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RED2, a Brain-specific Member of the RNA-specific Adenosine Deaminase Family*

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The mammalian RNA-specific adenosine deaminases DRADA/dsRAD (alias ADAR) and RED1 (alias ADARB1) have been implicated in the site-selective editing of brain-expressed pre-mRNAs for glutamate receptor subunits and of antigenomic RNA of hepatitis delta virus. These enzymes are expressed in many if not all tissues, predicting an as yet unappreciated significance for adenosine deamination-mediated recoding of gene transcripts in the mammalian organism. We now report the molecular cloning of cDNA for RED2 (alias ADARB2), a third member of the RNA-specific adenosine deaminase family in the rodent. RED2 is closely sequence-related to RED1 but appears to be expressed only in the brain, where expression is widespread reaching highest levels in olfactory bulb and thalamus. RED2 further differs from RED1 in having a 54-residue amino-terminal extension which includes an arginine-rich motif. Different from DRADA and RED1, recombinantly expressed RED2 did not deaminate adenosines in extended synthetic dsRNA or in GluR-B pre-mRNA. However, a chimera of RED1 and RED2 edited the GluR-B Q/R and R/G sites with moderate efficiency. Our data suggest that RED2 may edit brain-specific transcripts with distinct structural features.

RNA editing by nucleotide conversion occurs in some mammalian gene transcripts, altering the RNA's informational capacity (reviewed in Ref. 1). A cytosine to uridine deamination is observed in the transcript for intestinal apolipoprotein B (apoB[1]; reviewed in Ref. 2), and adenosine deamination occurs in pre-mRNAs for subunits of glutamate-gated receptor channels (GluR) mediating excitatory synaptic transmission in the central nervous system (reviewed in Refs. 3 and 4). Whereas apoB editing involves linear sequence recognition, GluR pre-mRNA editing requires *in vitro* (5–7) and *in vivo* (8) the formation of a double-stranded (ds) RNA configured from exonic and intronic sequences. This requirement for a dsRNA structure and the observed conversion of adenosine to inosine in particular GluR pre-mRNA codons (9–11) predicts catalysis by dsRNA-specific adenosine deaminases. Indeed, molecular cloning has revealed the existence of two dsRNA adenosine deaminases, expressed in many if not all tissues. These enzymes, termed DRADA (dsRNA adenosine deaminase; also termed dsRAD or ADAR) (12–14) and RED1 (dsRNA-specific editase 1 or ADARB1; Ref. 15), share a common domain architecture with several dsRNA binding domains (dsRBDs; Ref. 16) being located amino-terminal to a catalytic deamination domain. This latter domain is defined by conserved amino acid residues containing the putative Zn^{2+}- chelating and proton-transferring residues necessary for adenosine deamination (13, 15, 17). These are also found in other nucleoside deaminases (18), including the cytidine deaminase APOBEC1 (apoB editing catalytic component 1) for apoB editing (19). Recombinantly expressed DRADA and RED1 deaminate *in vitro* up to 50% of the adenosines in extended, synthetic dsRNA, used as an artificial substrate (15, 20–23). Furthermore, DRADA edits the R/G site as well as several intronic adenosines in GluR-B pre-mRNA but not the adenosine of the GluR-B Q/R site whereas RED1 edits the GluR-B Q/R and R/G sites (15, 23). DRADA was further shown to edit the amber/W site in antigenomic hepatitis delta virus RNA *in vitro* (24). These findings suggest that RED1 and DRADA display distinct but overlapping substrate specificities *in vivo*.

The potential significance for mammalian gene expression of selective nucleotide changes in pre-mRNAs and hence of amino acid changes in gene-encoded proteins led us to search for additional dsRNA adenosine deaminases. We identified RED2 (ADARB2), a protein with high sequence identity to RED1 but apparently different substrate specificity and expression pattern.

MATERIALS AND METHODS

Isolation of RED2 cDNA—Rat brain total RNA of postnatal day 42 was reverse-transcribed as described (5), and products of PCR reactions using the primers pCHAE, pPCG, pMSCS, and pQGAL were size-fractionated on agarose gels. Products approximately corresponding in size to that calculated for RED1 and DRADA cDNAs were directionally cloned in *Eco*RI/*Kpn*I-digested M13mp18 RF-DNA, and dual filter lifts of recombinant phage plaques were hybridized as described (9) with ^{32}P-labeled oligonucleotides red1is1 (15) and rdrada (14). Filters were washed in 0.5xSSC at 60 °C and single-stranded DNAs of not hybridizing recombinant phage plaques were sequenced (ABI Sequencer 373A). The DNA insert of one clone displaying high sequence identity to the catalytic domains of DRADA and RED1 was ^{32}P-labeled and used as probe to screen a rat hippocampal cDNA library in phage λgt10 as described (15). The entire RED2 cDNA sequence was determined from two cDNA clones.

Northern Blot and in Situ Hybridization—A rat multitissue RNA blot (Clontech) was hybridized with a ^{32}P-labeled DNA probe encoding RED2 residues 253–542, and *in situ* hybridization with oligonucleotide red2is1 was performed as described (15).

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[1] The abbreviations used are: apoB, apolipoprotein B; ADAR, adenosine deaminase, RNA-specific; DRADA and dsRAD, dsRNA adenosine deaminase; dsRBD, dsRNA binding domain; dsRNA, double-stranded RNA; GluR, L-glutamate-activated receptor channel; HIV, human immunodeficiency virus; RED1, dsRNA-specific editase 1; PCR, polymerase chain reaction; RF-DNA, replicative form DNA; RT, reverse transcription.

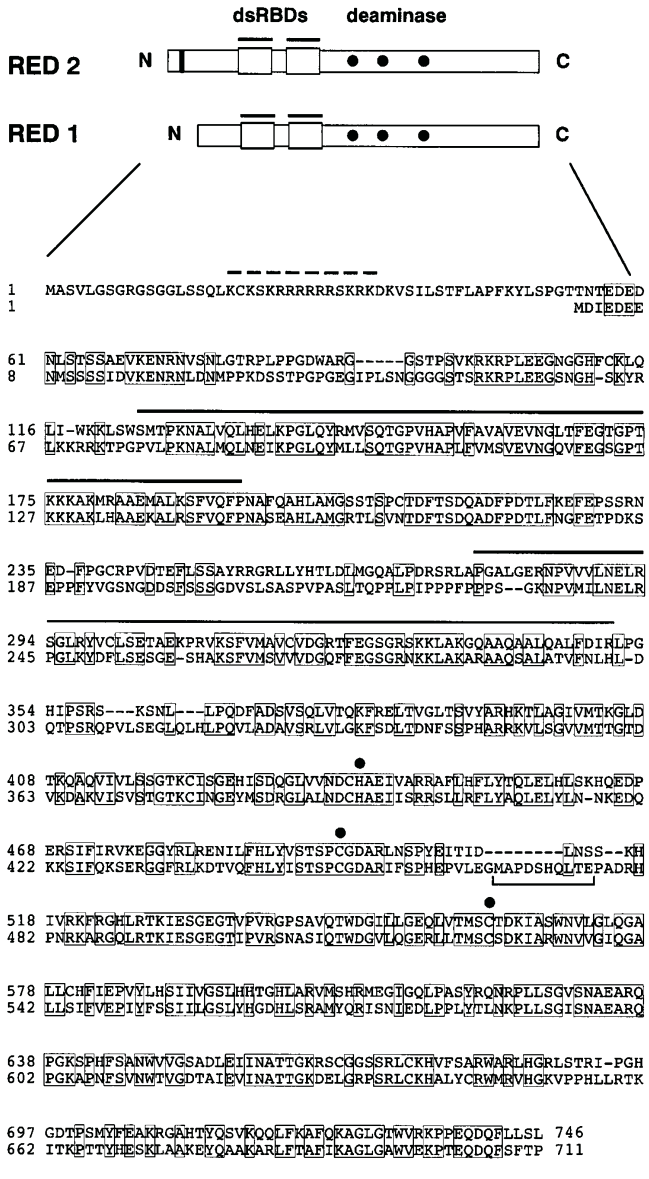

FIG. 1. **The amino acid sequence of RED2 as predicted from cloned cDNA.** Domain maps on top show the location of an arginine-rich motif in RED2 (*vertical bar*), the dsRBDs (*boxed* and *overlined*; Ref. 16), and the deaminase domain with its putative Zn^{2+} chelating residues (*filled circles*; Refs. 13, 15, and 17) in RED2 and RED1. In the numbered sequence alignment, *gaps* are introduced for optimal alignment, identical residues are *boxed*, the dsRBDs are *overlined*, and the arginine-rich RNA binding motif in RED2 is *highlighted by a broken line*. The *bracket* spans the 10 residues (amino acids 467–476) in the deaminase domain of RED1 which are missing from a RED1 splice variant. The GenBank™ accession number for RED2 cDNA is U74586.

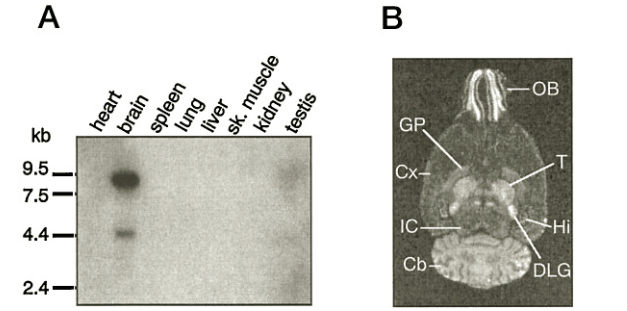

FIG. 2. **Expression of RED2 mRNA.** *A*, Northern blot analysis of RNAs from different rat tissues, as indicated on *top* (*sk*, skeletal). Size markers in kilobases are shown on the *left*. Rehybridization of the blot with a β-actin probe indicated that RNA amounts in lanes differed by no more than 2-fold (not shown). *B*, *in situ* hybridization in a horizontal brain section of a 3-week-old rat. *Cb*, cerebellum; *Cx*, cortex; *DLG*, dorsolateral geniculate nucleus; *GP*, globus pallidus; *Hi*, hippocampus; *IC*, inferior colliculus; *OB*, olfactory bulb; *T*, thalamus.

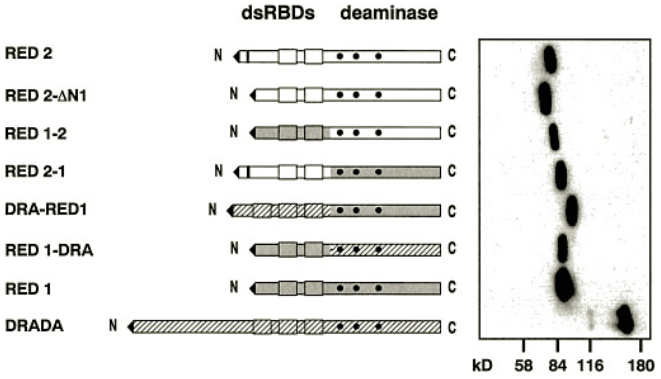

FIG. 3. **Domain maps and recombinant expression of wild type and chimeric dsRNA adenosine deaminases.** Domain maps for the different proteins are depicted with the dsRBDs *boxed* and the putative Zn^{2+} chelating residues in the deaminase domain indicated by *filled circles*. The *vertical bar* in the amino-terminal region of RED2 and RED2-1 denotes the putative arginine-rich RNA binding motif. *Open*, *stippled*, and *hatched areas*, respectively, denote RED2, RED1, and DRADA sequences. The DRA-RED1 chimera contains the amino-terminal region of DRADA mutant ΔN5 (23). The vector (26) for recombinant expression in HEK 293 cells encoded the proteins amino-terminally tagged by the FLAG epitope (*filled triangles*). Protein expression in transfected HEK 293 cells is documented by Western blot analysis using an anti-FLAG antibody. Size markers are indicated on the *bottom*.

DNA Constructs—Cloned full-length cDNA for RED2 engineered to encode an amino-terminal FLAG epitope (25) was inserted into a mammalian expression vector (26). Deletion mutant RED2-ΔN1 (deletion of RED2 residues 2–53) was constructed by PCR-mediated mutagenesis. Chimeras (RED1–2, RED1 residues 1–306 fused to RED2 residues 358–746; RED2–1, RED2 residues 1–357 fused to RED1 residues 307–711; DRA-RED1, DRADA residues 393–769 to RED1 residues 307- 711; RED1-DRA, RED1 residues 1–306 to DRADA residues 770-1175) were constructed by exchanging restriction enzyme fragments after PCR-supported introduction in the cDNAs of a *Xba*I recognition site in the codons for the SR dipeptide located between the dsRBDs and the deaminase domain of the three deaminases. The DRA-RED1 chimera contains the amino-terminally truncated region of DRADA mutant ΔN5 which displayed the same activity profile as full-length DRADA (23). Expression vectors for DRADA and RED1 are described (15).

Cell Transfections and Analysis—GluR-B minigenes B13 (5) and pBgl (6) were cotransfected with deaminase constructs in HEK 293 cells, and Western blots, preparation of nuclear extracts, and dsRNA conversion assay were as described (23).

Analysis of GluR-B Pre-mRNA Editing—RT-PCR amplification of GluR-B sequences from minigene-transfected HEK 293 cells was performed (5, 6) with the primer sets rsp23/MH50 (GluR-B Q/R site and hotspot1) and rsp23/Bint1 (GluR-B R/G site). Approximately 80 ng of the gel-purified PCR fragments were cycle-sequenced (ABI Dye Terminator *Taq*-FS Kit) with 10 pmol of the nested primers MH36 (GluR-B Q/R site and hotspot1) or Bint2 (GluR-B R/G site). Products were resolved by 6% polyacrylamide-urea gels (ABI Sequencer 373A), and data were collected and analyzed by ABI software Data Collection 1.2.0 and Analysis 1.2.0. Peak heights of the adenosine and guanosine channel were used to calculate the editing efficiency of the respective positions. PCR amplifications using as templates B13 (5) and B13-R (carrying a CGG codon at the Q/R site) plasmids mixed in known proportions revealed a close correspondence of calculated and experimental editing efficiency in the range of 10–90% editing. Sequencing of PCR fragments for which the degree of editing at Q/R, hotspot1, and R/G positions in GluR-B pre-mRNA had been determined by primer extension in previous studies (15, 23) documented the reliability of the sequencing-based assay in the range of 10–90% editing (see Table I).

Oligonucleotides—The oligonucleotides used in this study were: pCHAE, 5′-GCGAATTCAATGACTG(TC)CATGC(AT)GA(AG)-3′; pPCG, 5′-GCGGTACCACA(CTG)GG(ATG)G(CA)(CAG)GTGCTGAT(GA)TA-3′; pMSCS, 5′-GCGGTACCA(TG)(TC)TT(AG)(GC)(AT)(AG)TC(AG)CA-

TABLE I
Activity profiles of wild type and chimeric dsRNA adenosine deaminases

The activity of the different deaminases in converting adenosines in extended, synthetic dsRNA as measured in nuclear extracts of transfected HEK 293 cells is given in units per μg of total extract protein (unit definition in Ref. 15). Editing of the Q/R, hotspot1, and R/G adenosines in GluR-B pre-mRNA was assayed by direct fluorescent sequencing of RT-PCR products from cotransfected cells. For comparison, data for RED1, DRADA and mock transfections from previous studies obtained by a primer extension assay are also listed. All values are given as mean ± S.D. with the number of independent cell transfections in parentheses; ND, not determined. Editing values between 0% and 10% and between 90% and 100% were not resolved reliably by the sequencing assay and are listed as <10% or >90%.

Construct	dsRNA deamination	Editing		
		Q/R site	hotspot1	R/G site
	units/μg		%	
RED2	<0.01 (4)	<10 (8)	<10 (8)	<10 (5)
RED2-ΔN1	<0.01 (4)	<10 (4)	<10 (4)	<10 (4)
RED1–2	<0.01 (4)	<10 (6)	<10 (6)	<10 (6)
RED2–1	<0.01 (4)	16 ± 4 (6)	18 ± 4 (6)	17 ± 7 (4)
DRA-RED1	0.05 ± 0.02 (4)	<10 (4)	21 ± 3 (4)	29 ± 4 (4)
RED1-DRA	0.04 ± 0.01 (4)	<10 (4)	70 ± 2 (4)	69 ± 3 (4)
RED1	ND	>90 (5)	37 ± 5 (5)	89 (2)
	2.84 ± 0.49 (13)[a]	89 ± 1 (8)[a]	41 ± 4 (8)[a]	85 ± 4 (8)[a]
DRADA	ND	<10 (2)	75 (2)	ND
	0.93 ± 0.62 (17)[a]	9.5 ± 1.2 (16)[b]	59.7 ± 7.5 (15)[b]	67.5 ± 9.5 (23)[b]
Mock	ND	<10 (3)	<10 (3)	<10 (2)
	<0.01 (11)[a]	4.0 ± 0.4 (7)[b]	8.9 ± 0.5 (9)[b]	6.8 ± 1.6 (22)[b]

[a] Ref. 15.
[b] Ref. 23.

(AG)(GC)(AT)CAT-3′; pQGAL, 5′-GCGGTACCA(AG)GGC(AT)CC(TC)-TGCA(GCA)(AT)CC(TCA)A(GCA)(CTA)AC-3′; red2is1, 5′-GGGGTCC-TCTTGATGCTTGCTCAAGTGCAGCTCCAGCTG-3′; rsp23 (5); Bint1 (9); MH50, Bint2 (15); MH36 (23).

RESULTS AND DISCUSSION

In searching for other dsRNA adenosine deaminases, we designed degenerate oligonucleotides to conserved amino acid residues in the deaminase domains of DRADA (dsRAD, ADAR) (Refs. 13 and 14) and RED1 (ADARB1) (Ref. 15) and amplified rat brain RNA-derived RT-PCR products of the appropriate lengths. Sequence analysis of the cloned DNA products revealed the existence of a transcript in rat brain with high sequence similarity to both DRADA and RED1 (not shown). Full-length cDNA for this putative dsRNA adenosine deaminase isolated from a rat hippocampal cDNA library encoded a protein of 746 amino acids with a calculated molecular mass of 82 kDa which we termed RED2, short for dsRNA-specific editase 2 (alias ADARB2). The open reading frame was defined from two independent clones by the presence of three in-frame stop codons 5′ to a methionine codon with a consensus sequence for translational initiation (27) and a single stop codon. RED2 shares its domain structure with RED1 to which it is 50% sequence identical (Fig. 1). Both proteins contain two dsRBDs followed by the deaminase moiety. In this latter domain, the sequence alignment revealed nonlinearity around RED1 positions 465–475. RT-PCR analysis (not shown) documented that in this region RED1, but not RED2, can exist in two splice forms which differ by 10 amino acid residues (Fig. 1) and occur at comparable levels in the rat brain. RED2 further differs from RED1 in carrying a 54-residue amino-terminal extension. This includes an arginine-rich sequence found in certain nucleic acid-binding proteins, such as protamins (nuclear DNA-binding proteins of spermatids; Ref. 28), and also in some viral and phage RNA binding proteins of which HIV Tat and Rev constitute well-known examples (29). RED2 appears to be the first protein to carry dsRBDs and an arginine-rich motif. This motif might mediate recognition of particular stem-loop structures in RNA substrates (29) or could be involved in DNA binding (28). DNA binding is unexpected for a putative dsRNA adenosine deaminase but has been observed for DRADA (30). Furthermore, the transcription factor NF90, which binds to the IL2 gene enhancer, contains two dsRBDs with close sequence identity to the dsRBDs of RED1 and RED2 (31).

We determined the expression profile of the RED2 gene by Northern blot analysis and oligonucleotide-mediated *in situ* hybridization of rat brain sections. In the tissues tested, RED2 mRNA was expressed exclusively in the brain with a dominant transcript of 8.5 kilobases and a minor 4.4-kilobase transcript (Fig. 2*A*). The brain-restricted expression sets RED2 apart from DRADA and RED1 which are also expressed in many non-neuronal tissues (14, 15). *In situ* hybridization in rat brain revealed differential expression of RED2 mRNA with high transcript levels in the olfactory bulb, thalamus, dorsolateral geniculate nucleus, dentate gyrus, globus pallidus, and cerebellum and lower levels in neocortical areas (Fig. 2*B*). This pattern is similar to that of RED1 mRNA (15) but differs in globus pallidus and thalamic expression. Furthermore, RED1 mRNA levels in brain exceed those of RED2, as independently assessed by RT-PCR using common primers (not shown). *In situ* hybridization in prenatal rat brain (embryonic day 17) revealed RED2 expression during brain development (not shown).

To study the functional properties of RED2 and compare them to those of DRADA and RED1 we expressed each protein transiently in HEK 293 cells, monitoring expression of the FLAG epitope-tagged (25) proteins by Western blot (Fig. 3). Nuclear extracts from transfected cells were assayed for dsRNA adenosine deaminase activity, using as a substrate extended synthetic dsRNA. No activity was observed for RED2 whereas DRADA and RED1 catalyzed adenosine to inosine conversion as described previously (15, 23) (Table I). Site-selective RNA editing was determined by co-expressing in HEK 293 cells each enzyme with editing-competent minigenes for the Q/R and R/G sites in GluR-B pre-mRNA (5, 6) and analyzing the extent of adenosine conversion at these sites by direct DNA sequencing of the RT-PCR products (see "Materials and Methods"). Whereas DRADA and RED1 acted on these substrates as reported (15, 23), RED2 showed no detectable activity (Table I). Neither did a 54-residue amino-terminal deletion mutant (RED2-ΔN1), suggesting that this sequence which includes the arginine-rich motif is not responsible for the observed lack of RED2 activity. We then constructed reciprocal chimeras between RED1 and RED2 by fusing the cDNA sequences encoding the dsRBDs of one protein with those encoding the deaminase domain of the other (Fig. 3). Justification for the particular crossover point was derived from functional chi-

meras between the more distantly related DRADA and RED1 sequences. When crossed at the homologous position, the resultant DRA-RED1 and RED1-DRA proteins displayed clearly detectable adenosine to inosine conversion in extended, synthetic dsRNA and edited in GluR-B pre-mRNA the R/G site as well as the intronic hotspot1 but not the Q/R site (Table I). The homologous RED1-2 and RED2-1 chimeras lacked deaminase activity on synthetic extended dsRNA. With GluR-B pre-mRNA as a substrate, RED1-2 was similarly inactive but the RED2-1 chimera converted the adenosines of the Q/R site, the R/G site, and hotspot1 significantly above background levels defined by mock transfection (Table I).

These results indicate that the dsRBDs of RED2 can cooperate with the deaminase domain of RED1. We cannot explain absence of all activity for the reciprocal chimera in which the deaminase domain is that of RED2. Lack of the longer splice form for the RED2 deaminase domain (see above and Fig. 1) is not a possible explanation, as the two splice forms of RED1 were found to display indistinguishable activity profiles (not shown). We further conclude that measurable activity on synthetic extended dsRNA may not be a hallmark of all dsRNA adenosine deaminases. This conclusion seems to have been foreshadowed by the amino-terminally severely truncated DRADA mutant ΔN7 which still edited efficiently the R/G site in GluR-B pre-mRNA but had very low activity on extended dsRNA (23). We speculate that RED2, by sequence a member of the RNA-specific adenosine deaminase family, edits as yet unknown brain-expressed transcripts.

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