cellular processes by regulating the stability, translation efficiency and trafficking of targeted RNAs. Moreover, ADAR2 has also been shown to affect siRNA and microRNA pathways. Therefore, the function and regulation of ADAR2 is under extensive study. However, as more findings are made, even more questions have been raised. The molecular mechanisms underlying ADAR2 nucleolar localization are not fully understood since some data suggests that dsRBMs are not sufficient to localize protein to the nucleolus. The dsRBMs have

been shown to bind to GluR-2 (Q/R site) RNA specifically, yet whether such specific binding is common to all substrates is unknown. ADAR2 contains two dsRBMs and these motifs are required for editing activity and nucleolar localization. Their sequence and structural similarity however, has raised questions regarding the role(s) that each dsRBM plays in ADAR2 function. In an effort to address these questions, this dissertation has focused upon the following specific aims.

I. Identification of C-terminal localization signal in ADAR2

II. Structure and specific RNA binding of ADAR2 dsRBMs

III. Differential roles of dsRBMs in regulating ADAR2 function

CHAPTER II

IDENTIFICATION OF C-TERMINAL LOCALIZATION SIGNAL IN ADAR2

Introduction

The conversion of A-to-I by RNA editing is a widespread posttranscriptional modification, resulting in the hydrolytic deamination of targeted adenosine residues to change the sequence of RNA from that encoded by the genome. Most of the well-characterized A-to-I editing events result in non-synonymous codon changes in the mRNA, to produce functionally distinct protein isoforms (Berg et al., 2001; Bhalla et al., 2004; Burns et al., 1997; Hoopengardner et al., 2003; Kohler et al., 1993; Lomeli et al., 1994; Niswender et al., 1999). A-to-I modifications have also been described in non-translated RNA species and non-coding regions of RNA transcripts, suggesting that editing may also affect other aspects of cellular RNA function including the splicing, trafficking, stability or translation efficiency (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004; Morse et al., 2002; Yang et al., 2006).

A-to-I RNA editing in mammals is mediated by a family of enzymes known as ADARs, including ADAR1, ADAR2 and ADAR3 (Bass *et al.*, 1997). ADAR2 is an 80kD protein, containing one NLS and two dsRBMs at the N-terminus and one conserved catalytic domain at the C-terminal portion. ADAR2 catalyzes the majority of known codon-altering A-to-I modifications, and the ADAR2-deficient mice die by three weeks after birth primarily due to the lack of editing at the Q/R

site of GluR-2 transcripts (Higuchi *et al.*, 2000).

While A-to-I RNA editing is thought to take place co-transcriptionally in the nucleoplasm, ADAR2 has been found to be highly concentrated to the nucleolus at steady-state and shuttles rapidly between nucleolus and nucleoplasm (Desterro *et al.*, 2003; Sansam *et al.*, 2003). Increased translocation of ADAR2 to the nucleoplasm correlates with the increased editing activity, suggesting that ADAR2 is functionally sequestered by the nucleolus (Sansam *et al.*, 2003). Both functional dsRBMs and rRNA synthesis are required for ADAR2 nucleolar localization, indicating that ADAR2 may bind to the duplex regions within rRNAs. (Sansam *et al.*, 2003).

Although dsRBMs are required for nucleolar localization, whether other nucleolar localization signals are required for this subnuclear targeting is unknown. Preliminary data have indicated that deletion of the C-terminal region of ADAR2 will result in a loss of nucleolar localization. This effect may be due to the loss of a specific nucleolar localization signal or a change in the overall protein structure. In this study, subcellular localization and enzymatic activity of a series of ADAR2 mutants are assessed to examine whether a specific localization signal exists in the C-terminus.

Materials and Methods

Plasmids

Construction of all eGFP-ADAR2 mutants was performed using the wild-type eGFP-ADAR2 plasmid as a template for run-around PCR (Coolidge and Patton, 1995), using specific primer sets as following : VU1144 and VU1152 for eGFP-ADAR2(1-528), VU1120 and VU1068 for eGFP-ADAR2(532-711), VU1069 and VU1068 for eGFP-ADAR2(Δ392-531), VU1144 and VU1145 for eGFP-ADAR2(Δ529-590), VU1146 and VU1147 for eGFP-ADAR2(Δ591-649), VU1148 and VU1152 for eGFP-ADAR2(Δ648-711), VU1221 and VU1152 for eGFP-ADAR2(1-301).

Cell culture and transfection

Human embryonic kidney (HEK293) cells and NIH/3T3 mouse fibroblasts (American Type Culture Collection) were maintained in AMEM and DMEM (Life Technologies, Grand Island, NY), respectively, and supplemented with 10% (vol/vol) bovine calf serum (HyClone, Logan, UT).

HEK293 cells (about 50% confluent in 100mm culture dish) were transiently transfected by calcium phosphate coprecipitation with 8 μg of plasmid encoding wild-type or mutant eGFP-ADAR2 fusion proteins as previously described (Rueter *et al.*, 1999).

For analysis of subcellular fluorescence, NIH/3T3 cells were plated on 35mm dishes and transiently transfected with 1μg of plasmids encoding wild-type or

mutant eGFP-ADAR2 fusion proteins using Fugene 6, according to the manufacture's instruction (Boehringer Mannheim).

Fluorescence microscopy

Twenty-four hours after transfection, the subcellular localization of eGFP fusion proteins was determined by fluorescence microscopy (Axiovert S100, Carl Zeiss Inc). All images were acquired using a 20X/0.40 LD ACHROPLAN objective lens.

In vitro editing assay

The two strands of D79N dsRNA were transcribed in the presence of [α-^{32}P]-ATP individually and hybridized, and the concentration was determined by scintillation spectrometry as described previously (Dawson *et al.*, 2004). For preliminary studies (Figure 25A), HEK293 nuclear extracts containing the wild-type eGFP-ADAR2 fusion protein were diluted 15-fold for a time-course study to determine the linear range of enzymatic activity for D79N RNA substrate. 100 fmoles of RNA substrate was incubated with nuclear extracts containing equivalent amounts of eGFP-ADAR2 protein at 30^{o} C, in a total volume of 50 µl, for varying time periods and the reactions were terminated by freezing on dry ice. Subsequent analyses of editing activity for equivalent amounts of wild-type and mutant eGFP-ADAR2 fusion proteins were performed in the same system using 60mins as reaction time. Quantification of editing activity for [α-^{32}P]-labeled D79N dsRNA was performed by thin-layer chromatography (Rueter *et al.*, 1995).

Results

Altered localization by C-terminal region deletion

Based on its amino acid sequence and biological function, ADAR2 is predicted to contain two dsRBMs at the N-terminus and a catalytic adenosine deaminase domain at the C-terminus. However, the precise boundaries of each domain have not been well defined until recently (Macbeth et al., 2005; Stefl et al., 2006). At the time this project was initiated, it was well accepted that the deaminase domain extended from amino acid D^{392} to I^{530} in ADAR2, based upon sequence similarity to *E.coli* CDA, and the sequences from L^{301} to N^{391} and from A^{531} to P^{711} did not correspond to the structure of any characterized domain (Figure 11).

A fusion protein in which ADAR2 was subcloned in-frame with the coding region of eGFP displayed a similar subcellular localization and comparable enzymatic activity to endogenous ADAR2 (Sansam *et al.*, 2003), providing an ideal tool to monitor the localization and activity changes of ADAR2. A fusion protein (1-528) lacking the region immediately following the deaminase domain (C-terminal region) showed diffuse nuclear localization in transiently transfected NIH/3T3 cells (Figure 11). This effect may due to the loss of a specific nucleolar localization signal in the last 183 amino acids, but may also result from an overall change in the protein structure which impedes dsRBM function.

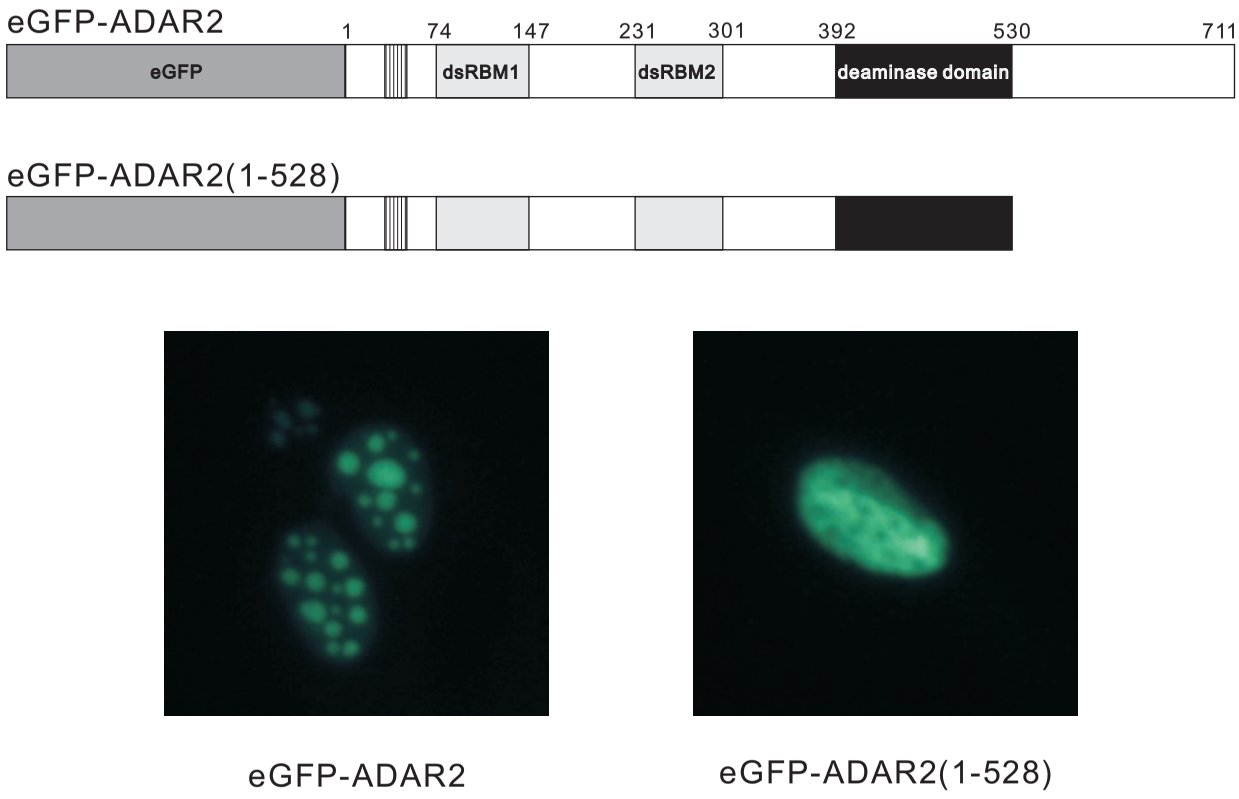

Figure 11. Loss of nucleolar localization by deleting the C-terminal region. A schematic diagram indicating the domain structure of wild-type eGFP-ADAR2 and the C-terminal deletion construct, eGFP-ADAR2(1-528) is shown. The coordinates for the domains are relative to the normal ADAR2 start codon. Representative micrographs of the subcelluar localization of each protein transiently expressed in NIH/3T3 cells are shown.

Insufficiency of C-terminal region for nucleolar localization

If a peptide contains a functional nucleolar localization signal, it should be able to target a heterologous protein such as eGFP to the nucleolus. To test whether the C-terminal region of ADAR2 contains such a localization signal, the fusion protein eGFP-ADAR2(532-711) was made (Figure 12), yet this protein was detected in both the nucleus and cytoplasm (Figure 13). In the nucleus, a diffuse fluorescence pattern was observed, suggesting that there is no specific nucleolar localization signal in the last 180 amino acids of ADAR2.

Altered localization by deleting the deaminase domain

If the altered localization by deletion of the C-terminal region is due to the loss of specific nucleolar localization signal, we thought this effect should be rather specific. The deaminase domain is known to contain no nucleolar localization signal. Surprisingly an eGFP-ADAR2 fusion protein solely lacking the deaminase domain (Δ392-531) was localized diffusely throughout the nucleus (Figure 12,13),suggesting that the altered localization is not specific to the C-terminal region. These data is more consistent with the hypothesis that the C-terminal deletion resulted in an overall change in the protein structures.

Lack of dissectible localization signal in the C-terminal region

No domain structure was identified in the C-terminal region of ADAR2 based upon the primary amino acid sequence. To further examine the identify of specific nucleolar targeting sequences, the C-terminal region was equally divided into

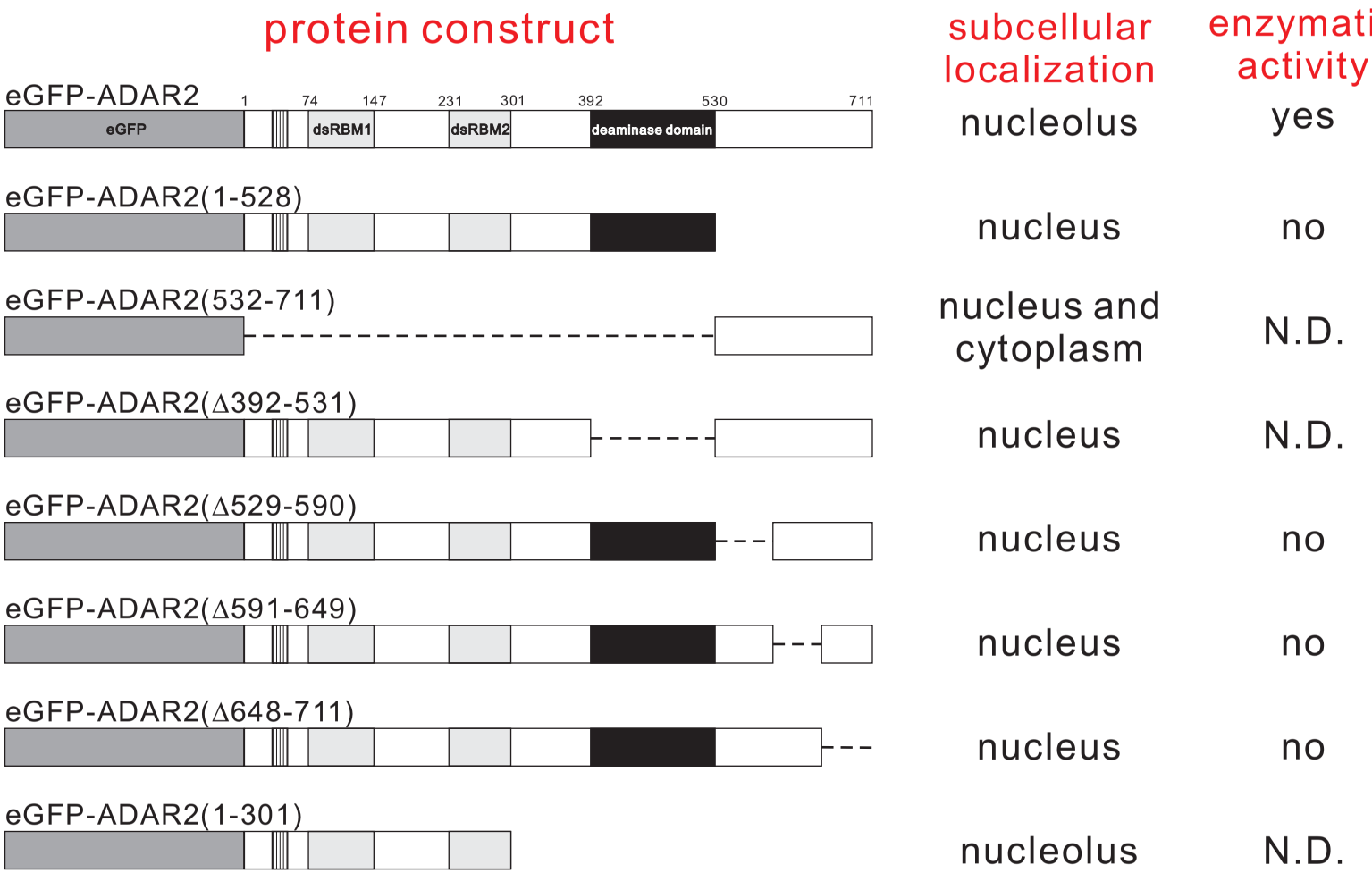

Figure 12. A summary of the subcelluar localization and enzymatic activity of C-terminal mutants. A schematic diagram indicating the domain structure of each mutant is shown; all the coordinates are relative to normal ADAR2 start codon. N.D., not determined.

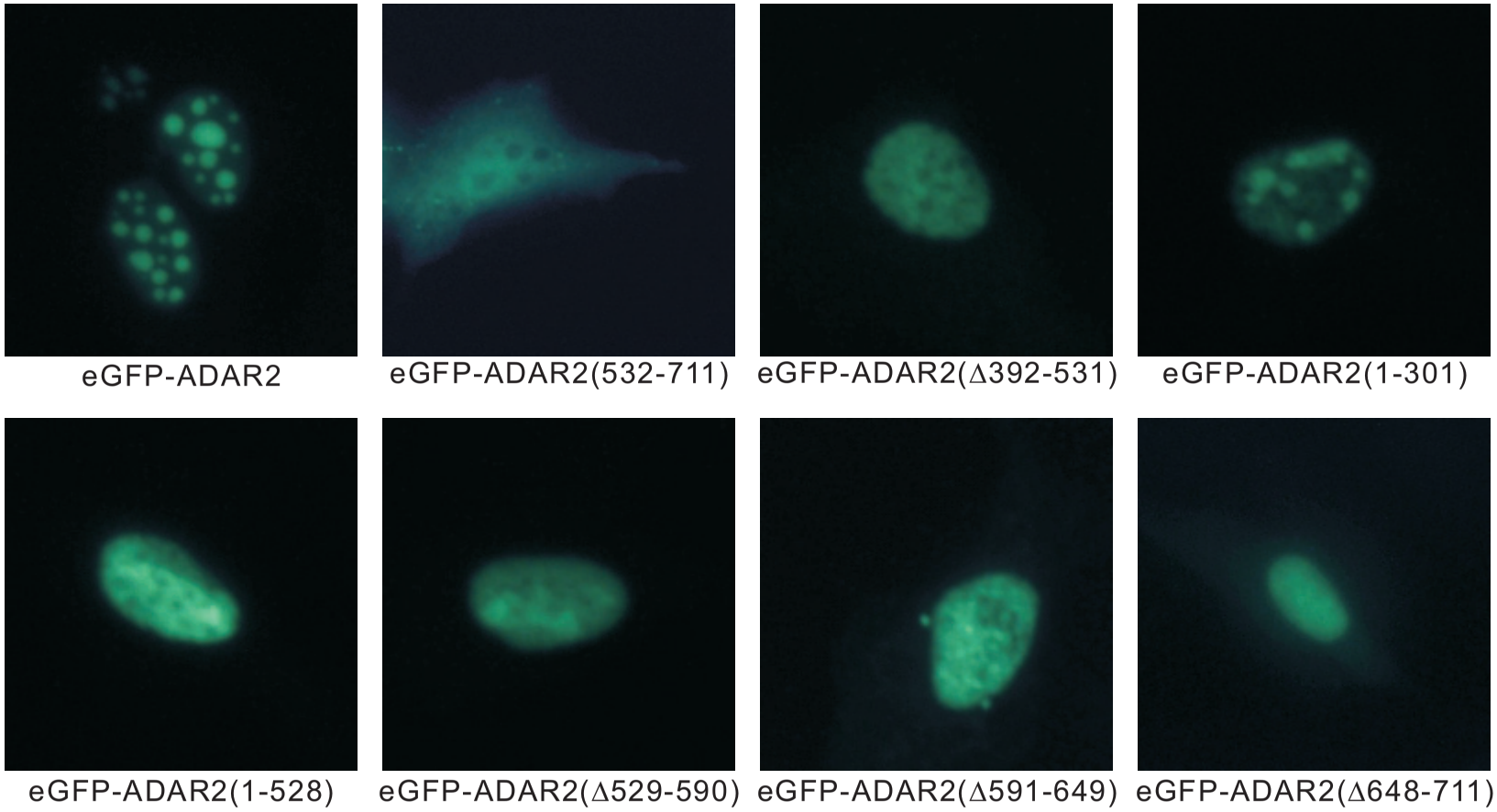

Figure 13. Subcellular localization of C-terminal mutants. Representative micrographs of the subcellular localization of mutant eGFP-ADAR2 fusion proteins are shown.

three portions and each was deleted from the eGFP-ADAR2 fusion protein (Figure 12). All three mutant eGFP-ADAR2 proteins, eGFP-ADAR2(Δ529-590), eGFP-ADAR2(Δ591-649), eGFP-ADAR2(Δ648-711), displayed a diffuse pattern in the nucleus (Figure 13), demonstrating the absence of a dissectible nucleolar localization signal (<60aa) in this region of ADAR2.

The sufficiency of the dsRBMs for nucleolar localization

While the dsRBMs of ADAR2 are required for nucleolar localization (Sansam *et al.*, 2003), whether these domains are sufficient for subnuclear targeting has not been tested. This fact, together with the altered localization observed for the C-terminal region deletion, leads us to examine a putative C-terminal nucleolar localization signal. However, the fact that no dissectible region was identified in the C-terminus, urged us to go back and examine the sufficiency of the dsRBMs for nucleolar localization. An eGFP-ADAR2(1-301) mutant was made by deletion of region immediately after dsRBM2. Surprisingly, this mutant protein was highly concentrated in the nucleolus, similar to wild-type eGFP-ADAR2, suggesting that the dsRBMs are sufficient for nucleolar localization in the absence of the C-terminal region.

Loss of enzymatic activity by mutations in the C-terminal region

The subcellular localization of eGFP-ADAR2 mutants indicated that deletion of C-terminal region most likely caused an overall change in protein structure, thereby interfering with dsRBM function and altering subnuclear localization. To

further test this hypothesis, the enzymatic activity of C-terminal deletion mutants was examined using a synthetic RNA duplex in vitro. While the wild-type eGFP-ADAR2 converted 30% of the adenosine residues into inosine, both the entire C-terminal region deletion (1-528) and the three smaller deletions in this region displayed no editing activity (Figure 14). The sequence between amino acid R^{648} and P^{711} is far from the active site, yet deletion of this region totally ablated editing activity and the nucleolar localization, strongly suggesting that the C-terminal region is highly structured and that disruption of this region can cause an widespread change in protein structure, which in turn, can impede the dsRNA-binding activity of the dsRBMs.

Discussion

Deletion of the C-terminal 183 amino acids of ADAR2 altered its subcellular localization, leading us to hypothesize the existence of a specific nucleolar localization signal in the C-terminal region. However, the C-terminal region is not sufficient to localize eGFP to the nucleolus and the deletion of deaminase domain causes a similar change in subnuclear targeting, suggesting this effect is not specific to the C-terminal region. Three smaller deletions in the C-terminal region all eliminated nucleolar accumulation, indicating that there is no dissectible nucleolar localization signal in this region. A fusion protein lacking both the deaminase domain and C-terminal region retained its nucleolar accumulation, suggesting that the C-terminal region is not required for localization. Furthermore, all deletions in the C-terminal region ablated editing activity, suggesting that

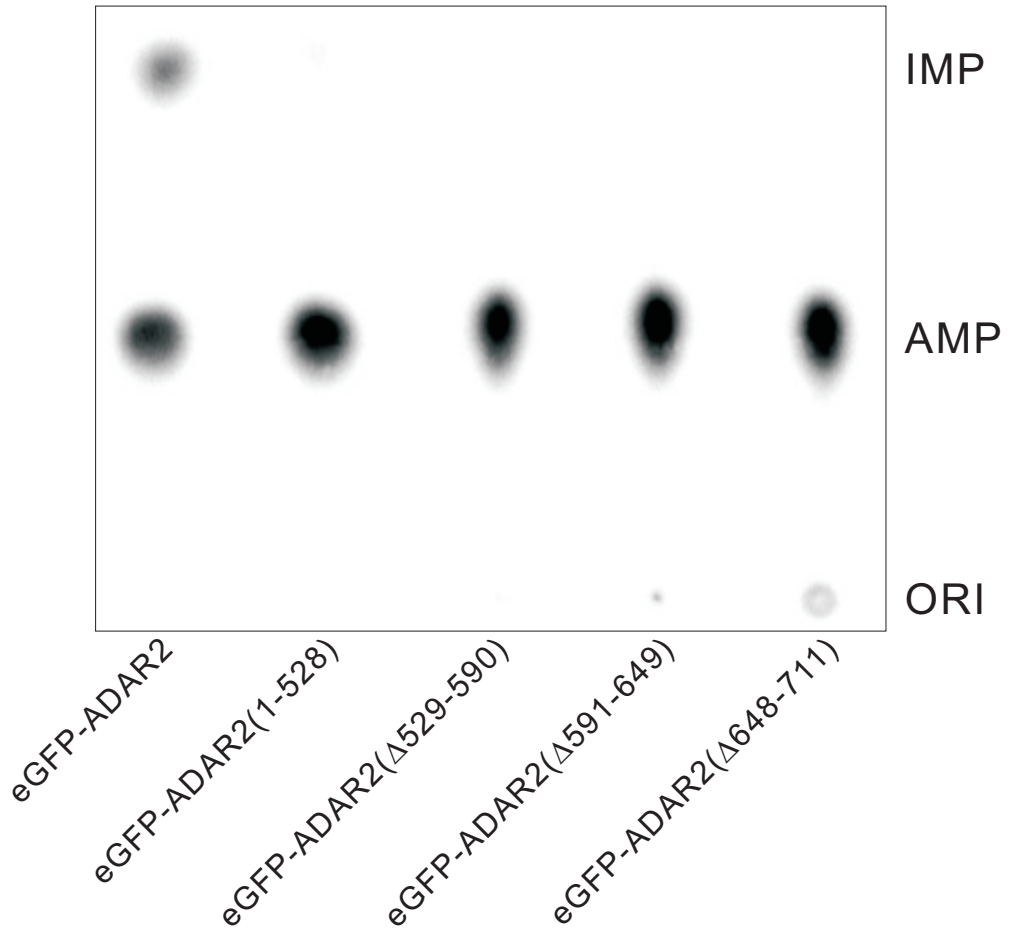

Figure 14. Loss of enzymatic activity by C-terminal deletions. Representative autoradiograms from in vitro analyses of editing activity for a perfect dsRNA mediated by eGFP-ADAR2 fusion proteins are shown. Editing activity for a synthetic RNA duplex (D79N) was determined by thin-layer chromatography (TLC); the TLC origin (ORI) and the migration positions of adenosine 5'-monophosphate (AMP) and inosine 5'-monophosphate (IMP) are indicated.

altered localization of eGFP-ADAR2(1-528) is mostly likely due to an overall change in protein structure which impedes dsRBM function rather than the loss of a specific nucleolar localization signal.

The highly structured C-terminal region

Based on the sequence similarity to *E.coli* CDA, the deaminase domain of ADAR2 is previously defined as the sequence between D^{392} and I^{531}, and the C-terminal region is thought to be sequence without tertiary structure. However, our mutagenesis studies strongly suggested that the C-terminal region is highly structured, since small deletions (~60aa) within this region, which are far from the dsRBMs and catalytically active site, eliminate both ADAR2 enzymatic activity and nucleolar targeting. Recent X-ray crystallographic studies have confirmed this finding (Macbeth *et al.*, 2005) (Figure 5A). The actual deaminase domain, extending from amino acid Q^{321} to T^{710}, is much larger than predicted. There is an IP_6 molecule buried in the enzyme core that has been shown to be required for editing activity, presumably by defining and stabilizing the folding of the deaminase domain. The sequence after L^{560} forms a number of helices and provides most of the IP_6 binding sites (Macbeth *et al.*, 2005). Therefore, the C-terminal region is highly structured and folds as an integrated part of the deaminase domain.

The IP_6 and dsRBM-mediated nucleolar localization

The dsRBMs of ADAR2 can fold correctly and are sufficient for nucleolar localization in the absence of the deaminase domain (Stefl et al., 2006) (Figure 23) and the deaminase domain can fold correctly with IP_6 in the enzyme core in the absence of dsRBMs (Macbeth *et al.*, 2005). The deletions in C-terminal region cause a misfolding of the deaminase domain, so it is not surprising that all of these mutants were enzymatically inactive. However, if we assume that functional domains fold independently, how the misfolding of the deaminase domain interferes with dsRBM-mediated nucleolar localization remains unknown.

The IP_6 binding residues are highly conserved in all ADARs and yeast ADAT1, but not found in yeast ADAT2, ADAT3 and *E.coli* ADAT2 and CDA (Macbeth *et al.*, 2005). Therefore, IP_6 seems not to be essential for deaminase activity or the substrate preference of adenosine over cytidine. Then what is the evolutionary advantage of IP_6 and its folding into ADAR deaminase domain?

Sequence alignments indicate that ADAT1 is the evolutionary link between ADAT2/3 and ADARs (Gerber and Keller, 2001). ADAT2/3 deaminates specific adenosines in anticodon loop of tRNAs. Since the substrate is relatively small, without any RNA binding domain, the ADAT2/3 deaminase domain can accurately modify the targeted adenosines. To evolve to ADARs, which are designed to catalyze specific adenosines out of thousands of mRNAs, the presence of dsRBMs are required for defining the substrate specificity (Stefl et al., 2006; Stephens et al., 2004). We reason that the deaminase domain of ADAT2/3 has the intrinsic binding activity for the dsRBMs, similar to the dsRBM binding to the

catalytic domain observed in PKR and RNase III, hence interfering both dsRNA binding and catalytic activity, preventing the evolution from ADAT2/3 to ADARs directly. ADAT1 happened to obtain the ability to bind to IP_6 with the extended C-terminal region through spontaneous mutations and this change may mask the binding activity of the original deaminase domain to the dsRBMs, making it possible to have the dsRBMs and the deaminase domain existing in the same molecule and finally evolve to ADARs. This is consistent with the fact that dsRBM only presents in a deaminase containing the IP_6 binding residues.

This hypothesis well explains the evolutionary advantage of the IP_6 and the sequence conservation of IP_6 binding residues in all ADARs. It is also consistent with the experimental evidence. When the IP_6 binding is abolished by mutations in the C-terminal region, the original deaminase domain with the dsRBM binding activity is exposed. The resulting intramolecular interaction blocks the dsRBM accessibility to the rRNA, thus, altered the subcellular localization. Although the hypothesis is consistent with the experimental data, the validity needs to be tested directly.

CHAPTER III

STRUCTURE AND SPECIFIC RNA BINDING OF ADAR2 DSRBMS

Introduction

ADARs convert adenosine to inosine by hydrolytic deamination in cellular and viral RNA transcripts containing either perfect or imperfect regions of double-stranded RNA (Bass, 2002; Emeson and Singh, 2001; Gerber and Keller, 2001; Keegan et al., 2001). A-to-I modification is non-specific within perfect dsRNA substrates, deaminating up to 50% of the adenosine residues (Bass, 2002; Emeson and Singh, 2001). The majority of non-selective editing occurs in UTRs and introns where large regular duplexes are formed (Levanon et al., 2004; Morse et al., 2002; Morse and Bass, 1999; Rueter et al., 1999).

A-to-I editing can also be highly specific within imperfect dsRNA regions containing bulges, loops, and mismatches, modifying a single or limited set of adenosine residues (Bass, 2002; Emeson and Singh, 2001). In the pre-mRNA encoding the GluR-2, several such specific editing sites have been found (Seeburg *et al.*, 1998). One of these locations is the R/G site, where an arginine codon is converted to a glycine codon. This change affects the biophysical properties of the ion channel in which the edited isoform recovers faster from desensitization (Lomeli *et al.*, 1994).

ADARs display a modular domain organization, containing from one to three tandem copies of dsRBMs in its N-terminal region, and an adenosine deaminase

domain in its C-terminal portion. The dsRBMs of ADARs may play an important role in modulating the editing selectivity of ADARs (Carlson et al., 2003; Doyle and Jantsch, 2002; Stephens et al., 2004). The structural studies of several dsRBMs revealed a highly conserved αβββα protein topology in which the two α-helices are packed along a face of a three-stranded anti-parallel β-sheet. (Bycroft *et al.*, 1995; Kharrat *et al.*, 1995; Nanduri *et al.*, 1998) Furthermore, structures of the dsRBMs in complex with non-natural or natural RNA substrates have been solved (Blaszczyk et al., 2004; Ramos et al., 2000; Ryter and Schultz, 1998; Wu et al., 2004). These structures revealed not only how dsRBMs can bind any dsRNA, regardless of base composition, but also how structure-specific recognition of RNA hairpins is achieved (Stefl *et al.*, 2005a).

While the enzymatic activity of ADARs and their biological role(s) have extensively been studied (Bass, 2002; Emeson and Singh, 2001), the determinants that control site-selective RNA modification are poorly understood. Here, we report the solution structure of the two dsRBMs of ADAR2 and their interactions with the conserved 71 nt RNA stem-loop containing the GluR-2 R/G site (R/G stem-loop). Our structural study demonstrates that the dsRBMs of ADAR2 have the ability to discriminate specific structural features of RNA, suggesting their importance for the editing site selectivity.

Materials and Methods

Plasmids

For the expression of His-dsRBM1 and His-dsRBM2, the DNA fragments corresponding to rat dsRBM1 (74-147) and dsRBM2 (231-301) were PCR amplified from pEGFP-ADAR2b plasmid by specific primers (VU990, VU991 for dsRBM1; VU1024, VU1092 for dsRBM2) to introduce NdeI and BamHI restriction sites for in-frame subcloning into pET16b expression vector (Novagen). The dsRBM1/2 cDNA was cloned into pET30-GBFusion1 vector yielding N-terminal GB1-tagged and C-terminal His-tagged construct of GB1-dsRBD1/2. A 116 bp fragment containing a portion of the mouse GluR-2 gene with the complete R/G duplex was amplified from mouse genomic DNA using sense (VU682) and antisense (VU683) primers and subcloned into pBSKII$^-$ (Stratagene). Alterations in the sequence for the R/G stem-loop (GCUCUA, GCAA, GCACA) were made by PCR-based mutagenesis from the wild-type R/G stem-loop construct using the same antisense primer (VU1239) and individual sense primers (VU1245, VU1244, VU1238).

Recombinant protein expression and purification

His-dsRBM proteins were expressed in BL21 (DE3) *E.coli*, and cells were grown at 37^{o}C in minimal medium (2L) containing [$^{13}C_6$] glucose and $^{15}NH_4Cl$ to OD_{600}=0.8. After induction with 0.1mM IPTG for 7.5 hours at 30 ^{o}C, the His-dsRBM proteins were purified under natural condition using TALON resin.

The protein elution was subjected to dialysis to remove imidazole, followed by centrifugal concentration using MILLIPORE UFV2BCC10 system. BL21 expressing GB1-dsRBM1/2 were grown at 37 ^{o}C in minimal medium (0.5L) to OD_{600}=1. After 1mM IPTG induction, the protein was purified under denaturating conditions and refolded into native buffer on Ni-NTA affinity column. All proteins are concentrated to more than 0.8mM in 50mM sodium phosphate buffer (pH=8) containing 200mM NaCl.

Cell culture and protein preparation for in vitro editing assay

Human embryonic kidney (HEK293) cells (American Type Culture Collection) were maintained in AMEM (Life Technologies, Grand Island, NY), and supplemented with 10% (vol/vol) bovine calf serum (HyClone, Logan, UT).

HEK293 cells (about 50% confluent in 100mm culture dish) were transiently transfected by calcium phosphate coprecipitation with 8 μg of plasmid encoding wild-type or mutant eGFP-ADAR2 fusion proteins as previously described (Rueter *et al.*, 1999).

RNA preparation for in vitro RNA editing assay

The plasmids containing wild-type and mutant R/G stem-loop were all linearized with BamHI, transcribed in vitro using T3 RNA polymerase (Promega) and the resultant RNAs were subjected to digestion with RQ1 DNase (Promega) to remove the template DNA. For synthesis of the R/G stem (no loop) RNA, two strands were synthesized separately as follows: a 247 bp fragment was amplified

by PCR from the wild-type R/G stem-loop construct, using sense (VU1265) and antisense (VU1264) primers, to serve as a template for in vitro transcription of the first RNA strand, followed by digestion with RQ1 DNase. The second, synthetic RNA strand (VU1272) was obtained from Integrated DNA Technologies (Coralville, IA). The first and second strands were hybridized together at a 1:10 ratio in RNA annealing buffer incubating at 85^{o}C for 10mins, followed by slow cooling to room temperature (Dawson *et al.*, 2004).

In vitro editing assay and quantification of R/G site editing

Analyses of editing activity for equivalent amounts of wild-type and mutant eGFP-ADAR2 fusion proteins (1:90 dilution for wilt-type protein) on each RNA substrate were performed for 30mins. For quantification of RNA editing, the in vitro reaction product was subject to RT-PCR amplification using sense (VU682) and antisense (VU1264) primers, followed by primer-extension analysis as described previously (Dawson *et al.*, 2004).

More materials and methods

RNA preparation for NMR, NMR spectroscopy, structural calculations, model for RNA binding, uses of structure/sequence databases and sequence alignment, light scattering experiment, coordination and NMR restraints were performed by Dr. Richard Stefl in ETH, Switzerland, and have been described extensively in the paper (Stefl et al., 2006).

Results

ADAR2 dsRBMs are independent domains

We investigated the N-terminal region of rat ADAR2 (74-301) that includes the dsRBM1 (74-147), the interdomain linker (148-230) and dsRBM2 (231-301) using NMR spectroscopy. This protein construct was amino-terminally fused with non-cleavable solubility-enhancement tag, GB1, to improve its expression and solubility (Stefl et al., 2005b; Zhou et al., 2001). GB1-dsRBM1/2 and two isolated His-tagged dsRBMs, His-dsRBM1 and His-dsRBM2, were expressed in *E.coli* and purified to homogenerity (Figure 15). 0.8~1 mM of purified protein samples were used to generate the NMR spectra. The comparison between the spectrum of GB1-dsRBM1/2 and the spectra of His-dsRBM1 and His-dsRBM2 shows that the dsRBMs are identical in both contexts, except for a few N- and C-terminal residues. This comparison also indicates that the interdomain linker is flexible. In addition, when the isolated dsRBM1 and dsRBM2 were mixed in *trans*, the spectrum showed no change compared to the two spectra of the isolated domains. These results indicate that the ADAR2 dsRBMs are independent domains separated by a flexible linker, similar to the two dsRBMs of PKR (Nanduri *et al.*, 1998). Thus, we used separate dsRBM1 and dsRBM2 constructs to determine their structures by NMR.

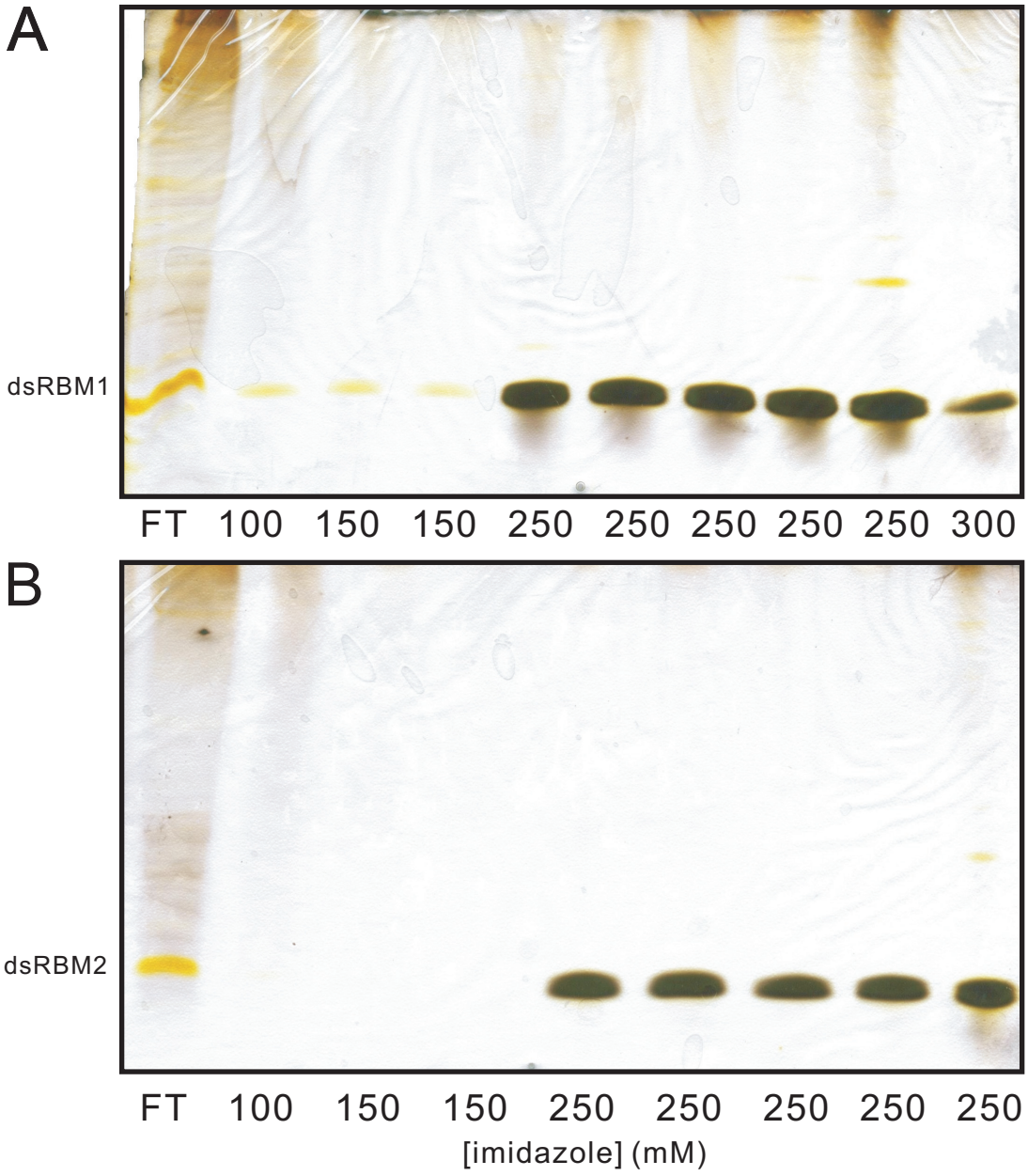

Figure 15. Purification of His-dsRBM1 and His-dsRBM2 protein to homogenerity. Aliquots of flow through (FT) or elutions of the proteins from TALON beads by increasing concentration of imidazole were loaded on 4-20% gradient gel for SDS-PAGE, followed by silver staining analysis.

ADAR2 dsRBMs structures are not identical

Both ADAR2 dsRBM1 and dsRBM2 structures adopt the same fold as all other members of the dsRBM family, with an αββββα topology in which the two α-helices are packed along a face of a three-stranded anti-parallel β-sheet (Figure 16). A central hydrophobic core stabilizes the fold of the domain. Although the two dsRBMs of ADAR2 have 50% amino acid identity, the two structures differ slightly in the orientation of α-helix 1 relative to the other secondary structure elements. This altered orientation is a result of a protein sequence difference in two amino acids at the C-terminus of α-helix 2 where Phe 142 and Val 143 in dsRBM1 are replaced by Val 296 and Phe 297 in dsRBM2. Phe 297, compared to Val 143, is more bulky leading to a different interaction between the two α-helices (Figure 16, blue in dsRBM2). We found another difference between the two dsRBMs in the conformation of the β1-β2 loop. The β1−β2 loop of dsRBM1 is well defined, whereas the β1−β2 loop of dsRBM2 is conformationally heterogeneous, which is probably due to the presence of two prolines in dsRBM1, not found in dsRBM2. Flexible β1−β2 loops were also observed in other dsRBM structures (Leulliot et al., 2004; Ramos et al., 2000). Altogether, the longer α-helix 1 and the conformationally preorganized β1−β2 loop of dsRBM1 might be an important factor for ADAR2 RNA-recognition.

Mapping of RNA-binding surface on the ADAR2 dsRBMs

To investigate how ADAR2 dsRBMs bind RNA, we performed an NMR chemical shift perturbation study (looking for changes in NMR spectrum upon

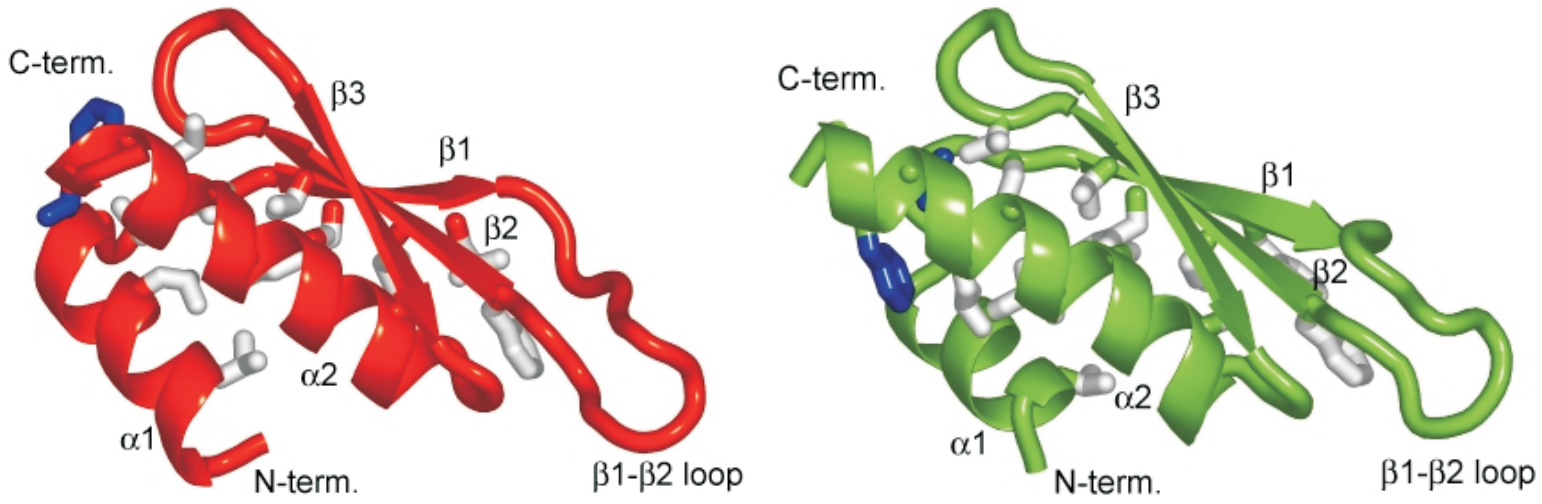

Figure 16. ADAR2 dsRBM protein structure. Ribbon representation of rat ADAR2 dsRBM1 (left; in red) and dsRBM2 (right; in green) with conserved hydrophobic core residues (sticks, in white) are shown.

RNA binding) with a 71 nt R/G stem-loop RNA (Figure 17A). This RNA is a 33 bp helix containing three mismatches (two A·C and one G·G) that is capped by a structured pentaloop (Stefl and Allain, 2005). A8 of this RNA can be specifically edited (up to 74 %) by ADAR2 in vitro, but if the mismatches are replaced by Watson-Crick base-pairs the editing efficiency is reduced substantially (Kallman et al., 2003; Ohman et al., 2000).

First we studied the interaction between GB1-dsRBM1/2 and the 71 nt R/G stem-loop. Upon RNA titration up to an equimolar ratio, significant changes were observed. However, we could not map the RNA-binding residues using this 55 kD GB1-dsRBM1/2·R/G stem-loop complex due to low signal/noise ratio. As the dsRBMs are independent in the free form, we presumed that they could have different binding sites on the R/G stem-loop. Therefore, we used two truncations of the R/G stem-loop, a 52 nt “stem” and a 41 nt “loop”. We prepared four complexes (the two truncated RNAs bound to each dsRBMs) and measured the spectrum. The spectra of dsRBM1-loop and dsRBM2-stem complexes were virtually identical to the one of full-length complex. These observations indicate that the dsRBMs are bound in the same manner in these two subcomplexes and in the full-length complex (GB1-dsRBM1/2 bound to the R/G stem-loop). The two dsRBMs of ADAR2 bind two distinct locations on the R/G stem-loop, dsRBM1 binding close to the pentaloop and dsRBM2 close to the editing site. The NMR data of the reciprocal complexes (dsRBM2-loop and dsRBM1-stem complexes) indicated that such subcomplexes are formed; however their spectra do not resemble the one observed in the full-length complex. These observations

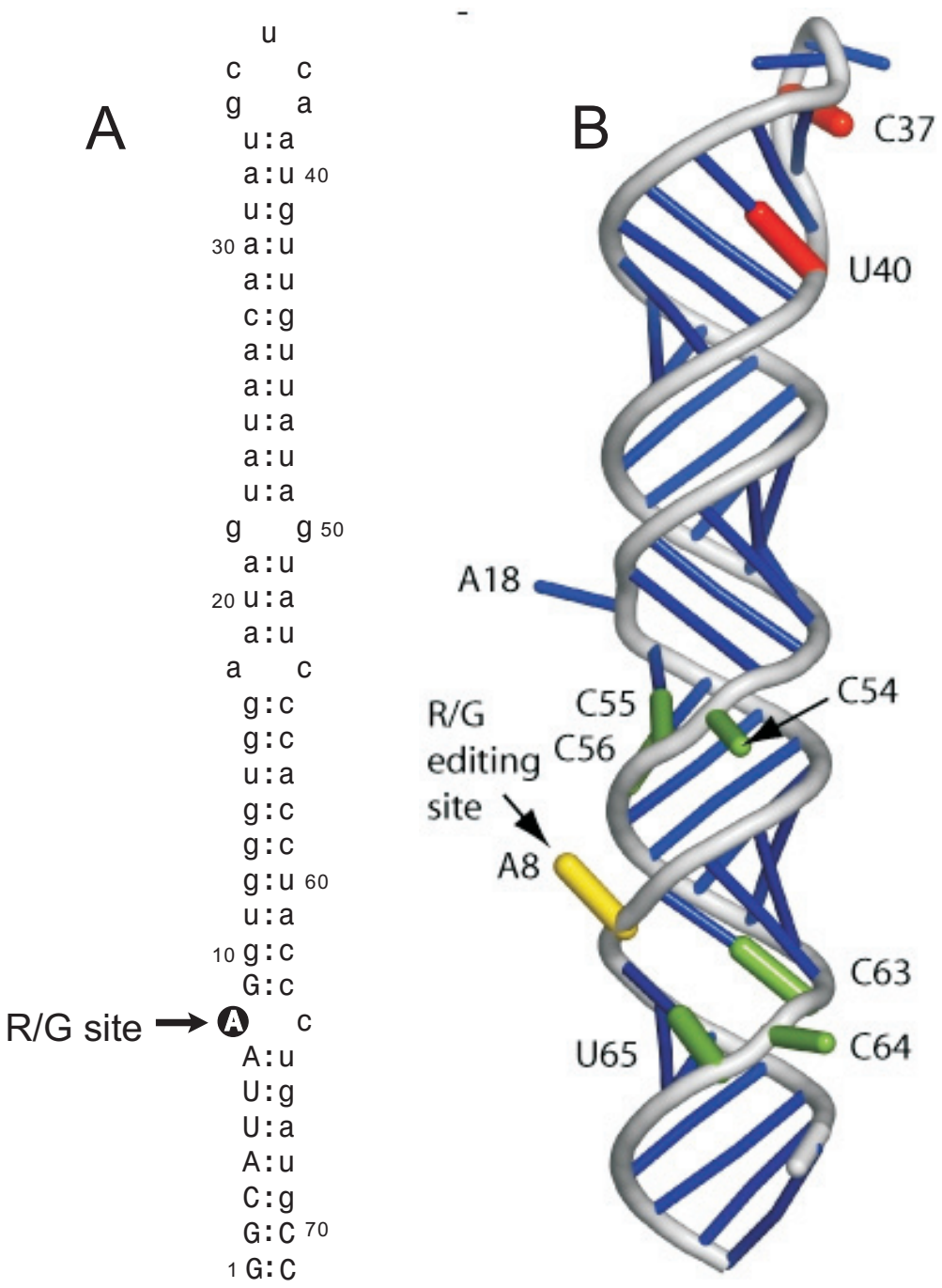

Figure 17. The structure and protein binding surface of R/G stem-loop. (A) The predicted secondary structure of R/G stem-loop, a 71 nt stem-loop that includes the 67 nt of the rat GluR-2 mRNA stem-loop and is closed by two GC base pairs to improve the yield from in vitro transcription. (B) Chemical shift changes upon proteins binding mapped to NMR model of R/G stem-loop. The nucleotides in red and green are affected upon dsRBM1 and dsRBM2 binding, respectively.

confirmed the relative position of the dsRBMs on the full length R/G stem-loop, yet more surprisingly indicated that the binding of both dsRBMs is specific.

In both dsRBMs, the largest changes between the free and the bound forms were observed in α-helix 1 and the β1−β2 loop. In addition, changes were also observed for the β3−α2 loop and the N-terminus of α-helix 2 of dsRBM1, whereas no large changes were observed for the dsRBM2 in these two regions. These results are surprising since these regions of dsRBM1 and dsRBM2 are similar in sequence with the presence of three conserved lysines. Taken together, the patterns of chemical shift perturbations indicate that the protein-RNA interactions are different between dsRBM1 and dsRBM2 reflecting the structural differences already observed in the free dsRBMs structures. The RNA-binding surfaces of ADAR2 dsRBM1 and dsRBM2, although not identical, are similar to the ones observed in other dsRBMs·RNA complexes (Blaszczyk et al., 2004; Ramos et al., 2000; Ryter and Schultz, 1998; Wu et al., 2004).

Mapping of protein-binding surface on the R/G stem-loop

To investigate the protein-binding surface, the NMR spectrum of the 71 nt R/G stem-loop were measured. The NMR data showed the presence of a G22·G50 mismatch and two “open” A·C mismatches (A8·C64 and A18·C54). Based on these data together with our NMR structure of the central pentaloop region of human R/G stem-loop (Stefl and Allain, 2005), we built a structural model of the rat 71 nt R/G stem-loop (Figure 17B). Upon protein binding to the 71 nt R/G stem-loop, no significant changes of the spectrum were observed, indicating that

no changes in the RNA secondary structure take place upon complex formation. In the two subcomplexes of dsRBM1-loop and dsRBM2-stem, a precise chemical shift perturbations study upon protein binding was done. In the course of the protein titrations, the spectra move from their initial positions, corresponding to the free form, in a stepwise directional manner until they reached their final positions that correspond to the fully bound state. These data indicates that in both subcomplexes, the RNAs are in fast exchange between their free and bound forms relative to the NMR time scale. The binding of dsRBM1 to the loop RNA induces a significant change of C37 and U40, and the binding of dsRBM2 to the stem RNA causes changes of C54, C55, C56, C63, C64, and U65, where C54 and C64 experience the largest changes (Figure 17B). These data strongly suggest that the above-mentioned RNA bases are interacting with the proteins upon protein binding.

NMR model of ADAR2 dsRBM1/2 in complex with R/G stem-loop

To understand the basic principles of this recognition, we constructed a model of ADAR2 dsRBMs in complex with the 71 nt R/G stem-loop based on our precise NMR identification of both the protein and RNA interaction surfaces and on the knowledge of the basic structural elements controlling dsRBMs·RNA recognition (Stefl *et al.*, 2005a). The NMR model with the lowest energy of the ADAR2 dsRBM1/2·R/G stem-loop complex is shown (Figure 18). In a similar manner to what was observed for Rnt1p dsRBM·AGNN tetraloop-containing RNA complex (Wu *et al.*, 2004), the dsRBM1 contacts the minor groove of GCUCA pentaloop

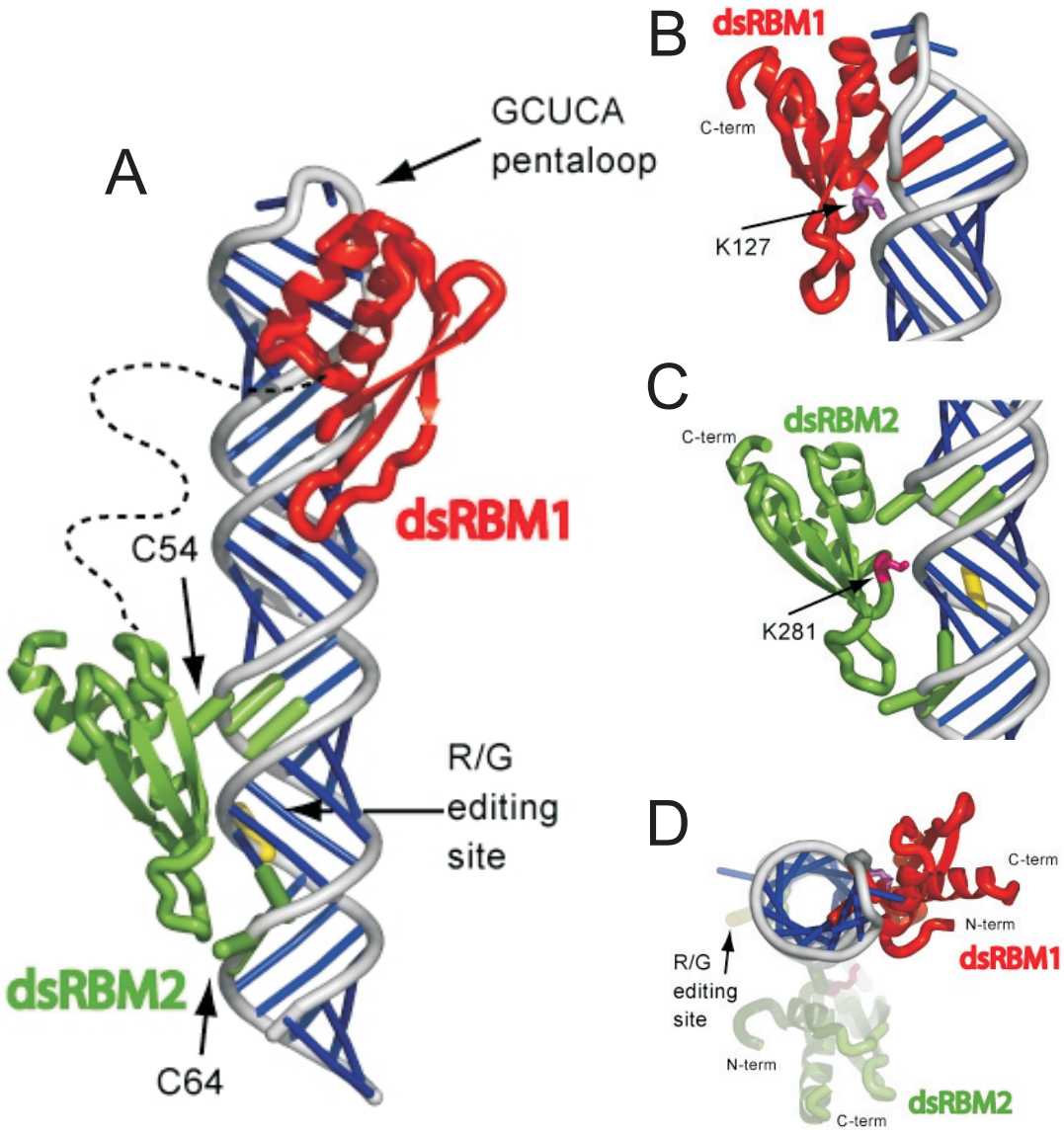

Figure 18. Model for the interaction between ADAR2 dsRBMs and R/G RNA duplex. (A) Overall NMR model of ADAR2 dsRBM1/2 in complex with R/G stem-loop. (B) dsRBM1 (in red) interacts with the central part of the R/G stem-loop: α -helix 1 contacts the pentaloop and adjacent G·U base-pair. (C) dsRBM2 interacts with bulged cytosines, opposite the editing site. K127 and K281, residues mutated to alanine in our later functional studies, are shown in magenta and pink, respectively. (D) Overall NMR model (top view).

and the adjacent G·U mismatch of the central region of R/G stem-loop with the α-helix 1. The dsRBM2 interacts with the bulged C54 and C64 opposite to the editing site. Among the dsRBM·dsRNA complexes determined to date, the interaction of ADAR2 dsRBM2 is unique since dsRBM2 appears to recognize two bulged cytosines.

Both dsRBMs are important for efficient editing of the R/G site

To investigate whether both ADAR2 dsRBM-RNA interactions are important for ADAR2-mediated editing of the R/G site, either dsRBM1 or dsRBM2 were deleted from an eGFP-ADAR2 fusion protein (Figure 19), which has been previously shown to have a comparable enzymatic activity to wild-type ADAR2 protein (Sansam *et al.*, 2003). We took advantage of an in vitro editing system using the R/G editing substrate and wild-type or mutant eGFP-ADAR2 proteins in HEK293 nuclear extracts. Preliminary time course analyses with wild-type eGFP-ADAR2 protein were used to define the linear range of the in vitro editing reaction and equivalent amounts of wild-type and mutant proteins, as determined by quantitative Western blotting, were incubated with an in vitro transcribed R/G editing substrate (Dawson *et al.*, 2004). Nuclear extracts from eGFP transfected cells defined background editing levels for the in vitro system, while the wild-type eGFP-ADAR2 protein demonstrated robust editing of the R/G site (Figure 19). Deletion of either dsRBM1 or dsRBM2 dramatically decreased the editing on R/G site by 3-10 fold, while deletion of both dsRBMs (eGFP-ΔdsRBM1/2) completely eliminated A-to-I conversion at the R/G site. In addition, simultaneous mutations

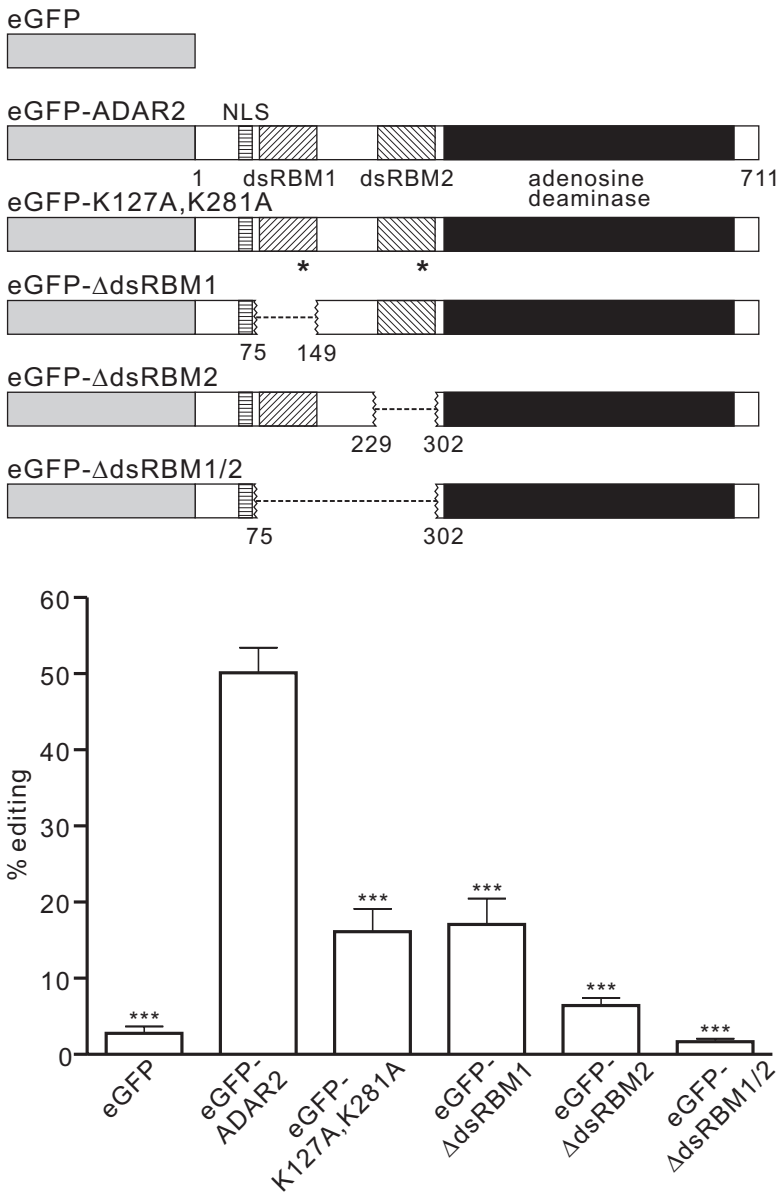

Figure 19. The importance of dsRBMs for R/G site editing. Both dsRBMs are required for R/G site editing. The domain structures of wild-type and mutant proteins used for this analysis are shown on the top. Bottom, quantitative analysis of RNA editing for the R/G site from in vitro editing analyses with wild-type and mutant eGFP-ADAR2 fusion constructs (mean±SEM; n=5); ***, $p<0.001$ compared to wild-type eGFP-ADAR2 using Student's t-test.

of the two highly conserved K127 (dsRBM1) and K281 (dsRBM2) displayed significantly lower editing activity at the R/G site further confirming the importance of RNA binding of both domains for editing, as both side chains are predicted in our NMR-model to interact with the sugar-phosphate backbone (Figure 18).

Functional importance of R/G stem-loop secondary structure

Our NMR study shows that both dsRBM1 and dsRBM2 bind specific region of the R/G stem-loop, dsRBM1 binding near the pentaloop and dsRBM2 binding the stem with two A•C mismatches in the neighborhood of the R/G editing site (Figure 18). The functional importance of the A•C mismatches was previously shown as their replacement by Watson-Crick base-pairs decreases the editing from 74% to 41% (Ohman *et al.*, 2000) and its selectivity for the R/G site from 80% to 30% (Kallman *et al.*, 2003). To assess the functional importance of the pentaloop, we created several mutants in the loop region of the R/G stem-loop (Figure 20) and assayed them for editing activity at the R/G site in vitro. These mutants include a variation in the GCUCA pentaloop sequence (GC**A**CA; a single mutation that changes the fold of the loop) (Stefl and Allain, 2005) and variations in the loop size (GCAA tetraloop and GCUCUA hexaloop). All these mutants display lower editing efficiency at the R/G site compared to the wildtype (Figure 20), indicating that the pentaloop sequence GCUCA and its specific structure is a functional determinant of the editing at the R/G site. In addition, an R/G stem-loop mutant lacking the entire GCUCA pentaloop (R/G stem) has also lower editing activity at the R/G site. Altogether, changes in the sequence or in the size of pentaloop that

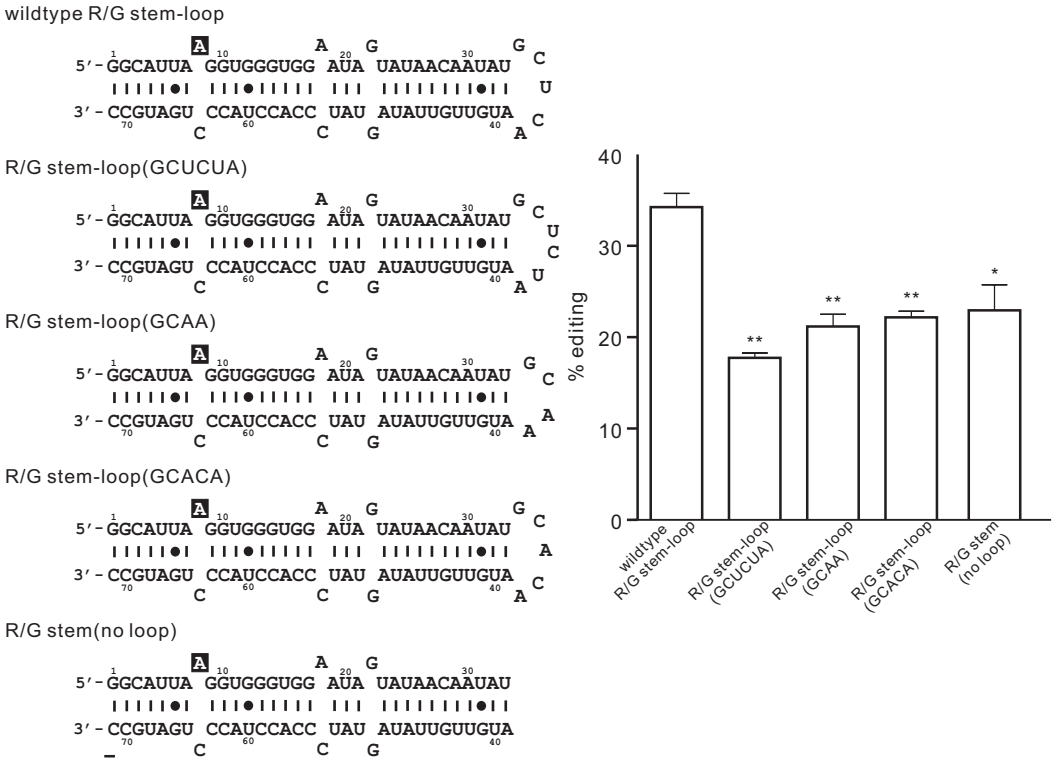

Figure 20. The importance of the pentaloop structure. The pentaloop structure is required for R/G site editing. Various mutations introduced in the loop region of the R/G stem-loop and their role on editing efficiency at the R/G site was examined. Mutations include: GCUCA pentaloop replaced by a GCUCUA hexaloop, by a GCAA tetraloop, and by a GCACA pentaloop. All these mutations change the conformation of the loop. In addition, we used an RNA where the pentaloop of the R/G stem-loop is removed and where the stem sequence is conserved. All mutants were assayed for editing activity in vitro using wild-type eGFP-ADAR2, (mean±SEM; n=3); **, $p<0.01$; *, $p<0.03$ compared to wild-type.

leads to a different pentaloop topology result in lower editing efficiency at the R/G site, indicating the functional importance of the pentaloop structure (Stefl and Allain, 2005)

Discussion

Structure of ADAR2 dsRBMs

In comparison to other dsRBMs, ADAR2 dsRBM1 and dsRBM2 differ from the canonical dsRBM fold like the ones of Xlrbpa2 (Ryter and Schultz, 1998) and *Aquifex aeolicus* RNase III (Blaszczyk *et al.*, 2004). Interestingly, ADAR2 dsRBM1 resembles the dsRBM of Rnt1p (Leulliot *et al.*, 2004) albeit it lacks the α-helix 3, an additional element that imposes the conformation of the "recognition" α-helix 1 in the dsRBM of Rnt1p. ADAR2 dsRBM2 appears to be unique among other members of the dsRBM family. This structural difference in the relative orientation of the α-helix 1 may be functionally important as it may be a key element that modulates the RNA-binding specificity of dsRBMs (see below) (Ramos et al., 2000; Stefl et al., 2005a; Wu et al., 2004).

ADAR2 dsRBMs specifically recognize the R/G stem-loop

With dsRBM-containing proteins, questions regarding binding specificities have always been difficult to answer as this abundant RNA binding domain is considered to bind any dsRNA in a non-sequence specific manner. Structures of single dsRBM in complex with dsRNA indeed revealed that dsRBMs are not

sequence-specific RNA binders but raised the question of whether dsRBMs would rather recognize certain RNA-structures, like stem-loops or irregular duplexes (Ramos et al., 2000; Stefl et al., 2005a; Wu et al., 2004). Our extensive binding study of ADAR2 dsRBMs with the GluR-2 R/G stem-loop and our structural model extends further our understanding of how ADAR2 dsRBMs recognize their targets and more generally how dsRBMs recognize RNA.

Double-stranded RNA binding motifs (dsRBMs) are often present in multiple non identical copies in proteins. In studying the two dsRBMs of ADAR2, we provide one of the first structural studies on how two domains work together. Surprisingly, although both dsRBM are essential for efficient RNA editing, they apparently bind the RNA independently as the interdomain linker (147-231) that bridges the dsRBMs of ADAR2 is found unstructured in both the free and bound forms of the protein and does not appear to participate in the interaction with the R/G stem-loop. Another surprising result is that both dsRBMs are bound in a well-defined location on the R/G stem-loop (dsRBM1 and dsRBM2 close to the pentaloop and the editing site, respectively), indicating that ADAR2 dsRBMs recognize this RNA substrate by themselves without the deaminase domain. This specific binding apparently originates from dsRBM2 that prefer an RNA duplex containing mismatches over a regular A-form duplex or a stem-loop, and from dsRBM1 that prefers a stem-loop over a regular duplex. The binding preference of ADAR2 dsRBM1 for a stem-loop containing a stable GCU(A/C)A pentaloop is reminiscent of Rnt1p dsRBM structure-specific recognition of AGNN tetraloop (Wu *et al.*, 2004) and to Staufen dsRBM3 bound to a stem-loop capped by a

UUCG tetraloop (Ramos *et al.*, 2000). Interestingly, all three dsRBMs have similar structures especially regarding the position of α-helix1. This suggests that dsRBMs binding preference for stem-loop over regular RNA duplexes might be more general than previously expected. In contrast, dsRBM2 favors RNA duplex substrates that contain mismatches and more particularly here two cytosines involved in A·C mismatches. Although we cannot tell if this recognition is base-specific or structure-specific (the backbone deformation around the A·C mismatch), this is the first structural indication that some dsRBMs specifically recognize RNA mismatches.

Deletion of dsRBM1 from ADAR2 decreased the editing of R/G site by 3-fold, and deletion of dsRBM2 decreased the editing of R/G site by 10-fold. This highlights the importance of dsRBM2 and its exclusive binding to an RNA helix containing two A·C mismatches separated by ten base-pairs adjacent to the R/G site. The essential role of dsRBM2 interaction with A·C mismatches are consistent with several biochemical experiments showing that ADAR2 forms multiple non-specific complexes when bound to the R/G stem-loop lacking mismatches, resulting in a dramatically reduced editing efficiency and selectivity at the R/G site (Kallman et al., 2003; Ohman et al., 2000). The binding of dsRBM1 to the stem-loop region that contains structured GCUCA pentaloop is also important as the variations in the loop sequence and size have effect on editing efficiency. This interaction is likely to contribute to the overall binding affinity (Macbeth *et al.*, 2004). In conclusion, this structural study suggests that the dsRBMs of ADAR2 appear to recognize preferentially certain structural elements (the stem-loop and

the mismatches) of the R/G stem-loop, explaining why the secondary structure of the R/G stem-loop is very well conserved (Aruscavage and Bass, 2000).

Implication for ADAR editing

Our structural study of the ADAR2 dsRBMs demonstrates that dsRBMs can specifically recognize certain secondary structure elements of the R/G stem-loop, a natural ADAR2 substrate encoding the 2-subunit of the AMPA-subtype of glutamate receptor. These observations indicate that the R/G stem-loop recognition by the ADAR2 dsRBMs is an important determinant for directing the enzyme to the R/G editing site. How is this related to other editing sites? Recent bioinformatics analyses have predicted more than 12,000 new A-to-I editing sites, located predominantly in Alu repetitive elements in the human transcriptome (Athanasiadis *et al.*, 2004; Blow *et al.*, 2004; Levanon *et al.*, 2004). These analyses showed that A-to-I editing is clearly more frequent at adenosines involved in A·C mismatches than at any other mismatches or base-pairs. These findings correlate well with the binding preferences of ADAR2 dsRBM2 observed in our study and suggests that the dsRBM2 of ADAR2 may play a more general role in A-to-I editing site selection that previously expected. Of course, not all A·C mismatches are edited by ADAR2 indicating that dsRBM2 is not the only determinant for the specificity of A-to-I conversion. Our data showed that the dsRBM1 prefers to bind irregular RNA elements like stem-loops or non-Watson-Crick base-pairs over regular RNA duplexes (in contrast to the dsRBM2 that binds mismatches but not loop regions). The dsRBM1 of ADAR2

may serve to anchor the protein on long irregular RNA, consistent with the observation that most A-to-I editing sites are embedded within irregular RNA duplexes.

CHAPTER IV

DIFFERENTIAL ROLES OF DSRBMS IN REGULATING ADAR2 FUNCTION

Introduction

A-to-I RNA editing is mediated by a family of deaminases known as ADARs (Bass *et al.*, 1997). ADAR1 and ADAR2 can catalyze the hydrolytic deamination of multiple sites in synthetic dsRNAs, or mediate the site-specific modification of naturally-occurring viral and cellular mRNA transcripts (Bass, 2002; Emeson and Singh, 2001).

ADAR2 is responsible for the majority of the codon-altering A-to-I editing events. It displays a modular organization with two tandem dsRBMs connected by a flexible linker in the amino-terminal region and a conserved adenosine deaminase domain at the carboxyl-terminus (Macbeth et al., 2005; Stefl et al., 2006). The two dsRBMs of ADAR2, with >80% amino acid sequence similarity, adopt the same fold as all other members of the dsRBM family, although the two domains differ slightly from one another in the orientation of α1 helix relative to the other secondary structural elements (Stefl et al., 2006). Like other dsRBM-containing proteins, ADAR2 demonstrates a high affinity for dsRNA, and can edit up to 50% of the adenosine moieties in perfect dsRNA (Cho et al., 2003; Dawson et al., 2004; Lehmann and Bass, 2000; Liu et al., 1999). However, ADAR2 can also demonstrate site-specific A-to-I conversion in mRNA transcripts (Bass, 2002; Emeson and Singh, 2001), providing a paradox by which such

specificity can be achieved in the absence of sequence-specific dsRBM-RNA contacts. Recent studies have indicated that the dsRBMs of ADAR2 bind selectively to imperfect RNA duplexes in a manner distinct from that of an PKR-derived dsRBM, suggesting that individual dsRBMs possess intrinsic binding selectivity that influence substrate specificity for the parent protein (Stephens *et al.*, 2004).

In addition to their role(s) in substrate recognition, the dsRBMs of ADAR2 also have been shown to be critical for the nucleolar localization, representing an important mechanism by which RNA editing can be modulated by the sequestration of enzymatic activity from RNA substrates in the nucleoplasm (Desterro *et al.*, 2003; Sansam *et al.*, 2003).

The sequence similarity and conserved tertiary structures of two dsRBMs in ADAR2 have raised questions regarding the role(s) that each dsRBM plays in ADAR2 function. In this study, by deleting or introducing point mutations in each dsRBM, we demonstrated that each dsRBM plays a differential role in both the site-selective RNA editing, as well as in the localization of ADAR2 to the nucleolus. The observation of substrate-dependent contributions of dsRBM to ADAR2 editing activity is consistent with a molecular mechanism by which the dsRBMs interact with distinct structural determinants on different target mRNAs and thus contribute to the editing specificity.

Materials and Methods

Plasmids

To generate fusion constructs encoding eGFP-ADAR2, the rat ADAR2b cDNA (Genbank accession # NM_012894) was subcloned into pEGFP-C1 (Clontech) as previously described (Sansam *et al.*, 2003). Mutation of lysine to alanine at positions 127 and 281 of the open reading frame was performed by PCR-mediated overlap extension (Warrens *et al.*, 1997), using primer sets as follows: VU1127, VU777, VU778 and VU1128 for K127A; VU1127 and VU776 for K281A; VU1127, VU777, VU778 and VU776 for K127A, K281A. Construction of eGFP-ΔdsRBM mutants was performed using the wild-type eGFP-ADAR2 plasmid as a template for "run-around" PCR (Coolidge and Patton, 1995) to delete amino acids 76-148 (ΔdsRBM1 with VU1190 and VU1191), 230-301 (ΔdsRBM2 with VU1192 and VU1193) and 76-301 (ΔdsRBM1/2 with VU1191 and VU1192) from the ADAR2b open reading frame.

Plasmids expressing eYFP-dsRBM fusion proteins were constructed by PCR amplification of a region of the ADAR2b open reading frame encoding dsRBM1 or dsRBM2, corresponding to amino acids 74-147 (VU853 and VU991) or 231-301 (VU1024 and VU1092), respectively, and subcloned into pEYFP-C1 (Clontech).

Construction of plasmids encoding eGFP-ADAR2b isoforms with tandem copies of a single dsRBM was performed using PCR-mediated mutagenesis to delete the region encoding dsRBM2 (234-300, run-around PCR with VU1247 and VU1248) and replace it with amino acids 73-146 (VU1246 and VU1249)

(dsRBM1/1) or by deleting the region encoding dsRBM1 (76-148, run-around PCR with VU1190 and VU1191) and replacing it with amino acids 231-301 (VU1220 and VU1221) (dsRBM2/2). For the dsRBM1/1 plasmid, a single amino acid change was introduced (L301V relative to the wild-type ADAR2b sequence) from subcloning dsRBM1 into the position initially occupied by dsRBM2. The construction of dsRBM2/1 plasmid is the same with that of dsRBM1/1 except for using pEGFP-ADAR2-dsRBM2/2 as the template.

Nucleolin cDNA was amplified by PCR with sense (VU1072) and antisense (VU1073) primers using pSport6-nucleolin (Open Biosystems) as a template and introduced EcoRI and BglII restriction sites, and were used for subsequent subcloning into the pEYFP-C1 vector (Clontech).

Construction of the GluR-2 (R/G site) mini-gene for transfection analysis was made by PCR amplification of mouse genomic DNA with sense (VU1209) and antisense (VU1210) primers to introduce HindIII and XbaI restriction sites for the subcloning into pRC/CMV2 (Invitrogen).

Cell culture and transfection

Human embryonic kidney (HEK293) cells and NIH/3T3 mouse fibroblasts (American Type Culture Collection) were maintained in AMEM and DMEM (Life Technologies, Grand Island, NY), respectively, and supplemented with 10% (vol/vol) bovine calf serum (HyClone, Logan, UT).

HEK293 cells (about 50% confluent in 100mm culture dish) were transiently transfected by calcium phosphate coprecipitation with 8 μg of plasmid encoding

wild-type or mutant eGFP-ADAR2 fusion proteins and 2 μg of either a mouse GluR-2 (R/G site) or rat ADAR2 (-1 site) mini-gene reporter plasmid, as previously described (Rueter *et al.*, 1999). For preparing the nuclear extracts used in vitro editing assay, 8 μg of plasmid encoding wild-type or mutant eGFP-ADAR2 fusion proteins was transfected using similar method.

For analysis of subnuclear fluorescence of dsRBM point mutation or deletion constructs, NIH/3T3 cells were plated on 35 mm glass bottom MatTek dishes (MatTek Corp.) and transiently transfected (when 50% confluent) with 1μg of plasmids encoding wild-type or mutant eGFP-ADAR2 fusion proteins and 1μg of the eYFP-nucleolin plasmid using Fugene 6, according to the manufacturer's instruction (Boehringer Mannheim). For eYFP-dsRBM and eGFP-ADAR2 mutants with tandem dsRBMs, only 1μg of each plasmid is transiently transfected.

Quantitative fluorescence microscopy

Twenty-four hours after transfection, the subcelluar localization of eGFP and eYFP fusion proteins was determined by multi-spectral confocal microscopy (LSM510 Meta, Carl Zeiss Inc); eGFP and eYFP signals were simultaneously excited at 488 nm and the total fluorescence emission in the range of 510-628 nm was passed through a spectral grating and discriminated with 10.7 nm resolution using a multi-anode (multi-channel) array detector. Multi-channel signals for eGFP-only and eYFP-only samples were recorded and subsequently used as reference "signatures" to apply with a linear unmixing algorithm (Zimmermann *et al.*, 2003) for clear discrimination of both fluorophores when mixed in the same

specimen. All images were acquired using a 40x/1.3 Plan Neofluar objective lens. Average nucleolar eGFP fluorescence intensity (Fn) was defined as the eGFP signal overlapping with eYFP-nucleolin and was compared to the average eGFP fluorescence intensity from a comparable area of the nucleoplasm (Fo) using the Image J image analysis software (http://rsb.info.nih.gov/ij/ ; National Institute of Mental Health, Bethesda, Maryland). For eYFP-dsRBM or eGFP-ADAR2 mutants with tandem dsRBMs, the eYFP or eGFP fluorescence images were acquired using the appropriate filters. The relative positions of nucleolus and nucleoplasm were determined based on the cell shape.

Quantitative analysis of editing on transfected ADAR substrates

Total RNA was isolated from HEK293 cells, 60 hours after transient transfection, using TRI Reagent (Molecular Research Center, Inc.). First-strand cDNA was synthesized using AMV reverse transcriptase (Promega) with minigene-specific primers for ADAR2 -1 site (VU343) and GluR-2 R/G site (VU683) using 5 μg of total RNA.

To quantify the relative expression of ADAR2 mRNA splice variants resulting from editing at the -1 site, the ADAR2 cDNA was amplified by PCR with a 6-carboxyfluorescein (6-FAM) labeled sense primer (VU1201) and a non-labeled antisense primer (VU343). Amplicons corresponding to alternatively spliced ADAR2 variants were resolved by 2.5% agarose gel electrophesis, and quantified by phosphorimager analysis (Amersham Biosciences).

To quantify the editing on GluR-2 R/G site, the cDNA derived from the

mini-gene was PCR amplified by primer set: VU682 and VU683. The amplicons were used to was perform a modified primer-extension analysis, as described previously (Dawson *et al.*, 2004).

Western blotting analysis

Crude nuclear extracts were prepared from HEK293 cells, 60 hours after transient transfection, as previously described (Schreiber *et al.*, 1989) and diluted with dialysis buffer to maintain enzymatic activity (30mM HEPES pH 7.6, 300mM NaCl, 10% glycerol, 1mM EDTA, 0.5mM EGTA, 1mM DTT, 1mM PMSF, 2mg/ml leupeptin, 0.1% aprotinin) (Rueter *et al.*, 1999). Equivalent volumes for each protein sample were resolved by polyacrylamide gel electrophoresis (7.5-12% SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond-C Super; Amersham Biosciences). The membrane was probed with an affinity-purified ADAR2-specific antiserum raised against amino acids 6-66 of the rat ADAR2 open-reading frame (Sansam *et al.*, 2003), detected with an Alexa Fluor 680-labeled donkey anti-sheep IgG secondary antibody (0.4ng/ml) and quantified using an Odyssey infrared imaging system (LI-COR Biotechnology).

In vitro editing assay

The plasmids containing the editing substrate minigenes (Burns et al., 1997; Dawson et al., 2004; Rueter et al., 1995; Rueter et al., 1999) were linearized with restriction eyzymes, followed by in vitro transcription using the Megascript T7 transcription kit (Ambion) or T3 RNA polymerase (Promega) (Table 3). The

Table 3. Summary of experimental conditions for in vitro RNA editing and primer extension analysis.

RNA fragment	site	cut by	transcription	ADAR2 dilution	time	RT primer	PCR primer	PE primer	ddNTP, dNTP	nonedited	edited
D79N Rueter *et al.*, 1995		XhoI NotI	T7 T3	1:15	60mins			Thin Layer Chromatography			
rat GluR-2 Rueter *et al.*, 1995	Q/R	BamHI	T3	1:30	20mins	VU35	VU3 VU35	VU97(15mer)	ddATP dCTP,dGTP,dTTP	19mer	22mer
mouse GluR-2 Dawson *et al.*, 2004	R/G	EcoRI	T3	1:270	30mins	VU683	VU682 VU683	VU788(17mer)	ddTTP dCTP,dATP	21mer	22mer
mouse $5HT_{2C}R$ Burns *et al.*, 1997	D	XhoI	T7	1:30	8mins	VU168	VU183 VU168	VU1183(21mer)	ddCTP,ddGTP dATP,dTTP	24mer	26mer
mouse ADAR2 Dawson *et al.*, 2004	-1	MscI	T7	1:30	5mins	VU477	VU331 VU38	VU356(17mer)	ddTTP dCTP	19mer	20mer

template DNA was removed by RQ1 DNase treatment (Promega) and the concentration of the resultant RNAs was determined by UV absorbance spectrometry at 260 nm. The two strands of D79N dsRNA were transcribed in the presence of [α-^{32}P]-ATP individually and hybridized, and the concentration was determined by scintillation spectrometry as described previously (Dawson *et al.*, 2004).

For preliminary studies (Figure 25), HEK293 nuclear extracts containing the wild-type eGFP-ADAR2 fusion protein were variably diluted for a time-course study to determine the linear range of enzymatic activity for each RNA substrate. 100 fmoles of each RNA substrate was incubated with nuclear extracts containing equivalent amounts of eGFP-ADAR2 protein at 30^{o} C, in a total volume of 50 µl, for varying time periods and the reactions were terminated by freezing on dry ice.

Subsequent analyses of editing activity for equivalent amounts of wild-type and mutant eGFP-ADAR2 fusion proteins were performed in the same system using the empirically determined conditions based on the time course study (Table 3).

Quantitative analysis of in vitro RNA editing activity

Quantification of editing activity for [α-^{32}P]-labeled D79N dsRNA was performed by thin-layer chromatography (Rueter *et al.*, 1995). 20 fmoles of the in vitro reaction products for other substrates were RT-PCR amplified and subjected to a modified primer-extension analysis, as described previously (Table 3) (Burns et al., 1997; Dawson et al., 2004; Feng et al., 2006; Rueter et al., 1995; Rueter et

al., 1999).

Some RT-PCR products were also used for bulk DNA sequencing to inspect multiple editing sites on the same transcript, with the template specific primers (VU3 for Q/R site, VU183 for D site, VU331 for -1 site).

Results

Differential roles of dsRBMs in nucleolar localization

Previous analyses of ADAR2 subcellular localization have taken advantage of enzymatically active eGFP-ADAR2 fusion proteins to demonstrate rapid shuttling between the nucleolus and nucleoplasm, yet deletion of both dsRBMs, or the mutation of highly conserved lysine moieties (K^{127} and K^{281}) in each dsRBM, results in the translocation of ADAR2 from the nucleolar to the nucleoplasmic compartment (Desterro *et al.*, 2003; Sansam *et al.*, 2003). To further examine the role of individual dsRBM in maintaining the steady-state nucleolar localization of ADAR2 in living cells, we employed multicolor fluorescence microscopy using a series of eGFP-ADAR2 mutants in transfected NIH/3T3 mouse fibroblasts (Figure 21), along with an eYFP-nucleolin fusion protein that was included to define the nucleolar compartment. The relative nucleolar localization of wild-type and mutant eGFP-ADAR2 proteins was quantified by measuring the average fluorescence intensity of eGFP-ADAR2 that overlapped with the eYFP-nucleolin signal in nucleoli (Fn) compared to the average fluorescence intensity of eGFP-ADAR2 from a corresponding area of the nucleoplasm (Fo). Because eGFP and eYFP

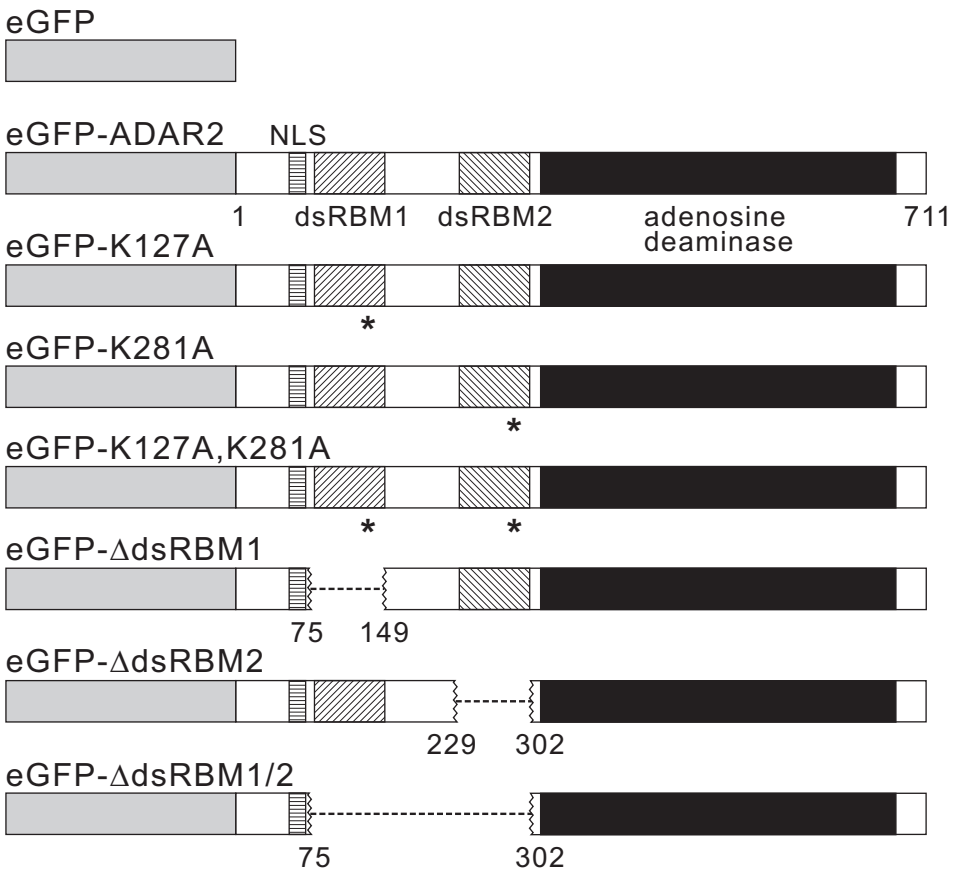

Figure 21. eGFP-ADAR2 fusion constructs for analysis of subnuclear localization and site-specific RNA editing. A schematic diagram indicating the domain structures of eGFP, wild-type (eGFP-ADAR2) and mutant fusion proteins is presented showing deletion or mutation of the double-stranded RNA-binding motifs (dsRBMs). The positions of lysine-to-alanine (KA) mutations at positions 127 and 281 are indicated with asterisks and the coordinates for each deletion are indicated relative to the normal ADAR2 start codon. NLS, nuclear localization signal.

have strongly overlapping emission spectra, we applied a computational spectral separation technique to resolve the signals from each fluorophore (Zimmermann *et al.*, 2003). Transfection of eGFP alone resulted in a diffuse pattern of fluorescence in the cytoplasm and nucleus, with no preferential concentration in nucleoli, whereas the pattern of eYFP-nucleolin fluorescence was highly restricted to the nucleolar compartment (Figure 22A). The fluorescence pattern for cells co-transfected with both eGFP and eYFP-nucleolin was identical to that observed when each fluorophore was transfected independently, with little observable bleed through between the emission channels for these simultaneously excited fluorescent proteins (Figure 22A).

Transient expression of wild-type eGFP-ADAR2 in NIH/3T3 cells demonstrated the previously observed pattern of nucleolar localization (Fn/Fo = 13.8±2.4), and deletion of both dsRBM domains and the intervening 81 aa linker [ΔdsRBM1/2 (Δ76-301)] produced the expected pattern of diffuse nuclear fluorescence (Figure 22A), suggesting that dsRNA-binding was required to maintain the steady-state localization of ADAR2 in nucleoli (Desterro *et al.*, 2003; Sansam *et al.*, 2003). Expression of a mutant eGFP-ADAR2 fusion protein (K127A, K281A) containing substitutions for highly conserved amino acids in the loop between the β3 and α2 regions for all dsRBMs (Tian *et al.*, 2004), resulted in a diffuse pattern of nuclear fluorescence nearly identical to the pattern observed when the region containing both dsRBMs was deleted from the fusion protein (Figure 22A). These results further demonstrate that the localization of ADAR2 depends upon its ability to bind dsRNA, since analogous mutations in PKR and

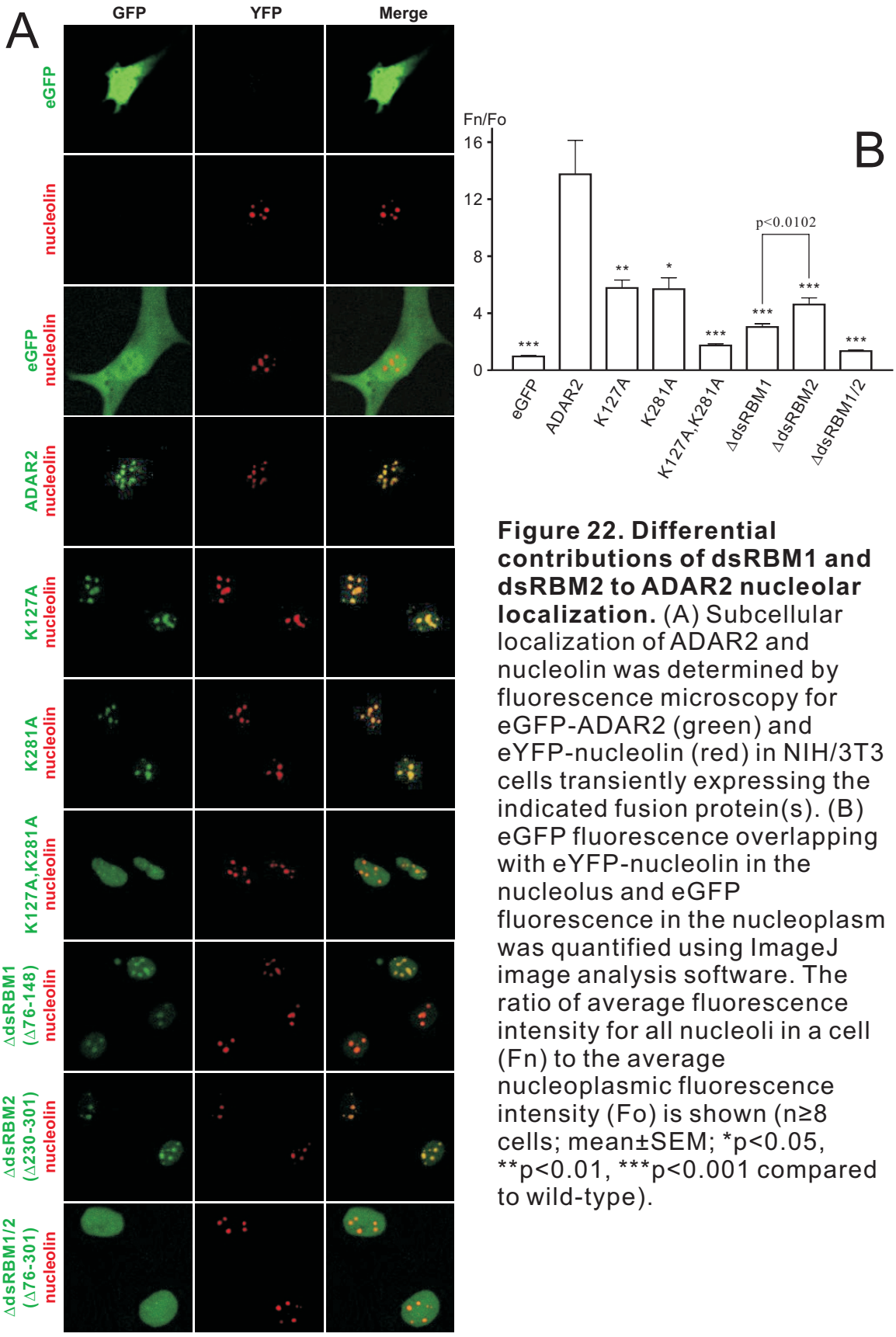

Figure 22. Differential contributions of dsRBM1 and dsRBM2 to ADAR2 nucleolar localization. (A) Subcellular localization of ADAR2 and nucleolin was determined by fluorescence microscopy for eGFP-ADAR2 (green) and eYFP-nucleolin (red) in NIH/3T3 cells transiently expressing the indicated fusion protein(s). (B) eGFP fluorescence overlapping with eYFP-nucleolin in the nucleolus and eGFP fluorescence in the nucleoplasm was quantified using ImageJ image analysis software. The ratio of average fluorescence intensity for all nucleoli in a cell (Fn) to the average nucleoplasmic fluorescence intensity (Fo) is shown (n≥8 cells; mean±SEM; *p<0.05, **p<0.01, ***p<0.001 compared to wild-type).

Staufen have been shown to ablate dsRNA binding activity (McMillan *et al.*, 1995; Ramos *et al.*, 2000). The expression of eGFP-ADAR2 mutants containing independent deletions of dsRBM1 [ΔdsRBM1 (Δ76-148)] or dsRBM2 [ΔdsRBM2 (Δ230-301)] also resulted in reduced nucleolar fluorescence for both fusion proteins (Figures 21, 22), indicating that both dsRBMs are essential for the normal steady-state localization of ADAR2 in nucleoli. The relative nucleolar fluorescence for the ΔdsRBM1 mutant was significantly less than for the fusion protein lacking dsRBM2 ($p<0.02$), suggesting a greater role for dsRBM1 in the subnuclear compartmentalization of ADAR2. Further observation that independent substitutions of K^{127} and K^{281} decreased relative nucleolar fluorescence to the same extent (K127A and K281A), yet the magnitude of this effect was less than that observed for the dsRBM deletions (Figure 22), suggested that additional contacts between the dsRBMs and nucleolar dsRNA target(s) are required for normal nucleolar localization.

Since deletion or even subtle point mutations of ADAR2 dsRBMs could result in structural alterations that cause ADAR2 mislocalization, we also examined the subcellular localization of eYFP when expressed as a fusion protein with either dsRBM1 (aa 74-147) or dsRBM2 (aa 231-301) alone (Figure 23); the precise borders for each motif were based upon amino acid sequence homology to other dsRBM-containing proteins and the recently resolved NMR structure for these domains in ADAR2 (Fierro-Monti and Mathews, 2000; Stefl et al., 2006). Both eYFP-dsRBM1 and eYFP-dsRBM2 were localized to the cytoplasm and nucleus, presumably due to the absence of a nuclear localization signal and the passive

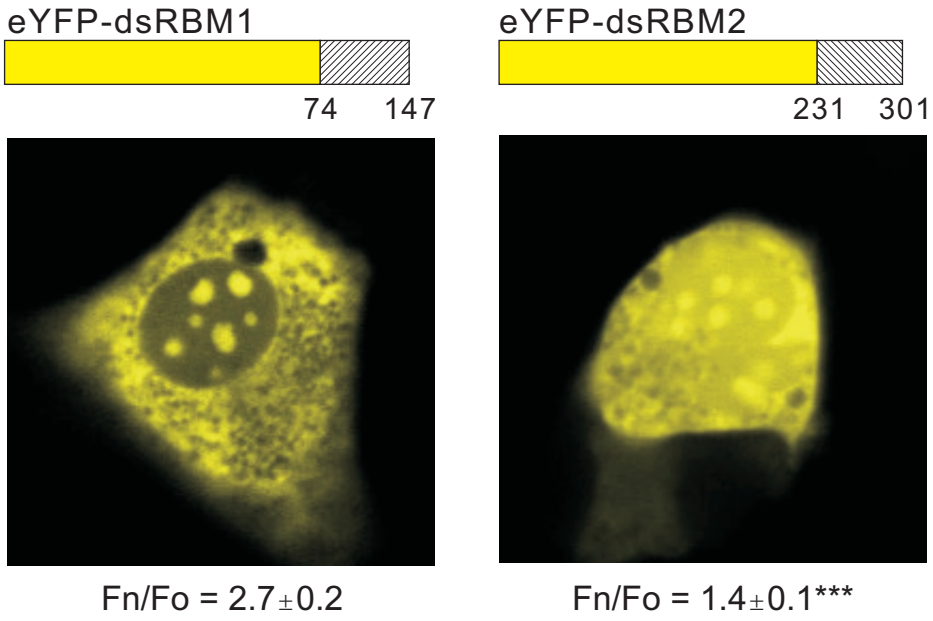

Figure 23. Ability of ADAR2 dsRBMs to localize eYFP to the nucleolus. A schematic diagram indicating the domain structures of eYFP-dsRBM1 and eYFP-dsRBM2 fusion proteins is presented; the coordinates of the dsRBM domain are indicated relative to the normal ADAR2 start codon. A representative micrograph of the subcellular localization of each fusion protein in NIH/3T3 cells is shown with corresponding Fn/Fo values (n≥10 cells; mean±SEM; ***, $p<0.001$)

diffusion of in these small fusion proteins (~40 kD) through the nuclear pore (Suntharalingam and Wente, 2003); however, eYFP-dsRBM1 was localized to nucleoli significantly better than eYFP-dsRBM2 ($p<0.001$; Figure 23), further confirming a non-equivalent role for these highly conserved domains in the maintenance of ADAR2 nucleolar localization.

Differential roles for dsRBM in site-selective editing

Given the differential effects that mutation or deletion of dsRBMs have on the nucleolar localization of ADAR2, it is unclear to what extent these domains also control the site-selective editing of ADAR2 substrates. To further examine the roles of individual dsRBMs, we employed a transiently transfected tissue culture model system in which a series of eGFP-ADAR2 mutants were assessed for their ability to catalyze site-selective A-to-I conversion in two well characterized ADAR2 targets, the -1 and R/G sites of ADAR2 and GluR-2 transcripts, respectively (Dawson et al., 2004; Lomeli et al., 1994; Rueter et al., 1999). HEK293 cells were chosen for this analysis as previous studies demonstrated a low level of endogenous editing activity in this cell line (Burns et al., 1997; Maas et al., 1996; Rueter et al., 1999; Schaub and Keller, 2002).

Editing of the -1 site within ADAR2 pre-mRNAs represents a negative autoregulatory mechanism by which ADAR2 can modulate its own level of expression by generating a proximal 3'-splice site within intron 4 to direct the inclusion of an additional 47 nucleotides in the ADAR2 open reading-frame (Dawson et al., 2004; Feng et al., 2006; Rueter et al., 1999). As an indirect index

of -1 site editing, we quantified the relative abundance of minigne-derived ADAR2 splice variants, containing (+47) or lacking (-47) this alternatively spliced cassette, using an RT-PCR-based strategy with a 6-carboxyfluorescein (6-FAM) labeled sense PCR primer (Feng *et al.*, 2006). Cotransfection of eGFP and the minigene resulted in sole expression of the -47 RNA isoform, indicative of the absence of -1 site editing, whereas coexpression of wild-type eGFP-ADAR2 generated the +47 isoform almost exclusively (Figure 24A). Deletion of dsRBM1 (ΔdsRBM1) or substitution of the conserved lysine in this domain (K127A) had little effect on the extent of editing-dependent alternative splicing, yet deletion of dsRBM2 (ΔdsRBM2) or deletion of both dsRBMs (ΔdsRBM1/2) completely ablated use of the proximal 3'-splice site. These results indicate that dsRBM1 is not required, while dsRBM2 is critical, for site-specific editing at the -1 site of ADAR2 pre-mRNA transcripts. Further substitution of a conserved lysine in dsRBM2 (K281A) or conserved lysines in both dsRBMs (K127A,K281A), reduced the extent of alternative splicing by 44-61%, indicating K^{281} contributes only partially to dsRBM2-dependent editing at the -1 site (Figure 24A). Similar analyses, using a minigene derived from the GluR-2 transcript, demonstrated that while deletion of dsRBM1 (ΔdsRBM1) caused a 19% decrease in R/G site editing ($p<0.01$), deletion of dsRBM2 resulted in a 57% reduction of ADAR2 activity ($p<0.001$) (Figure 24B). These results were quite distinct from those observed for the -1 site, where dsRBM1 was completely dispensable and dsRBM2 was essential for A-to-I conversion (Figure 24A), demonstrating the differential roles that these dsRBMs play in the editing of specific sites.

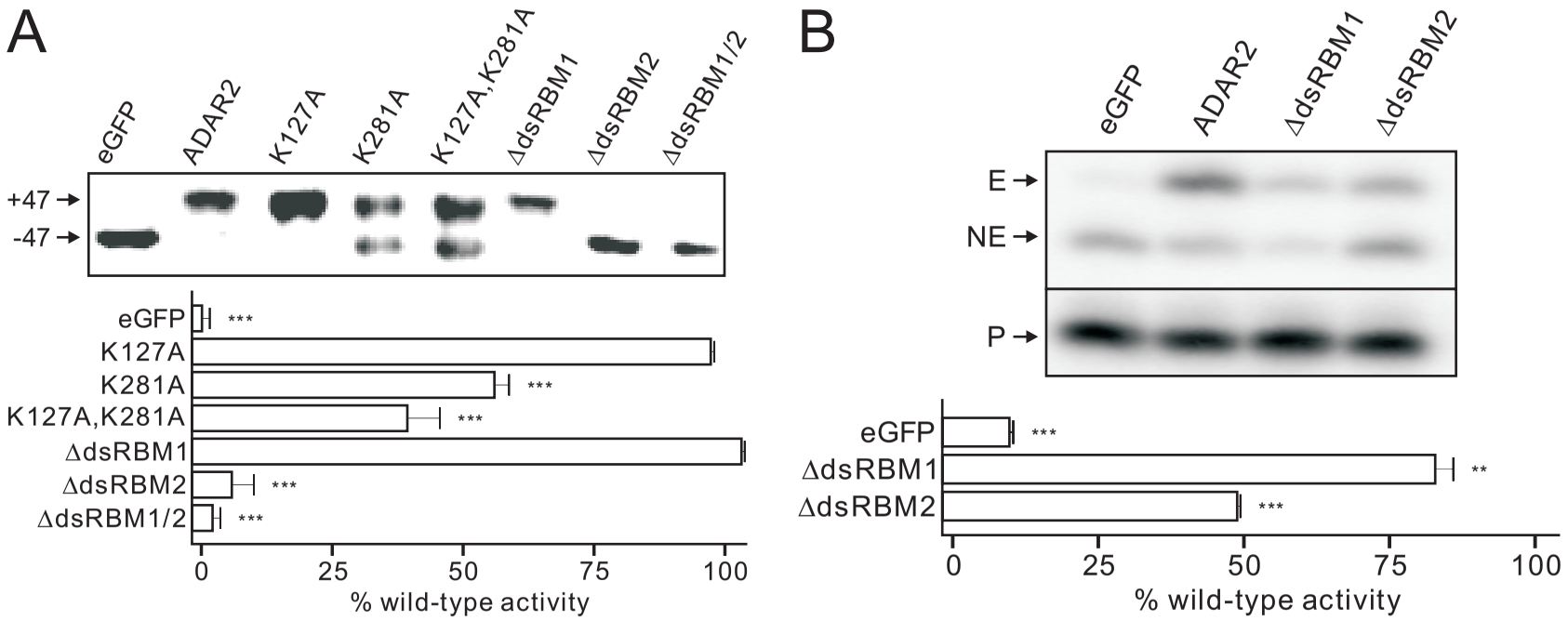

Figure 24. Differential roles for dsRBM1 and dsRBM2 on editing of transfected ADAR substrates. (A) Quantitative analysis of ADAR2 alternative splicing is presented from HEK293 cells transiently co-transfected with a genomic ADAR2 minigene and eGFP-ADAR2 fusion constructs. The migration positions of RT-PCR amplicons generated from alternatively spliced ADAR2 transcripts, resulting from the presence (+47) or absence (-47) of A-to-I editing, are indicated. Relative editing activity was normalized to the extent of alternative splicing (+47) observed for the wild-type eGFP-ADAR2 fusion construct (n≥3, mean±SEM;***p<0.001 compared to wild-type). (B) Modified primer-extension analysis of GluR-2 (R/G) editing from HEK293 cells transiently co-transfected with a GluR-2 (R/G) minigene and eGFP-ADAR2 fusion constructs. The migration positions for the primer (P) and primer-extension products corresponding to edited (E) and non-edited (NE) RNAs are shown. A summary of GluR-2 (R/G) editing activites for eGFP-ADAR2 mutants is shown with all values normalized to wild-type (eGFP-ADAR2) activity (n=4, mean±SEM; **p<0.01, ***p<0.001 compared to wild-type).

Substrate-specific contributions from each dsRBM

While preliminary transfection studies provided insights into distinct roles for ADAR2 dsRBMs in defining substrate-specific A-to-I conversion, this model system has a number of disadvantages for comparing the relative activities of different eGFP-ADAR2 mutants. These disadvantages include differences in the levels of expression for transfected fusion proteins and minigene-derived RNA substrates and the fact that such cellular RNA processing events do not necessarily occur in the linear range for ADAR2 enzymatic activity. In addition, previous studies have demonstrated that A-to-I conversion takes place in the nucleoplasm (Desterro et al., 2003; Raitskin et al., 2001; Rueter et al., 1999; Sansam et al., 2003) and that translocation of ADAR2 to the nucleoplasm results in increased editing activity (Sansam *et al.*, 2003), making comparisons of editing activity for mutations that simultaneously affect subnuclear accumulation and site-specific editing difficult to interpret.

To circumvent these problems, we employed an in vitro editing system using crude nuclear extracts from HEK293 cells transiently transfected with different eGFP-ADAR2 mutants. The relative protein level for wild-type and mutant eGFP-ADAR2 proteins in HEK293 nuclear extracts was determined by quantitative Western blotting analysis using an affinity-purified antiserum directed against amino acids 6-66 of wild-type ADAR2 (Figure 25) (Dawson et al., 2004; Sansam et al., 2003), and all proteins were diluted to achieve the same final concentration in the in vitro editing reaction. A band corresponding to the expected molecular weight for each eGFP-ADAR2 protein was observed, along

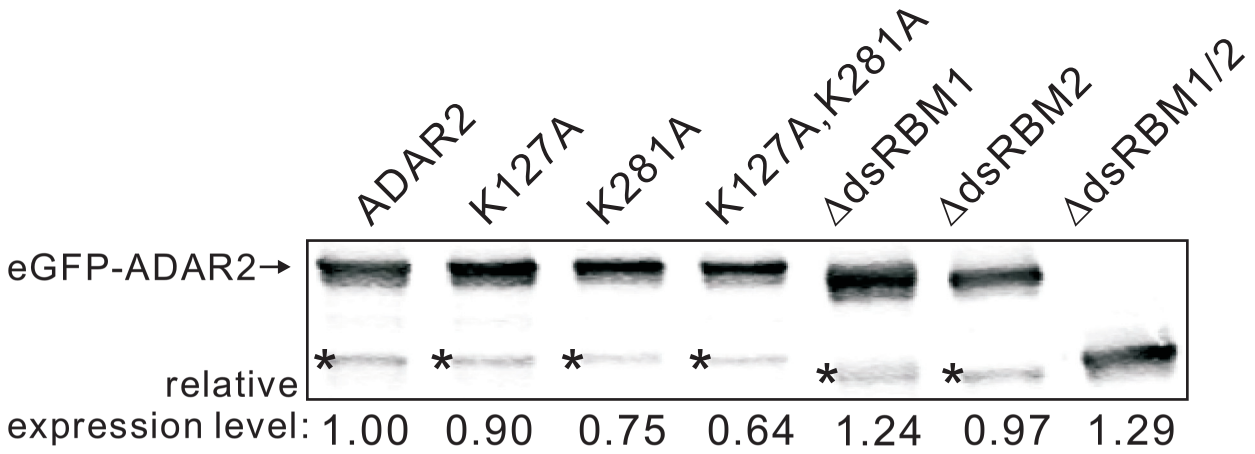

Figure 25. Quantification of wild-type and mutant eGFP-ADAR2 protein levels in HEK293 nuclear extracts. A representative Western blot is presented for quantitative analysis of eGFP-ADAR2 fusion protein levels; partially degraded protein fragments are indicated with an asterisk. A determination of relative protein expression in each nuclear extract was performed in triplicate and normalized to the expression level for wild-type eGFP-ADAR2.

with a minor band representing a stable degradation product (Figure 25), and no signal was seen for mock-transfected HEK293 cells. In addition to the RNA substrates previously used for transfection studies (Figure 24), the in vitro analyses also took advantage of a variety of ADAR2 substrates that are distinct at the level of both nucleotide sequence and predicted RNA secondary structure (Dawson *et al.*, 2004), including RNA targets containing the D-site of 5-HT_{2C}R transcripts (Burns *et al.*, 1997), the Q/R site of GluR-2 RNAs (Higuchi et al., 1993; Sommer et al., 1991) and a perfect, synthetic dsRNA (D79N) derived from a portion of the α2A adrenergic receptor (Burns *et al.*, 1997; Lakhlani *et al.*, 1997). For each RNA editing substrate (100 fmoles), the amount of the eGFP-ADAR2 protein and the duration of the reaction were empirically determined to assure that all of the editing reactions were performed in the linear range of the assay (Figure 26). The extent of total inosine production for the perfect dsRNA was determined by thin-layer chromatography (Rueter *et al.*, 1995) and site-specific editing of the remaining ADAR2 substrates was quantified using a modified primer-extension analysis (Dawson et al., 2004; Feng et al., 2006; Sansam et al., 2003).

Using crude nuclear extracts from mock-transfected HEK293 cells, only background levels of editing were observed for any of the RNA substrates (Figure 27), consistent with previous observations regarding the low level of endogenous editing activity in this cell line (Burns et al., 1997; Maas et al., 1996; Rueter et al., 1999; Schaub and Keller, 2002). Deletion of the region containing both dsRBM domains and the intervening linker (ΔdsRBM1/2) reduced editing in all RNA targets to near background levels, as did deletion of dsRBM2 alone (ΔdsRBM2),

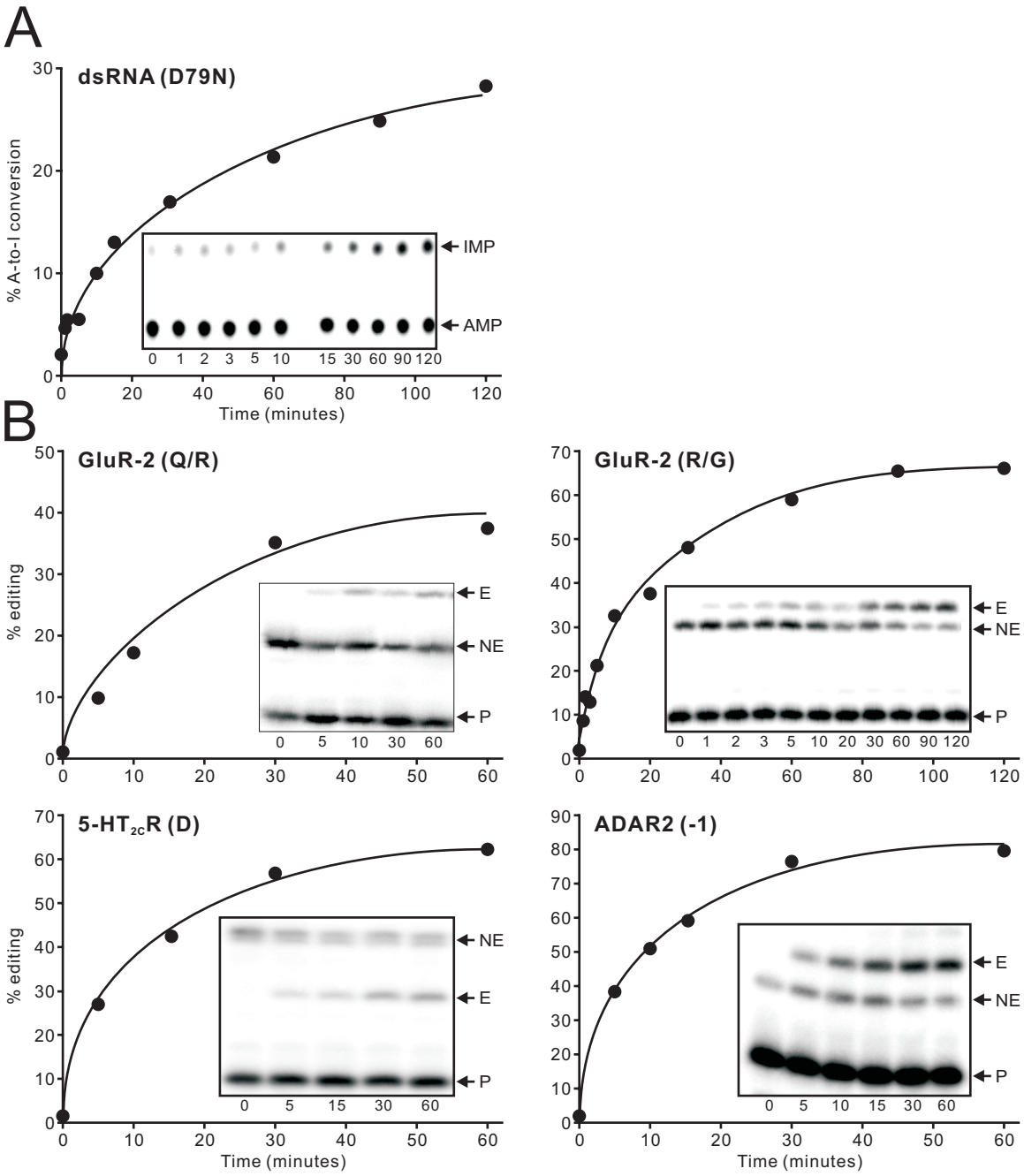

Figure 26. In vitro time course analysis of site-specific editing for the wild-type eGFP-ADAR2 protein. A time course analysis for eGFP-ADAR2 from HEK293 nuclear extracts was incubated with 100 fmol of in vitro transcribed ADAR substrates. (A) The extent of editing for the perfect dsRNA duplex (D79N) was determined by thin layer chromatography; the migration positions of adenosine 5'-monophosphate (AMP) and inosine 5'-monophosphate (IMP) are indicated. (B) Modified primer-extension analyses of editing are shown using in vitro reactions with eGFP-ADAR2 and RNA transcripts. The migration positions for the primer (P) and extension products corresponding to edited (E) and non-edited (NE) RNAs are shown.

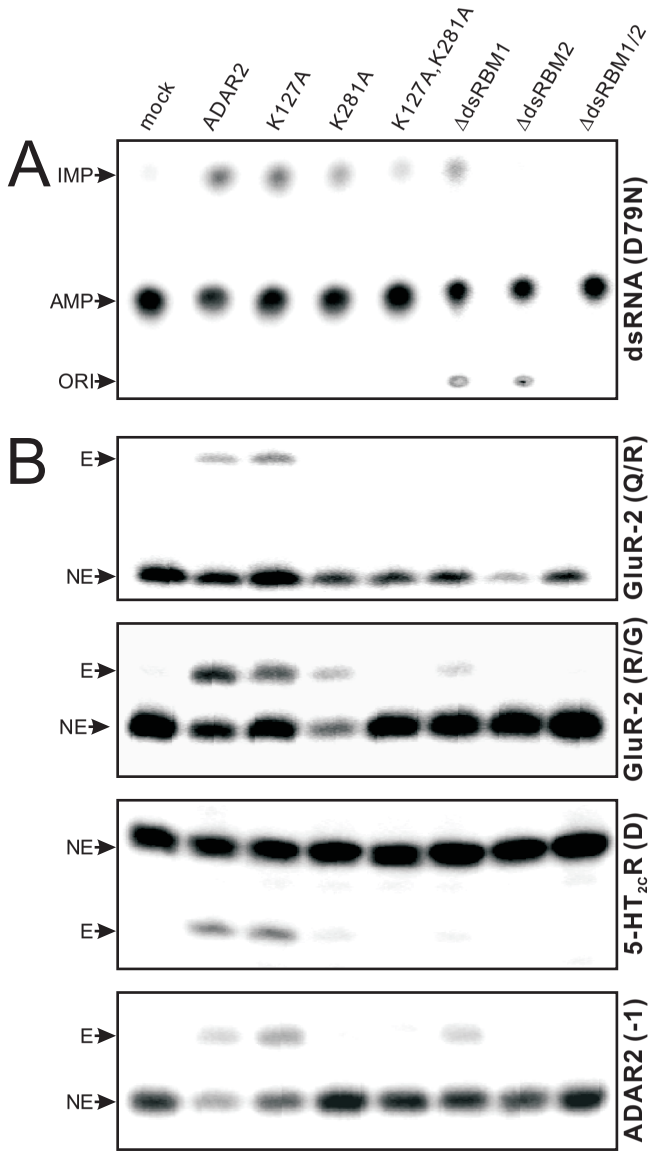

Figure 27. Differential contributions of dsRBM1 and dsRBM2 to site-specific editing activity. Representative in vitro analyses of editing activity for multiple ADAR substrates mediated by eGFP-ADAR2 fusion proteins are shown. (A) Editing activity for a synthetic, perfect RNA duplex (D79N) was determined by thin-layer chromatography (TLC); the TLC origin (ORI) and the migration positions of adenosine 5'-monophosphate (AMP) and inosine 5'-monophosphate (IMP) are indicated. (B) Modified primer-extension analyses of site-specific editing are shown using in vitro reactions with eGFP-ADAR2 fusion proteins and minigene transcripts. The migration positions for primer-extension products corresponding to edited (E) and non-edited (NE) RNAs are shown.

demonstrating that dsRBM2 is essential for the editing of perfect dsRNAs as well as naturally occurring ADAR2 substrates containing imperfect inverted repeats. Interestingly, deletion of dsRBM1 (ΔdsRBM1) decreased editing in a substrate-dependent fashion, as the extent of editing for GluR-2 (Q/R site) and 5-HT_{2C}R (D-site) transcripts was less than 10% of that observed for wild-type eGFP-ADAR2 (Figure 27, 28), whereas editing of the remaining ADAR2 substrates was variably reduced by 30 to 70% (Figure 27, 28). These results not only show that the dsRBMs of ADAR2 are required for editing activity, but also demonstrate that the dependence upon dsRBM1 varies in a substrate-specific manner.

Substrate-specific contributions from K^{281}

Independent (K127A and K281A) or simultaneous (K127A, K281A) lysine substitutions also demonstrated differential effects on the various ADAR2 targets with the K281A mutation causing a modest decrease (13-25%) in the extent of editing for the perfect dsRNA (D79N) and the GluR-2 (R/G) transcript, while editing efficiency for the GluR-2 (Q/R), 5-HT_{2C}R (D) and ADAR2 (-1) sites was reduced by greater than 85% (Figure 27, 29B). The observation that dsRBM2 was critical for efficient editing of all targets examined, yet only a subset of these substrates was affected by the K281A mutation, demonstrates the differential role that specific amino acid residues in dsRBM2 play in site-specific adenosine deamination. By contrast with the substrate-dependent effect seen in K281A, the K127A mutation produced a minimal decrease in editing for all RNAs examined

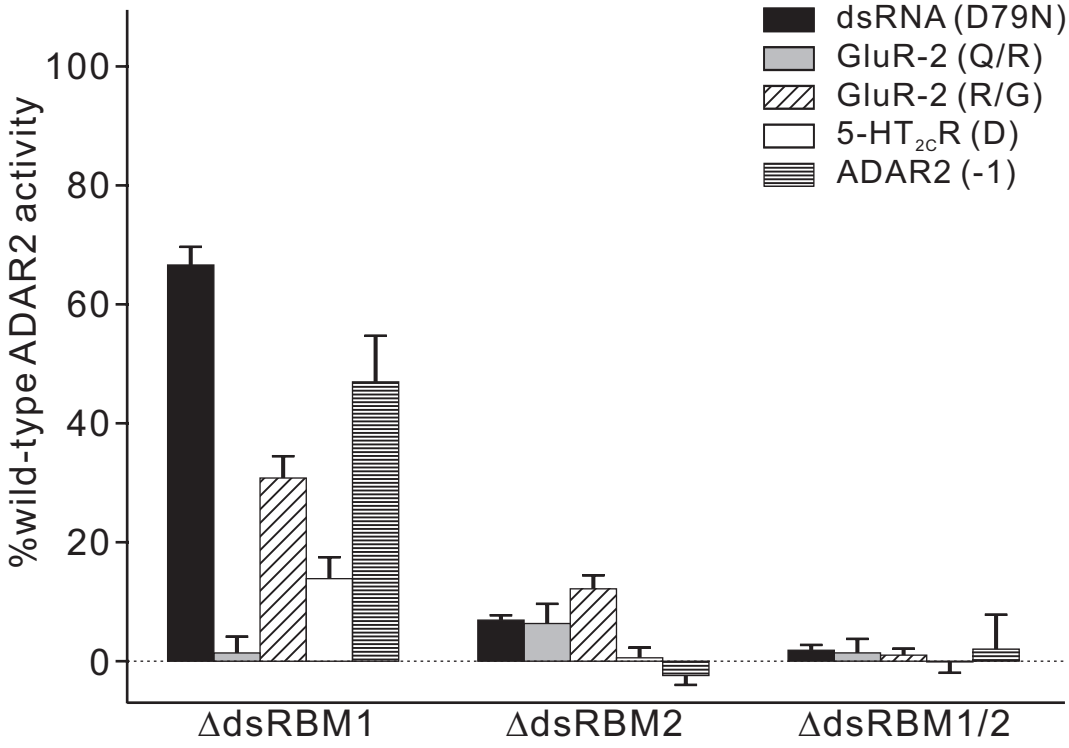

Figure 28. Quantitative analysis of dsRBM1 and dsRBM2 contributions to site-specific editing activity. For each substrate the relative activity is shown by normalizing mutant editing activity to the wildtype eGFP-ADAR2 editing activity after background subtraction. (mean±SEM; n≥3)

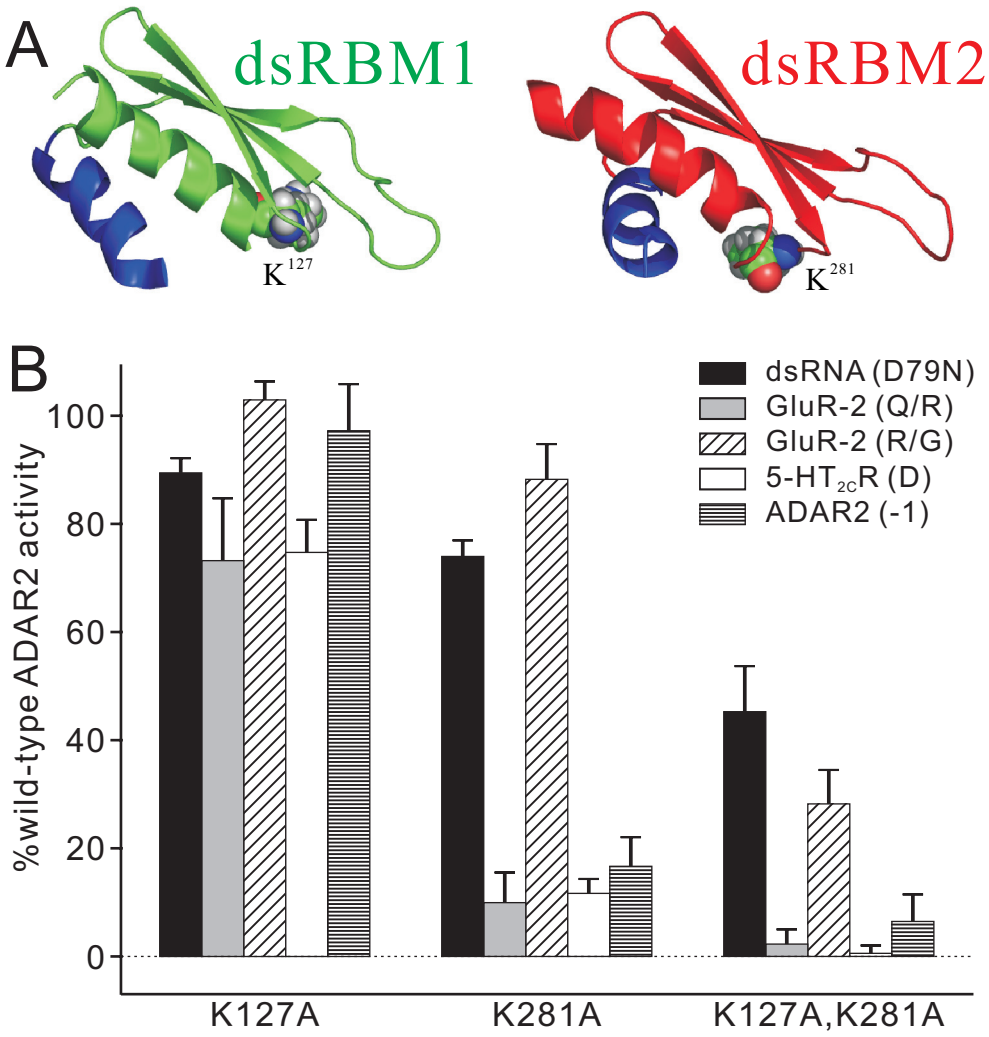

Figure 29. Quantitative analysis of K^{127} and K^{281} contributions to site-specific editing activity. (A) The positions of K^{127} and K^{281} within the structures of dsRBM1 and dsRBM2 are shown. (B) For each RNA substrate, the relative activity for ADAR2 mutants is shown by normalizing mutant editing activity to the wild-type eGFP-ADAR2 value after background subtraction (mean±SEM; n≥3).

(Figure 29). As K^{127} and K^{281} share the same relative position in each dsRBM (Figure 29A), these results further demonstrate the differential role of each dsRBM in site-specific A-to-I editing.

Function-dependent interchangeability of ADAR2 dsRBMs

The contribution of dsRBM1 to the site-selective editing of ADAR2 appears to be substrate-dependent, while dsRBM2 is critical for enzymatic activity with all substrates examined (Figure 28), despite the high degree of conservation at both the levels of amino acid sequence and protein structure (Tian *et al.*, 2004). This functional inequality between dsRBM1 and dsRBM2 could result from subtle sequence or structural differences affecting RNA-protein interactions or the relative position of each motif in the ADAR2 protein. To determine if the unique properties of each dsRBM regarding site-specific editing and nucleolar localization are a function of their relative position, three additional eGFP-ADAR2 mutants (Figure 30A, 31A) containing tandem copies of either dsRBM1 (dsRBM1/1) or dsRBM2 (dsRBM2/2), or a mutant in which the positions of the dsRBMs were interchanged (dsRBM2/1), were used for in vitro editing analysis of the same five ADAR2 substrates. To assess the level of mutant eGFP-ADAR2 expression in crude nuclear extracts from HEK293 cells, quantitative Western blotting analysis was performed (as in Figure 26) to adjust the enzyme input to equivalent levels for the in vitro reaction (Figure 30B). If the properties of each dsRBM are dependent simply upon its relative position, replacement of either dsRBM with the alternate motif should maintain wild-type editing activity, yet such

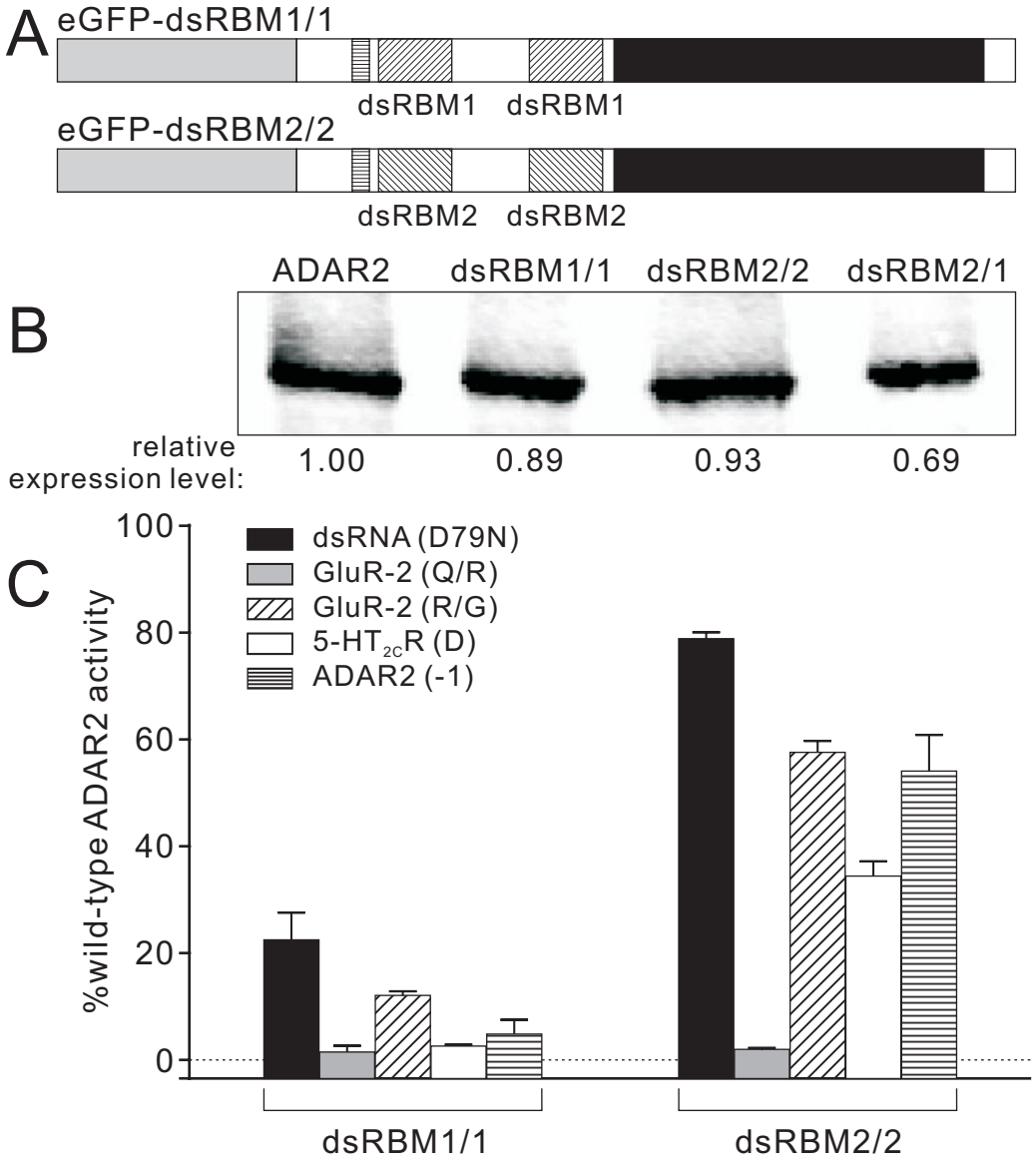

Figure 30. Lack of interchangeability between ADAR2 dsRBMs for editing activity. (A) A schematic diagram is presented indicating the domain structure of eGFP-ADAR2 mutants containing two copies of dsRBM1 (eGFP-dsRBM1/1) or dsRBM2 (eGFP-dsRBM2/2). (B) Quantitative Western blot analysis of eGFP-ADAR2 fusion proteins in nuclear extracts prepared from transiently transfected HEK293 cells; the relative protein expression levels of eGFP-ADAR2 mutants, compared to wild-type eGFP-ADAR2, are indicated. (C) Quantitative analysis of in vitro editing activity for ADAR substrates using eGFP-dsRBM1/1 and eGFP-dsRBM2/2 mutants with all values normalized to wild-type (eGFP-ADAR2) activity (n=3, mean±SEM).

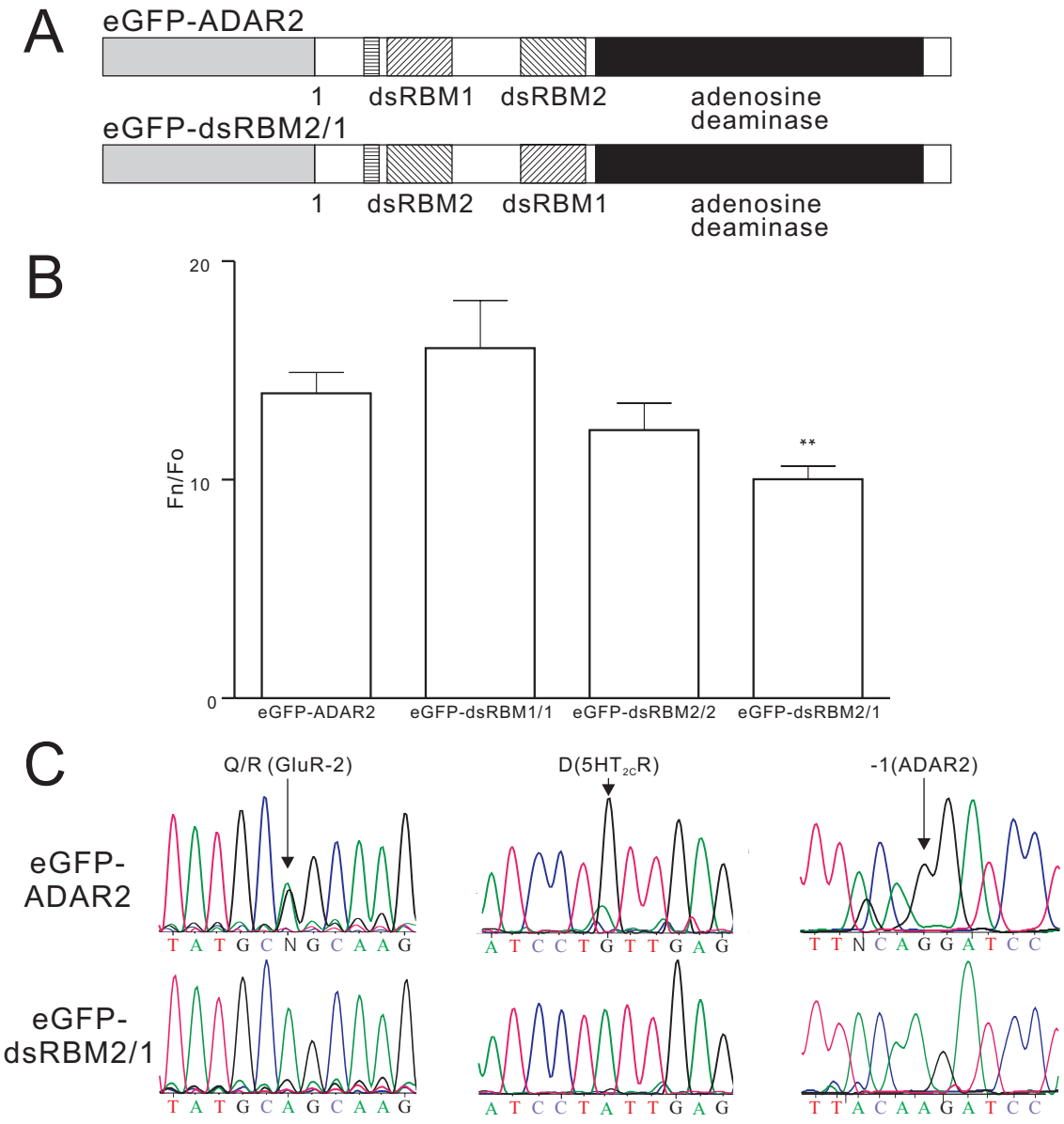

Figure 31. Analysis of eGFP-dsRBM2/1 function. (A) A schematic diagram showing the domain structure of mutant eGFP-ADAR2 whose dsRBMs are interchanged (eGFP-dsRBM2/1). (B) Quantitative analysis of subnuclear localization of eGFP-ADAR2 mutant constructs. The Fn/Fo value are shown (mean±SEM; n≥15;**p<0.01 compared to wild-type). (C) Bulk sequence analysis of in vitro reaction products. Editing sites are indicated by arrows. At the editing site, the A peak represents non-edited RNA, while the G peak represents the edited RNA.

replacement mutations significantly reduced site-specific A-to-I conversion (Figure 30). Replacement of dsRBM2 with dsRBM1 (dsRBM1/1) resulted in a near complete loss of editing for all substrates examined, providing a pattern of activity that was quite similar to the dsRBM2 deletion mutant (ΔdsRBM2). Similarly, replacement of dsRBM1 with dsRBM2 (dsRBM2/2) resulted in a pattern of editing that was nearly identical to protein lacking dsRBM1 (ΔdsRBM1), suggesting that replacement of either RNA-binding motif was no better than deleting the domain. Consistent with these findings, an eGFP-ADAR2 mutant in which the positions of the dsRBMs were interchanged (dsRBM 2/1) also had negligible editing activity with any of the RNA substrates tested (Figure 29C). These results demonstrate that the differential properties of dsRBM1 and dsRBM2 to site-selective editing are related to their unique sequences/structures rather than their relative positions within ADAR2.

To assess whether alterations in the identities of the dsRBMs also affected nucleolar accumulation of ADAR2, we used confocal microscopy to compare fluorescence intensity in nucleoli and the nucleoplasm (as in Figure 22) of wild-type eGFP-ADAR2 and the corresponding dsRBM1/1, dsRBM2/2 and dsRBM2/1 mutants. Although transposition or replacement of the dsRBMs significantly reduced site-specific editing on naturally-occurring ADAR2 substrates (Figure 30C, 31C), such changes had little effect on the extent of nucleolar localization (Figure 31B), suggesting that the precise nature of the dsRBM-RNA interactions associated with editing and localization were distinct.

Discussion

The recognition of double-stranded RNA is a key event for a variety of biological processes including the production of small interfering RNAs and microRNAs from hairpin precursors (Sontheimer and Carthew, 2005), the interferon-mediated antiviral response (Williams, 2001), and the deamination of specific adenosine moieties as a consequence of RNA editing (Bass, 2002; Emeson and Singh, 2001). A majority of the proteins involved in the recognition of duplex RNA contain multiple copies of a highly conserved 65-75 aa double-stranded RNA-binding motif, providing a molecular mechanism to facilitate the interaction of these proteins with their dsRNA targets (Carlson et al., 2003; Doyle and Jantsch, 2002; Fierro-Monti and Mathews, 2000; Tian et al., 2004). ADAR2 is a double-stranded RNA-specific adenosine deaminase that also contains two tandem copies of this highly conserved motif (Tian *et al.*, 2004). To determine whether these highly similar dsRBMs play equivalent roles in the nucleolar localization of ADAR2 and their ability to promote the deamination of selective adenosine residues, we have examined the subcellular localization of a series of wild-type and mutant eGFP-ADAR2 fusion proteins and their ability to catalyze site-specific editing events on multiple ADAR2 substrates. Results from these analyses have demonstrated a functional inequality between the two highly conserved dsRBMs, where dsRBM1 tends to play a greater role in localizing ADAR2 to the nucleolus and dsRBM2 is critical for the editing of ADAR2 targets. The relative nucleolar localization of ADAR2 seems to depend primarily upon the number of functional dsRBMs present, while site-specific A-to-I conversion is also

strongly affected by the nature and the organization of the dsRBMs, indicating that each dsRBM possesses an intrinsic ability to recognize specific determinants in duplex RNAs. In addition, the contributions of dsRBM1 and K^{281} to editing activity are substrate-dependent, consistent with previous findings that dsRBMs can specifically recognize distinct structural determinants on naturally-occurring ADAR2 targets (Stefl et al., 2006; Stephens et al., 2004).

dsRBMs and ADAR2 substrate specificity

ADAR2 has been shown to bind to perfect dsRNA regions and catalyze non-specific editing duplexes (Cho et al., 2003; Dawson et al., 2004; Lehmann and Bass, 2000; Liu et al., 1999), bind to specific editing sites (Ohman et al., 2000; Stefl et al., 2006; Stephens et al., 2004), or bind to other duplex regions with no productive A-to-I conversion (Klaue *et al.*, 2003). All of these binding events contribute to macroscopic measurements of ADAR2 affinity for an RNA substrate but they do not necessarily contribute to specific editing (Klaue *et al.*, 2003), thereby uncoupling binding affinity from site-selective editing. Consistent with these observations, previous studies have demonstrated that a mutation in dsRBM1 (K127A) decreased binding affinity to a GluR-2 (R/G site) substrate (Macbeth *et al.*, 2004), yet this mutation had no effect on editing of the R/G site (Figure 29). Given the disparity between site-specific editing and dsRBM binding, we focused on dsRBM-RNA interactions related to site-selective editing, rather than ADAR2-substrate affinity, using both deletion and substitution analyses to compare the role(s) that each dsRBM plays in the editing of multiple ADAR2

substrates. While deletion analysis represents a common strategy for defining the functional relevance of specific domains within a protein, a caveat to this approach is the potential for mutant protein misfolding which can complicate interpretation of experimental results. To minimize this possibility, we defined each dsRBM deletion based upon the recently solved NMR structure of these domains in ADAR2 (Stefl et al., 2006). Previous analyses of the deaminase domain and individual dsRBMs have demonstrated that these regions can fold independently from one another (Macbeth et al., 2005; Stefl et al., 2006), arguing against the potential for broad alterations in ADAR2 protein topology resulting from the deletion of individual domains. Further evidence in support of this idea is the fact that nucleolar localization (Figure 22) and editing of the GluR-2 transcripts (R/G site) are only reduced by ~50% for the dsRBM2 mutant in transfected cells (Figure 24B). Similarly, the extent of editing for the dsRBM1 mutant protein is reduced in a substrate-dependent fashion (Figure 28), demonstrating that deletion of this domain does not simply alter structure to generate a non-functional protein.

Previous observations that dsRBMs bind to perfect dsRNA, in a sequence-independent manner (Blaszczyk et al., 2004; Ramos et al., 2000; Ryter and Schultz, 1998), have suggested that the dsRBMs of ADAR2 may recognize their RNA targets in a similar fashion. Consistent with this idea, ADAR2 has been shown to non-specifically deaminate adenosine moieties in a wide range of synthetic RNA duplexes (Cho et al., 2003; Dawson et al., 2004; Lehmann and Bass, 2000; Liu et al., 1999). However, the substrate-specific contribution of dsRBM1 to editing activity (Figure 28) not only indicates that this motif makes

specific contacts with imperfect dsRNA targets, but also suggests that each dsRBM can interact with different structural determinants on each naturally-occurring substrate. Further support for this model of ADAR2-RNA interaction has been provided by recent biochemical and NMR analyses demonstrating that both dsRBM1 and dsRBM2 can bind to unique structural determinants in different RNA targets encoding either the R/G or Q/R sites of GluR-2 transcripts (Stefl et al., 2006; Stephens et al., 2004).

How can the same dsRBMs in ADAR2 specifically recognize distinct sequence/structural determinants in multiple RNAs? Deletion analyses have demonstrated that dsRBM2 is critical for the editing of all RNA substrates examined, yet substitution (K281A) of a highly conserved lysine residue that resides on the RNA-binding surface of dsRBM2 only affects editing activity on a subset of these transcripts (Figure 28, 29). Using a substrate containing the Q/R site, the K281A mutation reduced editing to background levels, suggesting that K^{281} is a key residue mediating dsRBM2-RNA interactions. By contrast, the same mutation had little effect on editing of the R/G site (Figure 29), indicating that dsRBM2 may utilize distinct amino acid residues to contact different RNA targets. The lack of universal importance for K^{281} has been further confirmed by NMR analysis where no chemical shift change in this residue was observed upon binding of a GluR-2 transcript containing the R/G site. If the ability of dsRBMs to utilize unique complements of amino acids to make specific RNA contacts is ubiquitous, this represents a powerful mechanism by which to achieve site-selective recognition for a broad range of potential targets. Although we

cannot eliminate other possible interpretations, such as K281A-mediated structural alterations that affect binding in a substrate-specific manner, this possibility is unlikely since the corresponding lysines in other dsRBMs have been shown to make direct contact with dsRNA (Ramos et al., 2000; Ryter and Schultz, 1998) and that analogous mutations in PKR and Staufen ablated dsRNA binding (McMillan *et al.*, 1995; Ramos *et al.*, 2000).

The distinct effects observed for the deletion of dsRBM1 and dsRBM2 on editing activity (Figure 28) could result from the precise sequence/structure of each domain or their relative location within the ADAR2 protein. Replacement of dsRBM2 with dsRBM1 (dsRBM1/1) resulted in a near complete loss of editing for all substrates examined (Figure 6C), providing a pattern of activity that was quite similar to the dsRBM2 deletion mutant (ΔdsRBM2). Similarly, replacement of dsRBM1 with dsRBM2 (dsRBM2/2) resulted in a pattern of editing that was nearly identical to protein lacking dsRBM1 (ΔdsRBM1), indicating that functional differences in editing activity come from subtle sequence/structure difference between these motifs. The lack of interchangeability between these domains further suggests that the unique binding preferences of each dsRBM contribute to site-selective editing activity.

dsRBMs and nucleolar localization

The steady-state accumulation of ADAR2 in nucleoli has been suggested to represent a regulatory mechanism by which to modulate editing activity at its site of action in the nucleoplasm (Desterro *et al.*, 2003; Sansam *et al.*, 2003). The

nucleolar localization of ADAR2 is dependent upon its dsRBMs and the presence of rRNA, suggesting that ADAR2 is targeted to the nucleolus by directly binding extended duplex regions in mature or pre-rRNA transcripts (Sansam *et al.*, 2003). Several other dsRBM-containing proteins including ADAR1, PKR, Staufen, RNA helicase A, RNA helicase II/Gu and NF-kappaB repressing factor (NRF) have also been shown to accumulate in nucleoli (Desterro et al., 2003; Macchi et al., 2004; Niedick et al., 2004; Tian and Mathews, 2001; Valdez et al., 2002; Zhang et al., 2004b), suggesting that dsRBM-mediated binding may serve as a general mechanism for nucleolar localization. Observations that deletion or mutation of either dsRBM can significantly affect the extent of nucleolar targeting (Figure 22) suggest that either both motifs are required for binding to specific dsRNA target(s) or that each motif binds to duplex RNAs that represent only a subset of the total nucleolar ADAR2 binding sites. Unlike the significant reductions in editing activity observed by replacement or interchange of the dsRBMs, such modifications had little effect on ADAR2 subnuclear localization (Figure 31B), suggesting that the nucleolar localization of ADAR2 results from a general dsRNA-binding activity for one or multiple nucleolar targets rather than the specific interactions required for site-selective adenosine deamination.

The inequality of dsRBMs

The lack of obvious sequence requirements for the binding of dsRBM-containing proteins led to the initial conclusion that these motifs only confer general dsRNA-binding affinity with little sequence preference, yet most of

the data regarding this hypothesis focused upon model RNA substrates that contained a perfect RNA duplex (Blaszczyk et al., 2004; Ramos et al., 2000; Ryter and Schultz, 1998). By contrast, more recent studies using naturally-occurring dsRNAs, formed by intramolecular base-pairing between imperfect, inverted repeats, have indicated that dsRBMs have unique functional properties based upon intrinsic binding preferences and affinities (Spanggord et al., 2002; Stefl et al., 2006; Stephens et al., 2004). While the common features of the dsRBMs provide them with a general ability to interact with extended RNA duplexes, the subtle differences in amino acid sequence and structure between these domains also allow individual motifs to play diverse, substrate-specific roles that ultimately define the function(s) of their parent proteins.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Adenosine to inosine RNA editing is the most widespread RNA modification identified in animals. A-to-I editing has been shown to alter coding potential of the targeted RNA transcripts, generating protein isoforms with different functional properties, including GluR subunits, 5-HT_{2C}R, K_v1.1 and *Drosophila* dADAR (Burns et al., 1997; Hoopengardner et al., 2003; Keegan et al., 2005; Lomeli et al., 1994; Sommer et al., 1991). In addition, A-to-I conversion has also been demonstrated to change the splicing pattern of pre-mRNAs encoding ADAR2 and a non-receptor protein tyrosine phosphatase (Beghini et al., 2000; Rueter et al., 1999). Moreover, the studies from *C. elegans* and mammals have indicated that A-to-I editing may also intersect with the RNAi pathway and modulate microRNA biogenesis (Tonkin and Bass, 2003; Yang et al., 2006; Yang et al., 2005). Recent systematic screening for novel A-to-I editing events revealed that a majority of editing events occur within non-coding regions of RNA transcripts in humans, mice and chickens (Blow *et al.*, 2004; Levanon *et al.*, 2004; Levanon *et al.*, 2005). Previous studies have demonstrated that hyper-edited RNA may be selectively retained in the nucleus in mammalian cells and subsequently degraded by an inosine-specific ribonuclease (Kumar and Carmichael, 1997; Scadden and O'Connell, 2005; Scadden and Smith, 2001), suggesting that the extensive A-to-I RNA editing found in UTRs may be involved in controlling the stability and

localization of the edited transcripts.

A-to-I RNA editing is catalyzed by a family of ADARs (Bass *et al.*, 1997). All ADARs contains one to three copies of dsRBMs in the N-terminal region and one conserved deaminase domain at the C-terminus. The structures of dsRBMs and deaminase domain of ADAR2 have been recently solved (Macbeth et al., 2005; Stefl et al., 2006), providing more insight into the catalytic mechanism and substrate specificity of ADARs. Unexpectedly, an IP_6 molecule is found buried in the core of the deaminase domain and is necessary for the enzymatic activity (Macbeth *et al.*, 2005). However, why the IP_6 binding residues are only conserved in ADARs, but not ADATs, and whether IP_6 plays a regulatory role in modulating ADAR cellular activity remains unknown.

Although the functional consequences of A-to-I RNA editing have been extensively studied, the molecular mechanisms of ADAR substrate specificity are poorly understood. Because dsRBMs exhibit sequence-independent interactions with A-form dsRNA, it is postulated that dsRBMs in ADARs are only used to increase the affinity to the substrate RNA duplexes and do not contribute to the substrate specificity. However, recent biochemical study suggested that dsRBMs of ADAR2 bind specifically to GluR-2 Q/R site RNA (Stephens *et al.*, 2004). Later, ADAR2 dsRBMs were demonstrated to bind also specifically to GluR-2 R/G site RNA by a structural study (Stefl et al., 2006). Although both studies emphases that dsRBMs can specifically bind to ADAR2 substrates and contribute to the specificity, there is a discrepancy of which determinant dsRBM recognize on the substrates. For example, on the Q/R site RNA, the dsRBM1 binds near the

adenosine of an A-C mismatch (Figure 7), but on R/G site RNA, the same motif recognizes the structurally conserved pentaloop (Figure 18). This recognition discrepancy is supported by the observation that dsRBM1 displayed substrate-dependent contribution to the editing activity (Figure 28), where the deletion of dsRBM1 decreased the Q/R editing by 50-fold and decreased R/G editing by only 3-fold. We reason that interactions with different determinants by dsRBM1 provide ADAR2 differential affinity to the substrates and hence facilitate the editing to different extents. Then how can the same motif recognized totally different determinants on RNA? The dsRBM2 is required for the editing activity on all substrates, however, mutation of K^{281} to alanine causes 10-fold decrease in Q/R site editing but has almost no effect on R/G site editing (Figure 30). It seems possible that dsRBM2 uses one set of amino acids, including K^{281}, to contact the determinant on Q/R site and uses another set of residues that do not include K^{281} to contact the determinant on R/G site. The differential usage of amino acids should provide dsRBM the capability to specifically recognize multiple determinants. An emerging view on ADAR substrate specificity is that dsRBMs specifically recognize all natural substrates, and through the alternative usage of different residues the dsRBM binds to different determinants on each substrate.

Potential rRNA editing by ADARs

The nucleolus is the site of rRNA transcription and processing. The initial transcript, 45S pre-rRNA, contains 5.8S, 18S and 28S rRNAs, which represent important components of the ribosome that are critical for protein translation. In

addition, pre-rRNA contains two external transcribed spacers (5'-ETS and 3'-ETS) and two internal transcribed spacers (ITS-1 and ITS-2), that are known to play an important role in pre-rRNA processing. The relative nucleolar concentration of ADAR2 in NIH/3T3 cells is 14 times higher than in the nucleoplasm. Given the high levels of ADAR2 in the nucleolus, it is conceivable that ADAR2 can bind to pre-rRNA and catalyze the deamination of specific adenosines in pre-rRNA transcripts.

ADAR2 is thought to maintain its nucleolar localization by the interaction between dsRBMs and rRNA precursors (Sansam *et al.*, 2003). Using two complementary methods, we demonstrated that dsRBM1 is more important in maintaining the nucleolar localization of ADAR2 at steady-state. One possibility is that there are multiple binding sites, some for dsRBM1 and some for dsRBM2, but the average binding affinity of dsRBM1 is greater. Another possibility, which is more intriguing, is that there are one or several specific binding sites on pre-rRNA and that specific dsRBM1 binding provides more affinity than dsRBM2-specific binding. When the positions of dsRBM1 and dsRBM2 were interchanged, however, the Fn/Fo value had only a small decrease (Figure 31B), suggesting that overall affinity, rather than site-specific binding was responsible for nucleolar localization. In this case, it is unlikely that pre-rRNAs will be selectively modified by A-to-I conversion, unless the presence of a change in the Fn/Fo ratio upon dsRBM transposition represents a small percentage of ADAR2 protein involved in site-selective binding.

Proposed model for ADAR substrate recognition

While the effect of the dsRBM1 deletion on ADAR2 editing activity is highly substrate-dependent, the deletion of dsRBM2 ablated editing activity on all five substrates tested (Figure 28). Similar analyses of ADAR1 suggested that dsRBM3 is critical for editing activity on multiple substrates, whereas the other two dsRBMs plays less important roles (Lai et al., 1995; Liu et al., 1999; Liu and Samuel, 1996; Liu and Samuel, 1999). The critical importance of the dsRBM immediately adjacent to the deaminase domain indicates that there may be a common mechanism for ADAR substrate recognition.

ADARs can convert up to 50% of the adenosines to inosine in synthetic, long dsRNAs until the duplex structure is sufficiently disrupted by the formation of unstable I-U base pairs (Bass and Weintraub, 1988; Lehmann and Bass, 2000), indicating that the deaminase domain lacks specificity. The 5'- and 3'- nearest neighbor preference for the ADARs (Lehmann and Bass, 2000), is likely to be derived from the deaminase domain *per se* because the nucleotides next to the editing site can strongly affect the ability of the targeted adenosine moiety to flip out of the RNA duplex and fit into the active site. Although the deaminase domain is very active, it lacks the ability to efficiently bind to the double-stranded RNA substrates (Figure 28). Therefore, the motif targeting the deaminase domain to the substrates plays a critical role in defining substrate specificity.

dsRBMs of ADARs can bind to perfect dsRNA with high affinity (Kim *et al.*, 1994a; Ohman *et al.*, 2000). However, work from several laboratories has shown that the ADAR2 dsRBMs bind specifically on naturally-occurring imperfect dsRNA

substrates (Stefl et al., 2006; Stephens et al., 2004). So the dsRBM has the ability to target other domains to the certain region on a natural RNA substrate or any position of a perfect dsRNA.

In ADAR2, dsRBM2 and deaminase domain are connected by a relatively short (20aa), protease-inaccessible linker (Macbeth *et al.*, 2004), indicating these two domains are physically restricted and dsRBM2 is likely to serve as the targeting motif for the deaminase domain. The binding of dsRBM2 to the RNA will presumably bring the enzymatically active deaminase domain to the RNA substrate, allowing the catalytic site to perform the deamination reaction on any feasible adenosine. In contrast, dsRBM1 and dsRBM2 are connected by a long and flexible linker (>83aa), which is protease sensitive and not visible in NMR structure (Macbeth et al., 2004; Stefl et al., 2006). Therefore, the dsRBM1-RNA interaction will not strictly restrain the location of dsRBM2 and the deaminase domain and will therefore contribute less to enzyme specificity. Taken together, we hypothesize that dsRBM1-specific binding increases the chances of dsRBM2-specific binding through the 83aa linker. As soon as dsRBM2 binds to the RNA, the deaminase domain will be targeted near the dsRBM2 binding site, and a local movement defined by the 20aa linker will allow the editing of all feasible adenosines (Figure 32A).

In this model, the dsRBM closest to the deaminase domain (core dsRBM: dsRBM3 for ADAR1 and dsRBM2 for ADAR2) defines the substrate specificity by physical restraining the deaminase domain. In agreement with this hypothesis, the loss of dsRBM2 ablated ADAR2 editing on all substrates tested due to the fact

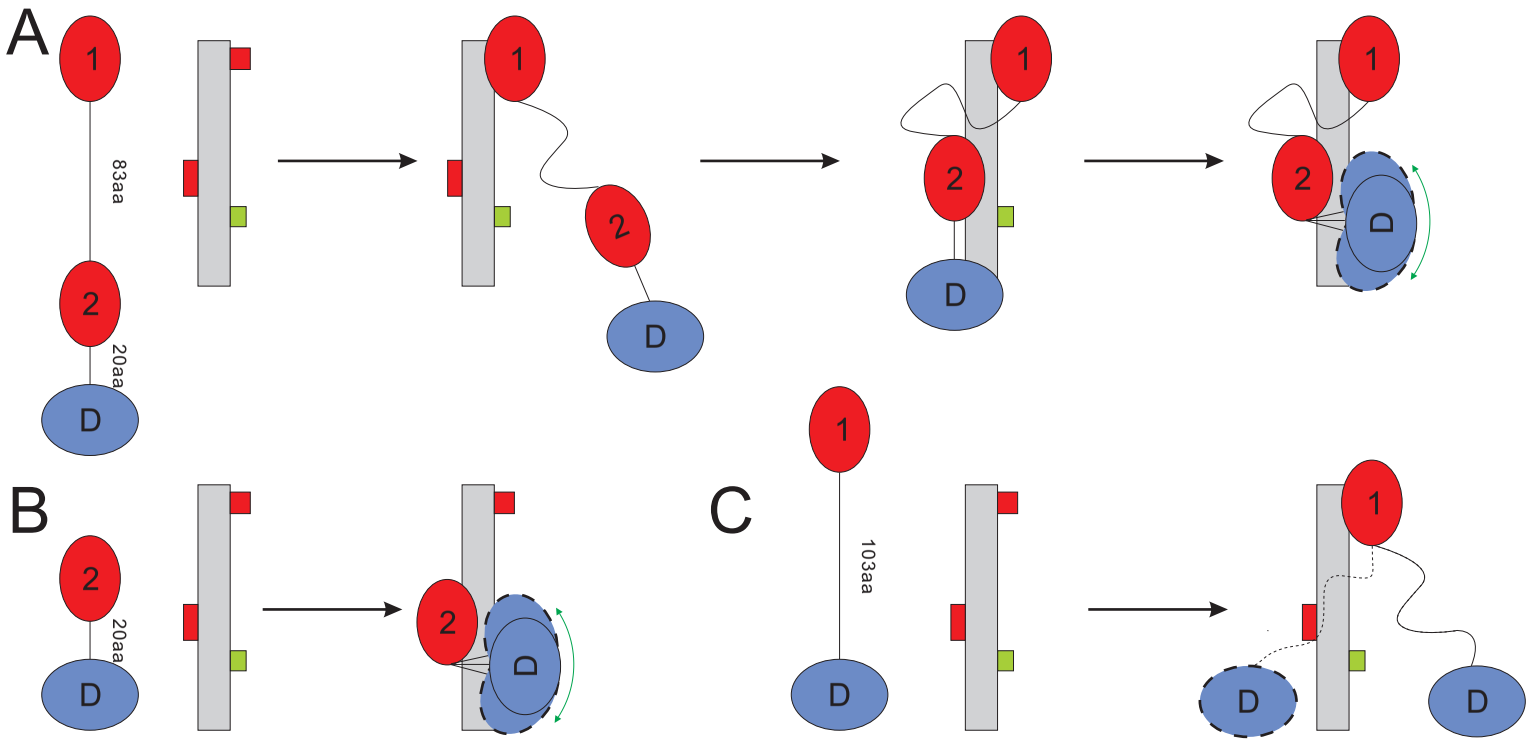

Figure 32. Proposed model for ADAR2 site-selective targeting. The dsRBMs of ADAR2 are labeled in red and deaminase domain is labeled in blue. The RNA backbones are in gray and the structural determinants recognized by the dsRBMs and the editing site are labeled in red and green, respectively. (A) Model for wild-type ADAR2. The binding of dsRBM1 increases the chance of dsRBM2 binding; dsRBM2 binding anchors the deaminase domain to the RNA and the 20aa linker allows a local movement of deaminase domain on the substrates. (B) In the absence of dsRBM1, dsRBM2 binds and allows deaminase domain to catalyze A-to-I conversion. The difference in relative affinity for dsRBMs result in a differential dsRBM1 contribution for each RNA substrate. (C) In the absence of dsRBM2, the 103aa long linker does not immobilize the deaminase domain on the RNA.

that the 100aa linker between dsRBM1 and the deaminase domain was not structurally adequate to position the deaminase domain on the RNA (Figure 32C). The requirement for dsRBM3 for ADAR1 activity can be explained by the same rationale (Lai *et al.*, 1995; Liu and Samuel, 1996), and is further supported by the fact that ADAR1, containing only dsRBM3 and deaminase domain, still retains the editing selectivity (Maas *et al.*, 1996).

In this model, the length of the linker between the core dsRBM and deaminase domain is critical. If the linker is too short, then it prevents accessibility of the active site to any adenosines on RNA. If the linker is too long, then it will not sufficiently restrain the deaminase domain on the RNA, which can lead to a loss of editing activity and specificity. ADAR3 shares 50% amino acid identity with ADAR2, and the sequences of the dsRBMs and deaminase domain are highly conserved. However, ADAR3 failed to show activity on any known substrates or a perfect RNA duplex. One possibility is that, ADAR3 has only a 14aa linker which prevents the active site to contact any adenosines. A chimera protein, ADAR3-2, which has a 20aa linker, recovers some of the editing activity, while ADAR2-3, which has 14aa linker is still inactive (Melcher *et al.*, 1996a). Naturally occurring ADAR1 splice variants, which differ in the length of the linker between dsRBM3 and the deaminase domain, displayed different editing specificity (Liu et al., 1999; Liu et al., 1997; Liu et al., 2000), further supporting the importance of the linker length.

When the core dsRBM binds to a specific site on a naturally occurring RNA, the deaminase domain can move locally to make contact with all the adenosines

within the range defined by the linker. This is further supported by the fact that the editing sites are always found in cluster and most of them are not functionally important (Burns et al., 1997; Dawson et al., 2004; Levanon et al., 2004; Maas et al., 1996; Rueter et al., 1999; Yang et al., 2006). More interestingly, there are two editing sites on the opposite side of the duplex in ADAR2 pre-mRNA and mutations affecting the editing on one site always have the same effect on editing of the other site (Dawson *et al.*, 2004), suggesting these two editing events result from the same dsRBM2-RNA interaction. There is a possibility that some adenosines within the range, defined by dsRBM2 and the linker, cannot be edited because the local RNA structure/sequence does not allow the adenosine to flip out and fit into the active site of the deaminase domain (Hart *et al.*, 2005).

In this model, the function of other dsRBMs is to target ADARs to the transcripts which are going to be edited and increase the chance of specific binding of the core dsRBM. In ADAR2, dsRBM1 will bind to different determinants on each RNA substrate, and the strength of these interactions is likely to be substrate-dependent. For Q/R site, this interaction maybe is very strong, which can increase dsRBM2 binding by 50 times, for R/G site, this interaction is relatively weak, which only increase dsRBM2 binding by 3-fold. These scenarios would explain the substrate-dependent contribution of dsRBM1. Because dsRBM2 mainly defines the specificity, for some substrates, even without dsRBM1, ADAR2 still can direct significant editing (Figure 32B).

The proposed model is consistent with most of the published data, but its validity needs to be specifically addressed. To test the importance of the linker

length between dsRBM2 and deaminase domain in ADAR2 editing specificity, further studies might involve a series of ADAR2 mutant proteins in which the length of the linker between dsRBM2 and deaminase domain varied. Each construct can be transfected into HEK293 cells and the nuclear extracts prepared from transfected cells can be used as the protein resource for in vitro editing analysis. An in vitro transcribed RNA substrate can then be incubated with wild-type or mutant ADAR2 proteins, and the resultant RNA can be sequenced to detect A-to-I RNA editing on multiple sites. If the working model is correct, when the linker is shortened, the deaminase domain will be anchored closer to the dsRBM2 binding site and may catalyze adenosine deamination closer to the binding site; when the linker length is increased, however, the deaminase domain may be allowed to reach more adenosines and may edit adenosines that are relatively far from the dsRBM2 binding site. If an increase or decrease in linker length generates new editing sites in close proximity to the original editing site, the result would suggest that dsRBM2 anchors the deaminase domain and the linker between dsRBM2 and deaminase domain contributes to site-selective A-to-I conversion.

To illustrate the role of linker length between dsRBM1 and dsRBM2, a series of mutant ADAR2 protein with a varied linker length between the dsRBMs can be made. If our working model is correct, dsRBM1 and dsRBM2 will recognize different structural determinants on each RNA substrate, hence the linker length required to achieve both dsRBM-RNA interactions is likely to be substrate-dependent. For example, on a substrate where dsRBM1 and dsRBM2

binding sites are far from one another, a shortened linker may not allow the simultaneous binding of both dsRBMs, hence the editing activity will be similar to the ΔdsRBM1 protein; yet on another substrate where two dsRBM binding sites are relatively close, a shortened linker may still be enough to achieve both dsRBM-RNA interactions at the same time, hence the editing activity will be similar to the wild-type protein. When the linker between the dsRBMs is shortened further, the editing activity will be increasingly similar to the ΔdsRBM1 mutant. These results would further support that each dsRBM recognizes distinct structural determinants on different naturally-occurring RNA substrates. With efforts from many laboratories, I hope that the mysteries of ADAR substrate specificity will be clarified in the near future.

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APPENDIX

SEQUENCE OF PRIMERS USED IN THE STUDY

VU3: 5’- CTATCATGATCAAGAAGCCTCAGAAG -3’
VU35: 5’- CGAAATATCGCATCCTT -3’
VU38: 5’- AATCCGGATTGCCCGGAACGT -3’
VU97: 5’- CCTTGGGTGCCTTTA -3’
VU168: 5’- GACAACCGATCAAACGC -3’
VU183: 5’- TATTTGTGCCCCGTCTGG -3’
VU331: 5’- GCGTTGAGGAAGGAAATATGC -3’
VU343: 5’- GGATCCCCCGGGCTGCAG -3’
VU356: 5’- CGCCTTCGTTGCAGGAT -3’
VU477: 5’- AATCCGGATTGCCCGGAACGTGGAATACCCTCGCCAGGAC -3’
VU682: 5’- CCGGGAGCTCATCGCCACACCTAAAGGATCC -3’
VU683: 5’- GGCCGAATTCTACAAACCGTTAAGAGTCTTA -3’
VU776: 5’- CATGAAGCTTGGCGTTTCTCCCTGAGCCCTC -3’
VU777: 5’- AAGGCTCCGGCCCTACAGCCAAGAAGGCAAAGCTGCATG -3’
VU778: 5’- TGGCTGTAGGGCCGGAGCCTTCAAAGA -3’
VU788: 5’- GTTATACTATTCCACCC -3’
VU853: 5’- GGAGGATCCCCAGGGCCCGTTCTACCC -3’
VU990: 5’- GGAATTCCATATGCCAGGGCCCGTTCTAC -3’
VU991: 5’- CGGGATCCTTAGTTGGGAAACTGGACAAAA -3’
VU1024: 5’- GGAATTCCATATGCCAAGTGGGAAGAACCCCG -3’
VU1068: 5’- CGCTGGAACGTGGTGGGC -3’
VU1069: 5’- ATTGAGAGCCAGGCCACGGT -3’
VU1072: 5’- CGGAATTCCTATTCAAACTTCGTCTTCTTTCCTT -3’
VU1073: 5’- GAAGATCTGCATCCGCCACCATGGTGAAG -3’
VU1092: 5’- CGGGATCCTTACAAGTGCAAATTGAAGACAG -3’
VU1120: 5’- GGTAAAGCGGCCGCCAG -3’
VU1127: 5’- GCTCAAGCTTCCAATTCTGCAGATATCCATCACAC -3’
VU1128: 5’- TGGCAAGCTTCTTGTTTCTC -3’
VU1144: 5’- GTCACTGCAGGACATGGTGAGC -3’
VU1145: 5’- CTCAGCGGTATCAGCAATGCA -3’
VU1146: 5’- CAGGGGCTTGTTGAGGGTGTA -3’
VU1147: 5’- CACGGAAAGGTGCCCCC -3’
VU1148: 5’- CATCCAGCGACAGTACAGCG -3’
VU1152: 5’- TGAGCCAGGCGGAGTCGA -3’
VU1183: 5’- CGAATTGAACCGGCTATGCTC -3’
VU1190: 5’- TCTGAGGCCCACCTGG -3’
VU1191: 5’- CCCTGGCGTTTTCCTTCG -3’
VU1192: 5’- GACCAAACGCCATCTCGCC -3’

VU1193: 5’- TGGGAATGGTGGTGGGATG -3’
VU1201: 5’- FAM-CCGCCAGTCAAGAAGCCC -3’
VU1209: 5’- CCGGAAGCTTATCGCCACACCTAAAGGATCC -3’
VU1210: 5’- GGCCTCTAGATACAAACCGTTAAGAGTCTTA -3’
VU1220: 5’- CCAAGTGGGAAGAACCCCG -3’
VU1221: 5’- CAAGTGCAAATTGAAGACAGTAGC -3’
VU1238: 5’- CGGGATCCTCATTAAGGTGGGTGG
AATAGTATAACAATATGCACAATGTTGT -3’
VU1239: 5’- GATAAGCTTCATATCGAATTCTACAAACCGTTAAGAG -3’
VU1244: 5’- CGGGATCCTCATTAAGGTGGGTGG
AATAGTATAACAATATGCAAATGTTGT -3’
VU1245: 5’- CGGGATCCTCATTAAGGTGGGTGG
AATAGTATAACAATATGCTCTAATGTTGT -3’
VU1246: 5’- AGCTGGTACCCCAGGCCCCGTTCTACCCAAGAATGC -3’
VU1247: 5’- AGCTGGTACCGCTTGGGGGTGGG -3’
VU1248: 5’- AGCTGTCGACCAAACGCCATCTCGC -3’
VU1249: 5’- AGCTGTCGACGGGAAACTGGACAAAAGACC -3’
VU1264: 5’- ATATTGTTATACTATTCCACCCA -3’
VU1265: 5’- CGGGCAGTGAGCGCAAC -3’
VU1272: 5’- AUGUUGUUAUAGUAUCCCACCUACCCUGAUG -3’ (RNA)

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