Substrate-dependent Contribution of Double-stranded RNA-binding Motifs to ADAR2 Function[□]

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ADAR2 is a double-stranded RNA-specific adenosine deaminase involved in the editing of mammalian RNAs by the site-specific conversion of adenosine to inosine (A-to-I). ADAR2 contains two tandem double-stranded RNA-binding motifs (dsRBMs) that are not only important for efficient editing of RNA substrates but also necessary for localizing ADAR2 to nucleoli. The sequence and structural similarity of these motifs have raised questions regarding the role(s) that each dsRBM plays in ADAR2 function. Here, we demonstrate that the dsRBMs of ADAR2 differ in both their ability to modulate subnuclear localization as well as to promote site-selective A-to-I conversion. Surprisingly, dsRBM1 contributes to editing activity in a substrate-dependent manner, indicating that dsRBMs recognize distinct structural determinants in each RNA substrate. Although dsRBM2 is essential for the editing of all substrates examined, a point mutation in this motif affects editing for only a subset of RNAs, suggesting that dsRBM2 uses unique sets of amino acid(s) for functional interactions with different RNA targets. The dsRBMs of ADAR2 are interchangeable for subnuclear targeting, yet such motif alterations do not support site-selective editing, indicating that the unique binding preferences of each dsRBM differentially contribute to their pleiotropic function.

INTRODUCTION

The conversion of adenosine to inosine (A-to-I) by RNA editing is mediated by a family of double-stranded RNA (dsRNA)-binding proteins referred to as adenosine deaminases that act on RNA (ADARs) (Bass *et al.*, 1997). Two mammalian enzymes in this family, 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 containing extended duplex regions formed by the presence of imperfect inverted repeats (Rueter and Emeson, 1998; Bass, 2002). The most extensively studied substrates of A-to-I conversion are transcripts encoding ionotropic glutamate receptor (GluR) subunits and the 2C-subtype of the serotonin receptor (5-HT_{2C}R), in which editing leads to non-synonymous codon changes that generate channels with altered electrophysiologic and ion permeation properties (Seeburg and Hartner, 2003) and receptors with decreased G protein-coupling efficiency (Burns *et al.*, 1997; Niswender *et al.*, 1999; Berg *et al.*, 2001). A-to-I modifications have also been described in nontranslated RNAs and noncoding regions of mRNA transcripts, suggesting that such RNA modifications affect other aspects of RNA function, including splicing, translation efficiency, nuclear retention, and transcript stability (Rueter *et al.*, 1999; Athanasiadis *et al.*, 2004;

Blow *et al.*, 2004; Kim *et al.*, 2004; Levanon *et al.*, 2004; DeCervo and Carmichael, 2005; Prasanth *et al.*, 2005).

ADAR2 displays a modular organization with two tandem dsRNA-binding motifs (dsRBMs) connected by a flexible linker and a conserved adenosine deaminase domain toward the carboxy terminus, for which the structures have recently been determined (Macbeth *et al.*, 2005; Stefl *et al.*, 2006). The dsRBM is a highly conserved 65–75 amino acid (aa) domain, present in many eukaryotic proteins with diverse cellular functions, that forms an α 1- β 1- β 2- β 3- α 2 topology in which the two α -helices are packed along a face of a three-stranded antiparallel β -sheet, with most of the potential RNA-binding residues exposed on one surface (Fierro-Monti and Mathews, 2000; Tian *et al.*, 2004). Structural analyses of dsRBMs complexed to short, synthetic RNA duplexes have suggested that dsRBM binding is independent of RNA sequence, because the majority of dsRBM–RNA interactions involve direct contact with the 2'-hydroxyl groups of the ribose sugars and direct or water-mediated contacts with nonbridging oxygen residues of the phosphodiester backbone (Ryter and Schultz, 1998; Ramos *et al.*, 2000; Blaszczyk *et al.*, 2004). 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 α 1 helix relative to the other secondary structural elements (Steffl *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 (Melcher *et al.*, 1996; Liu *et al.*, 1999; Lehmann and Bass, 2000; Cho *et al.*, 2003; Dawson *et al.*, 2004). However, ADAR2 can also demonstrate site-specific A-to-I conversion in mRNA transcripts (Emeson and Singh, 2001; Bass, 2002), 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

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to imperfect RNA duplexes in a manner distinct from that of an RNA-dependent protein kinase (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 ADAR1 and ADAR2 also have been shown to be critical for the nucleolar localization of these enzymes, 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 localization of ADAR2 to the nucleolus is dependent not only on the ability of ADAR2 to bind to RNA duplexes via its dsRBMs but also on the presence of rRNA, suggesting that ADAR2 is targeted to the nucleolus by directly binding duplex regions in mature or pre-rRNA transcripts.

In this study, we demonstrate that deletion or the introduction of point mutations in each dsRBM of ADAR2 has a differential effect on both the site-selective modification of adenosine moieties in distinct ADAR2 substrates as well as in the localization of ADAR2 to the nucleolus. Although the dsRBMs of ADAR2 can functionally replace one another for subnuclear targeting, such alterations cannot support site-selective editing activity, emphasizing that the unique sequence/structure of each motif subserves specific roles in substrate recognition and subsequent ADAR2 function. More interestingly, observations that dsRBM point mutations have substrate-dependent effects upon site-specific editing in different RNA targets suggests that the precise amino acid residues involved in ADAR2–RNA interactions are unique for each RNA substrate, providing a molecular mechanism by which dsRBMs can specifically recognize multiple RNA structural determinants and enable ADAR2 to mediate the selective deamination of adenosine residues in a broad range of heterogeneous RNA targets.

MATERIALS AND METHODS

Plasmid Construction

To generate fusion constructs encoding enhanced green fluorescent protein (eGFP)-ADAR2, the rat ADAR2b cDNA (GenBank accession no. NM_012894) was subcloned into pEGFP-C1 (Clontech, Mountain View, CA) as described previously (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). 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), 230–301 (ΔdsRBM2) and 76–301 (ΔdsRBM1/2) from the ADAR2b open reading frame. Plasmids expressing enhanced yellow fluorescent protein (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 or 231–301, 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 (Ausubel *et al.*, 1998) to delete the region encoding dsRBM2 (aa 234–300) and replace it with amino acids 76–146 (dsRBM1/1) or by deleting the region encoding dsRBM1 (aa 76–148) and replacing it with amino acids 231–301 (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. Nucleolin cDNA was amplified by PCR with sense (5′-CGGAATTCTCTAATCAAACTTCGCTTCTTCTT-3′) and antisense (5′-GAAGATCTGATGCCACCATGGTGAAG-3′) primers using pSport6-nucleolin (Open Biosystems, Huntsville, AL) as a template, and introduced EcoRI and BglII restriction sites (underlined) were used for subsequent subcloning into the pEYFP-C1 vector (Clontech). Construction of the GluR-2 (R/G site) minigene for transfection analysis was made by PCR amplification of mouse genomic DNA with sense (5′-CCGAAGCTTACGCACACTAAG-GATCC-3′) and antisense (5′-GGCTCTAGATACAAACCGTTAA-GAGTCTTA-3′) primers to introduce HindIII and XbaI restriction sites (underlined) for subcloning into pRC/CMV2 (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells and NIH/3T3 mouse fibroblasts (American Type Culture Collection, Manassas, VA) were maintained in alpha minimum essential medium and DMEM (Invitrogen), respectively, and supplemented with 10% (vol/vol) bovine calf serum (Hyclone Laboratories, Logan, UT). HEK293 cells were transiently transfected by calcium phosphate coprecipitation (Ausubel *et al.*, 1998) with 8 μg of plasmid encoding wild-type or mutant eGFP-ADAR2 fusion proteins and 2 μg of either a rat ADAR2 (–1 site) or GluR-2 (R/G site) minigene reporter plasmid, as described previously (Rueter *et al.*, 1999; Dawson *et al.*, 2004). For analysis of subnuclear fluorescence, NIH/3T3 cells were plated on 35-mm glass-bottomed MatTek dishes (MatTek, Ashland, MA) and transiently transfected with 1 μg of plasmids encoding wild-type or mutant eGFP-ADAR2 fusion proteins and 1 μg of the eYFP-nucleolin plasmid using FuGENE6, according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN).

Quantitative Fluorescence Microscopy

Twenty-four hours after transfection, the subcellular localization of eGFP and eYFP fusion proteins was determined by multispectral confocal microscopy (LSM510 Meta; Carl Zeiss, Thornwood, NY); 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 (multichannel) array detector. Multichannel 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 sample. All images were acquired using a 40×/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 with 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, MD).

Western Blotting Analysis

Crude nuclear extracts were prepared from HEK293 cells, 60 h after transient transfection, as described previously (Schreiber *et al.*, 1989) and diluted with dialysis buffer to maintain enzymatic activity (30 mM HEPES, pH 7.6, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 0.1% aprotinin) (Rueter *et al.*, 1999). Equivalent volumes for each protein sample were resolved by PAGE (7.5–12% SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond-C Super; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). 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 (0.4 ng/μl), and quantified using an Odyssey infrared imaging system (LI-COR Biotechnology, Lincoln, NE). For transient cotransfection analysis with ADAR2 and GluR-2 minigenes, relative ADAR2 protein expression was also normalized to the level of a constitutive nuclear protein, histone deacetylase 2 (HDAC2), using an affinity-purified goat polyclonal antibody (4 ng/μl; Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative Analysis of RNA Editing

Analysis of A-to-I conversion, using nuclear extracts from HEK293 cells transfected with wild-type and mutant eGFP-ADAR2 fusion proteins, was performed with *in vitro*-transcribed RNA substrates using the MEGascript T7 transcription kit (Ambion, Austin, TX) or T3 RNA polymerase (Promega, Madison, WI), as described previously (Burns *et al.*, 1997; Dawson *et al.*, 2004); the concentration of transcribed RNA was determined by UV absorbance spectrometry at 260 nm. On hundred femtomoles of each RNA substrate was incubated with the nuclear extracts containing equivalent amounts of each eGFP-ADAR2 protein at 30°C, in a total volume of 50 μl, for varying times, and the reactions were terminated by freezing on dry ice. For preliminary studies (Supplemental Figure S1), HEK293 nuclear extracts containing the wild-type eGFP-ADAR2 fusion protein were variably diluted to determine the linear range of enzymatic activity for each RNA substrate. Subsequent analysis of editing activity for wild-type and mutant eGFP-ADAR2 fusion proteins was performed using the empirically determined conditions, as follows: dsRNA (D79N): extract dilution, 15; incubation time, 60 min; GluR-2 (Q/R site): extract dilution, 30; incubation time, 20 min; GluR-2 (R/G site): extract dilution, 270; incubation time, 30 min; 5-HT_{2C}R (D-site): extract dilution, 30; incubation time, 8 min; and ADAR2 (–1 site): extract dilution, 30; incubation time, 5 min. Quantification of editing efficiency for [α -³²P]ATP-labeled D79N dsRNA was performed by thin layer chromatography (TLC) (Rueter *et al.*, 1995), and analysis of editing for the remaining *in vitro* reaction products was performed using a modified primer-extension analysis, as described previously (Burns *et al.*, 1997; Dawson *et al.*, 2004; Feng *et al.*, 2006).

Total RNA was isolated from HEK293 cells, 60 h after transient transfection, using TRI Reagent (Molecular Research Center, Cincinnati, OH). First-strand

Figure 1. 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 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.

cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega) with minigenome-specific primers for ADAR2 (5'-GGATCCCCGGGCTGCAG-3') and GluR-2 (5'-GGCCGAATCTACAAACCGTTAAGAGTCTTA-3') using 5 μ g 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 and a nonlabeled antisense primer (Feng *et al.*, 2006). Amplicons corresponding to alternatively spliced ADAR2 variants were resolved by 2.5% agarose gel electrophoresis and quantified by PhosphorImager analysis (GE Healthcare). Quantification of R/G site editing was performed using a modified primer-extension analysis, as described previously (Dawson *et al.*, 2004).

Statistical Analysis

Student's *t* test was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Values are reported as mean $\pm$ SEM; *p* < 0.05 was considered significant.

RESULTS

The Role of dsRBMs in the Nucleolar Localization of ADAR2

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 (K¹²⁷ and K²⁸¹) in each dsRBM, resulted 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 dsRBMs in maintaining the steady-state nucleolar localization of ADAR2 in living cells, we used multi-color fluorescence microscopy using a series of eGFP-ADAR2 mutants in transfected NIH/3T3 mouse fibroblasts (Figure 1), 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 with the average fluorescence intensity of eGFP-ADAR2 from a corresponding area of the nucleoplasm (Fo). Because eGFP and

eYFP 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 2A). The fluorescence pattern for cells cotransfected 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 2A).

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 [Δ dsRBM1/2 (Δ 76–301)] produced the expected pattern of diffuse nuclear fluorescence (Figure 2A), 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 β 3 and α 2 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 2A). Because analogous mutations in PKR and Staufen have been shown to ablate dsRNA-binding activity (McMillan *et al.*, 1995; Ramos *et al.*, 2000), these results further demonstrate that the localization of ADAR2 depends upon its ability to bind dsRNA. The expression of eGFP-ADAR2 mutants containing independent deletions of dsRBM1 [Δ dsRBM1 (Δ 76–148)] or dsRBM2 [Δ dsRBM2 (Δ 230–301)] also resulted in reduced nucleolar fluorescence for both fusion proteins (Figures 1 and 2, A and B), indicating that both dsRBMs are essential for the normal steady-state localization of ADAR2 in nucleoli. The relative nucleolar fluorescence for the Δ dsRBM1 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. Independent substitutions of K¹²⁷ and K²⁸¹ decreased relative nucleolar fluorescence to the same extent (K127A and K281A); however, the magnitude of this effect was less than that observed for the dsRBM deletions (Figure 2), suggesting that additional contacts between the dsRBMs and nucleolar binding site(s) are required for normal nucleolar localization.

Because 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 2C); 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 because of the absence of a nuclear localization signal and the passive diffusion of in these small fusion proteins (~40 kDa) through the nuclear pore (Suntharalingam and Wenthe, 2003); however, eYFP-dsRBM1 was localized to nucleoli significantly better than eYFP-dsRBM2 (*p* < 0.001; Figure 2C), further confirming a nonequivalent role for these highly

Figure 2. 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

conserved domains in the maintenance of ADAR2 nucleolar localization.

Substrate-dependent dsRBM Contribution to Site-selective Editing Activity

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 used 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 (Supplemental Figure S2), respectively (Lomeli *et al.*, 1994; Rueter *et al.*, 1999; Dawson *et al.*, 2004). HEK293 cells were chosen for this analysis because previous studies demonstrated a low level of endogenous editing activity in this cell line (Maas *et al.*, 1996; Burns *et al.*, 1997; Rueter *et al.*, 1999; Schaub and Keller, 2002).

Editing of the -1 site within ADAR2 pre-mRNAs generates a proximal 3'-splice site within intron 4 to direct the inclusion of an additional 47 nucleotides in the ADAR2 open reading frame (Rueter *et al.*, 1999; Dawson *et al.*, 2004; Feng *et al.*, 2006). As an indirect index of -1 site editing, we quantified the relative abundance of minigene-derived ADAR2 splice variants, containing (+47) or lacking (-47) this alternatively spliced cassette, using a reverse transcription (RT)-PCR-based strategy with a 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 3A). Deletion of dsRBM1 (Δ dsRBM1) or substitution of the conserved lysine in this domain (K127A) had little effect on the extent of editing-dependent alternative splicing for the minigene, yet deletion of dsRBM2 (Δ dsRBM2) or deletion of both dsRBMs (Δ dsRBM1/2) completely ablated use of the proximal 3'-splice site. These results indicate that dsRBM1 is not required, whereas 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 K²⁸¹ contributes only partially to dsRBM2-dependent editing at the -1 site (Figure 3A). Similar analyses, using a minigene derived from the GluR-2 transcript, demonstrated that although deletion of dsRBM1 (Δ dsRBM1) 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 3B). These results were distinct from those observed

Figure 3. Substrate-dependent dsRBM contributions to the editing of transfected ADAR substrates. (A) Quantitative analysis of ADAR2 alternative splicing is presented from HEK293 cells transiently cotransfected 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 ($n \geq 3$; mean $\pm$ SEM; *** $p < 0.001$ compared with wild-type activity). (B) Modified primer-extension analysis of GluR-2 (R/G) editing from HEK293 cells transiently cotransfected 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 nonedited (NE) RNAs are shown. A summary of GluR-2 (R/G) editing activities for eGFP-ADAR2 mutants is shown with all values normalized to wild-type (eGFP-ADAR2) activity ($n = 4$; mean $\pm$ SEM; ** $p < 0.01$, *** $p < 0.001$ compared with wild-type activity).

Figure 2 (cont). 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 ($n \geq 8$ cells; mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with wild type). (C) 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 ($n \geq 10$ cells; mean $\pm$ SEM; *** $p < 0.001$).

for the -1 site, where dsRBM1 was completely dispensable and dsRBM2 was essential for A-to-I conversion (Figure 3A), yet such a disparity could not be explained by differences in the relative expression of wild-type or mutant fusion proteins in transfected cells (Supplemental Figure S3). Overall, these results suggest that substrate-dependent dsRBM recognition of RNA targets is a critical component of site-specific adenosine deamination.

Although transfection studies (Figure 3) provided preliminary insight into substrate-dependent roles for ADAR2 dsRBMs in defining A-to-I conversion, this model system has a number of disadvantages for comparing the relative

Figure 4. 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 in nuclear extracts prepared from transiently transfected HEK293 cells; 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.

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 (Rueter *et al.*, 1999; Raittskin *et al.*, 2001; Desterro *et al.*, 2003; 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 both subnuclear accumulation and site-specific editing difficult to interpret.

To circumvent these problems, we used 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 4) (Sansam *et al.*, 2003; Dawson *et al.*, 2004), 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 with minor band representing a stable degradation product (Figure 4), and no signal was seen for mock-transfected HEK293 cells (our unpublished data). In addition to the RNA substrates previously used for transfection studies (Figure 3), 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 (Emeson and Singh, 2001; 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 (Sommer *et al.*, 1991; Higuchi *et al.*, 1993), and a perfect, synthetic dsRNA (D79N) derived from a portion of the α2A adrenergic receptor (Burns *et al.*, 1997; Lakhani *et al.*, 1997) (Supplemental Figure S2). For each RNA editing substrate (100 fmol), 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 (Supplemental Figure S1). The extent of total inosine production for the perfect dsRNA was determined by TLC (Rueter *et al.*, 1995), and site-specific editing of the remaining ADAR2 substrates was quantified using modified primer-extension analyses (Sansam *et al.*, 2003; Dawson *et al.*, 2004).

Using crude nuclear extracts from mock-transfected HEK293 cells, only background levels of editing were observed for any of the RNA substrates (Figure 5, A and B),

Figure 5. Substrate-dependent dsRBM contributions to the *in vitro* editing of ADAR substrates. Representative *in vitro* analyses of

Figure 6. Function-dependent interchangeability between ADAR2 dsRBMs. (A) Schematic diagram is presented indicating the domain structure of wild-type eGFP-ADAR2 and eGFP-ADAR2 mutants in which the dsRBMs are transposed (eGFP-dsRBM2/1) or that contain tandem 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 with 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 $\pm$ SEM). (D) Representative fluorescence micrographs demonstrating the subcellular localization of wild-type and mutant eGFP-ADAR2 fusion proteins and the corresponding Fn/Fo values ($n \geq 15$ cells; mean $\pm$ SEM; ** $p < 0.01$).

consistent with previous observations regarding the low level of endogenous editing activity in this cell line (Maas *et al.*, 1996; Burns *et al.*, 1997; Rueter *et al.*, 1999; Schaub and Keller, 2002). Deletion of the region containing both dsRBMs and the intervening linker (Δ dsRBM1/2) reduced editing in all RNA targets to near background levels, as did deletion of dsRBM2 alone (Δ dsRBM2), 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 (Δ dsRBM1) decreased editing in a substrate-dependent manner, because the extent of editing for GluR-2 (Q/R site) and 5-HT_{2C}R (D-site) transcripts was $<10\%$ of that observed for wild-type eGFP-ADAR2 (Figure 5, B and C), whereas editing of the remaining ADAR2 substrates was variably reduced by 30–70% (Figure 5). These results not only show that the dsRBMs of

ADAR2 are required for editing activity but also confirm previous observations in transfected cells (Figure 3) regarding the substrate-dependent contributions of each motif to site-specific editing.

Highly Conserved Lysine Residues in each dsRBM Contribute to Substrate-specific Editing Activity

The roles of dsRBMs are substrate dependent, possibly resulting from the precise amino acids in each motif that promote dsRBM–RNA interactions with each substrate. To explore this possibility, independent (K127A and K281A) or simultaneous (K127A, K281A) lysine substitutions were introduced, and mutant proteins were assayed for their editing activity on the same five ADAR substrates. The K127A mutation produced a minimal decrease in editing for all RNAs examined (Figure 5). This result was particularly surprising, because editing at the Q/R and D-sites of the GluR-2 and 5-HT_{2C}R transcripts, respectively, was highly dependent upon the presence of dsRBM1 in ADAR2, whereas mutation of the analogous lysine residue in PKR or Staufen eliminated dsRNA-binding (McMillan *et al.*, 1995; Ramos *et al.*, 2000). These results suggest that although dsRBM1 is critical for the editing of specific ADAR2 substrates, the precise amino acid residues required for A-to-I conversion may be distinct from those necessary for dsRNA binding. By contrast, the K281A mutation caused a modest decrease (13–25%) in the extent of editing for the perfect dsRNA (D79N) and the GluR-2 (R/G) transcript, whereas editing efficiency for the GluR-2 (Q/R), 5-HT_{2C}R (D), and ADAR2 (–1) sites was reduced by greater than 85% (Figure 5C). The observation that dsRBM2 was critical for efficient editing of all targets examined, yet only a subset of these

Figure 5 (cont). 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 TLC of an RNA substrate uniformly labeled with [α -³²P]ATP (see Materials and Methods); the TLC origin (ORI) and the migration positions of AMP and 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 nonedited (NE) RNAs are shown for Q/R and R/G sites in GluR-2 transcripts and the D- and –1 sites in 5-HT_{2C}R and ADAR2 transcripts, respectively. (C) A summary of the site-selective editing activities for eGFP-ADAR2 mutants is shown with all values normalized to wild-type (eGFP-ADAR2) activity ($n \geq 3$; mean $\pm$ SEM).

substrates was affected by the K281A mutation, demonstrates the relative importance of specific amino acid residues in dsRBM2-dependent editing activity.

Function-dependent Replacement and Transposition of ADAR2 dsRBMs

The contribution of dsRBM1 to the site-selective editing of ADAR2 seems to be substrate dependent, whereas dsRBM2 is critical for enzymatic activity with all substrates examined (Figure 5), despite the high degree of conservation at both the levels of amino acid sequence and protein structure (Tian *et al.*, 2004; Stefl *et al.*, 2006). 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 whether 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 6A) 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 with the same five ADAR2 substrates presented in Figure 5. 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 4) to adjust the enzyme input to equivalent levels for the *in vitro* reaction (Figure 6B). If the properties of each dsRBM in ADAR2 are dependent simply upon its relative position, replacement of either dsRBM with the alternate motif should maintain wild-type editing activity, yet such replacement mutations significantly reduced site-specific A-to-I conversion (Figure 6C). 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 very similar to the dsRBM2 deletion mutant (Δ dsRBM2). Similarly, replacement of dsRBM1 with dsRBM2 (dsRBM2/2) resulted in a pattern of editing that was nearly identical to protein lacking dsRBM1 (Δ dsRBM1), 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 (our unpublished data). 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 each dsRBM also affected nucleolar accumulation of ADAR2, we used confocal microscopy to compare fluorescence intensity in nucleoli and the nucleoplasm (as in Figure 2) 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 6C), such changes had little effect on the extent of nucleolar localization (Figure 6D), suggesting that the precise nature of the dsRBM-RNA interactions associated with editing and localization is distinct.

DISCUSSION

The recognition of dsRNA is a key event for a variety of biological processes, including the production of small interfering RNAs and micro-RNAs 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

(Emeson and Singh, 2001; Bass, 2002). A majority of the proteins involved in the recognition of duplex RNA contain multiple copies of a highly conserved 65- to 75-aa dsRNA-binding motif, providing a molecular mechanism to facilitate the interaction of these proteins with their dsRNA targets (Fierro-Monti and Mathews, 2000; Doyle and Jantsch, 2002; Carlson *et al.*, 2003; Tian *et al.*, 2004). ADAR2 is a dsRNA-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 depends primarily upon the number of functional dsRBMs present, whereas site-specific A-to-I conversion is also strongly affected by the nature and the organization of these motifs, indicating that each dsRBM possesses an intrinsic ability to recognize specific determinants in duplex RNAs. More importantly, the contributions of dsRBM1 and K²⁸¹ to editing activity are substrate dependent, consistent with previous findings that dsRBMs can specifically recognize distinct structural determinants on naturally occurring ADAR2 targets (Stephens *et al.*, 2004; Stefl *et al.*, 2006).

ADAR2 has been shown to bind to perfect dsRNA regions and catalyze nonspecific editing (Melcher *et al.*, 1996; Liu *et al.*, 1999; Lehmann and Bass, 2000; Cho *et al.*, 2003; Dawson *et al.*, 2004), bind to specific editing sites (Ohman *et al.*, 2000; Stephens *et al.*, 2004; Stefl *et al.*, 2006), 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 activity. 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 5). 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.

Previous observations that dsRBMs bind to perfect dsRNA, in a sequence-independent manner (Schreiber *et al.*, 1989; Ryter and Schultz, 1998; Ramos *et al.*, 2000; Blaszczyk *et al.*, 2004), have suggested that the dsRBMs of ADAR2 may recognize all of their RNA targets in a similar manner. Consistent with this idea, ADAR2 has been shown to non-specifically deaminate adenosine moieties in a wide range of synthetic RNA duplexes (Melcher *et al.*, 1996; Liu *et al.*, 1999; Lehmann and Bass, 2000; Cho *et al.*, 2003; Dawson *et al.*, 2004). However, the substrate-dependent contribution of dsRBM1 to editing activity (Figure 5C) not only indicates that this motif makes specific contacts with imperfect dsRNA targets but also suggests that this dsRBM can interact with different structural determinants in 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 (Stephens *et al.*, 2004; Stefl *et al.*, 2006).

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 5). Using a substrate containing the Q/R site, the K281A mutation reduced editing to background levels, suggesting that K²⁸¹ is a key residue mediating dsRBM2–RNA interactions. By contrast, the same mutation had little effect on editing of the R/G site (Figure 5), indicating that dsRBM2 may use distinct amino acid residues to contact different RNA targets. The lack of universal importance for K²⁸¹ has been further suggested by NMR analysis where no chemical shift change in the amide proton for this residue was observed upon binding of a GluR-2 transcript containing the R/G site (Steff *et al.*, 2006). If the ability of dsRBMs to use 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 because the corresponding lysines in other dsRBMs have been shown to make direct contact with dsRNA (Ryter and Schultz, 1998; Ramos *et al.*, 2000) 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 5) could result from the precise sequence/structure of each domain or their relative location within the ADAR2 protein. Replacement of one dsRBM with another (Δ dsRBM1/1 and Δ dsRBM2/2) provided patterns of editing activity that were similar to mutants lacking the replaced dsRBM, indicating that functional differences in A-to-I conversion 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.

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- κ B repressing factor have also been shown to accumulate in nucleoli (Tian and Mathews, 2001; Valdez *et al.*, 2002; Desterro *et al.*, 2003; Macchi *et al.*, 2004; Niedick *et al.*, 2004; Zhang *et al.*, 2004), 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 2) suggest that either both motifs are required for binding to specific dsRNAs 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 6), suggesting that the nucleolar localization of ADAR2 results from a general dsRNA-binding activity for one or multiple nucleolar binding sites rather than the specific dsRBM–RNA interactions required for site-selective adenosine deamination.

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 (Ryter and Schultz, 1998; Ramos *et al.*, 2000; Blaszczyk *et al.*, 2004). 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 (Spangord *et al.*, 2002; Stephens *et al.*, 2004; Stefl *et al.*, 2006). Although 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.

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