Editing of Glutamate Receptor Subunit B Pre-mRNA by Splice-site Variants of Interferon-inducible Double-stranded RNA-specific Adenosine Deaminase ADAR1*

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The interferon-inducible RNA-specific adenosine deaminase (ADAR1) is an RNA-editing enzyme that catalyzes the deamination of adenosine in double-stranded RNA structures. Three alternative splice-site variants of ADAR1 (ADAR1-a, -b, and -c) occur that possess functionally distinct double-stranded RNA-binding motifs as measured with synthetic double-stranded RNA substrates. The pre-mRNA transcript encoding the B subunit of glutamate receptor (GluR-B) has two functionally important editing sites (Q/R and R/G sites) that undergo selective A-to-I conversions. We have examined the ability of the three ADAR1 splice-site variants to catalyze the editing of GluR-B pre-mRNA at the Q/R and R/G sites as well as an intron hotspot (+60) of unknown function. Measurement of GluR-B pre-mRNA editing in vitro revealed different site-specific deamination catalyzed by the three ADAR1 variants. The ADAR1-a, -b, and -c splice variants all efficiently edited the R/G site and the intron +60 hotspot but exhibited little editing activity at the Q/R site. ADAR1-b and -c showed higher editing activity than ADAR1-a for the R/G site, whereas the intron +60 site was edited with comparable efficiency by all three ADAR1 splice variants. Mutational analysis revealed that the functional importance of each of the three RNA-binding motifs of ADAR1 varied with the specific target editing site in GluR-B RNA. Quantitative reverse transcription-polymerase chain reaction analyses of GluR-B RNA from dissected regions of rat brain showed significant expression and editing at the R/G site in all brain regions examined except the choroid plexus. The relative levels of the alternatively spliced flip and flop isoforms of GluR-B RNA varied among the choroid plexus, cortex, hippocampus, olfactory bulb, and striatum, but in all regions of rat brain the editing of the flip isoform was greater than that of the flop isoform.

RNA editing by site-selective conversion of adenosine to inosine (1, 2) plays a significant role in generating functional diversity of ionotropic glutamate receptor channel subunits (GluR)3 (3, 4). Multiple glutamate receptor forms have been identified that mediate rapid excitatory neurotransmission in the vertebrate central nervous system (5). Within the non-N-methyl-D-aspartate receptor family, including the AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate) and kainate receptor subtypes, a total of eight adenosine positions so far have been identified that undergo A-to-I editing in five different receptor subunit genes. These editing events result in altered RNA coding and subsequently altered biophysical properties of the receptor proteins (3, 4).

The AMPA subtype of the glutamate receptors consists of four subunits, GluR-A, -B, -C, and -D, that associate in homomeric and heteromeric combinations (6, 7). Each GluR subunit possesses four hydrophobic domains predicted to either span (TM1, TM3, and TM4) or loop into (TM2) the cytoplasmic membrane (8). Alternative splicing of a 38-amino acid region between TM3 and TM4, termed the flip/flop module specified by exons 14 and 15 of the GluR-B gene (9), generates two distinct receptor isoforms that differ from each other in the amplitude and kinetics of glutamate-gated responses (10). A genome-encoded glutamine (Q) codon (CAG) in exon 11, located in TM2 of AMPA receptor subunits, has been identified as a key residue that controls Ca2+ permeability (11, 12). The CAG codon is a target for A-to-I editing of the GluR-B subunit (13). Since an arginine (R) CIG codon is generated by editing, the site is designated as the Q/R site (1, 2). Editing at the Q/R site occurs almost completely in GluR-B transcripts (>99%), as demonstrated by RT-PCR analysis of brain RNA isolated from adult rats (14). Q/R site editing results in reduced permeability to Ca2+ ions of AMPA receptors containing the GluR-B subunit (11, 15). A second A-to-I editing site, termed the R/G site due to a conversion of an arginine (R) codon (AGA) to a glycine (G) codon (IGA), occurs in the GluR-B, -C, and -D subunits; the R/G site is the last codon of exon 13, immediately upstream of the flip/flop module (9, 16). Editing at the R/G site controls the kinetic properties of AMPA receptor channels because alteration of this single amino acid leads to faster recovery rates from receptor desensitization (16).

Elucidation of the RNA elements dictating the GluR-B editing specificity at the Q/R site revealed a unique cis-acting inverted repeat sequence located in the proximal portion of intron 11, which is predicted to form an imperfect duplex structure with the exon 11 sequence spanning the Q/R site (13, Fig. 1A). Similar duplex RNA structures also exist within exon 13 containing the R/G site and the proximal portion of intron 13 in the genes of the GluR-B, -C, and -D subunits (Fig. 1A) but are absent in the gene encoding GluR-A subunit (16). Mutational analyses established that these exon-intron dsRNA structures are absolutely essential for efficient editing at the Q/R and R/G sites (13, 16). In addition, multiple editing sites within intron 11 have been identified that also undergo A-to-I modification; these are referred to as hotspots (13, 17). The dsRNA structure

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requirement for the editing of GluR subunit transcripts suggested the involvement of RNA-specific adenosine deaminases (ADAR) (18) as the candidate editing enzymes.

ADAR deaminase, originally identified as an RNA unwinding activity, catalyzes the C6 deamination of adenosine in double-stranded (ds) structures present within viral RNAs and cellular pre-mRNAs as well as synthetic dsRNA substrates (19–24). Molecular cloning studies established that the ADAR deaminases constitute a multi-gene family of enzymes (18). So far two human ADAR genes, ADAR1 and ADAR2, have been shown to encode functional deaminases (18, 25–28). We isolated ADAR1 as an interferon-inducible enzyme (25, 29) that possesses both dsRNA binding and Z-DNA binding properties (30–32). The ADAR1 cDNA specified a 1226-amino acid protein that contains in the central region three functionally distinct copies of the highly conserved double-stranded RNA-binding motif (dsRBM), designated as dsRBM1, dsRBMII, and dsRBMIII. These dsRBM copies constitute the RNA-binding domain (dsRBD) and are implicated in the recognition of dsRNA structures within the substrate RNAs (25–27). The core amino acid residues of the dsRBM, the prototype of which was first described in the interferon-inducible RNA-dependent protein kinase PKR (33), are essential for the dsRNA binding activity of PKR (34, 35). These residues are exactly conserved in each of the three repeated dsRBM copies found in ADAR1 (25, 32).

Interestingly, characterization of ADAR1 and PKR genomic clones revealed that the codon phasing is precisely conserved at the junctions of the three exons that specify the three dsRBM copies of ADAR1 and the junctions of the two exons that specify the two dsRBM copies of PKR (36). A repeated domain present in the N-terminal region of ADAR1, homologous to the N-terminal region of the vaccinia virus E3L protein (25), specifies two Z-DNA binding domains within ADAR1 designated as Zα and Zβ (37). Two immunologically related forms of the human ADAR1 deaminase that differ in size, intracellular location, and interferon inducibility are present in a variety of human cell lines; an interferon-inducible ~150-kDa protein is present in both the cytoplasm and nucleus, and a constitutively expressed ~110-kDa truncated protein is present predominantly if not exclusively in the nucleus (25). The gene encoding these ADAR1 proteins maps to human chromosome 1q21.1–21.2 (38).

A second ADAR gene, ADAR2, maps to human chromosome 21q22.3 (39, 40) and encodes an ~80-kDa protein (28, 41). Unlike ADAR1, ADAR2 has a shorter N-terminal region and only two dsRBM-binding subdomains encoded by one single large exon (39).

Multiple ADAR1 and ADAR2 deaminase isoforms are generated by alternative splicing. For human ADAR1, we described three naturally occurring splice variant isoforms that show tissue-specific expression (36). In comparison to the full-length 1226 amino acid ADAR1 protein (25–27), designated ADAR1-a, ADAR1-b is a 5′-splice-site variant that has 26-amino acid deletion within exon 7 between the dsRBMII motif and the catalytic (C) domain. The ADAR1-c variant has an additional deletion of 19 amino acids within exon 6 located between dsRBM1 and dsRBMII resulting from an alternative 3′-splice-site selection (36).

Although the three ADAR1 isoforms exhibit comparable deaminase activity measured with a synthetic dsRNA substrate, site-directed mutagenesis revealed that the three dsRBM copies of the ADAR1 variants are functionally distinct (36). Similar to ADAR1, multiple splice variant isoforms of ADAR2 have been identified, some of which show altered deaminase activities (28). In contrast to the case of ADAR1, the alternative splice-site variants identified for ADAR2 differ within the catalytic deaminase domain by insertion of an Alu-like sequence, or at the C terminus (28, 39, 42) and the N terminus (43), rather than in the central RNA-binding region as seen for ADAR1.

The ADAR1 and ADAR2 deaminases show different activities for GluR-B RNA transcripts as indicated by editing analyses in vitro (28, 41). Recombinant ADAR1-a enzyme possesses little editing activity at the Q/R site but efficiently modifies the intron hotspot adenosine at the +60 site of GluR-B pre-mRNA (Fig. 1A). In contrast, recombinant ADAR2 enzyme efficiently edits the Q/R site of GluR-B transcripts (28, 41). Both ADAR enzymes are capable of editing the R/G site of GluR-B pre-mRNA (28, 41).

We previously demonstrated, using a synthetic dsRNA substrate, that the three dsRNA-binding motifs of ADAR1 are functionally distinct within the three splice-site variant isoforms (36). Here we describe the site-dependent editing of GluR-B RNA in vitro by the splice variant isoforms of ADAR1 and the editing patterns in vivo in RNA from dissected brain regions. Although the three recombinant ADAR1 variants a, b, and c were comparably active in vitro for editing the intron +60 hotspot of GluR-B pre-mRNA, none was active for the adjacent Q/R exon site. The R/G exon site was edited by all three variants, but ADAR1-b and -c displayed higher editing activity than ADAR1-a. Quantitative reverse transcription (RT)-PCR analyses of the RNA transcripts from different rat brain regions revealed differential expression levels of the GluR-B flip and flop receptor isoforms, with region-specific differences in editing efficiency at the R/G site.

MATERIALS AND METHODS

Expression and Analysis of Recombinant ADAR1 Proteins—Construction of ADAR1 expression vectors in pcDNA I/Neo was as described previously (31, 32, 36), including the full-length (FL) form in which ADAR1 translation initiates at Met-1 or the N-terminally truncated (M296) form which initiates at Met-296. Wild-type FL and M296 constructs included the three naturally occurring splice-site variants ADAR1-a, -b, and -c (36). Mutant ADAR1-b constructs likewise have been described (32, 36), both in the FL and M296 form, in which the dsRBM motifs possess a site-directed amino acid substitution at a highly conserved and critical lysine residue required for RNA binding. These substitution mutants include the three single mutants, each with one of the three dsRBM motifs mutated: dsRBM1(K665E), dsRBM2(K665E), or dsRBM3(K776E) designated as the R1, R2, or R3 mutant, respectively (32). A double substitution mutant of ADAR1 deficient in catalytic activity, designated as the C mutant and constructed in the M296 form of ADAR1-b, possesses H910Q and E912A substitutions within the highly conserved CHAE sequence of the C-terminal deaminase catalytic domain (32).

Monkey kidney COS-1 cells, maintained in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone), were transfected with wild-type or mutant cDNA expression vectors by the DEAE-dextran-chloroquine phosphate method for the expression of ADAR1 proteins (44). Recombinant ADAR1 proteins were prepared from cytoplasmic extracts as described previously (31, 36). ADAR1 protein levels were monitored by Western immunoblot analysis carried out with antisera (1.500 dilution) generated against recombinant ADAR1 protein expressed in Escherichia coli as described previously (25). Antibody-antigen complexes were subsequently detected with 125I-labeled protein A (0.05 mCi/ml; ICN) by autoradiography, and quantitation was done using a molecular imager (Bio-Rad GS-520). The dsRNA-specific adenosine deaminase activity of the expressed recombinant ADAR1 proteins was routinely measured with a 32P-labeled synthetic dsRNA substrate as described previously (25, 31, 36). Conversion of adenosine to inosine was analyzed by thin-layer chromatography (TLC) on cellulose NM 300 glass plates (Macherey & Nagel) to separate IMP and AMP, following hydrolysis of the deaminated RNA product with nuclease P1 (Amersham Pharmacia Biotech). Quantitation was carried out with the molecular imaging system.

Oligonucleotides—The following oligonucleotides were used for subcloning and construction of mouse GluR-B minigenes (9, 13, 16) that span the Q/R or R/G editing sites and for the primer extension analyses (Fig. 1B): The oligonucleotides derived from exons 13, 14, and 15 were also used for quantitative RT-PCR analysis of the alternatively spliced
flip and flop isoforms of GluR-B RNA transcripts from rat brain RNA since these sequences are identical between mouse and rat GluR-B genes. MBE11(+)/BglII, 5'-gaacAGATCTGATGGCCTGTTG-3' (nt 186–187 upstream of the Q/R site in exon 11, with the A at the Q/R site referred to as 0 position); MBII1(--)/XbaI, 5'-gaacCTCTAGATCGAT-3' and GATCATGACGTTGATGATCTTCCAG-3' (nt 3–26 of intron 13); and MBE15(--)/StuI, 5'-GTTCAACAGGCTTGTTTCG-3' (nt 35–52 of exon 11); MBI3(+)/BglII, 5'-gaacAGACATCTAAATTGAGCAGT-3' (nt 1–18 of exon 13); MB14(--)/StuI, 5'-GACATTGCCCTATCCTTGTCG-3' (nt 2–25 downstream of the Q/R site); MB(-)/HS, 5'-GAACGACATGATCTACCGAG-3' (nt 64–86 downstream of the Q/R site); MB(-)/R/G, 5'-GATCTTCTAATGCTCTCAGC-3' (nt 3–26 of intron 13) and MBE15(--), 5'-CAGCTAGGTTACCGTGC-3' (nt 17–35 of exon 15). The symbol (+) indicates the sense primer, and (-) the antisense primer. The underlined sequences indicated the restriction sites utilized, and the lowercase sequences were included to facilitate restriction digestion.

Construction of GluR-B Minigenes and RNA Editing Assays—A 741-nt GluR-B genomic DNA fragment spanning 211 nt of exon 11 containing the Q/R site and 530 nt of the proximal sequence of intron 11 has been shown to be editing-competent (13). This fragment was amplified by PCR with Taq DNA polymerase (Fisher) under conditions supplied by the manufacturer from a mouse genomic DNA library (CLONTECH) using primers MBE11(+)/BglII and MBII1(--)/XbaI. After digestion with BglII and XbaI, the PCR product was subcloned into pcDNA INeo vector that had been digested with BamHI and XbaI to generate GluR-B-Q/R minigene. Likewise, an approximately 1.1-kilobase pair GluR-B genomic fragment spanning the R/G site and the entire intron 13 was amplified with primers MBE13(+)/BglII and MBE14(--)/StuI. The PCR product digested with BglII and StuI was subcloned into pcDNA INeo vector that had been cut by BamHI and EcoRI and subsequently used as GluR-B-R/G minigene. GluR-B RNA transcript was made by in vitro transcription and used as the substrate of editing assays.Briefly, XbaI-linearized plasmid DNA (5 μg) with the Glur-B-Q/R or -R/G minigenes was transcribed in vitro using phage T7 RNA polymerase (New England Biolabs) according to the manufacturer's instructions. The RNA transcript was recovered by precipitation with ethanol after extraction with phenol and chloroform. In a standard editing assay, the GluR-B RNA transcript was incubated at a final concentration of about 1 nM in 100 μl of reaction mixture containing recombinant ADAR1 protein from the cytoplasmic fraction of transfected COS cells. An equivalent amount of wild-type ADAR1 deaminase activity was added for each isoform as measured previously with a synthetic dsRNA substrate; the wild-type ADAR1-a, -b, and -c splice variants have a comparable specific deaminase activity on the dsRNA substrate (36). For the ADAR1 substitution mutants proteins with altered enzymatic activity, an equivalent amount of mutant protein determined by Western blot assay to that of the wild-type protein was added. After incubation in the presence of 20 units of RNase inhibitor (Promega) for 3 h at 30 °C, the edited RNA was recovered again and subjected to quantification of editing by primer extension analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT)-PCR—The edited GluR-B RNA containing the Q/R site or the R/G site was directly subjected to primer extension analysis using a reverse transcription (RT) reaction as shown in Fig. 1B. Three primers, MB(-)/Q/R, MB(-)/HS, and MB(-)/R/G, which were 5′-end labeled by T4 polynucleotide kinase (New England Biolabs) using [γ-32P]ATP (Amer- sham Pharmacia Biotech), were annealed to edited GluR-B RNA by heating at 70 °C for 10 min and gradually cooling to 42 °C. Three extension reactions were established as indicated in Fig. 1B to measure the editing efficiency at the Q/R site, the intron hotspot +60 site, and the R/G site, using avian myeloblastosis virus reverse transcriptase according to the manufacturer's instructions (Promega). Each reaction contained a combination of deoxythymidine 5′-triphosphate at 1 mM and the other three deoxyxynucleoside 5′-triphosphates (dNTPs) each at 0.1 mM. After incubation at 42 °C for 40 min, extended products were resolved on denaturing 16% polyacrylamide gels with 7 M urea and quantified using a molecular imaging system.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—The GluR-B RNA transcripts edited in vivo at the R/G site for the alternatively spliced flip and flop transcripts and their expression levels were determined using the total RNA isolated from different rat brain regions (kindly provided by Dr. R. B. Emeson, Vanderbilt University) by quantitative RT-PCR with Taq DNA polymerase (Fisher) under conditions recommended by the manufacturer. The total RNA from dissected brain areas of adult 8–12-week-old rats (24) included choroid plexus, cortex, hippocampus, olfactory bulb, and striatum. Reverse transcription was carried out at 37 °C with random hexamer oligonucleotides using Moloney murine leukemia virus-reverse transcriptase (New England Biolabs) for the brain-derived RNA. Limiting amounts of the resultant cDNA were then subjected to PCR analyses, using end-labeled primer MBE13(+)/BglII and primer MBE14(--)/StuI to detect the flip isoform or primer MBE15(--) the flop isoform of GluR-B (Fig. 2A). The PCR products were resolved on a 1.5% agarose gel with the R/G site as an internal control. In both cases, the bands at 140 bp were excised from the gel and electroeluted using the manufacturer's instructions (Promega). The PCR products were resolved on native 6% polyacrylamide gels and end-labeled 1-kilobase pair DNA ladder (New England Biolabs) was used as the marker.

**RESULTS**

Site-selective Editing of GluR-B RNA in Vitro by the ADAR1-a, -b, and -c Proteins—To understand better the basis of the site selectivity of the GluR-B editing process (Fig. 1A), we examined the ability of the three isoforms of the interferon-inducible ADAR1 protein to catalyze the site-specific editing of the GluR-B RNA substrate in vitro. A primer extension strategy was employed as shown in Fig. 1B to measure GluR-B editing. Oligonucleotide primer MB(-)/Q/R was used to measure editing at the Q/R site, primer MB(-)/HS for the +60 intron hotspot, and primer MB(-)/R/G for the R/G site.

The three recombinant ADAR1 proteins were all capable of modifying the two GluR-B RNA substrates in vitro in a site-selective fashion (Fig. 2A). The ADAR1-a, -b, and -c isoforms all showed a preference for the intron hotspot +60 site of the 741-nt exon 11-intron 11 GluR-B RNA (Fig. 2B). None of the three ADAR1 splice variants were able to efficiently edit the exon 11 Q/R site (data not shown). However, the R/G site of the 1100-nt exon 13-intron 13-exon 14 GluR-B RNA served as an effective substrate for all three ADAR1 variant proteins (Fig. 2A). When the specific editing activities obtained for the hotspot +60 and the R/G site were normalized to the deam-

Functionally Distinct RNA Binding and Catalytic Domains of ADAR1 Associated with Editing of GluR-B RNA—Site-directed mutagenesis earlier revealed that the three copies of the dsRBM motif and the catalytic deaminase domain are functionally distinct from each other in ADAR1 (32) and that the functional importance of each individual dsRBM varies among the three ADAR1 splice variants when synthetic dsRNA is used as the substrate as compared with a fully complementary synthetic dsRNA substrate. For each of three dsRNA substrates, the dsRBMII(11) motif of ADAR1-b was the most important of the...
three motifs for dsRNA deamination, and the dsRBMII motif was dispensable for enzyme activity. The functional significance of the dsRBMIII motif was intermediate between that of the dsRBMII motif and dsRBMIII motif measured with dsRNA (Fig. 3A).

When the GluR-B RNA substrates were tested, the relative functional importance of each of the three dsRBM motifs was found to be dependent upon the particular editing site examined. Mutation of any one of the three dsRBM motifs of ADAR1 significantly reduced editing at the intron 160 hotspot (Fig. 3B). However, for editing at the R/G site, the dsRBM III motif was the most important of the three dsRBM motifs, and the dsRBMI and dsRBMII motifs were much less important (Fig. 3C), similar to earlier findings obtained with a synthetic dsRNA substrate (32, 36). Curiously, mutation of the dsRBMII motif resulted in an increased editing efficiency at the R/G site, especially with the FL form of the protein (Fig. 3C). Comparison of our results obtained with different substrates revealed that the dsRBMII and dsRBMIII motifs of ADAR1-b were more important for editing the +60 hotspot than for editing either the R/G site or for deamination of synthetic dsRNA. The double substitution mutation introduced into the catalytic domain in the M296 form of ADAR1-b protein abolished deaminase activity for synthetic dsRNA substrate (32), and also resulted in the loss of editing activity for the +60 hotspot and the R/G site of the GluR-B RNA substrate (Fig. 3).

Expression and Editing Patterns of GluR-B RNA Flip and Flop Isoforms in Dissected Rat Brain Regions—Alternative splicing of the GluR-B transcript characterized by skipping either exon 14 or exon 15 produces two functionally different GluR-B isoforms, designated flip and flop (10), as illustrated by the schematic diagram shown in Fig. 4A. To assess the expression patterns in the brain of the alternatively spliced flip and flop isoforms of GluR-B RNA, quantitative RT-PCR was carried out utilizing RNA isolated from five dissected rat brain regions (24) including choroid plexus, cortex, hippocampus, olfactory bulb, and striatum. The flip and flop isoforms were differentially expressed in the five regions of the brain examined (Fig. 4B). Relative to α-tubulin as an internal standard, the lowest relative expression levels of both GluR-B isoform transcripts were found in the choroid plexus. The flip isoform was most efficiently expressed and more abundant than the flop isoform in cortex and hippocampus, whereas the flop isoform was expressed at comparable levels to the flip isoform in the choroid plexus, olfactory bulb, and striatum.

PCR analyses with primers specific for exon 14 and 15 sequences yielded the two predicted products that correspond to the flop and the flip isoforms of GluR-B. The identification of the products as flip and flop was confirmed by mobility of the products following digestion by MseI restriction endonuclease (Fig. 5A). By using the additional MseI site present in the flip isoform as an internal control for complete digestion (Fig. 5A), the efficiency of editing at the R/G site was determined based on the fact that the non-edited R/G site sequence constitutes an MseI restriction site (TTA). The efficiency of editing at the R/G site was generally higher for the flip than for the flop isoform.
isoform in all five of the brain regions examined (Fig. 5B). The lowest level of editing for the flop isoform was found in choroid plexus (≈ 10%) among the five dissected brain regions examined (Fig. 5B).

**DISCUSSION**

The RNA-editing enzyme ADAR1 is an interferon-inducible, double-stranded RNA-binding protein (25, 29, 47). Although the three recombinant ADAR1 splice-site variants possess comparable adenosine deaminase activity determined with a synthetic dsRNA substrate (36), little is known regarding their editing preferences for the sites of GluR-B pre-mRNA, one of the postulated natural substrates of ADAR enzymes (1). Several important points emerge from our results reported herein on the editing of the GluR-B pre-mRNA by ADAR1 splice site variants in vitro and the expression and editing in vivo of the flip and flop isoforms of GluR-B transcripts in dissected regions from rat brain.
Recombinant ADAR1-a, -b, and -c splice-site variant proteins displayed quantitatively different A-to-I modifying activities for the Q/R, R/G, and intron 160 hotspot sites within GluR-B substrate RNAs in vitro. All three of the human ADAR1 splice variants showed efficient editing activity for the 160 hotspot and R/G sites of GluR-B but were nearly inactive for the Q/R site (Fig. 2). The inability of ADAR1-a, -b, or -c to edit the Q/R site is consistent with the notion that ADAR2 is most likely responsible for editing the Q/R site of GluR-B (28, 41). Although the ADAR1-a, -b, and -c isoforms did not display any significant difference in their ability to edit the +60 hotspot, they showed different editing activities for the R/G site (Fig. 2B). Our finding that human ADAR1-a did catalyze editing at the R/G site by the primer extension assay is consistent with recent reports for the rat and human ADAR1-a proteins (41, 49), the isoform that we and others initially cloned (18). However, among the splice variants of ADAR1 that are now known (36), we observed that the ADAR1-b and especially the ADAR1-c isoforms were substantially more active than the ADAR1-a isoform for editing the exon R/G site but not the +60 intron hotspot in GluR-B substrates. These results suggest that the two deletions introduced in the ADAR1-b and -c isoforms by
alternative splicing do not qualitatively affect the site selectivity of ADAR1 for editing the GluR-B RNA substrates in vitro, but indeed do affect how efficiently an adenosine at a particular site is presented to the catalytic center of ADAR1 for subsequent deamination. Most likely the functional importance of the spacer region between the RNA-binding domain and the catalytic domain of the ADAR1 splice-site isoforms depends upon the particular overall structure of the substrate RNA bound by the dsRBM motifs.

Previously we established by site-directed mutagenesis that the three repeated copies of the dsRBM in ADAR1-b behaved in a functionally distinct manner when enzyme activity was measured using a synthetic dsRNA substrate (32, 36). Herein we observed, using minigene RNA versions of the natural GluR-B substrate RNA, that all three dsRBM copies of ADAR1-b were required for efficient editing of the +60 intron hotspot. However, for editing at the R/G site, the dsRBMIII motif was uniquely important among the three dsRBM copies; mutation of the dsRBMII and dsRBMIII motifs revealed that they contributed far less relative to dsRBMIII for R/G editing activity (Fig. 3). Thus, the relative functional importance of the individual dsRBM motifs for editing at the R/G site appears comparable to their importance for editing a synthetic dsRNA substrate. The differences in behavior of the individual dsRBM copies for editing at the +60 hotspot and the R/G site likely reflect the distinct structural features of the GluR-B RNA surrounding the +60 hotspot and R/G editing sites.

Quantitative RT-PCR analyses of RNA from dissected regions of rat brain revealed that the expression of the alternatively spliced flip and flop isoforms of GluR-B transcripts varied relative to one another in different regions of brain. The lowest levels of GluR-B transcripts were found in choroid plexus and the highest levels in the cortex and hippocampus (Fig. 4B). Estimation of the editing efficiency at the R/G site in different regions of rat brain, using an Msel-cleavage method of analysis, revealed that the extent of editing of the flip form was consistently higher than that of the flop form (Fig. 5B). The lowest relative level of editing at the R/G site was found in the choroid plexus, the region of lowest GluR-B expression. The ADAR1and ADAR2 proteins are expressed in all five of the rat brain regions.

Our results obtained for GluR-B editing can be compared with those observed for serotonin receptor 5-HT2c-R RNA, which also undergoes A-to-I editing in the brain (24). Analysis of the mature mRNA encoding 5-HT2c-R as well as the 5-HT2c-R pre-mRNA revealed a region-specific pattern of expression and editing of 5-HT2c-R in rat brain (24). In contrast to the relatively low level of GluR-B transcripts in the choroid plexus (Fig. 4B), the 5-HT2c-R RNA transcripts were most abundant in choroid plexus. But, similar to the low relative extent of editing seen for the R/G site of GluR-B transcripts in choroid plexus, editing of 5-HT2c-R RNA at the A and B sites is also less efficient in choroid plexus as compared with cortex, hippocampus, olfactory bulb, and striatum regions (24). Most likely a large number of RNA species serve as substrates for ADAR-catalyzed editing in the brain, based on a recent estimate from analysis of inosine-containing RNA transcripts that one adenosine out of every 17,000 nucleotides in mRNA from the brain undergoes A-to-I editing (50). Some of these RNAs likely encode GluR subunits other than GluR-B (3, 4), but the vast majority of the I-containing mRNAs are as yet unidentified. Other than the induction of ADAR1 by the cytokine interferon (25, 29, 47), little is known about the basis of regulation of ADAR gene expression and modulation of the amount of ADAR enzyme activity in the context of the total pool of potential RNA substrates that includes the GluR-B and 5-HT2c-R pre-mRNAs. Both biochemical analyses in vitro and transfection studies in vivo show that ADAR1 is capable of efficiently editing the A and B sites of the 5-HT2c-R pre-mRNA (24). Curiously, the expression levels of the ADAR1-a and -b isoforms relative to α-tubulin are highest in the choroid plexus even though editing at the R/G site of the GluR-B RNA (Fig. 5B) and the A and B sites of the 5-HT2c-R RNA (24) was the lowest in choroid plexus relative to other brain regions. This suggests the possible involvement of trans-acting factors in choroid plexus that modulate the editing of both GluR-B and 5-HT2c-R RNAs by ADAR1. Such factors could include either RNAs (51) or proteins (49) that modulate ADAR1 activity. Alternatively, an enzyme other than ADAR1 may be primarily responsible for editing the R/G site of GluR-B pre-mRNA and the A and B sites in 5-HT2c receptor pre-mRNA under certain conditions in the brain.

The results shown herein that the wild-type ADAR1-a, -b, and -c variants and the dsRBM motif mutants of the ADAR1-b variant differ in their ability to edit the natural cellular GluR-B RNA substrate, together with the finding that the Xenopus ADAR1 dsRBM motifs exhibit different RNA-binding behaviors (48), support the notion that functionally distinct dsRNA-binding domains of ADAR1 (32, 36) reflect the occurrence of different structures among potential RNA substrates that are bound and edited by ADAR1 isoforms. Our results indicate that changes in the region between the dsRBD domain and the catalytic domain of ADAR1 affects the editing efficiency of GluR-B RNA in a site-specific fashion but without altering site-selectivity per se. The deletions possessed by the ADAR1-b and -c splice-site variants relative to ADAR1-a between the dsRBM motifs and the catalytic domain may alter the manner in which RNA substrates are recognized and bound by one or more of the dsRBMs that constitute the RNA-binding domain prior to deamination. Our results also imply that multiple isoforms of ADAR enzymes, including those of ADAR1 (36) and ADAR2 (28, 41), may exist which act in combination with each other on a single RNA substrate that possesses multiple sites for A-to-I editing.

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