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A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains

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ABSTRACT

Members of the double-stranded RNA- (dsRNA) specific adenosine deaminase gene family convert adenosine residues into inosines in dsRNA and are involved in A-to-I RNA editing of transcripts of glutamate receptor (GluR) subunits and serotonin receptor subtype 2C (5-HT_{2C}R). We have isolated hADAR3, the third member of this class of human enzyme and investigated its editing site selectivity using in vitro RNA editing assay systems. As originally reported for rat ADAR3 or RED2, purified ADAR3 proteins could not edit GluR-B RNA at the "Q/R" site, the "R/G" site, and the intronic "hot spot" site. In addition, ADAR3 did not edit any of five sites discovered recently within the intracellular loop II region of 5-HT_{2C}R RNAs, confirming its total lack of editing activity for currently known substrate RNAs. Filter-binding analyses revealed that ADAR3 is capable of binding not only to dsRNA but also to single-stranded RNA (ssRNA). Deletion mutagenesis identified a region rich in arginine residues located in the N-terminus that is responsible for binding of ADAR3 to ssRNA. The presence of this ssRNA-binding domain as well as its expression in restricted brain regions and postmitotic neurons make ADAR3 distinct from the other two ADAR gene family members, editing competent ADAR1 and ADAR2. ADAR3 inhibited in vitro the activities of RNA editing enzymes of the ADAR gene family, raising the possibility of a regulatory role in RNA editing.

Keywords: ADAR; adenosine deamination; double-stranded RNA; RNA binding proteins; RNA editing; single-stranded RNA

INTRODUCTION

RNA editing changes the sequence of mRNAs, which results in synthesis of proteins not encoded in the gene (Smith et al., 1997). Transcripts of certain genes such as glutamate receptor (GluR) ion channels (Sommer et al., 1991; Lomeli et al., 1994), serotonin receptor 2C subtype (5-HT_{2C}R) (Burns et al., 1997), and K_V2K^+ channels (Patton et al., 1997) are subjected to one type of RNA editing that involves the conversion of adenosine residues into inosine. A-to-I RNA editing may be a widespread phenomenon: as many as one out of every 10 (brain) to 100 (skeletal muscle) mRNAs may contain an inosine residue converted from adenosine by the A-to-I editing mechanism (Paul & Bass, 1998). The amino acid changes introduced by this A-to-I RNA editing result in significant alterations in the physiological properties of gene products. For instance, editing of the "Q/R" site of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) GluR-B subunit dramatically decreases the Ca^{2+} permeability of the channel (Sommer et al., 1991; Köhler et al., 1993). Dramatic changes in the G-protein coupling efficiency of 5-HT_{2C}R (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000) as well as in the rates of K_V2K^+ channel closure (Patton et al., 1997) have been reported to be consequences of A-to-I RNA editing also. In addition, creation of an alternative splice acceptor site via editing of its own mRNA by ADAR2 has been reported (Rueter et al., 1999). The A-to-I RNA editing mechanism requires: (1) a double-stranded RNA (dsRNA) structure, usually formed between the exonic editing site and a downstream intron sequence (Higuchi et al., 1993; Lomeli et al., 1994; Herb et al., 1996;

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Burns et al., 1997) and (2) dsRNA-specific adenosine deaminases (Melcher et al., 1995, 1996b; Dabiri et al., 1996; Maas et al., 1996; Gerber et al., 1997; Lai et al., 1997a), originally called DRADA (Kim et al., 1994b) or dsRAD (O'Connell et al., 1995; Patterson & Samuel, 1995; Hough & Bass, 1997) and more recently termed ADAR (adenosine deaminases that act on RNA) (Bass et al., 1997). The members of this ADAR gene family appear to share structural similarity, containing two to three repeats of dsRNA-binding domains and a separate deaminase or catalytic domain (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995; Melcher et al., 1996b; Bass et al., 1997; Gerber et al., 1997; Hough & Bass, 1997; Lai et al., 1997a). The presence of functional domains for both substrate binding and catalysis within each ADAR gene family member contrasts with the requirement for other subunits by APOBEC-1, a C-to-U deaminase involved in RNA editing of apolipoprotein B mRNAs (Smith et al., 1997). APOBEC-1, which contains only a catalytic domain, requires a separate substrate-binding subunit and possibly additional auxiliary factors for in vitro editing of apolipoprotein B RNAs (Smith et al., 1997). When purified ADAR1 and ADAR2 recombinant proteins were tested in vitro, they proved self sufficient in carrying out A-to-I editing of GluR-B RNAs (Melcher et al., 1995, 1996b; Dabiri et al., 1996; Maas et al., 1996; Gerber et al., 1997; Lai et al., 1997a). Although these previous studies have suggested that the A-to-I RNA editing can be carried out by ADARs alone, at least in vitro (Dabiri et al., 1996; Maas et al., 1996; Melcher et al., 1996b; Gerber et al., 1997; Lai et al., 1997a), the requirements of regulatory cofactors or the presence of inhibitors in vivo have been also suggested (Saccomanno & Bass, 1994; Dabiri et al., 1996; Lai et al., 1997b).

Through human expressed sequence tag (EST) cDNA databases and cDNA library screening, we have isolated the third member of this ADAR gene family, ADAR3, a human homolog of the recently cloned rat RED2 (Melcher et al., 1996a). The expression of ADAR3 is restricted to certain regions of human brain such as amygdala and thalamus. Purified recombinant ADAR3 proteins could not edit any previously known sites of GluR-B and 5-HT_{2C}R RNAs, nor were they capable of deaminating adenosine residues of a synthetic long dsRNA substrate. Deletion mutagenesis and filter-binding analysis have revealed that ADAR3 can bind to not only dsRNAs via two dsRNA-binding motifs (DRBM) but also single-stranded RNA (ssRNA) via an arginine- and lysine-rich domain (R-domain) located at the N-terminus region. Because of the presence of this R domain, ADAR3 appears to interact distinctively with GluR-B and 5-HT_{2C}R RNAs, both of which contain not only dsRNA regions but also ssRNA regions of loops and bulges. When tested for its potential competitive inhibitory effects on other ADAR members, ADAR1 and ADAR2, ADAR3 did display strong inhibitory activities at least in vitro. Our results suggest the possibility that ADAR3 may play a role in the regulation of substrate-specific RNA editing in mammalian brains.

RESULTS

Isolation of human ADAR3 cDNAs

A search through human EST databases (Boguski et al., 1993; Adam et al., 1995) using the sequence of the catalytic domain of the human ADAR1 (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995) or ADAR2 (Melcher et al., 1996b; Gerber et al., 1997; Lai et al., 1997a) led to identification of ADAR3, a human homolog of the rat RED2 (Melcher et al., 1996a) (Fig. 1A). The nucleotide sequence of 3,655 bp corresponding to the longest overlapping cDNA sequence was determined. This sequence contained a 5′ untranslated region (UTR) (334 bp), an open reading frame (2,220 bp), and a 3′ UTR (1,101 bp), including a polyadenylation signal AATAAA (nt position 3628). The overall similarity and identity of rat and human ADAR3 protein sequences were 87% and 84%, respectively. The human ADAR3 protein is more similar to ADAR2a with 72% sequence similarity than to ADAR1 with 53% sequence similarity (Fig. 1B). Whereas ADAR1 contains three repeats of DRBMs, ADAR2 and ADAR3 consist of only two repeats of DRBMs. In addition, ADAR3 contains a unique arginine- and lysine-rich region (R domain) including six consecutive arginine residues at the N-terminus as originally pointed out by Melcher et al. (1996a).

Northern blot analysis detected human ADAR3 transcripts as a major 9.5-kb mRNA (Fig. 2A). ADAR3 mRNA expression appears to be restricted to only brain, in contrast to the ubiquitous expression of ADAR1 and ADAR2 (Fig. 2A). Furthermore, certain regions of brain such as thalamus and amygdala contain relatively higher concentrations of ADAR3 mRNA than other regions (Fig. 2B). Because the coding region and 5′ UTR of ADAR3 are approximately 2.2 and 0.3 kb, respectively, we anticipate that the major 9.5-kb mRNA contains a long 3′ UTR (~7 kb) with a separate alternative polyadenylation site(s). Both ADAR1 and ADAR2 also contain long 3′ UTR regions (Kim et al., 1994b; Patterson & Samuel, 1995; Lai et al., 1997a). The ADAR3 protein expression was detected as an 81-kDa protein by western immunoblot analysis of total mouse brain extracts using a monoclonal antibody (MAb) raised against the N-terminal region (Fig. 2C). Interestingly, a low level of ADAR3 expression was detected with the same antibody in a human teratocarcinoma cell line NT2 upon its terminal differentiation to NT2-N neurons (Fig. 2C). This postmitotic neuron specific expression of ADAR3 is in contrast to the constitutive expression of ADAR1 and ADAR2 detected in undifferentiated NT2 cells as well

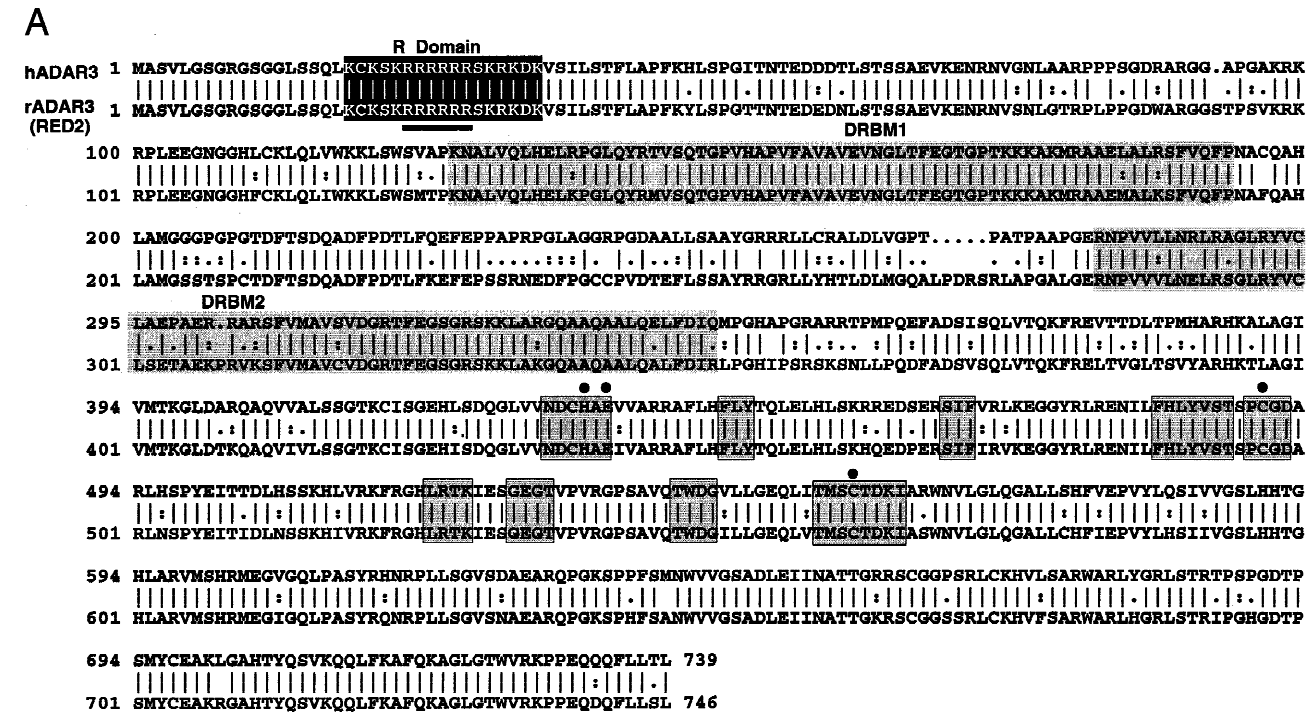


FIGURE 1. Amino acid sequence and domain structure of human ADAR3. **A**: Amino acid sequence alignment of human ADAR3 (GenBank accession number AF034837) and RED2 or rat ADAR3 (Melcher et al., 1996a; GenBank accession number U74586). The R-domain (filled box) consisting of six consecutive arginines (underlined) located within the region rich in lysines and arginines, and two DRBM repeats (lightly shaded), are indicated. Within the putative deaminase domain of ADAR3, there are nine stretches of amino acid residues (boxed and shaded) conserved in all three ADAR gene family members (core deaminase domain). Within this core deaminase domain, four amino acids, H, E, C, and C, likely to be essential for the formation of the catalytic center of ADAR gene family members (Lai et al., 1995), are marked by filled circles. **B**: Domain structure of three ADAR gene family members and two deletion mutants of ADAR3. Nuclear localization signals (NLS) present in ADAR1 and ADAR2, two Z-DNA binding domains (Zα and Zβ) (Herbert et al., 1997) present only in ADAR1, the dsRNA binding domain consisting of two or three DRBMs (Kim et al., 1994b), the core deaminase domain (Kim et al., 1994b; Lai et al., 1995; Patterson & Samuel, 1995), and the R-domain unique to ADAR3 (Melcher et al., 1996a) are schematically presented. Below the three ADARs, the structure of the wild-type (WT) and two deletion mutants (ΔR and ΔM1M2) of ADAR3 used in this study are schematically shown. The dashed lines depict the sequences deleted in the mutants. The numbers below each line indicate the amino acid residues present in the deletion mutant proteins.

as terminally differentiated NT2-N neurons (Lai et al., 1997b).

Absence of RNA editing activity in ADAR3

It has been previously reported by Melcher et al. (1996a) that RED2 (the rat ADAR3) lacks A-to-I modification activity and is incapable of editing GluR-B RNAs at any of three sites tested. These previous studies were carried out either by using crude extracts of HEK293 cells transfected with a rat ADAR3 expression construct as enzyme source or by cotransfecting an ADAR3 expression construct and a GluR-B mini gene construct into HEK293 cells (Melcher et al., 1996a). We wondered if the reported inactivity of ADAR3 might result from the assay system employed, for instance, because of the

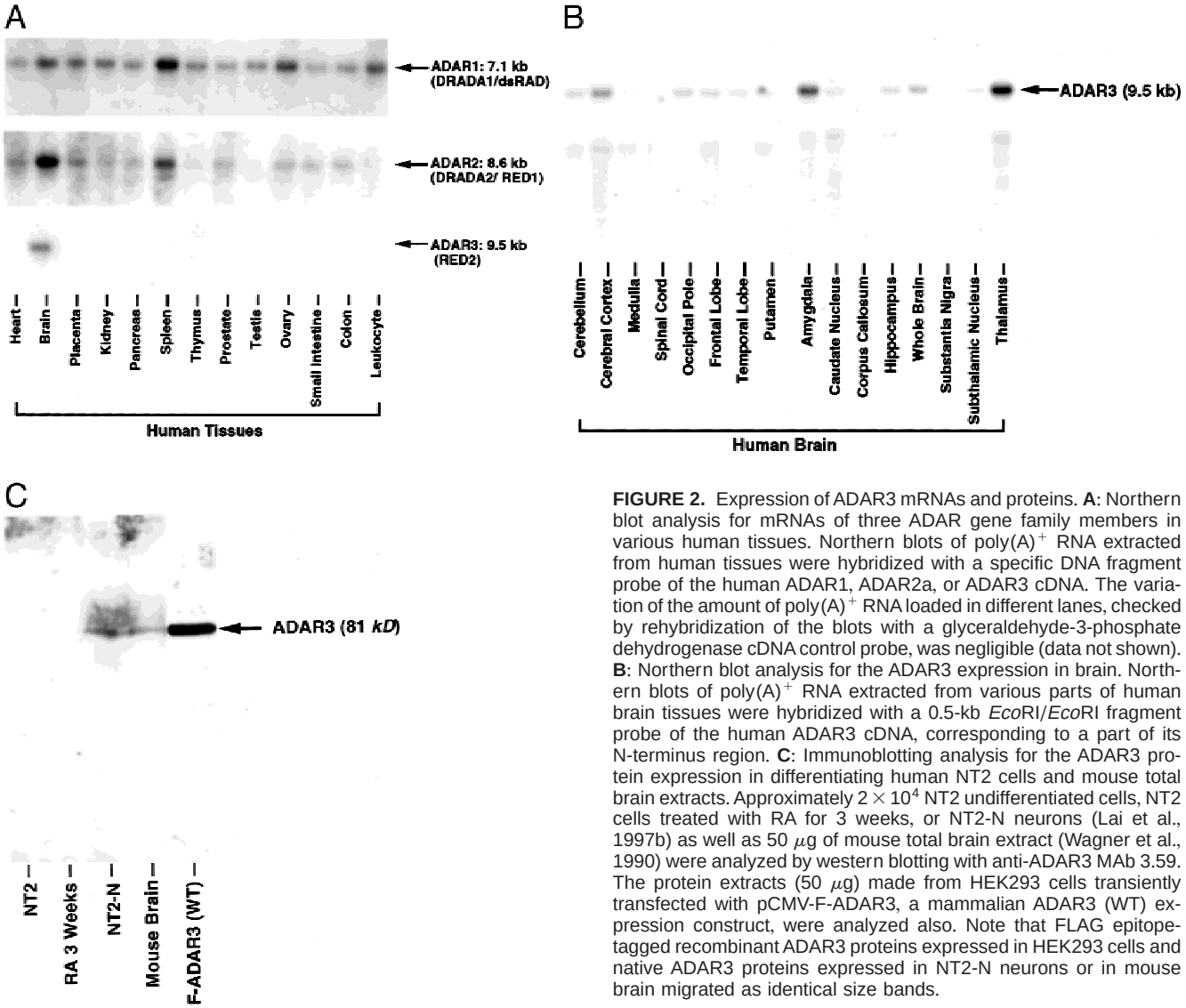


FIGURE 2. Expression of ADAR3 mRNAs and proteins. **A**: Northern blot analysis for mRNAs of three ADAR gene family members in various human tissues. Northern blots of poly(A)$^+$ RNA extracted from human tissues were hybridized with a specific DNA fragment probe of the human ADAR1, ADAR2a, or ADAR3 cDNA. The variation of the amount of poly(A)$^+$ RNA loaded in different lanes, checked by rehybridization of the blots with a glyceraldehyde-3-phosphate dehydrogenase cDNA control probe, was negligible (data not shown). **B**: Northern blot analysis for the ADAR3 expression in brain. Northern blots of poly(A)$^+$ RNA extracted from various parts of human brain tissues were hybridized with a 0.5-kb *Eco*RI/*Eco*RI fragment probe of the human ADAR3 cDNA, corresponding to a part of its N-terminus region. **C**: Immunoblotting analysis for the ADAR3 protein expression in differentiating human NT2 cells and mouse total brain extracts. Approximately 2×10^4 NT2 undifferentiated cells, NT2 cells treated with RA for 3 weeks, or NT2-N neurons (Lai et al., 1997b) as well as 50 μg of mouse total brain extract (Wagner et al., 1990) were analyzed by western blotting with anti-ADAR3 MAb 3.59. The protein extracts (50 μg) made from HEK293 cells transiently transfected with pCMV-F-ADAR3, a mammalian ADAR3 (WT) expression construct, were analyzed also. Note that FLAG epitope-tagged recombinant ADAR3 proteins expressed in HEK293 cells and native ADAR3 proteins expressed in NT2-N neurons or in mouse brain migrated as identical size bands.

presence of inhibitor(s) that block an otherwise active ADAR3 enzyme.

To test this, we reexamined the activities of ADAR3 by using purified recombinant protein. Recombinant ADAR3, carrying an epitope-tagged peptide FLAG at the N-terminus, was produced in Sf9 insect cells and purified (Fig. 3A). Purified recombinant ADAR3 protein was examined first for its adenosine deaminase activity using long synthetic c-*myc* dsRNA (575 bp) as a substrate RNA. ADAR3 did not deaminate any adenosine residues, in contrast to the very efficient A-to-I modification activities of ADAR1 and ADAR2a (Fig. 3B). We then investigated the RNA editing site selectivity of ADAR3 in vitro. Two separate GluR-B gene transcript fragments, B11 RNA containing the Q/R site within exon 11 and an intronic hot spot +60 site located in intron 11 (Dabiri et al., 1996), and B13 RNA containing the R/G site within exon 13 (Lai et al., 1997a), were investigated (Fig. 3C) by an in vitro RNA editing assay and subsequent dideoxynucleotide/primer extension analysis of in vitro edited RNAs. We found that purified ADAR3 protein does not edit any of these sites, which can be edited efficiently by ADAR1 and/or ADAR2a (Fig. 3C). Thus, our findings from experiments with purified recombinant human ADAR3 protein are the same as the previous report on the enzymatic inactivity of RED2 (the rat ADAR3) tested with crude nuclear extracts or the cotransfection system (Melcher et al., 1996a).

A set of A-to-I RNA editing sites has been recently identified within the transcripts of 5-HT$_{2C}$R gene (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000). The A-to-I RNA editing at these sites, which are all located within the intra-loop II region of this 5-HT receptor, results in a dramatic decrease of its G-protein coupling efficiency (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000). Therefore, we tested the possibility that ADAR3 might edit certain sites of 5-HT$_{2C}$R RNAs

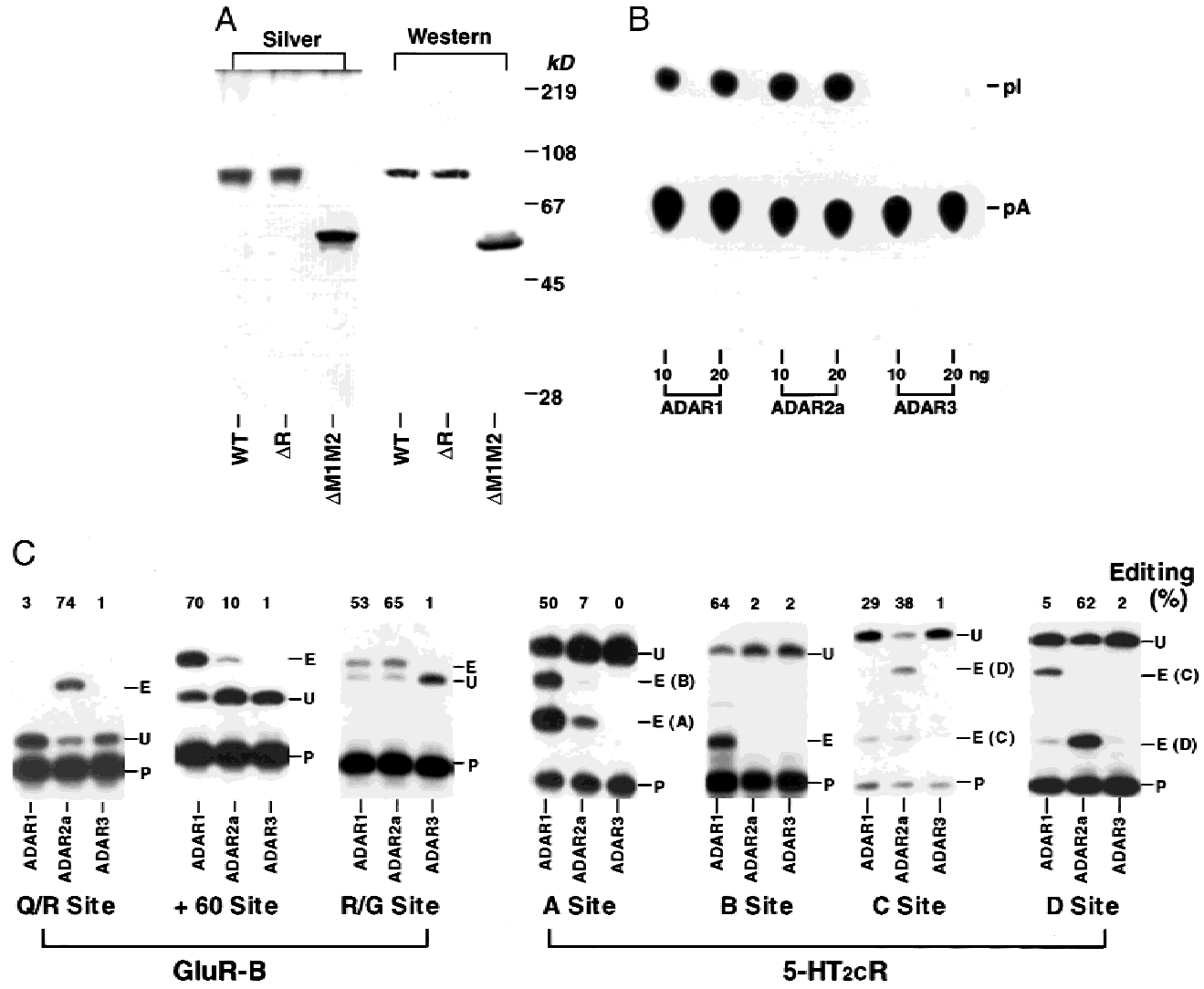


FIGURE 3. Absence of A-to-I conversion and RNA editing activities in purified ADAR3 proteins. **A**: Three recombinant ADAR3 proteins (WT, ΔR, and ΔM1M2) contained an epitope-tagged peptide FLAG at their N-terminus. Recombinant FLAG-ADAR3 fusion proteins, 50 ng for silver staining or 10 ng for immunoblotting, were fractionated by 8% SDS-PAGE. Immunoblotting hybridization was done with anti-FLAG M2 MAb. Molecular mass standards used were prestained myosin heavy-chain, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase. **B**: The A-to-I conversion activity of purified recombinant ADAR3 enzymes (10 or 20 ng) was determined by a base modification assay using 10 fmol of ^{32}P-ATP-labeled c-*myc* dsRNA (Kim et al., 1994a). For comparison, two other members of the ADAR gene family, ADAR1 and ADAR2a, were also examined. Following incubation for 2 h at 37 °C, the reaction products were deproteinized, digested with P1 nuclease, and analyzed by one-dimensional TLC (Kim et al., 1994a). **C**: RNA editing site selectivity of recombinant ADAR3. Editing site selectivity of purified recombinant ADAR3 proteins was examined in vitro at the Q/R, intronic +60, and R/G sites of GluR-B RNAs and A, B, C, and D sites of 5-HT$_{2C}$R RNA. For comparison, purified recombinant ADAR1 and ADAR2a proteins were tested simultaneously. The RNA editing efficiency (%) was estimated by quantifying the ratio of the primer (P) extended band specific for edited (E) and unedited (U) RNA with the PhosphorImaging System (Molecular Dynamics).

by using purified human ADAR3 recombinant protein and C5 RNA containing exon 5 and a part of intron 5 of human 5-HT$_{2C}$R (Xie et al., 1996). All five editing sites, A, B, C, D, and E (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000), are located in exon 5, and the region of intron 5 included within C5 RNA is sufficient for formation of the dsRNA structure essential for RNA editing of these sites (Burns et al., 1997). Preferential editing of the A site by ADAR1 and the D site by ADAR2a as well as overlapping site selectivity of ADAR1 and ADAR2a at the C site in co-transfected HEK293 cells has been previously reported (Burns et al., 1997). We confirmed these previous results with our in vitro RNA editing assay using purified recombinant ADAR1 and ADAR2a proteins (Fig. 3C). In addition, our assay revealed that the B site, not examined in the previous studies, was edited exclusively by ADAR1 (Fig. 3C). Most importantly, however, our

assay results indicated that purified ADAR3 was unable to edit 5-HT_{2C}R RNA at any of the A, B, C, and D sites (Fig. 3C) nor at the E site (Wang et al., 2000). Our results confirm the total absence of the RNA editing and A-to-I base modification activities in ADAR3, using currently known naturally occurring substrate RNAs.

The role of the R-domain in the ssRNA binding activity of ADAR3

The total lack of A-to-I modification and RNA editing activity prompted us to examine whether ADAR3 was capable of binding to substrate dsRNAs at all. We first investigated binding of ADAR3 wild-type and ADAR2a to GluR-B B11 and 5-HT_{2C}R C5 RNAs by a nitrocellulose filter-binding assay (Kim et al., 1994a; Lai et al., 1995). Two deletion mutants ADAR3ΔR and ADAR3 ΔM1M2 were tested also for binding (Fig. 1B). ADAR3 ΔR lacks the six consecutive arginine residues present within the R-domain, which is located at the N-terminus and highly conserved between human and rat ADAR3, suggesting its functional significance (Fig. 1A). ADAR3 ΔM1M2 lacks the entire dsRNA binding domain consisting of two repeats of dsRNA binding motifs (DRBM1 and DRBM2). The K_d values for binding, obtained by Scatchard plot analysis of the RNA binding assay data, are summarized in Table 1. The affinity of ADAR3 for both B11 and C5 RNA was found to be equivalent or slightly lower than that of ADAR2a; for instance, the K_ds for B11 RNA binding of ADAR2a and ADAR3 are 0.2 and 0.4 nM, respectively. We were puzzled that ADAR3ΔM1M2, lacking the entire dsRNA binding domain, was able to bind both B11 and C5 RNAs with comparable affinity to the wild-type protein. Our results suggested that ADAR3 may bind to substrate RNAs through the regions that are not double stranded. To test this possibility, the binding of WT-ADAR3 and ADAR2a to single-stranded and double-stranded c-*myc* RNAs were tested (Fig. 4). As expected, both ADAR2a and ADAR3 bound to c-*myc* dsRNA, although the affinity of ADAR3 is approximately 2.5-fold lower than that of ADAR2a. However, ADAR3 bound also to c-*myc* ssRNA, in contrast to the complete lack of the ssRNA binding capability in ADAR2a (Fig. 4). The binding of ADAR3 was also confirmed with β-globin ssRNA, showing that the binding is not restricted to the c-myc sequence. Binding of the two deletion mutants to c-*myc* ssRNA and dsRNA was then examined. The mutant ADAR3ΔR, but not ADAR3ΔM1M2, bound to c-*myc* dsRNA as expected. Interestingly, the mutant ADAR3 ΔM1M2 bound also to c-*myc* ssRNA with even higher affinity than WT-ADAR3 (Table 1). The ADAR3ΔR mutant, lacking the six consecutive arginine residues of the R-domain, did not bind to c-*myc* ssRNA at all. These results suggest that ADAR3 contains two separate domains that are involved in binding of substrate RNA; the R-domain rich in arginine and lysine residues for binding of ssRNA and the region consisting of two repeats of DRBMs for binding of dsRNA. In addition, our results explain why ADAR3ΔM1M2 still binds to B11 or C5 RNAs, as these GluR-B and 5-HT_{2C}R RNAs are thought to contain double-stranded as well as single-stranded regions.

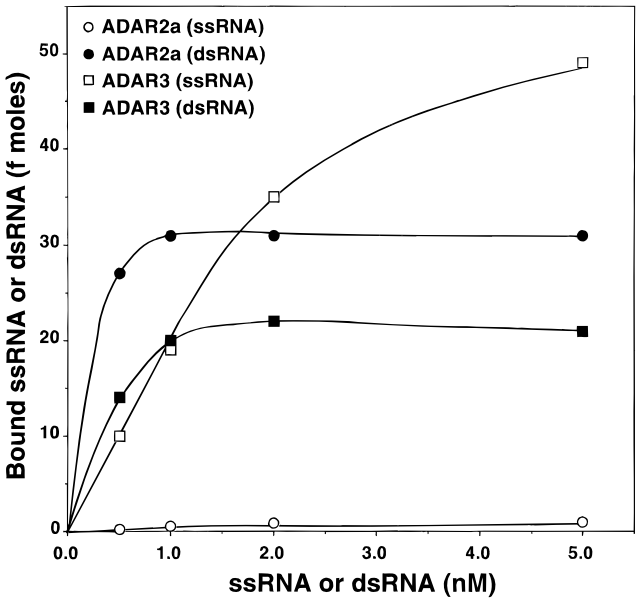


FIGURE 4. ADAR3 binds to both ssRNA and dsRNA. Binding of recombinant ADAR3 or ADAR2a proteins to [α-^{32}P]ATP labeled c-*myc* dsRNA (575 bp) or antisense ssRNA (605 nt) was analyzed by a nitrocellulose filter-binding assay. Ten nanograms of purified ADAR protein were incubated at 30 °C for 5 min in duplicate with various concentrations of dsRNA or ssRNA.

TABLE 1. RNA binding of ADAR proteins.

	K_d (nM)			
	GluR-B[a] (B11)	5-HT_{2C}R (C5)	ssRNA (c-*myc*)	dsRNA[a] (c-*myc*)
ADAR2a	0.2	0.5	NB[b]	0.2
ADAR3 WT	0.4	0.6	1.1	0.5
ADAR3ΔR	0.5	0.5	NB[b]	0.4
ADAR3ΔMIM2	0.5	0.7	0.7	NB[b]

Filter-binding assays were conducted in duplicate at various RNA concentrations. Variation of RNA bound between duplicate membranes at a given RNA concentration was less than 5% of the mean value.

[a] K_d values determined previously for ADAR1 are 0.3 nM for B11 RNA (Lai et al., 1997a) and 0.11 nM for c-*myc* dsRNA (Lai et al., 1995).

[b] NB: no binding.

In vitro inhibition of RNA editing by ADAR3

Having confirmed the capability of ADAR3 for binding GluR-B and 5-HT_{2C}R RNAs, we then tested the possibility that ADAR3 may interfere with the activities of

RNA editing competent enzymes, ADAR1 and ADAR2a, as a competitive inhibitor. The potential inhibitory effects of ADAR3 were tested in the in vitro RNA editing assay by preincubating substrate 5-HT_{2C}R RNA with purified ADAR3 protein prior to addition of ADAR1 or ADAR2a. The site selective editing of both ADAR1 and ADAR2a was effectively inhibited by ADAR3. For instance, the presence of a threefold excess of ADAR3 inhibited editing of 5-HT_{2C}R RNA by ADAR1 96% at the A site and by ADAR2a 83% at D site. When only an equal amount of ADAR3 was used for the inhibition assay, the RNA editing activities of ADAR1 and ADAR2a were still suppressed significantly (>80%) at most of the sites (Fig. 5A). The overall inhibitory effects of ADAR3 were unchanged when the time for RNA editing was increased to 2 h from 30 min (data not shown). We also tested the effects of ADAR3 on other ADAR activities by mixing all components (a substrate RNA, ADAR3, and ADAR1 or ADAR2a) together simultaneously or by premixing ADAR1 or ADAR2a first with various concentrations of ADAR3 prior to addition of a substrate RNA. We confirmed similar inhibitory effects of ADAR3 regardless of the mixing order (data not shown).

Reciprocal interference of ADAR1 and ADAR2a for RNA editing site selectivity

Because ADAR3 had inhibitory effects on the RNA editing activities of ADAR1 and ADAR2a, we examined the reciprocal effects of ADAR1 and ADAR2a for their RNA editing site selectivity. Interestingly, the presence of an equal amount of ADAR1 or ADAR2a modestly decreased, 15–70%, RNA editing efficiency at the preferred site of the other (Fig. 5B). These results suggest that the presence of ADAR1 and ADAR2a also can affect the site selectivity of each other in vitro.

DISCUSSION

The presence of both ssRNA and dsRNA binding domains in ADAR3

In this study, we have identified and characterized ADAR3, the third member of the dsRNA adenosine deaminase gene family and the human homolog of rat RED2 (Melcher et al., 1996a). The arrangement as well as the sequence of both dsRNA binding and deaminase domains of ADAR3 are very similar to those of ADAR2. For instance, phylogenetic analysis of DRBMs from three ADAR gene family members and four additional genes clearly indicates the most proximal relationship between the first DRBM (M1) of ADAR3 and the first DRBM (M1) of ADAR2. Similarly the second DRBM (M2) is most closely related to the second DRBM (M2) of ADAR2 (Fig. 6A). Their DRBMs are more distantly related to three DRBMs (M1–M3) of ADAR1. The deaminase domain sequence of ADAR3 is also more similar to that of ADAR2 than ADAR1 (Fig. 6B) indicat-

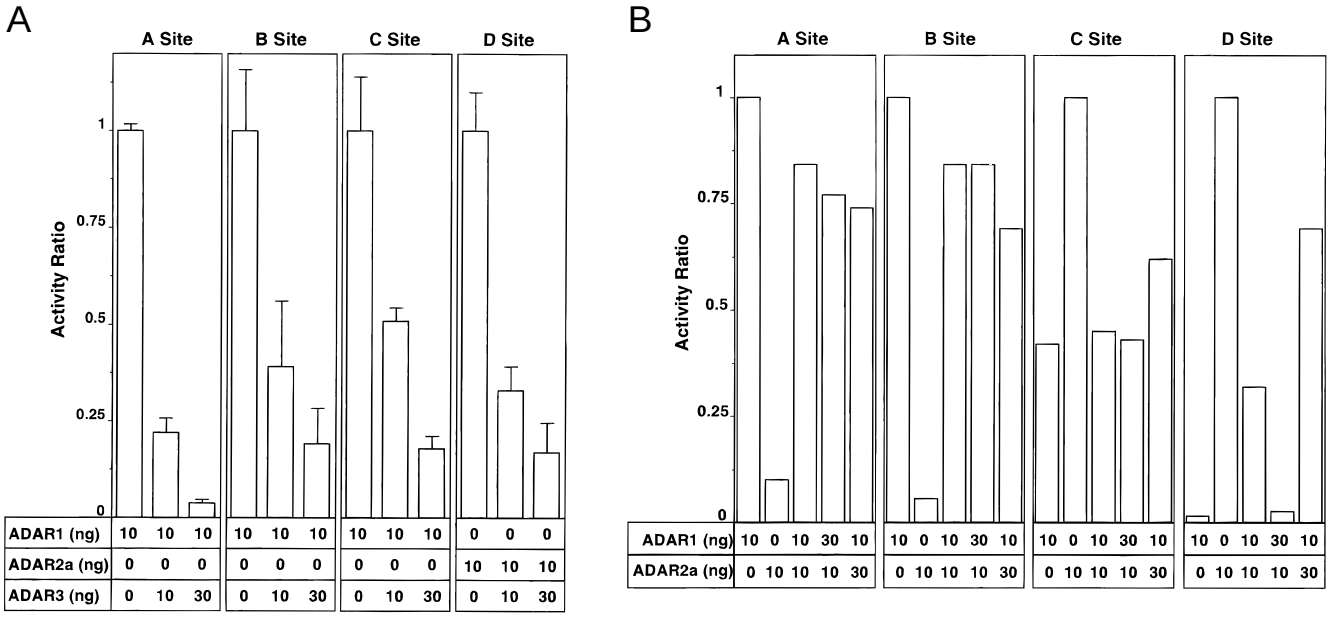


FIGURE 5. In vitro interference of different ADARs on RNA editing. A: Inhibitory effects of ADAR3 on RNA editing activities of ADAR1 and ADAR2a. The efficiency of 5-HT_{2C}R RNA editing by ADAR1 or ADAR2a (30 °C, 30 min) in the presence of a one- or threefold excess of ADAR3 (10 or 30 ng) was determined. The results were normalized as relative ratios to the values obtained without ADAR3. The editing efficiency of each site without ADAR3 was as follows: A site by ADAR1, 37%; B site by ADAR1, 38%; C site by ADAR1, 13%; D site by ADAR2a, 35%. Bars indicate standard error. Three independent experiments were conducted (n = 3). **B**: Interference of RNA editing site selectivity between ADAR1 and ADAR2a. The efficiency of 5-HT_{2C}R RNA editing was determined in the presence of mixtures of ADAR1 and ADAR2a in different ratios or ADAR1 or ADAR2a alone. ADAR1 and/or ADAR2a and 5-HT_{2C}R RNA were mixed together simultaneously.

A

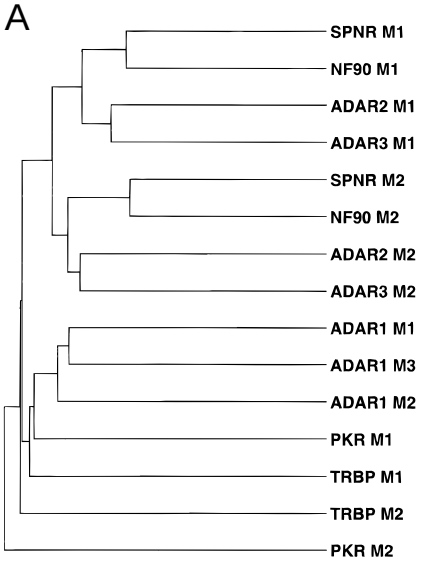
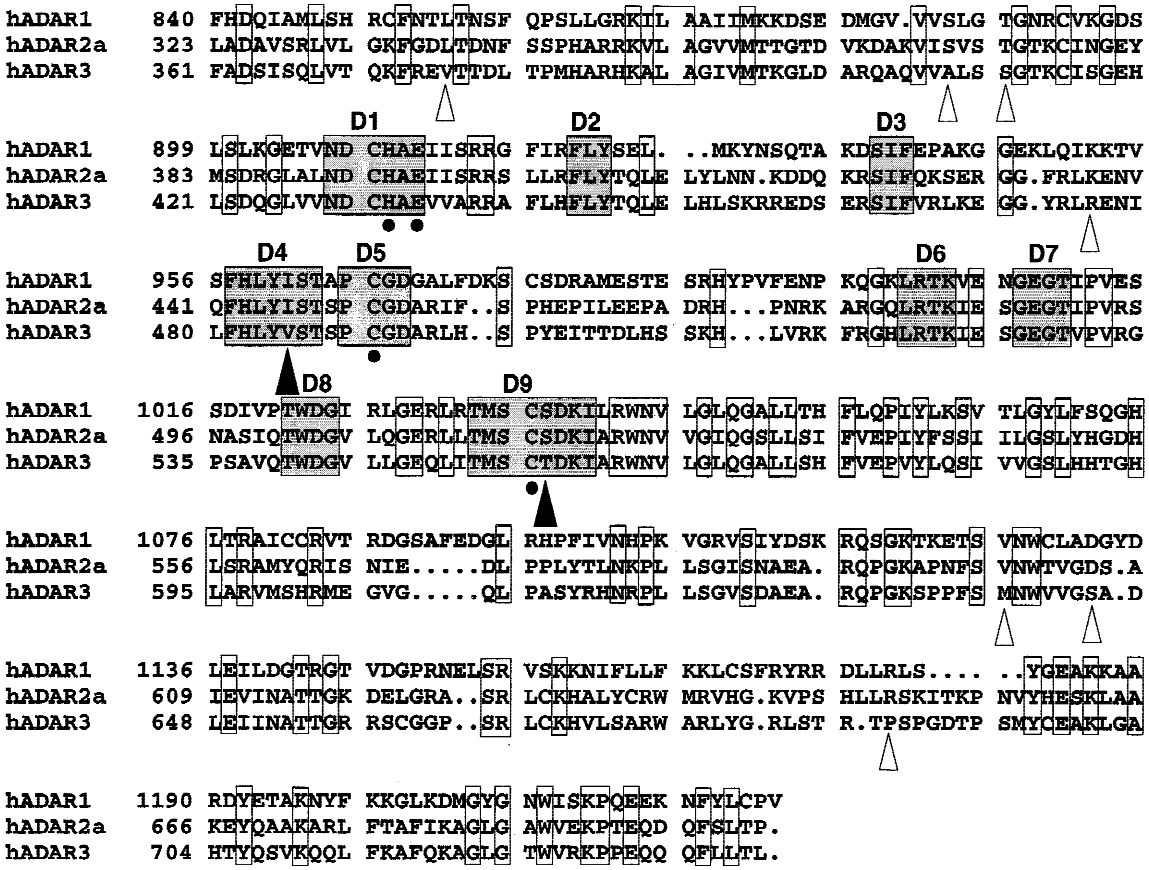


FIGURE 6. Comparison of functional domains of three human ADAR gene family members. **A**: Phylogenetic analysis of dsRNA-binding domains of three ADARs. The phylogenetic tree was constructed by the PILEUP program. Distance along the horizontal axis is inversely proportional to the percentages of homology between DRBMs. DRBM sequences of the following genes (accession number indicated in parenthesis) were analyzed. Human ADAR1 (U10439): M1: amino acids 502–572; M2: amino acids 613–683; M3: amino acids 725–795; human ADAR2a (U76420): Ml: amino acids 80–145; M2: amino acids 234–299; ADAR3 (AF034837): M1: amino acids 127–192; M2: amino acids 277–342; human dsRNA-activated PKR kinase (M35663): M1: amino acids 8–78; M2: amino acids 99–168; human TAR-binding protein TRBP (M60801): M1: amino acids 8–77; M2: amino acids 137–207; mouse spermatid perinuclear RNA binding protein SPNR (X8469L): M1: amino acids 389–454; M2: amino acids 512–577; human nuclear factor NF90 (U10324): M1: amino acids 404–469; M2: amino acids 531–596. **B**: Amino acid sequence alignment of the deaminase domains conserved among three human ADARs. In addition to nine stretches of amino acid residues highly conserved among different deaminases and considered as deaminase core sequences, D1–D9 (boxed and shaded), all amino acid residues conserved among three ADAR proteins are boxed. Two amino acid residues located within the core catalytic domain region and present in both ADAR1 and ADAR2a, but altered in ADAR3, are marked by filled arrowheads. The amino acid residues outside of the core catalytic domain regions and present in ADAR1 and ADAR2a as well as Tad1p [(not shown), a recently identified tRNA-specific deaminase (Gerber et al., 1998)], but altered in ADAR3, are marked by open arrowheads.

B



ing the possibility of a common evolutionary origin shared by ADAR2 and ADAR3. However, the presence of the arginine- and lysine-rich (R-domain) N-terminal extension makes ADAR3 distinctive from ADAR2 (Fig. 1B). Filter-binding analysis of two deletion mutants has indicated this region comprises the ssRNA binding domain. The six consecutive arginines located in the center of the R-domain play a dominant role in its affinity to ssRNA. Although the affinity of ADAR3 for dsRNA binding is lower than that of ADAR1 or ADAR2a, ADAR3 seems to interact uniquely with RNA substrates because of its dual capacity for binding not only to dsRNA via its DRBM repeats but also to ssRNA via its N-terminus R-domain.

Interestingly, an arginine-rich region of HIV-1 Tat protein has been reported to form a unique protein structure, the "arginine fork" made from arginine side chains, which interacts with adjacent pairs of phosphates via hydrogen bonds and is thus likely to bind to RNA loops and bulges but not to double-stranded A-form RNA (Calnan et al., 1991). HIV-1 Rev and bacteriophage λ N proteins utilize their arginine-rich domains also for their interaction with RNA (Lazinski et al., 1989; Calnan et al., 1991). It is possible that the R-domain of ADAR3 forms a similar protein structure and recognizes preferentially specific ssRNA structures of loops and bulges often found in the natural substrate RNAs known to be edited, such as GluR-B and 5-HT_{2C}R.

A possible role played by ADAR3 in the A-to-I RNA editing mechanism

When purified recombinant ADAR3 protein was tested by an in vitro RNA editing assay, it was found to be incapable of editing any of eight known sites of GluR-B (Q/R, +60 intronic, and R/G sites) and 5-HT_{2C}R (A, B, C, D, and E sites) RNAs. Similarly purified recombinant ADAR3 protein did not display any A-to-I conversion activity tested with a synthetic long dsRNA substrate (575 bp). It is difficult to explain the incompetence of ADAR3 in RNA editing activity. The amino acid residues highly conserved among ADAR gene family members and believed to make up the deaminase domain, including two triplet peptides HAE and PCG involved in the zinc chelating and electron transfer function (Lai et al., 1995), are all conserved in ADAR3 except for a few amino acid residues (Fig. 6B). Furthermore, the two repeats of dsRNA binding motif of ADAR3 are functional, as our RNA binding assay has proven its capability to bind to dsRNA. The structural features and domains present in ADAR3 strongly suggest that this third member of the ADAR gene family must be a functional deaminase enzyme. We have tested the possibility that the recombinant ADAR3 proteins prepared in Sf9 insect cells lack posttranslational modification critical for activation of the latent form enzyme. Recombinant ADAR3 proteins prepared from transiently transfected HEK293 mammalian cells did not exhibit enzymatic activity. In addition, mixing recombinant ADAR3 proteins with mouse brain nuclear extracts did not alter the latent state of the ADAR3 enzyme (C-X. Chen & K. Nishikura, unpubl. results).

Although these results all indicate ADAR3 is an inactive deaminase, we currently cannot exclude the possibility that ADAR3 may be converted to the active form upon necessary posttranslational modification or binding to a specific modulatory factor in brain, the only tissue where this third member of the ADAR gene family is expressed. Similarly, we cannot exclude the other possibility that ADAR3 may utilize its combined ssRNA- and dsRNA-binding activities to target a very selected set of RNA substrates that are different from the already known substrates such as GluR-B or 5-HT_{2C}R RNA tested in this study. Finally, the enzymatically inactive form of ADAR3 could affect the editing activities of ADAR1 and ADAR2 provided that they are coexpressed in individual cells. When ADAR3 was tested in vitro as a potential competitive inhibitor, it suppressed effectively editing of 5-HT_{2C}R RNA by ADAR1 and ADAR2a. An equal amount of ADAR3 decreased the RNA editing of ADAR1 or ADAR2a up to 70%–80%. At first glance, this in vitro inhibitory effect of ADAR3 on the activities of ADAR1 and ADAR2a appears to reflect its competitive binding to the substrate RNAs, in particular to their ssRNA regions. However, our preliminary inhibition experiments conducted with ADAR3ΔR and ADAR3ΔMIM2 mutants have revealed that their inhibitory activities are significantly less than those of the wild-type ADAR3, indicating a synergistic effect of the presence of both R-domain and DRBMs (data not shown). Future experiments will address the function(s) of the combined as well as the individual ssRNA- and dsRNA-binding domains of ADAR3 in the inhibition mechanism.

The efficiency of certain GluR-subunit or 5-HT_{2C}R-RNA editing varies significantly in different regions of the brain (Nutt & Kamboj, 1994; Paschen & Djuricic, 1995; Burns et al., 1997; Fitzgerald et al., 1999; Wang et al., 2000) and during terminal differentiation of clonal neuron cells (Lai et al., 1997b). Interestingly, our Northern hybridization analysis indicated that the expression of ADAR3 is limited to selected areas of brain such as amygdala and thalamus. Furthermore, the ADAR3 protein expression was detected only in postmitotic neurons. Thus, the enzymatically inactive deaminase, ADAR3, if simultaneously expressed in a single cell with the active and ubiquitous type deaminases such as ADAR1 and ADAR2, could affect the activities of the other enzymes and make the A-to-I RNA editing in brain and differentiating neurons more complicated than anticipated before. For instance, the previously observed regional variation in the editing site selection and efficiency could be explained by different ratios and/or combinations of these three distinctive ADAR gene family members. However, we have currently no information on the ADAR family protein expression levels in the different regions of brain, especially with regard to relative expression levels of different family members, and thus, the possible role of ADAR3 in the A-to-I RNA editing mechanism described here remains to be tested in vivo. Among the three ADAR enzymes, the newest member, ADAR3 may have many different facets yet to be discovered.

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides used for reverse transcription (RT), polymerase chain reaction (PCR), primer extension

assay, and in vitro mutagenesis were synthesized at the University of Pennsylvania, Cancer Center Nucleic Acid Facility and, if necessary, purified in 20% acrylamide-7 M urea gels. All ADAR3 oligonucleotides correspond to the human sequence. The nucleotide positions indicated in parentheses are based on the ADAR3 sequence (GenBank accession number AF034837) and are relative to the initiation codon ATG of ADAR3, in which A was assigned as position +1. The FLAG epitope-tagged sequence (Hopp et al., 1988) is underlined. All restriction sites within the oligonucleotides are shown in bold. All GluR-B oligonucleotides utilized in this study correspond to the murine sequence and were described previously (Dabiri et al., 1996; Lai et al., 1997a). 5-HT_{2C}R oligonucleotides correspond to the human sequence (Xie et al., 1996).

Bam-F-HCEUP: 5′-CGC**GGATCC**TATAAACATGGCTGACTACAAGGACGACGATGACAAGGCCTCGGTCCTGGGGAGCGGCAGAGGG-3′ (+4 to +30);
F-HCEDW: 5′-GGGCGCCACCGACCACGACAGC-3′ (+357 to +378);
MutΔR: 5′-CAGTCAACTCAAATGCAAGTCCAAGTCCAAGCGGAAAGATAAAGTAAGCA-3′ (+45 to +69 and +88 to +112);
RTSR2C-in: 5′-ACCAGTCGACGTCTGTA-3′;
PCHSR2CXb: 5′-GC**TCTAGA**GATTATGTCTGGCCACTACCTAGATAT-3′;
PCHSR2C-A3: 5′-CCC**AAGCTT**CCAGTCGACGTCTGTACGTTGTTCAC-3′;
EXSR2C-A: 5′-ATATCGCTGGAYCGGTATGTAG-3′;
EXSR2C-B: 5′-AACCGGCTATGCTCAAYAGGAYYAC-3′;
EXSR2C-C: 5′-ATTGAAMCGGCTATGCTCAAYA-3′;
EXSR2C-D: 5′-GCGAATTGAAMCGGCTATGCTC-3′.

For primer extension experiments oligonucleotides were labeled with [γ-^{32}P]ATP (6,000 Ci/mmol, Amersham, Arlington Heights, Illinois) and T4 polynucleotide kinase (Pharmacia, Piscataway, New Jersey) as described (Sambrook et al., 1989).

Searching databases and isolation of overlapping ADAR3 cDNA clones

Partial sequences displaying similarity to human ADAR1 (DRADA1 or dsRAD) (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995; Hough & Bass, 1997) and ADAR2a (DRADA2a or RED1) (Melcher et al., 1996b; Gerber et al., 1997; Lai et al., 1997a) were identified by searching two databases of human cDNA ESTs (Boguski et al., 1993; Adam et al., 1995) by the BLAST program (Altschul et al., 1990), using the deaminase domain sequence of human ADAR1 (amino acids 801–1226) or human ADAR2a (amino acids 350–701). A 1.9-kb insert of one of the EST cDNA clones, HCE3, was used as a starting library screening probe. Overlapping clones were isolated by hybridization screening of randomly primed human hippocampal, amygdala, cerebellum, and thalamus cDNA libraries (Clontech, Palo Alto, California) constructed in phage λgt10 as described previously (Sambrook et al., 1989). Inserts of cDNA clones were subjected to multiple rounds of sequencing in both directions after subcloning into pBluescript KSII+ (Stratagene, La Jolla, California).

Determination and analysis of the cDNA sequence

DNA sequencing was performed on an ABI377 DNA sequencer. The overlapping sequences were aligned and combined by the Fragment Assembly program (Devereux et al., 1984). Alignments of ADAR3 with other ADAR gene family members were determined by PILEUP, BESTFIT, and GAP programs, and identification of various protein sequence motifs was done by the MOTIFS program (Devereux et al., 1984). Phylogenetic analysis of DRBMs was carried out by the PILEUP program (Devereux et al., 1984).

ADAR3 expression constructs

Three cDNA clones, HCE1, HCE2, and HCE3, containing the 5′ end, center, and 3′ end regions of ADAR3, were merged to generate a full-length plasmid in pBluescript KSII+ vector (Stratagene). A FLAG epitope-tagged sequence (Hopp et al., 1988) was introduced into its N-terminus by PCR amplifying a 350-bp 5′-end fragment of HCE1 using Bam-F-HCEUP and F-HCEDW as a pair of primers and replacing the original 350-bp *Bam*HI/*Not*I (nt +1 to +350) region of a pBluescript clone with the PCR product. The resultant clone was termed pBS-IF-ADAR3, which contains a new Kozak (Kozak, 1989) sequence that is strongly preferred by baculovirus for protein translation initiation at the new N-terminus region. The region amplified by PCR was confirmed by sequencing. The *Bam*HI/*Xba*1 insert (nt +1 to +2632) of this pBluescript clone was then shifted to pFAST-Bac1 vector containing a baculovirus polyhedrin promoter (Life Technologies, Gaithersburg, Maryland), resulting in an expression construct, pBac-F-ADAR3. This baculovirus expression construct was then transformed in DH10Bac for transposition into the bacmid and subjected to blue/white screening. Production of recombinant baculoviruses, preparation of total cell extracts from infected Sf9 cells, and purification of recombinant proteins on anti-FLAG antibody M2 affinity column were carried out as described previously (Lai et al., 1995; Dabiri et al., 1996). The yield and purity of fractionated recombinant proteins were determined by electrophoresis on a 10% SDS-PAGE followed by silver staining. The presence of recombinant proteins was further confirmed by western blotting analysis using an anti-FLAG M2 MAb (Sigma, St. Louis, Missouri) as described previously (Lai et al., 1995; Dabiri et al., 1996).

Mutagenesis of ADAR3

Using the GeneEditor™ Site-Directed Mutagenesis Kit (Promega, Madison, Wisconsin) and a mutagenic primer MutPRΔR, a mutant expression construct pBac-F-ADAR3ΔR was made. This mutant had a deletion of 18 bp corresponding to six consecutive arginine residues located near the N-terminus of ADAR3. pBac-F-ADAR3ΔM1M2 was made by deleting a region encompassing both DRBM1 and DRBM2 (+385 to +1099). Two *Xcm*I restriction sites conveniently located at 5′ and 3′ ends of the dsRNA-binding domain were

utilized for preparation of this deletion mutant construct. The mutation site was confirmed directly by sequencing.

Preparation of ADAR3 specific antibodies

The N-terminal region of ADAR3 (amino acids 2–101) was constructed in pGEX-4T-3 vector (Pharmacia) as glutathione-S-transferase (GST) fusion protein (Guan & Dixon, 1991). The insert of this construct, *Stu*I/*Not*I DNA fragment (300 bp), was excised from pBS-IF-ADAR3 described above and ligated at *Sma*I and *Not*I sites of pGEX-4T-3 in frame. The GST-ADAR3 fusion proteins were expressed in *Escherichia coli* BL21 and purified by glutathione Sepharose 4B column chromatography (Guan & Dixon, 1991). MAbs were generated against the purified GST-ADAR3 fusion proteins. MAb 3.59 was selected for specific binding to ADAR3 and confirmed not to cross-react with different members of the ADAR family or GST proteins.

5-HT_{2C}R plasmid

Plasmid pHGSR-C5 contains a 614-bp DNA fragment, encompassing a part of intron 4, exon 5, and a part of intron 5 of human 5-HT_{2C}R gene (Xie et al., 1996), which are essential for RNA editing of five sites identified previously within exon 5 (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000). The insert of this plasmid was derived from a human 5-HT_{2C}R genomic P1 clone obtained by PCR screening of a human genomic P1 library (Genome Systems, Inc., St. Louis, Missouri).

RNA synthesis

GluR-B RNAs B11 and B13 were prepared as described previously (Dabiri et al., 1996; Lai et al., 1997a). For synthesis of 5-HT_{2C}R C5 RNA, pHGSR-C5, linearized with the restriction enzyme *Eco*RI, was transcribed at 37 °C for 1 h by 20 U of T7 RNA polymerase (Promega), in 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 200 μg/mL BSA, and 10 mM DTT. To monitor the RNA synthesis and also to estimate the yield, 2 μCi of [α^{32}P]ATP (400 Ci/mmol; Amersham) were included in the reaction in addition to 500 μM each of GTP, CTP, and UTP, and 250 μM ATP. The template DNAs were eliminated by treatment with RNase-free DNase I (Boehringer Mannheim, Indianapolis, Indiana) in the presence of RNasin (Promega) as described (Dabiri et al., 1996). Single-stranded antisense c-*myc* (605 nt) and c-*myc* dsRNA (575 bp) were prepared as described previously (Kim et al., 1994a).

In vitro RNA editing assay

Editing of a synthetic GluR-B RNA B11, B13, or 5-HT_{2C} RNA C5 was assayed in vitro using purified recombinant ADAR3 proteins as well as ADAR1 and ADAR2a proteins. The standard editing reaction contained 20 fmol of a synthetic B11, B13, or C5 RNA substrate, 10 ng of recombinant ADAR proteins, 0.02 M HEPES, pH 7.0, 0.1 M NaCl, 10% glycerol, 5 mM EDTA, 1 mM DTT, and 250 U/mL of RNasin (Promega). The reactions were incubated at 30 °C for various times. For the competitive inhibition assay, substrate RNA and 10 ng (1×) or 30 ng (3×) of recombinant ADAR3 protein were preincubated for 30 min at 30 °C, and then 10 ng of ADAR1 or ADAR2a was added to the editing reaction for 30 min or 2 h at 30 °C. The RNA was recovered by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The final RNA pellet was dissolved in DEPC-treated ddH_2O and stored at −70 °C.

Dideoxyoligonucleotide/primer extension assay

Quantitation of editing efficiency at three sites of GluR-B RNA (Q/R, +60 intronic, and R/G sites) was described already (Dabiri et al., 1996; Lai et al., 1997a). To quantitate the efficiency at four major sites of 5-HT_{2C}R RNA, A, B, C, and D sites (Burns et al., 1997), the in vitro-edited RNA was first amplified by RNA template-specific PCR. Ten femtomoles of RNA were dissolved in H_2O containing a specific RT primer, RTSR2Cin, and incubated at 70 °C for 10 min. Reverse transcription was carried out in a total reaction volume of 20 μL containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 40 U of RNasin, and 50 μM of each dNTP, and incubated with 2.5 U of AMV-RT (Boehringer Mannheim) for 1 h at 42 °C, followed by heating at 95 °C for 5 min. Amplification of cDNA was carried out in 50 μL of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM of each dNTP, 2 mM $MgCl_2$, 1 U of Taq DNA polymerase (Perkins-Elmer Cetus, Foster City, California), and 150 nM each of HSR2CXb and HSR2C-A3. The cycle conditions used were 94 °C, 2 min, and 35 cycles at 94 °C, 20 s; 55 °C, 1 min; 72 °C, 30 s, and finally, 72 °C, 10 min. The RT-PCR product was extracted with phenol-chloroform and then ethanol precipitated with 2 M NH_4OAc, pH 4, to remove the residual nucleotides. Approximately 20 ng of DNA was then used for primer extension with 10 fmol of ^{32}P-labeled extension oligonucleotide EXSR2C-A (A site analysis), EXSR2C-B (B site analysis), EXSR2C-C (C site analysis), or EXSR2C-D (D site analysis). Briefly, the DNA was heated at 95 °C for 2 min, chilled on ice, and annealed with 10 fmol of the primer in 1× Sequanase® buffer at 25 °C for 30 min. Extension was carried out for 5 min at 37 °C in the presence of 15 μM each of dNTP, and 1 mM of one specific dideoxy-NTP and the Sequanase® enzyme. Dideoxy-CTP was used for analysis at B, C, and D sites, whereas dideoxy-GTP was used for A site analysis. All primer-extended DNAs were fractionated on a 15% polyacrylamide–8 M urea gel. The ratio of the edited and unedited RNAs was estimated by quantifying the radioactivity of the primer-extended product with a PhosphorImaging System (Molecular Dynamics, Sunnyvale, California).

RNA-binding assay

Filter-binding assays were carried out in duplicate by using recombinant ADAR proteins and [α-^{32}P]ATP-labeled (hot) B11, C5, single-stranded antisense c-*myc*, and double-stranded c-*myc* RNA as described previously (Kim et al., 1994a; Lai et al., 1995) except that binding reaction was done at 30 °C for 5 min instead of 37 °C for 10 min.

Northern blotting analysis

Northern blots containing 2 μg of poly(A)$^+$ RNA from various human tissues (Multiple Tissue Northern Blots) were obtained from Clontech. Northern blot hybridization was carried out in buffer containing 5× SSC, 2.5× Denhardt's solution, 50% (v/v) formamide, 7% (w/v) dextran sulfate, 0.1% SDS, 1 mM EDTA, denatured salmon sperm DNA (0.1 mg/mL), and 1×10^6 DPM/mL probe at 42 °C for overnight. The membranes were washed with 0.2× SSC/0.1% SDS at 50 °C for 30 min twice.

Western immunoblot analysis

Proteins were fractionated on an SDS–8% polyacrylamide gel and transferred to a nylon membrane. Blots were blocked in a buffer containing PBS and 3% nonfat dry milk. Monoclonal antibodies, MAb 3.59 for detection of native ADAR3 proteins and MAb M2 for recombinant FLAG epitope-tagged ADAR3 proteins, were diluted 1:5,000, and incubated for 2 h at room temperature. ADAR3-specific protein bands were detected by peroxidase-conjugated goat antibodies directed against mouse immunoglobulins (Kirkegaard & Perry Lab., Gaithersburg, Maryland) and chemiluminescence staining using Renaissance® (New Life Science Products, Inc., Boston, Massachusetts).

GenBank accession number

The accession number for the human ADAR3 sequence reported in this paper is AF034837.

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