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 insertion (1, 2). In the transcript for intestinal apolipoprotein B nuclear transcripts and occurs by site-specific base modification (1, 2). In the transcript for intestinal apolipoprotein B nuclear transcripts and occurs by site-specific base modification (1, 2).

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, \(-\)amino-3-hydroxy-5-methylisoxazole-4-propionic acid; wt, wild type.

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GluR Constructs—GluR-B minigenes were B13 (GluR-B(Q/R) wt and hotspot1), ER3’s2 (GluR-B(Q/R) stop), 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)).

MATERIALS AND METHODS

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wt), mutant E1 (transferred to minigene pBgl) (GluR-B(R/G) A-U). GluR-B/R/G site minigene pBgl was referred to as BglI-BglII in Lonelli 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). Typically, 3 μg of each assay, 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 3Ha1 (9) for GluR 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 reassociated in 20 μl of 3 μg 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 μg each) and reverse-transcribed into cDNA (8). The RT primers contained at their 3’ end a 20 nt sequence with a 5’ probe 

\[ \text{5'}-\text{GCGAGTCTTTACTGCGGCTGCAA-3'} \]

on minigene B13; Bint2, 5’-ACCTGATGCACT-3’, antisense on minigene B13; MH50, 5’-GACCCCTTGATGAAATCCTAAC-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-GACCCCTTGATGAAATCCTAAC-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5’-ATCTCTGACCAACTCGTGAAGT-3’, antisense on minigene B13; Bint2, 5′-ATCTCTGACCAACTCGTGAAGT-3′, antisense on minigene B13.

To determine if

**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 human protein (17, 18) and another version (ΔN53) 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
RNA Editing of GluR Pre-mRNAs by DRADA

12223

sites may be substrates for related deaminases which differ in
substrate specificity. We determined by DNA sequencing of cloned RT-PCR prod-
ucts from the in vitro editing reactions with both DRADA forms whether the adenosine
deamination catalyzed by the recombi-
nant 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 aden-
osine 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 trans-
ected 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 re-
mained at cellular background levels (<5%) when co-transfect-
ing 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 mu-
tant 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 environ-
ments 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 ad-
osines (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

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 se-
quences (6,8,9) as a substrate for site-selective adenosine deamination. The 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 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 anal-
ysis 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.
the wild type with its A-C mismatch (Fig. 3). This may reflect the A-U-rich environment 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
intrinsic 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. 4A, 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 at the Q/R site 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 intrinsic 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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