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Structural Requirements for RNA Editing in Glutamate Receptor Pre-mRNAs by Recombinant Double-stranded RNA Adenosine Deaminase*

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Pre-mRNAs for brain-expressed ionotropic glutamate receptor subunits undergo RNA editing by site-specific adenosine deamination, which alters codons for molecular determinants of channel function. This nuclear process requires double-stranded RNA structures formed by exonic and intronic sequences in the pre-mRNA and is likely to be catalyzed by an adenosine deaminase that recognizes these structures as a substrate. DRADA, a double-stranded RNA adenosine deaminase, is a candidate enzyme for L-glutamate-activated receptor channel (GluR) pre-mRNA editing. We show here that DRADA indeed edits GluR pre-mRNAs, but that it displays selectivity for certain editing sites. Recombinantly expressed DRADA, both in its full-length form and in an N-terminally truncated version, edited the Q/R site in GluR6 pre-mRNA and the R/G site but not the Q/R site of GluR-B pre-mRNA. This substrate selectivity correlated with the base pairing status and sequence environment of the editing-targeted adenosines. The Q/R site of GluR-B pre-mRNA was edited by an activity partially purified from HeLa cells and thus differently structured editing sites in GluR pre-mRNAs appear to be substrates for different enzymatic activities.

The alteration of codons by RNA editing, leading to changes in protein structure and function, represents a newly recognized type of posttranscriptional modification in mammalian nuclear transcripts and occurs by site-specific base modification (1, 2). In the transcript for intestinal apolipoprotein B (apoB)[1], a translational stop codon is generated by cytidine deamination, generating the expression of a truncated protein with altered function (1). By contrast, specific adenosines are deaminated (2, 3) in pre-mRNAs for subunits of glutamate-gated receptor channels (GluR) (4). At the Q/R site (5) of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluR-B and the high-affinity kainate receptor subunits GluR5 and GluR6, a glutamine codon CAG is converted to the arginine codon CIG. At the R/G site of the AMPA receptor subunits GluR-B, -C, and -D (6) an arginine codon AGA is switched to the glycine codon IGA, and at the I/V and Y/C sites in GluR6, an ATT codon is changed to ITT and a TAC to TIC, respectively (7). Each of the amino acid changes generated by RNA editing alters functional properties of the glutamate-activated channel (3, 4).

Different from apoB RNA editing (1), the site-specific adenosine deamination in GluR transcripts requires a double-stranded (ds)RNA structure formed by the exonic sequence around the editing site and an intronic editing site complementary sequence (ECS) (3, 8), predicting that this type of RNA editing is catalyzed by an adenosine deaminase that operates on dsRNA. In addition to exonic adenosines, some intronic adenosines are also converted, including hotspot1 in GluR-B intron 11 (8) and in GluR6 pre-mRNA (9). The site-selective adenosine to inosine conversion in GluR-B pre-mRNA could be demonstrated *in vitro* (10–12). dsRAD (2, 13), also termed DRADA (14), is a dsRNA-specific adenosine deaminase that is widely expressed, both with respect to species and tissue. This enzyme lacks site-selective activity on extended dsRNAs but displays a sequence-dependent modification of specific adenosines in short synthetic dsRNAs (15). Although cloned human (16) and rat (17) cDNAs for DRADA have been isolated, the physiological substrates for this enzyme have yet to be identified. DRADA is currently viewed as a candidate enzyme for GluR pre-mRNA editing (2, 14), but this view lacks experimental support.

We now demonstrate that DRADA is indeed capable of editing specific adenosines in GluR pre-mRNAs *in vitro*. Indicating substrate selectivity for certain editing sites, the recombinantly expressed deaminase edited in synthetic pre-mRNAs the R/G site of GluR-B and the Q/R site of GluR6, but not the Q/R site of GluR-B. This latter site appears to be the substrate for a different activity, as indicated by fractionation of nuclear extract from HeLa cells. A comparison of the dsRNA structures for the different sites suggests that the structural environment of the to-be-edited adenosine may be one determinant for the substrate selectivity by different editing activities.

MATERIALS AND METHODS

GluR Constructs—GluR-B minigenes were B13 (GluR-B(Q/R) wt and hot spot1), ER3res2 (GluR-B(Q/R) stop) (8), B13 A-C (substitution by C of the T in position 319 of B13), (GluR-B(Q/R) A-C), pBgl (GluR-B(R/G)

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[1] The abbreviations used are: apoB, apolipoprotein B; GluR, L-glutamate-activated receptor channel; dsRNA, double-stranded RNA; ECS, editing site complementary sequence; DRADA and dsRAD, dsRNA specific adenosine deaminase; RT, reverse transcription; PCR, polymerase chain reaction; dsRBD, dsRNA binding domain; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; wt, wild type.

wt), mutant E1 (transferred to minigene pBgl) (GluR-B(R/G) A-U). GluR-B R/G site minigene pBgl was referred to as *Bgl*II-*Bgl*II in Lomeli *et al.* (6). GluR6 constructs GluR6 wt, M10, and M11 were as described (9).

Cell Transfections—For transfection of HEK 293 cells, minigene plasmids (2 μg each) were transfected (18) onto a half-confluent 14-cm culture dish of HEK 293 cells (ATCC CRL 1573) in the presence or absence of DRADA vectors (10 μg, see below). Sets of three GluR minigenes were transfected into 293 cells to analyze simultaneously editing at different sites.

In Vitro Editing of GluR Pre-mRNAs—RNAs were synthesized *in vitro* with SP6 RNA polymerase from linearized GluR minigenes, and *in vitro* editing assays were performed as described (10). For standard assays, a mixture of *in vitro* transcribed RNAs (5–10 fmol each) derived from wild type GluR minigenes (B13 for GluR-B Q/R site editing (8), pBgl for GluR-B R/G site editing (6), and 3ΔH1 (9) for GluR6 Q/R site editing) was incubated with purified recombinant DRADA. After incubation for 3 h at 30 °C, the reaction mixtures were treated with proteinase K and processed for RT-PCR (10).

RT-PCR Amplification for GluR Sequences—RT-PCR amplification of GluR-B sequences from minigene-transfected 293 cells was performed as described (6, 8). RNAs incubated with DRADA *in vitro* were resuspended in 20 μl of 3 μM RT primer mixture composed of primers KMH3 specific for the Q/R site in GluR-B, BFFK3 for the R/G site in GluR-B, and O3K3 for the Q/R site in GluR6 (1 μM each) and reverse-transcribed into cDNA (8). The RT primers contained at their 5′ end a 20 nt sequence with a *Kpn*I site, identical to the primer PCRK3. A two-step amplification procedure was applied with *Taq* polymerase under standard conditions. After a first amplification with primers cis55 and PCRK3, the second multiplex PCR was performed with primers cis55 and the nested primers MH50 and Bint2 and O3gPCRK2. The gel-purified PCR fragments were subjected to primer extension analysis (10). For the analysis of GluR-B hotspot1 (position 60 relative to the adenosine of the Q/R site, Ref. 8) editing during rat brain development, total RNA isolated from embryonic day 14 (E14) embryos and from the brains of postnatal day 0 (P0), P7, P14, P21, and P42 rats was reverse-transcribed as described (8), and the hotspot1 containing sequence was PCR-amplified with primers rspex10a and MH36. The gel-purified PCR fragments were analyzed by primer extension.

Primer Extension Analysis—The extent of editing at the different sites was determined by primer extension (10) with primers 6RT26 for the Q/R site in GluR6, B-RT for the Q/R site in GluR-B, and B-RTFF45 for the R/G site in GluR-B and PEBhot1 for hotspot1 in GluR-B intron 11.

DRADA: DNA Constructs, Characterization, and Purification—Cloned full-length cDNA for rat DRADA (17) engineered to encode a N-terminal FLAG epitope and six histidine residues at the C terminus was inserted into a mammalian expression vector (19). A DRADA mutant (SQAD) inactivated in the deaminase domain was constructed by substituting amino acid residues CHAE (amino acid positions 855–858) for SQAD and PCG (positions 911–913) for QSA. Deletion mutants of DRADA were constructed by PCR-supported mutagenesis: ΔN1, deletion of positions 2–158; ΔN5, deletion of positions 2–392; ΔN4, deletion of positions 2–480; ΔN6, deletion of positions 2–604; ΔN7, deletion of positions 2–663; ΔC1, deletion of positions 999-1175. Nuclear extract from 2–5 $\times$ 10^6 HEK 293 cells transfected with DRADA constructs was prepared as described (20), except that buffer A contained 0.7 μg/ml pepstatin A and 0.4 μg/ml leupeptin. Extracts were dialyzed against buffer D (21) and stored at −70 °C. The protein concentration of the extracts was determined by the BCA protein assay (Pierce) and ranged from 4 to 10 mg/ml. The correct size of the 293 cell-expressed DRADA forms was demonstrated by Western blotting (see below), and the enzyme activity was determined by a dsRNA conversion assay (22). One DRADA unit is defined as the amount required to produce 100 fmol inosine from 4 ng of dsRNA in 1 h at 30 °C. For purification of recombinant DRADA, whole cell extract was prepared from 5–10 $\times$ 10^7 HEK 293 cells transfected with DRADA vectors. Harvested cells washed in phosphate-buffered saline were homogenized (Ultraturrax, 60 s) in 4 ml of binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin A, and 0.4 μg/ml leupeptin). After centrifugation at 100,000 $\times$ *g* for 1 h, the supernatant was loaded onto a Ni^{2+}-NTA column (0.4 ml pack volume, QIAGEN). The column was washed with 5 ml of binding buffer and 3 ml of washing buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 0.2 M imidazole), and Ni^{2+}-NTA resin was transferred to a microcentrifuge spin column (InVitrogen). Residual liquid in the resin was removed by a quick spin. Protein was eluted from the resin by incubation with 200 μl of elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 0.5 M imidazole) at 4 °C for 20 min, and the eluate was collected (2,000 $\times$ *g*, 1 min). Elution was repeated four times, and the eluates were dialyzed extensively against buffer D. Fractions containing recombinant DRADA were combined and stored in aliquots at −70 °C. The size of the purified protein was analyzed by Western blotting with 2 μg/ml of the anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.). Signals were detected with the ECL system (Amersham Corp.).

Fractionation and Analysis of a GluR-B Q/R Site Editing Activity from HeLa Cells—An editing activity for the GluR-B Q/R site was partially purified in buffer A (50 mM Tris-HCl, pH 7.9, 10 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, and 0.4 μg/ml leupeptin), at variable KCl concentration, as indicated below. HeLa cell nuclear extract (21) was fractionated by chromatography over three columns. Nuclear extract (5.7 g) was first applied to a 1-liter Macro-Prep High Q column (Bio-Rad) and eluted with a 5 column volume salt gradient from 50 to 500 mM KCl. The Q/R editing activity was pooled (350 mg) and applied directly to a 100-ml Affi-Gel blue column (Bio-Rad), which was developed with a 5 column volume salt gradient from 300 to 1,000 mM KCl. The Q/R site editing activity (4 ml) was concentrated 4-fold with a Centricon 30 microconcentrator (Amicon) and applied to a HiLoad 16/60 Superdex 200 gel filtration column (Pharmacia Biotech Inc.). DRADA activity was assayed on extended dsRNA (22). GluR-B pre-mRNA editing assays were for 2 h as described (10). For the immunoblot, an aliquot (150 μl) of each fraction (1 ml) was precipitated with trichloroacetic acid (15%) and precipitates were washed twice with cold acetone and dissolved in SDS loading buffer. Protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Anti-DRADA serum (1:4,000) directed against dsRBD sequences of the bovine form (22) was used for DRADA detection (17).

Oligonucleotides Used in This Study—Vector primers were rsp23 (8) and cis55 (10). GluR-B oligonucleotides were rspex10a, 5′-GCGGATCCGGAATGAGCGTTACGAGGGCTAC-3′, sense, GluR-B gene exon 10; KMH3, BFFK3, B-RT, B-RTFF45 (10); MH36, 5′-TCACCAGGGAAACACATGATCAAC-3′, antisense on minigene B13; MH50, 5′-GACCCTGTAGGAAAAATCTAACC-3′, antisense on minigene B13; Bint2, 5′-ATCTCTAGACAAACCGTTAAGAGTC-3′, antisense on minigene pBgl; PEBhot1, 5′-ATGAATATCCACTTGAG-3′, antisense on minigene B13; O3K3, 5′-GACACGGTACCACACAACGGCTCCAGACTCTTGTCTACCAC-3′, antisense positions 2241–2217 of GluR6 minigene (9); 6RT26, 5′-AGGCTGAATCGTATACCTTG-3′ antisense, positions 22–3 of GluR6 minigene (9) and O3gPCRK2 (9). Common primer for PCR: PCRK3 (Ref. 10).

RESULTS

Recombinant DRADA Edits the GluR-B R/G and GluR6 Q/R Sites, but Not the Q/R Site of GluR-B—To determine if DRADA can edit GluR pre-mRNAs, we expressed the enzyme recombinantly in HEK 293 cells and purified it by a one-step procedure. We expressed two DRADA versions, one (wt) corresponding to the full-length rodent enzyme (17), and one (ΔN5) corresponding to an N-terminally shortened version (Fig. 1, see also Fig. 2). This shorter version, which in 293 cells was expressed 5–10-fold higher levels than the wt form (see Fig. 2), was similar in sequence extent to a human enzyme fragment of 88-kDa purified from HeLa cells (16) and was tested to exclude an effect on editing by the different N-terminal sequences of rat and human DRADA (16, 17). The recombinant enzyme preparations (Fig. 1) were incubated with a set of three *in vitro* transcribed pre-mRNAs, one for the GluR-B Q/R site and also containing intron 11 hotspot1 (Ref. 8), one for the R/G site in GluR-B (6), and one for the Q/R site in GluR6 (Ref. 9). As determined by primer extension on RT-PCR products, DRADA edited efficiently the adenosines of the R/G site and of hotspot1 in the GluR-B pre-mRNAs and also, to a lesser extent, the Q/R site in GluR6 pre-mRNA. The same extent of editing was obtained when incubating the pre-mRNAs individually with DRADA (not shown). Both DRADA forms edited these sites with comparable dose-dependent activities, but neither version edited the Q/R site of GluR-B (Fig. 1), even though the adenosine corresponding to hotspot1, converted in brain to >50% (8, 11), was edited to a high level, indicating proper folding of the RNA. These results suggest that *in vivo*, different GluR editing

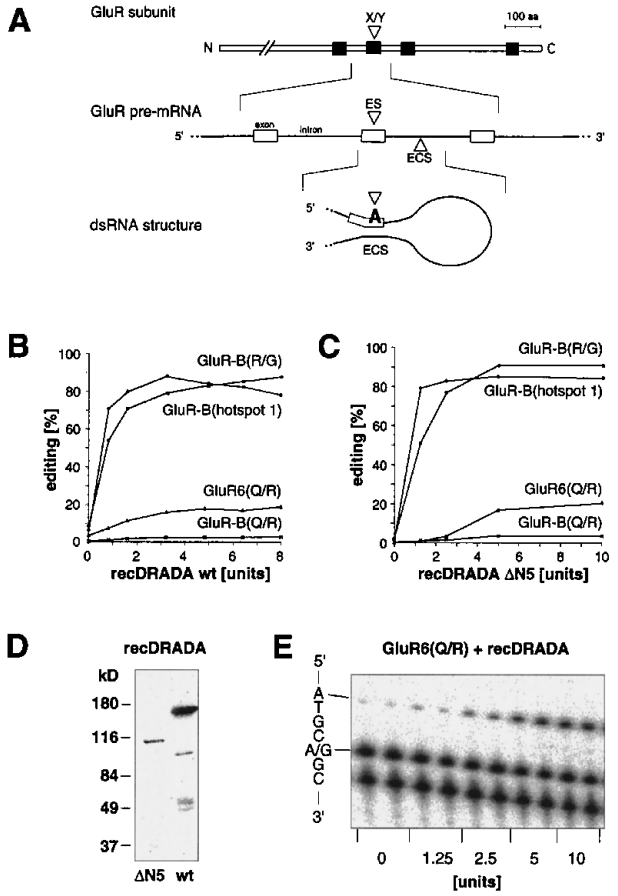

FIG. 1. **Recombinant DRADA edits selectively some sites in GluR pre-mRNAs.** *A*, schematic representation of a GluR subunit (4), its pre-mRNA, and the dsRNA structure of exonic and intronic sequences (6, 8, 9) as a substrate for site-selective adenosine deamination. A GluR subunit is depicted from N to C terminus with the *four black boxes* denoting segments for membrane insertion (4). *X*/*Y* indicates alternative amino acid residues, one (*X*) gene-encoded and the other (*Y*) introduced by site-selective RNA editing. Shown below is the pre-mRNA segment around the region containing the exonic editing site (*ES*) and the intronic ECS element essential for site selective RNA editing. Exonic and intronic RNA sequences form a dsRNA structure as schematically indicated, with the adenosine targeted for deamination by a dsRNA-dependent adenosine deaminase shown in bold. *B* and *C*, dependence of editing at four sites in GluR pre-mRNAs on the amount of recombinant DRADA in its full-length (wt) form (*B*) and an N-terminally truncated 88 kDa form (*C*) (*ΔN5*, see Fig. 2). The amount of enzyme is indicated in units determined by adenosine conversion on extended dsRNA (see "Materials and Methods"). *D*, Western blot analysis with anti-FLAG antibody of purified recombinant DRADA, full-length (wt) and N-terminally truncated (*ΔN5*). *E*, primer extension analysis of RT-PCR products from *in vitro* edited GluR6(Q/R) pre-mRNA. The exonic sequence around the editing site (nucleotides A/G) is shown on the *left*. The correspondence to adenosines of gel bands containing primer extension products is indicated. *Numbers* on *abscissa* are enzyme units.

sites may be substrates for related deaminases which differ in substrate specificity.

We determined by DNA sequencing of cloned RT-PCR products from the *in vitro* editing reactions with both DRADA forms whether the adenosine deamination catalyzed by the recombinant enzymes was site-selective or promiscuous. As a result (not shown), adenosine conversion in the stem-loop RNA structure for the R/G site (6) was limited to the correct position, indicating positional fidelity by DRADA. In GluR6 pre-mRNA, the Q/R site adenosine and additional intronic positions, which are also edited *in vivo*, were found to be modified (9). The analysis of products derived from pre-mRNA for the GluR-B Q/R site revealed that both DRADA versions had converted several adenosines other than that of the Q/R site, including positions −3, +3, +4, and 60 (hotspot1), but not the adenosine in the Q/R site itself (position 0). Collectively, our results indicate that recombinant DRADA can catalyze site-selective adenosine deamination in GluR pre-mRNAs and that at some sites this selectivity resembles that seen *in vivo*.

N-terminally Truncated DRADA Versions Retain Substrate Selectivity—To explore further the possibility that truncated DRADA forms as purified from various sources (22–24) might exhibit different substrate specificities, we constructed a set of N- and C-terminal deletion mutants of DRADA (Fig. 2), which were co-transfected into 293 cells with minigenes directing the expression of GluR-B pre-mRNAs containing the Q/R and R/G editing sites. HEK 293 cells were chosen because these cells edit to only low levels sites in transcripts derived from transfected GluR minigenes. This is in contrast to most other cell lines tested and appears to correlate with low DRADA expression in 293 cells (not shown). The expression of the DRADA mutants was documented by Western blot, RT-PCR products were analyzed by primer extension, and DRADA activity was monitored in nuclear extracts from the transfected cells with extended dsRNA as a substrate (Fig. 2). As a result, none of the N-terminally shorter DRADA forms edited the GluR-B Q/R site (<4%; not shown) but all edited efficiently the R/G site. Editing was catalyzed by DRADA, since adenosine conversion remained at cellular background levels (<5%) when co-transfecting a vector for a DRADA mutant (SQAD) incapacitated in the deaminase domain (Fig. 2). C-terminal DRADA deletions lacked activity on extended dsRNA and on GluR-B R/G pre-mRNA, indicating that this domain is critical for adenosine deamination. These results complement and extend a recent study on a different set of deletion mutants of DRADA (25). We observed that progressive N-terminal deletions sustained GluR-B R/G site editing better than adenosine conversion in extended dsRNA (Fig. 2). A severely truncated version of DRADA with only one remaining dsRBD edited the R/G site still efficiently but exhibited on extended dsRNA <10% of the activity of full-length DRADA (Fig. 2). Thus, this DRADA mutant still binds to dsRNA (26, 27), but appears to be restricted in its activity on extended dsRNA, perhaps catalyzing the deamination of only those adenosines located in a favorable sequence context (15).

DRADA-mediated Adenosine Conversion Correlates with Base Pairing Status—A comparison of exon-intron dsRNA structures required for site-selective editing in GluR pre-mRNAs (Fig. 3) documents that local sequence environment and base pairing status of the to-be-edited adenosines differ between the structures. The adenosine of the GluR-B Q/R site is base-paired (8), but the adenosine of the R/G site is mismatched (6), and the adenosine of the Q/R site of GluR6 is positioned in a loop (9). To determine if the extent of DRADA-mediated adenosine conversion in the different dsRNA structures might correlate with the base pairing status of the critical adenosine, we mutated each of the three wild type dsRNA structures (Q/R sites in GluR-B and GluR6; R/G site in GluR-B) in their intronic ECS element to either base pair or mismatch the critical adenosine (Fig. 3). Use of both the cellular and *in vitro* assays (Fig. 3) indicated that pre-mRNAs with base-paired adenosines for the editing sites were edited by DRADA to lower levels than the pre-mRNAs having mismatched adenosines (GluR6(Q/R) M10 *versus* GluR6(Q/R) M11 and wt; GluR-B(Q/R) wt *versus* GluR-B(Q/R) A-C). Thus, whereas the wild type configuration of the GluR-B Q/R site was not edited, the A-C mismatch mutant was an excellent substrate for DRADA. Base pairing the adenosine of the R/G site in GluR-B pre-mRNA resulted in only slightly lower editing levels than

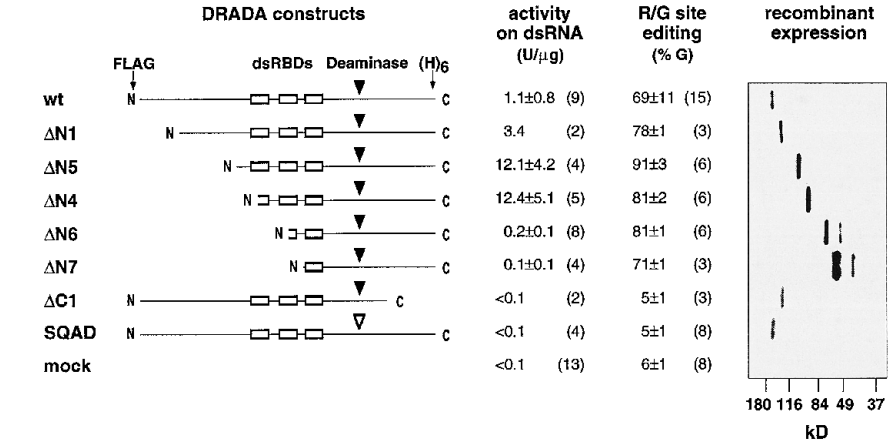

FIG. 2. **Expression and activity of truncated DRADA forms.** A series of N- and C-terminally truncated DRADA versions is depicted on the *left*. The domain map of DRADA shows the three dsRBDs (*boxed*), the deaminase domain (*arrowhead*), the N- and C-terminal tags (*arrows*). The activity (mean ± S.D.) of the different DRADA forms on extended dsRNA (measured in nuclear extracts of transfected 293 cells) and for converting the adenosine of the GluR-B(R/G) site as assayed by primer extension on RT-PCR products from co-transfected cells is given for each construct with the number of experiments in *parentheses*. The expression of the different DRADA forms is documented by Western blot analysis with an anti-FLAG antibody.

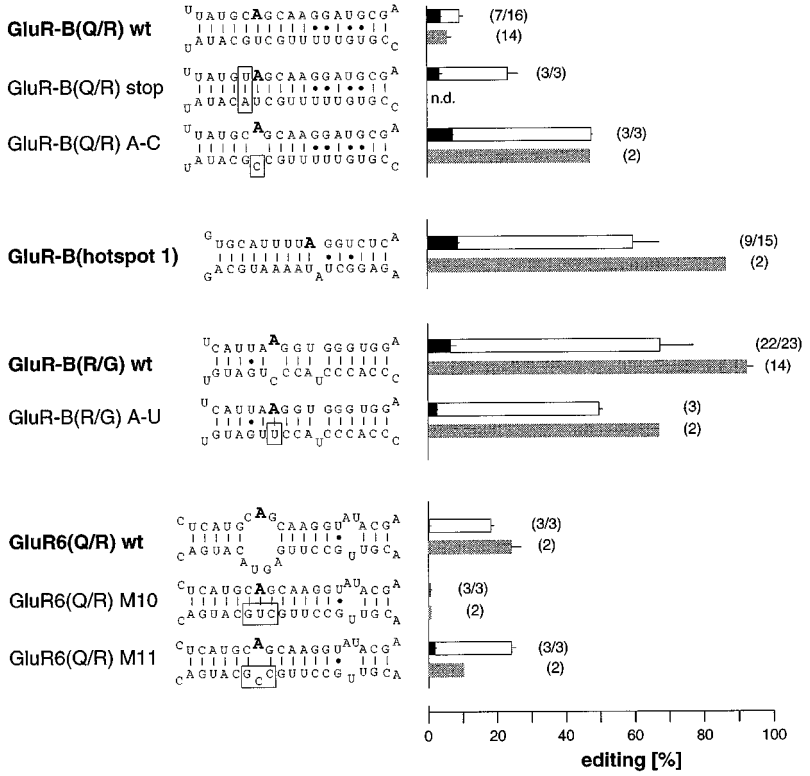

FIG. 3. **DRADA-mediated editing of individual dsRNA wild type and sequence-modified structures for different editing sites in GluR pre-mRNAs.** The dsRNAs tested as substrates for recombinant DRADA are the wild type (*bold*) and sequence-modified GluR pre-mRNAs for the Q/R site in GluR-B and GluR6, the GluR-B intron 11 hotspot1, and the GluR-B R/G site. The editing-targeted adenosine in the predicted dsRNA structures (6, 8, 9) is indicated in *bold*. In the mutated sites, sequence changes to the wild type structure are *boxed*. Minigenes for the pre-mRNAs were co-transfected into HEK 293 cells with or without a vector for recombinant DRADA, or pre-mRNAs were *in vitro* synthesized and incubated with purified recombinant DRADA. RT-PCR products were analyzed for RNA editing by primer extension. For each editing site tested, *bar graphs* (*upper bars*, cellular editing; *lower bars, shaded*, *in vitro* editing by 10 units of recombinant ΔN5 DRADA, see Fig. 1) indicate mean values of adenosine conversion, *line extensions* to *bars* give standard deviations, and the number of independent determinations is listed in *parentheses*. For cellular editing, values from co-transfection with DRADA are depicted by *open parts of bars*, control values (no DRADA vector) are indicated by *filled parts*. These cellular control values (only mean values are given) were lower for GluR6 constructs (~0.4% for wt, *M10*) than for GluR-B constructs (3–9%). Co-transfection data for GluR6 wt, M10 and M11, are from Ref. 9.

the wild type structure with its A-C mismatch (Fig. 3). This may reflect the A-U-rich environment 5′ of the targeted adenosine, permitting access by DRADA to the adenosine positioned in a destabilized dsRNA configuration (15, 28). A similar consideration might explain that the GluR-B(Q/R) stop mutant (8) is edited at higher efficiency than the corresponding wild type sequence, even though the adenosine is predicted to be paired in both structures (Fig. 3). The congruence in results obtained in the cellular and *in vitro* editing assays suggested that cellular factors may not be required for the DRADA-mediated adenosine conversion. Collectively, these data suggest that the base pairing status of the targeted adenosine may be a critical determinant for the substrate selection by DRADA.

An Activity Different from DRADA Edits the Q/R Site in GluR-B Pre-mRNA—An activity that can edit the Q/R site in GluR-B pre-mRNA has been separated from DRADA (12). We have partially purified this activity from HeLa cells by chromatography over three columns (see "Materials and Methods"). Fig. 4 shows the profile of a gel filtration column and documents the separation of the bulk of DRADA from the activity for Q/R site editing; the latter appears to be smaller in molecular weight. Selected column fractions were analyzed for activity on other editing sites in GluR-B pre-mRNAs. In agreement with the data obtained with recombinant DRADA (Fig. 1), the

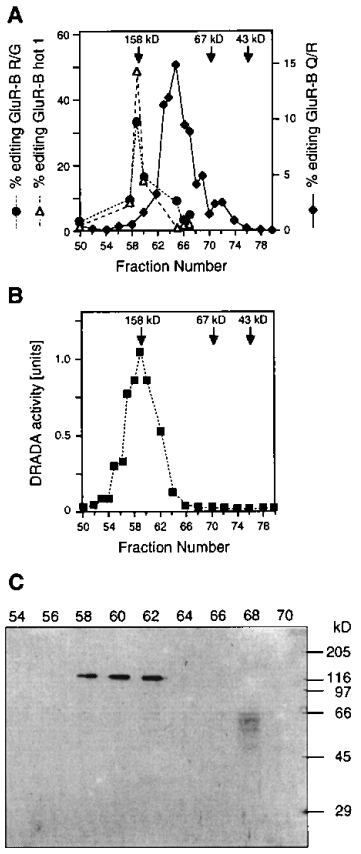

FIG. 4. **Gel filtration chromatography of DRADA and activities for GluR-B pre-mRNA editing.** *A*, elution profile of the HiLoad 16/60 Superdex 200 gel filtration column. The editing of three sites (Q/R, R/G, hotspot1) in GluR-B pre-mRNA was determined for selected fractions. *Arrows* indicate the position and molecular masses of the marker proteins aldolase (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). *B*, activity profile of DRADA in column fractions as assayed with dsRNA (22). *C*, immunoblot with anti-DRADA (dsRBD) serum (1:4,000) (Ref. 17) of even-numbered column fractions 54–70, detected by chemiluminescence. Size markers (kilodaltons) are indicated on the *right*.

intronic hotspot1 and the R/G site, but not the Q/R site, were edited by fractions containing HeLa cell DRADA. By contrast, the fractions with editing activity for the GluR-B Q/R site did not edit hotspot1 and, with the possible exception of the R/G site (Fig. 4*A, fraction 65*), failed to convert any adenosine in GluR-B pre-mRNA other than that of the Q/R site (Fig. 4). Indeed, none of 20 cloned GluR-B sequences derived from B13 transcripts, edited at the Q/R site by the activity in column fraction 65, had other adenosines deaminated. However, of 40 cloned sequences derived from incubation of B13 transcripts with fraction 59, the peak fraction for DRADA, 26 were edited in hotspot1 and none in the Q/R site. This suggests that the activity that edits the base-paired adenosine of the Q/R site may not, or not efficiently, convert adenosines in mismatched positions characteristic of other editing sites in GluR pre-mRNAs.

Western blotting of selected column fractions containing the Q/R site editing activity with antiserum against the first dsRBD of bovine DRADA (17) failed to detect DRADA. Moreover, the antiserum did not reveal a band corresponding in size to the Q/R site editing activity, which is smaller than DRADA (Fig. 4*C*). The <10% activity of adenosine conversion in extended dsRNA observed in the fraction with peak Q/R site editing activity compared with the DRADA peak fraction might derive from the Q/R site deaminase itself or from residual DRADA. However, at no point during purification could the Q/R site editing activity be enhanced by other fractions, including by peak fractions of DRADA (not shown). This appears to preclude the possibility that a factor from HeLa cells interacts with DRADA to generate Q/R site editing, as recently claimed for 293 cells (29). Collectively, these data provide suggestive evidence for the existence in HeLa cells of an enzyme different from DRADA, possibly the recently cloned RED1 deaminase (30), which can edit the Q/R site in GluR-B pre-mRNA.

DISCUSSION

DRADA is a candidate enzyme for mammalian nuclear transcript editing by adenosine deamination (2, 8, 14). In the absence of characterized natural substrates for DRADA, the enzyme's activity has been primarily characterized with artificial extended dsRNAs in which DRADA can deaminate up to 50% of the adenosines (2, 14). We tested the recombinant enzyme's activity at different editing positions in synthetic GluR transcripts and observed that DRADA edited to >90% the naturally mismatched adenosine of the GluR-B R/G site and the adenosine of hotspot1 in GluR-B intron 11, located in an A-U-rich environment next to a one-nucleotide bulge. The extent of DRADA-mediated editing at the GluR6 Q/R site with the adenosine positioned in an internal loop was lower, but this may reflect, in part, that the RNA tested for this site lacked a large segment of the native intron, potentially leading to inefficient RNA folding (9).

Importantly, the Q/R site in GluR-B pre-mRNAs was not edited, and thus, contrary to recent speculations (2, 8, 16), DRADA appears not to be involved in the editing of the GluR-B Q/R site. Fractionation of HeLa cell extracts suggests the existence of an editing activity distinct from DRADA, which can be separated from this enzyme by column chromatography, as reported by Yang *et al.* (12). As shown here, this activity converts the adenosine of the GluR-B Q/R site, but not the adenosine of hotspot1 on the same substrate RNA (8). Moreover, as predicted by the activity of recombinant DRADA on different GluR editing substrates, column fractions enriched in HeLa cell DRADA edited the R/G site and hotspot1, but not the GluR-B Q/R site, further substantiating the notion that these sites may serve as native substrates for DRADA. By testing truncated DRADA forms we largely excluded the possibility that the Q/R site editing activity of HeLa cells constitutes a smaller form of DRADA, generated by posttranslational or posttranscriptional processing. Additional differences between DRADA and the GluR-B Q/R site editing activity include the lack of cross-reactivity with a DRADA-specific anti-dsRBD serum (17) and the much smaller apparent size of the Q/R site editing activity, which would preclude the possibility that this activity represents DRADA complexed with a cellular factor. Therefore, the simplest explanation is that HeLa cells express a dsRNA-specific adenosine deaminase with distinct substrate specificity from DRADA.

A major determinant for the substrate selectivity by DRADA appears to be the local structure of the targeted adenosine, as revealed by mutational analysis. We observed that the Q/R site in GluR6 was not edited when the targeted adenosine was base paired (mutant M10), in analogy to the Q/R site of GluR-B. However, DRADA converted the GluR-B Q/R site adenosine at good efficiency when placed in an A-C mismatch configuration. These results are compatible with the view that DRADA can deaminate *in vivo* adenosines occupying mismatched positions in dsRNAs for the R/G site of AMPA receptor subunits GluR-B, -C, and -D and, possibly, the Q/R site in GluR5 and GluR6. Furthermore, DRADA may edit intronic hotspot1 in GluR-B pre-mRNA. Given the near ubiquitous expression of DRADA (31), the enzyme is likely to edit pre-mRNAs in addition to those encoding GluR subunits. While such genes need to be

characterized, the adenosine deamination in the intramolecular TAR stem-loop structure (32) may be generated by DRADA.

Notably, all sites putatively targeted by DRADA remain largely unedited in the embryonic brain. During postnatal stages, these sites, including the GluR-B intron 11 hotspot1 (embryonic day 14, ~20% edited; postnatal day 0, ~40%; P7, ~50%; P14, ~60%; P21 and P42, ~70%), undergo a comparable developmental progression in editing to an extent of 50–90% in the adult brain (6, 33, 34). Although DRADA expression in brain appears to increase during brain development (17), other gene products (35, 36) may also contribute to such progressive editing. However, editing at these sites is substantially below the >99% extent characteristic of the GluR-B Q/R site. The almost complete adenosine conversion at this position is essential for the low Ca^{2+} permeability of AMPA receptors (37, 38) and the physiology of the central nervous system (39). Conversely, the change in kinetic characteristics of AMPA receptor channels generated as a consequence of R/G site editing (6) may play a role in the developmentally regulated fine tuning of fast excitatory neurotransmission in central synapses. Hence, different RNA editing enzymes appear to participate in controlling the Ca^{2+} permeability and kinetic properties of AMPA receptor channels.

Based on the present study, we interpret the pattern of adenosine deamination in exonic and intronic GluR-B pre-mRNA sequences from brain (8, 11) as reflecting the combined activity of different editing enzymes. DRADA preferentially converts adenosines in mismatched positions, loops, and bulges, probably because the altered geometry of the RNA helix (28) permits access by this enzyme. The GluR-B Q/R site adenosine appears to be deaminated by a different activity, possibly RED1 (30), which is molecularly related to DRADA.

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