THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 1, Issue of January 3, pp. 473–478, 1997
Printed in U.S.A.

Purification of Human Double-stranded RNA-specific Editase 1 (hRED1) Involved in Editing of Brain Glutamate Receptor B Pre-mRNA*

(Received for publication, September 9, 1996)

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RNAs encoding subunits of glutamate-gated ion channel receptors are posttranscriptionally modified by RNA editing and alternative splicing. The change in amino acid sequence caused by RNA editing can affect both the kinetics and the permeability of the ion channel receptors to cations. Here, we report the purification of a 90-kDa double-stranded RNA-specific adenosine deaminase from HeLa cell nuclear extract that specifically edits the glutamine codon at position 586 in the pre-mRNA of the glutamate receptor B subunit. Site-specific deamination of an adenosine to an inosine converts the glutamine codon to that of arginine. Recently, a gene encoding a double-stranded-specific editase (RED1) was cloned from a rat brain cDNA library. Antibodies generated against the deaminase domain of its human homolog specifically recognized and inhibited the activity of the 90-kDa enzyme, indicating that we have purified hRED1 the human homolog of rat RED1. This enzyme is distinct from double-stranded RNA-specific adenosine deaminase which we and others have previously purified and cloned.

RNA editing, defined as an alteration in the coding capacity of mRNA other than splicing or 3′-end processing, was first observed in the kinetoplast of trypanosomes (1). Originally thought to be a mechanism of generating genetic diversity only in the mitochondria or chloroplasts of lower organisms and plants, the discovery of editing of the apolipoprotein B (apoB)[1] mRNA suggested that editing was also widespread in mammals (2, 3). To date there are several examples of mammalian RNA editing, the most dramatic perhaps being the editing of the pre-mRNA of glutamate-gated ion channel receptor subunits in the brain (see Ref. 4 for review).

L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. The ionotropic glutamate receptors (GluR) have been classified into three subtypes based on their selectivity to agonists and antagonists; the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainate receptors. The receptors are comprised of 17 sequence-related subunits (5–7). 5 out of the 17 are edited (see Refs. 8, 9 for reviews) and have a conversion from adenosine in the genomic sequence to inosine in the mRNA (10–12); inosine is read as if it were a guanosine by the translational machinery (13). This generates a substitution in an amino acid in the receptor subunit and can have major consequences on the properties of the ion channel (see Ref. 14 for review).

Arginine at position 586 of the GluR-B subunit controls the low Ca^{2+} permeability of the heteromeric AMPA receptors (15, 16). This arginine is generated by site-specific deamination of a CAG glutamine codon (Q) to a CIG arginine codon (R) (10–12). In the rat brain the Q/R site is edited at all developmental stages. Interestingly, the only known example where this site is not edited is in fish, where an arginine is already encoded in the genomic sequence (17). The formation of an intramolecular RNA duplex between portions of exon 11 and intron 11 of GluR-B pre-mRNA is required for RNA editing (18). Transgenic mice harboring a mutation on one of their alleles that disrupts this base pairing capacity are unable to edit the Q/R site, develop early onset epilepsy, and die within 3 weeks of birth (19). It is thought that this may be the result of increased Ca^{2+} influx, eventually leading to neuronal degeneration.

The dsRNA-specific adenosine deaminase (DRADA) is a ubiquitous enzyme that has been purified (20–23) and cloned from different sources (24–26). We investigated whether the DRADA we had isolated from calf thymus could edit the GluR-B pre-mRNA. We found that the purified enzyme was unable to edit the Q/R site in the GluR-B pre-mRNA to significant levels *in vitro* (27) but edited the R/G and hotspot 1 sites in addition to the Q/R site in the pre-mRNAs of the kainate receptor subunits GluR-5 and GluR-6 (27, 28). A comparison of the potential of these editing sites to form a double-stranded RNA structure suggested that DRADA could edit an adenosine if it was in a mismatched position, either a loop or a bulge, whereas the adenosine at the Q/R site is in a perfect duplex. These results suggested that the Q/R site in GluR-B pre-mRNA was very different from the sites edited by DRADA and was edited by a different activity (27).

Fractionation of HeLa cell nuclear extract separated an activity from DRADA that could specifically edit the GluR-B Q/R site (12, 27). One of the methods employed to separate these two activities was gel filtration, the molecular weight of the Q/R editing activity being lower than that of DRADA (27). It had been postulated that a complex between DRADA and a cofactor was responsible for Q/R editing (29–31), but we were unable to find such a complex. During fractionation of HeLa cell nuclear extract no fraction added to DRADA made it Q/R editing-competent nor could polyclonal antibodies raised

* This work was supported by the Cantons of Basel, the Swiss National Science Foundation, and Human Frontiers Science Program Grant 3/95. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a graduate student fellowship from the Boehringer Ingelheim Fonds.

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[1] The abbreviations used are: apoB, apolipoprotein B; GluR, glutamate-activated receptor channel subunits; RED1, double-stranded RNA-specific editase 1; ds, double-stranded; DRADA, dsRNA-specific adenosine deaminase; dsRBD, double-stranded RNA binding domain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; PCR, polymerase chain reaction.

against the dsRNA binding domain (dsRBD) of DRADA recognize the Q/R editing activity (27). Therefore, we concluded that another enzyme was responsible for editing the Q/R site in the GluR-B pre-mRNA.

The editing activity that specifically edits the GluR-B Q/R site was purified from HeLa nuclear extracts by chromatography over seven columns. A second dsRNA adenosine deaminase, dsRNA-specific editase 1 (RED1), that can specifically edit the GluR-B Q/R and R/G sites has recently been cloned from a rat brain cDNA library (32). This enzyme is 31% identical to DRADA and is comprised of two dsRBD and a deaminase domain (32). Polyclonal antibodies were generated against its deaminase domain that recognized and inhibited the deaminase activity of the purified 90-kDa enzyme but not DRADA. We therefore conclude that we have purified human dsRNA-specific editase 1 (hRED1).

EXPERIMENTAL PROCEDURES

GluR Constructs and Oligonucleotides—GluR-B minigenes used in this study were B13 (encoding the Q/R- and hotspot 1-edited sites) (18) and pBgl (encoding the R/G sites) (10); these constructs were linearized and the RNA transcribed as described previously (10). The antisense oligonucleotide used in the primer extension assay was B-RT; 5′-GGCGAAATATCGCATCCTTG-3′ which is complementary to the Q/R-edited site (10); BHS-RT, 5′-ACCATGAATATCCACTTGAG-3′ is the antisense primer used in the primer extension assay to measure editing at the hotspot 1 in GluR-B intron 11. The primers for PCR, reverse transcription, and sequencing were KMH3, 5′-GACACGGTACCACACAACGGCATTTCCATGAATTGATGTTAGAG-3′ which is a reverse transcriptase primer antisense on minigene B13; BFFK3, 5′-GACACGGTACCACACAACGGATTGTGAGTTACCTCATATCCG-3′ which is another reverse transcriptase primer antisense on minigene pBgl; PCRK3, 5′-GACACGGTACCACACA ACGG-3′, a PCR primer for cDNA primed with KMH3 or BFFK3; cis55, 5′-CTCTGCGAGCTCAGGTCCAACTGCACCTCGG-3′ a vector specific 5′-primer; MH50, 5′-GACCCTGTAGGAAAAATCTAACC-3′, antisense on minigene B13; MH36, 5′-TCACCAGGGAAACACATGATCAAC-3′ antisense on minigene B13; Bint1, 5′-GCGGTACCGTGAGTTACCTCATA TCCGTAT-3′ antisense on minigene pBgl; (10) and Bint2, 5′-ATCTCTAGACAAACCGTTAAGAGTC-3′, which is also antisense on minigene pBgl (27). The PCR primers used for cloning ESTs homologous to RED1 were R1, 5′-CTACAGGATCCAAATGTATTAATGG-3′; and R2, 5′-CCACCGTGCA AGCTTGTCACTGC-3′.

Purification of hRED1—The principal buffer used in this purification was buffer A (50 mM Tris-HCl, pH 7.9, 5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, and 0.4 μg/ml leupeptin). Most of the columns were developed with a KCl gradient so therefore the KCl concentration used is indicated below. All manipulations were carried out at 4 °C; fractions were frozen in liquid nitrogen and stored at −70 °C. Aliquots of column fractions were dialyzed against buffer A containing 200 mM KCl for 2 h before being assayed both for their ability to edit the Q/R and hotspot 1 sites of GluR-B by primer extension assay (10) and for deaminase activity (22). HeLa cell nuclear extract (14.67 g of protein which is the equivalent of approximately 1,500 liters of HeLa cells) was prepared by standard method (33). The nuclear extract was divided in two and loaded separately onto a 1.6-liter Macro-Prep High Q column (Bio-Rad), which had been equilibrated with buffer A containing 50 mM KCl. The column was washed with 1 column volume and developed with an 8-liter gradient from 50 to 500 mM KCl. The Q/R editing activity eluted with a broad peak from 130 to 280 mM KCl. The active pools from both Macro-Prep High Q columns were combined, the conductivity was adjusted to 300 mM KCl, the pool was then divided in two and loaded separately on a 300-ml Affi-Gel blue column (Bio-Rad). The column was washed with 2 column volumes of buffer A containing 300 mM KCl and developed with a 5-column KCl gradient 300-1000 mM KCl. The Q/R editing activity eluted between 480 and 800 mM KCl; the pools from both Affi-Gel blue columns were again combined; 10 mM $CaCl_2$ was added to the active pool, and it was loaded directly onto a 350-ml Ultrogel hydroxyapatite column (IBF Biotechnics). The hydroxyapatite column had been equilibrated with buffer B (20 mM Tris-HCl, pH 7.9, 50 mM KCl, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, and 0.4 μg/ml leupeptin). The column was washed with 2 column volumes and developed with an 8-column gradient of 0–500 mM potassium phosphate, pH 7.9, in buffer B. The Q/R editing activity eluted between 90 and 160 mM potassium phosphate. After extensive dialysis against buffer C (same as buffer A except for 50 mM HEPES-KOH, pH 7.9 replacing Tris-HCl) containing 50 mM KCl, the active pool was loaded onto an 8-ml Mono S column which had been equilibrated with buffer C. The column was washed with 3 column volumes, and the editing activity was eluted with a 30-column volume gradient from 50 to 500 mM KCl. The activity eluted between 190 and 330 mM KCl and was dialyzed against buffer A containing 50 mM KCl and 0.02% Nonidet P-40 (Nonidet P-40) which was added to all buffers from this stage on to increase protein stability. The active pool was loaded on an 8-ml Mono Q, washed with 2 column volumes, and developed with a 10-column volume gradient 50–500 mM KCl. The Q/R editing activity eluted both in the flow-through and between 50 and 280 mM KCl. Due to the presence of DRADA in the Mono Q flow-through, the final two columns were run separately with either the Mono Q flow-through or bound fraction, and these active pools were not combined. The conductivity of the Mono Q flow-through and bound fraction was adjusted to 500 mM KCl, and they were loaded separately onto a 5 ml poly(I)·poly(C) (Pharmacia Biotech Inc.) column. The column was washed with 2 column volumes and developed with an 8-column volume gradient from 500 to 2500 mM KCl. The Q/R editing activity eluted from both columns between 600 and 1800 mM KCl; the conductivity of the active pools was adjusted to 250 mM KCl by dilution with buffer A minus KCl and was loaded separately onto a 6-ml poly(G)·poly(C) column which had been prepared as described previously (22). The column was washed with 2 column volumes and developed with a 7-column volume gradient between 250 and 1000 mM KCl. The Q/R editing activity eluted between 370 and 500 mM KCl. Protein concentration was determined by the Bradford method (34) with BSA as reference standard, except for the pure protein which was quantified on an SDS-polyacrylamide gel with different amounts of BSA as reference standard.

The DRADA extract used was from a previous purification prepared in a similar manner as described above except that the DRADA activity was in the flow-through of the Mono Q column, and all the Q/R editing activity bound to the column. The DRADA activity from the Mono Q flow-through was concentrated 3-fold by Centricon 30 (Amicon), and the units used in experiments corresponded approximately to 100 fmol of inosine/min as measured on dsRNA.

In Vitro Editing—DsRNA was transcribed and assayed for dsRNA adenosine deaminase activity as described previously (22). The assay was performed at 30 °C. An analysis of the GluR-B minigene B13 was performed by primer extension assay with B-RT primer specific for the Q/R site and the BHS-RT primer specific for hotspot 1. In the standard assay, 10 fmol of *in vitro* transcribed RNA derived from minigene B13 was incubated with extract for 1–3 h at 30 °C. The reaction mixture was then treated with proteinase K, phenol chloroform-extracted, and ethanol-precipitated. 10 fmol of the appropriate radioactive primer was added and processed as described (10). The primer extension products were quantified on a PhosphorImager 425 (Molecular Dynamics).

Sequence Analysis of Edited GluR-B Pre-mRNAs—To determine the specificity of editing, reverse transcriptase-PCR amplification and subsequent sequencing of RNAs incubated with either hRED1 (60% conversion of adenosine to inosine per hour measured by primer extension assay) or DRADA (100 fmol of inosine/min as measured on dsRNA) was performed (10). After *in vitro* editing the RNAs were reverse-transcribed (Superscript™ ll RT, Life Technologies, Inc.), and the specific primers were KMH3 for the Q/R site and BFFK3 for the R/G site. The cDNAs were then amplified by a two-step PCR protocol; in the first amplification the primers were cis55 which is complementary to the vector into which the GluR transcripts were cloned and PCRK3 which primes at the 5′-end of the RT primers. The PCR was performed under standard conditions. In the second PCR, cis55 and MH50 were the primers used to specifically amplify the Q/R site and cis55 and Bint2 the primers for the R/G site. The PCR products were then gel-purified, quantified, and approximately 60 ng was used in a sequencing reaction on an Applied Biosystems 373A sequencer with dye terminators (Perkin-Elmer) according to the manufacturer's instructions. MH36 was the sequencing primer for minigene B13 and Bint1 for minigene pBgl.

Overexpression of Histidine-tag Fusion Protein and Antiserum Preparation—The ESTs, L25485 and T70335 are 81.3 and 80.7% homologous to RED1. They were amplified from HeLa cDNA with the complementary PCR primers R1 and R2 which contained restriction sites at their termini. The 575-base pair PCR product was subcloned into the *Bam*HI and *Hin*dIII sites in the polylinker of the histidine tag expression vector pTrcHisA (Invitrogen) and sequenced. *Escherichia coli* strain BL-21 was transformed with pTrcH-EST1/2 and subsequently induced at A_{600} = 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside for

TABLE I
Purification of hRED1 from HeLa cell nuclear extract

Fraction	Protein	Activity	Specific activity	Purification factor	Recovery
	mg	*units*[a]	*units/mg*		*%*
HeLa nuclear extract	14,670	87,349			
Macroprep Q pool	5,610	210,800	38	1	100
Affi-Gel Blue pool	657	100,800	153	4	48
Hydroxyapatite pool	147	73,946	503	13	35
Mono S pool	51	53,280	1,036	28	25
Mono Q pool	33	41,158	1,262	34	19
Poly(I) · poly(C) pool	1	38,868	38,868	1,034	18
Poly(G) · poly(C) pool	0.1	33,823	281,858	7,501	16

[a] One unit = 1% editing per min of the GluR-B pre-mRNA at the Q/R site.

3 h at 37 °C. The overexpressed fusion protein was purified under denaturing conditions on a nitrilotriacetic acid-Ni^{2+} affinity column as specified by the manufacturer (Qiagen). Aliquots from fractions of the nitrilotriacetic acid-Ni^{2+} affinity column were analyzed by electrophoresis on an SDS-polyacrylamide gel and detected with Anti-Xpress™ antibody 1:10,000 (Invitrogen). Approximately 100 μg of pure recombinant protein was gel-purified and injected subcutaneously into a New Zealand White rabbit which had first been bled to obtain preimmune serum. The antigen was emulsified with Specol adjuvant (Central Veterinary Institute, Lelystad, The Netherlands). The rabbit was boosted every 3 weeks with 50–100 μg of antigen, and blood was collected approximately 10 days after each boosting.

Immunoblot Analysis—Proteins were separated on an 8% SDS-polyacrylamide gel, blotted on nitrocellulose, and detected with chemiluminescence staining (ECL kit, Amersham) as described previously (25). The DRADA antiserum was diluted 1:4000, and the hRED1 antiserum was diluted either 1:1000 or 1:4000.

RESULTS

Purification of hRED1 That Edits the GluR-B Q/R Site—The partial separation of a Q/R editing activity that is distinct from DRADA has previously been reported (12, 27). Here, we demonstrate that this editing activity is the human homolog of RED1; hence, it will be referred to as hRED1. HeLa cell nuclear extract was chosen as the source for purification since it contains an editing activity analogous to that found *in vivo* in the brain that can specifically deaminate the adenosine at the GluR-B Q/R site. hRED1 was purified by chromatography over seven columns (Table I); ion exchange was the principal method used. Since the editing activity was capable of unspecifically deaminating adenosines in long dsRNA, the purification scheme chosen is very similar to the purification of DRADA from calf thymus (22).

The purification of hRED1 was greater than 7000-fold from 14.7 g of protein of HeLa cell nuclear extract (Table I), the equivalent of approximately 2 kg of packed HeLa cells. The large amount of protein necessitated the running of the first two columns twice. The presence of RNases made the accurate measurement of Q/R editing activity in nuclear extract impossible, and the units of activity are estimated from the Macro-Prep High Q pool. Aliquots of column fractions were dialyzed and assayed for both the presence of DRADA and hRED1 activity by primer extension (10) and for their ability to deaminate adenosine to inosine on extended dsRNA (22). The two editing activities eluted with overlapping peaks from the first four columns, but only hRED1 activity was pooled for further purification. The activities could be separated on a Mono Q column to which the HeLa DRADA activity does not bind. In general, hRED1 binds to Mono Q, but in this particular purification the activity split with half binding and the other half eluting in the flow-through. This was not the result of overloading the column since the protein did not bind when reapplied to a larger column. A similar problem had previously been encountered during the purification of DRADA from calf thymus (22). The hRED1 used in subsequent experiments was derived from the fraction that bound to Mono Q and contained no detectable DRADA activity.

A 30-fold purification of hRED1 was achieved by chromatography on a poly(I)·poly(C) column, and the enzyme was further purified and concentrated on a poly(G)·poly(C) column (Table I). The activity profile of this column shows a peak of activity in fractions 16–22 (Fig. 1*A*). This profile corresponds to Q/R editing analyzed by primer extension (Fig. 1*B*). The load fraction was too dilute, and only a faint band corresponding to editing activity is seen. No edited product was observed when the hotspot 1 site, which is edited by DRADA, was analyzed by the primer extension assay with these same column fractions (data not shown). A band of approximately 90 kDa co-migrated with Q/R editing activity when these fractions were electrophoresed on a 7.5% SDS-polyacrylamide gel (Fig. 1*C*). This band is the upper part of a doublet that could only be visualized by electrophoresis on a long SDS-polyacrylamide gel and allowing the 45-kDa marker to migrate out of the gel. The lower band of the doublet is present in the flow-through and in most fractions up to fraction number 24 and does not migrate with activity, neither does a smaller polypeptide of approximately 85 kDa which is also present in the flow-through. Moreover, antibodies generated to the deaminase domain of hRED1 specifically recognize only the upper band in the doublet (see below).

Substrate Specificity and Reaction Requirements of hRED1—To confirm the specificity of hRED1, reverse transcriptase-PCR was performed on the products of the editing reaction and were then sequenced (see "Experimental Procedures" for details). The trace data of the nucleotides adenosine and guanosine surrounding the Q/R, hotspot 1, and R/G site are depicted in Fig. 2, *A* and *B* (upon reverse transcription inosine appears as guanosine). Partially purified DRADA was very efficient at converting adenosine to guanosine at hotspot 1 in intron 11 but only converted approximately 25% of the adenosines at the Q/R site and 10% at position +4 (Fig. 2*A*). On the other hand hRED1 was capable of converting all adenosines at the Q/R site, approximately 20% at the +4 position and none at hotspot 1. Both enzymes converted adenosines at the R/G site (Fig. 2*B*), DRADA approximately 75% and the 90-kDa enzyme 80%. No other nucleotides were edited, as detected by sequencing the PCR products (data not shown), thereby confirming that hRED1 could specifically edit the Q/R and R/G sites *in vitro*.

Pure hRED1 is not as stable as DRADA and is very sensitive to freeze-thawing. Assays with the pure enzyme were performed in the presence of 20% glycerol to help stabilize the protein. Like DRADA it requires neither additional cofactors nor ATP for its editing activity. The temperature and KCl optimum of the enzyme were measured on extended dsRNA as this has a more stable conformation than GluR-B pre-mRNA. The editing activity has a temperature optimum between 35 and 37 °C but still retains 80% of its activity at 42 °C. The KCl

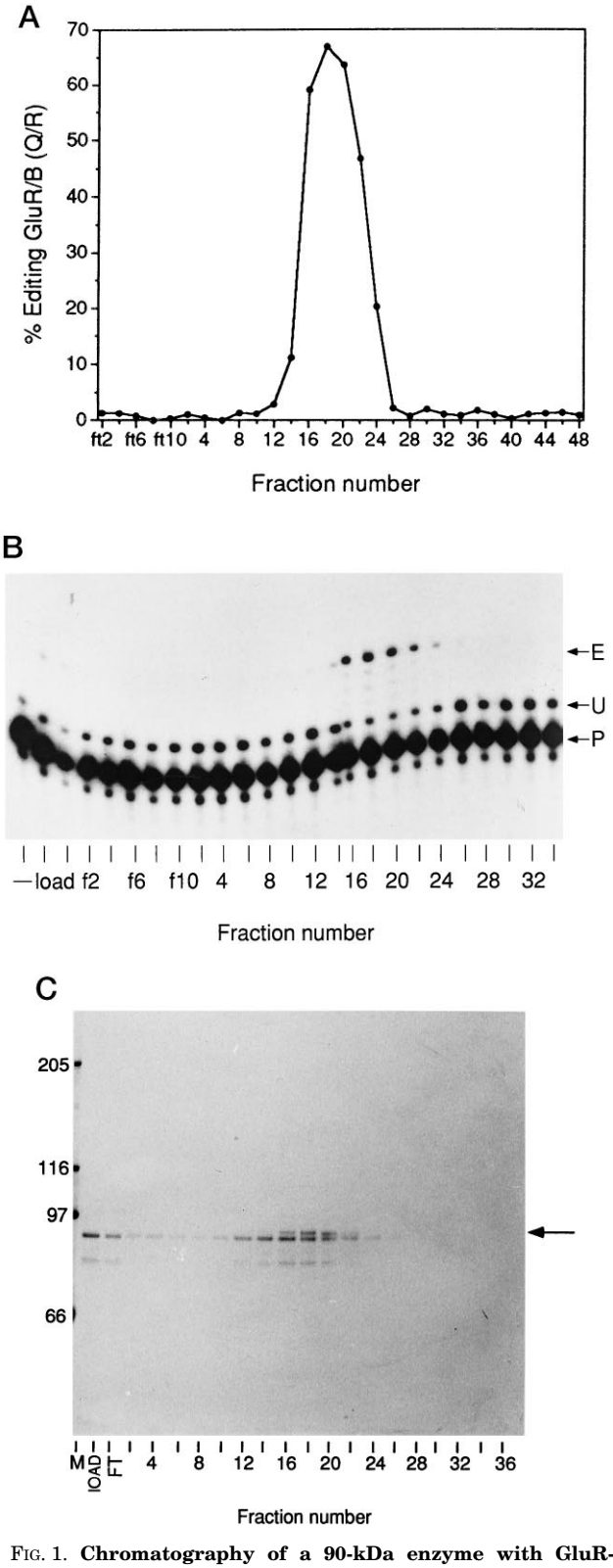

FIG. 1. **Chromatography of a 90-kDa enzyme with GluR-B (Q/R) editing activity on a poly(G)·poly(C) column.** *A*, the activity profile of the final poly(G)·poly(C) column measuring the conversion of adenosine to inosine by the primer extension assay. The units are expressed as the percent conversion of adenosine to inosine per hour. *B*, every other fraction of the poly(G)·poly(C) column was analyzed for Q/R editing by primer extension. The *arrows* indicate the position of *P*, the primer, *U*, the unedited product, and *E*, the edited product. The *first lane* shows the negative control without protein. *Lanes 2* and *3* contain the load fraction, 0.5 and 1 μl, respectively. The flow-through was collected in 10 fractions, and 0.25 μl of it (f2–f10) as well as fractions 2–34 were assayed. C, SDS-polyacrylamide gel electrophoresis of poly(G)·poly(C) column fractions. Aliquots of 15 μl were electrophoresed on a 7.5% SDS-polyacrylamide gel, and proteins were visualized by silver staining. Molecular masses of the size markers are indicated in kilodaltons on the *left*. The *arrow* on the *right* points to a protein of approximately 90 kDa.

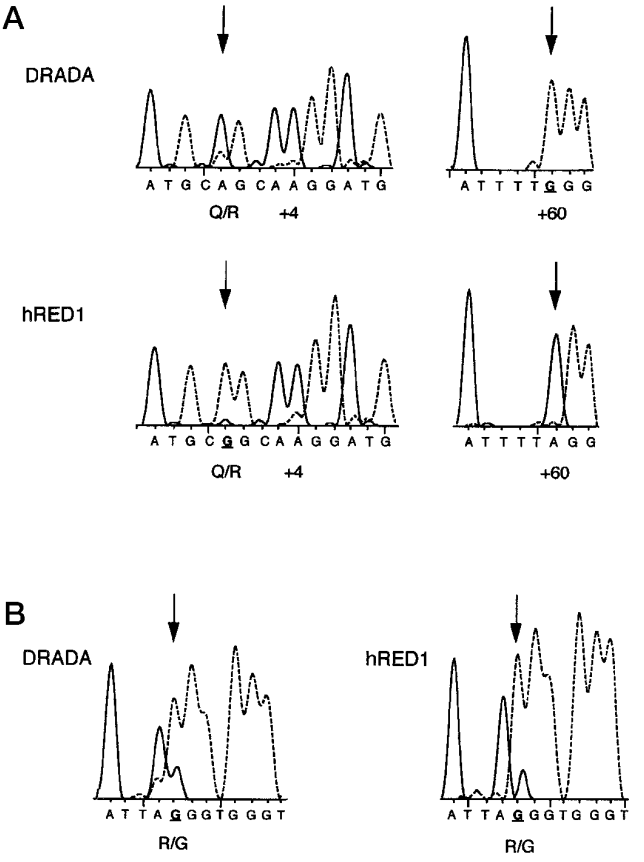

FIG. 2. **Sequence analysis of GluR-B at Q/R, hotspot 1, and R/G sites.** GluR-B pre-mRNA B13 (encoding the Q/R and +60 hotspot 1 sites) and pBgl (encoding the R/G site) were incubated with either DRADA or hRED1. The RNA was analyzed by reverse transcriptase-PCR, and the products were sequenced, as described under "Experimental Procedures." *A*, the trace data of the sequence surrounding GluR-B Q/R, +4, and hotspot (+60). Only the traces of adenosine and guanosine are shown. GluR-B was edited by both DRADA and hRED1; an *arrow* marks the Q/R and +60 sites. The edited nucleotides are in *bold* and *underlined*. *B*, the trace data of the sequence surrounding the GluR-B R/G site, which is edited by both DRADA and hRED1. An *arrow* indicates the edited nucleotides which are in *bold* and *underlined*.

optimum is 100 mM, but no significant decrease in activity is observed between 25 and 150 mM KCl (data not shown).

In competition experiments with 100 ng of poly(A), poly(U), poly(C), and poly(G), poly(U) inhibited the hRED1 activity by 97% (Fig. 3). The other polynucleotides either stimulated the enzyme [poly(A)] or inhibit only minimally by 5%. The affinity for poly(U) is very high, and when hRED1 is applied to a poly(U) column it is not possible to recover any activity from the column by high salt elution, which maybe due to binding of the enzyme irreversibly. This is in contrast to DRADA which is only inhibited by poly(G) (22) and unaffected by the addition of poly(U). The activity of DRADA is strongly inhibited by *o*-phenanthroline which probably chelates a Zn^{2+} ion that is required at its active site (25). At 5 mM *o*-phenanthroline DRADA is inhibited by 90% in comparison to the 30% inhibition of activity observed with hRED1 at the same *o*-phenanthroline concentration (Fig. 3). This result suggests that if there is an ion at the active site of hRED1, it is less accessible than in DRADA. Both enzymes are very sensitive to *N*-ethylmaleimide (NEM), at 5 mM the activity of hRED1 is inhibited

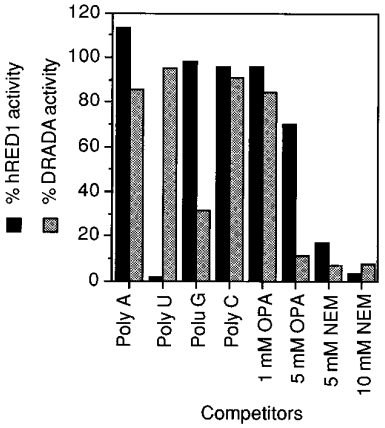

FIG. 3. **Inhibition of hRED1 and DRADA activity.** Competition experiments were performed with 100 ng of poly(A), poly(U), poly(C) and poly(G); *o*-phenanthroline (*OPA*), and *N*-ethylmaleimide (*NEM*) were tested for their inhibitory effects. Partially purified enzymes were preincubated for 15 min before addition of RNA substrate. DRADA (Mono Q flow-through) was assayed for adenosine deaminase activity for 1 h at 37 °C (22), and hRED1 (Mono S peak fraction) was assayed for editing of GluR-B pre-mRNA at the Q/R site for 1 h at 30 °C as described under "Experimental Procedures." The data shown here are results from two independent experiments.

by 85%, whereas DRADA is inhibited by 95% (Fig. 3).

The 90-kDa Protein Is the Human Homolog of RED1—Two ESTs, L25485 and T70335, listed in the data base have a high homology to the deaminase domains of RED1 and DRADA (32). A 575-base pair PCR fragment encoding both ESTs was fused to a histidine tag; the resulting protein was overexpressed in *E. coli*, and polyclonal antibodies were generated in a rabbit (see "Experimental Procedures" for details). This antiserum recognized the 90-kDa protein in fractions 16–20 of the poly(G)·poly(C) column (Fig. 4*A*). Therefore, we conclude that we have purified the human homolog of RED1 because the same band that co-eluted with editing activity was detected in these fractions on the silver-stained SDS-polyacrylamide gel (Fig. 1*C*).

The nucleotide sequence of hRED1 has a high homology to RED1 from rat[2] and contains two dsRBD. Polyclonal antibodies directed against the first dsRBD of HeLa DRADA (25) recognized both the HeLa and the calf thymus enzyme but not hRED1 (Fig. 4*B*). The anti-hRED1 serum, raised against the deaminase domain of hRED1 recognized hRED1 but not DRADA (Fig. 4*B*). This result suggests that even though the two adenosine deaminases contain similar dsRBDs and deaminase domains, they are sufficiently different to prevent cross-reaction of the specific serum. The hRED1 antiserum inhibited approximately 50% of the deaminase activity of hRED1 but did not inhibit DRADA (data not shown). This is additional proof that the purified 90-kDa enzyme is indeed hRED1 and not a new member of the dsRNA adenosine deaminase family.

DISCUSSION

Ca^{2+} permeability of the AMPA receptors is controlled by the presence in their GluR-B subunit of an arginine residue at the Q/R site that is generated by RNA editing of pre-mRNA (15, 16). There have been contradictory reports in the literature concerning the enzyme(s) that mediate this editing event (12, 27, 29–32). It was reported that DRADA edited the hotspot 1 site in intron 11 but required additional cofactors to edit the Q/R site (30, 31). Other investigators found that DRADA could be partially separated from the Q/R editing activity (12, 27).

[2] A. Gerber, M. A. O'Connell, and W. Keller, manuscript in preparation.

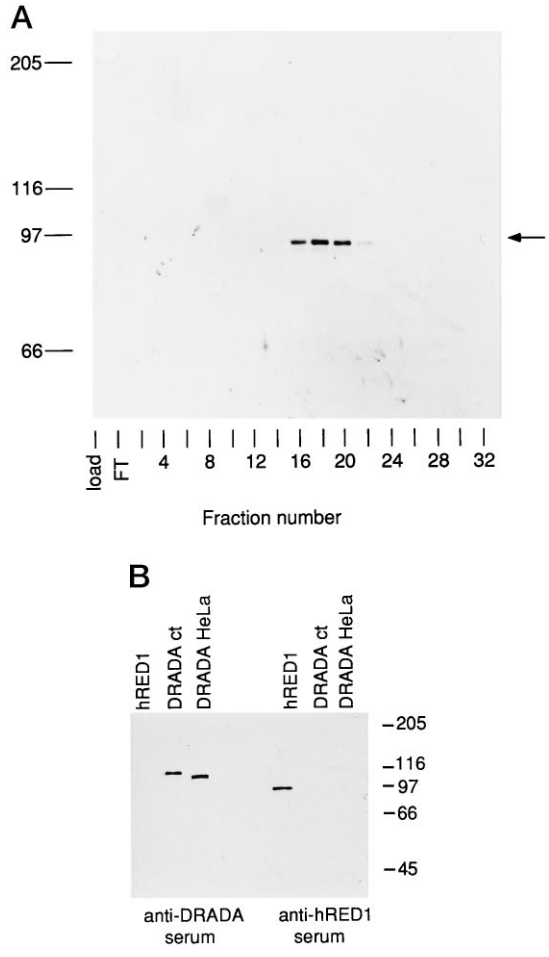

FIG. 4. **Immunoblot of fractions from the final poly(G)·poly(C) column with anti-hRED1 serum.** *A*, the same fractions (15 μl) from the final poly(G)·poly(C) as shown in Fig. 1*C* were electrophoresed on a 7.5% SDS-polyacrylamide gel and immunodetected with anti-hRED1 serum (1:1000). The molecular masses of the standards in kilodaltons are on the *left*. An *arrow* on the *right* points to a 90-kDa protein. *B*, immunoblot with anti-DRADA (1:3000) and anti-hRED1 (1:4000). Aliquots of 50 μl of pure calf thymus and partially purified HeLa DRADA were precipitated with trichloroacetic acid (15%), and a 10-μl aliquot of pure hRED1 was electrophoresed on an 8% SDS-polyacrylamide gel, and proteins were detected by chemiluminescence.

Melcher *et al.* (32) reported that a novel enzyme RED1 was responsible for the editing of the Q/R site. Here, we confirm the latter results by demonstrating that the Q/R site is edited *in vitro* by a 90-kDa enzyme from HeLa cells that represent the human homolog of RED1. The R/G site is edited to almost the same extent by hRED1 and DRADA, whereas the hotspot 1 in intron 11 is edited by DRADA only.

The 90-kDa homolog of RED1 was purified more than 7000-fold from HeLa cell nuclear extract by ion exchange chromatography. To ensure that the purified enzyme had the same fidelity of editing *in vitro* as was observed *in vivo* (11, 18), PCR products of the edited GluR-B RNA were sequenced directly so that populations instead of individual clones could be analyzed. A subset of the sequencing data is shown in Fig. 2. No nonspecific editing was observed with either hRED1 or DRADA. It is interesting to speculate why two similar enzymes are required to edit adenosines at two different sites in GluR-B pre-mRNA. The answer may lie in the different structural environment of the adenosines that are edited (27). Double-stranded RNA forms an A-type helix, and the amino group of the adenosine that is deaminated lies in the poorly accessible major groove (35). DRADA can deaminate the amino group of an adenosine

which is mismatched or in a bulge, such as at the R/G and hotspot 1 sites (27) since a bulged adenosine widens the narrow major groove to twice its normal width (36). In contrast, hRED1 is able to recognize and deaminate the adenosine at the Q/R site which is present in a perfect duplex and is less accessible (18). This appears to be the preferred substrate, since under the same editing conditions hRED1 can edit the Q/R site to 100% but edits the R/G site to only 80% (Fig. 2), suggesting that the adenosine at the R/G site is not in as favorable an environment.

There are many similarities between DRADA and hRED1. The purification scheme used for the purification of hRED1 is comparable with that of DRADA (22), which reflects their similarity in amino acid composition and the capacity of both enzymes to bind dsRNA. Both proteins perform the same enzymatic reaction in the absence of additional cofactors and are capable of deaminating up to 50% of the adenosines present in extended dsRNA, and in particular, they both edit the adenosine at the R/G site in GluR-B pre-mRNA. Even though editing of GluR-B pre-mRNA occurs in the brain, the enzymes responsible for this site-specific editing are present and active in HeLa cells suggesting the existence of additional RNA substrates for these enzymes in other cell types.

There is evidence that the two enzymes have differences in their active site which may underly their ability to deaminate adenosines in different editing sites. Both enzymes have opposite behaviors in competition experiments with poly(U) and poly(G); poly(U) completely inhibits the activity of hRED1, whereas poly(G) inhibits DRADA (Fig. 3). Differences were also observed when *o*-phenanthroline was added to the editing reaction; hRED1 was less sensitive to the inhibitor than DRADA (Fig. 3). This suggests that if there is a Zn^{2+} ion at the active site of hRED1, its environment is different to that in DRADA. Antibodies directed against the deaminase domain of hRED1 only recognize hRED1 (Fig. 4*B*) and are able to inhibit its activity but not that of DRADA. Taken together, these results suggest that even though the two enzymes share a high sequence homology (32),[2] the property of their active sites is different.

Apolipoprotein B (apoB) mRNA is edited by the deamination of a specific cytidine to a uridine, generating a new translational stop codon (2, 3). This editing is mediated by a cytidine deaminase termed APOBEC-1, which is the catalytic subunit of a multi-subunit editing complex (37). Even though the editing of the apoB transcript occurs by a very different enzymatic mechanism, APOBEC-1, DRADA and RED1 resemble each other in their active sites, as the amino acids thought to coordinate a Zn^{2+} ion are conserved (24, 25, 32). Recently, the mRNA of neurofibromatosis type 1 (NF1) was also found to be edited by cytidine deamination (38). This editing requires a tripartite motif that is highly homologous to the apoB mooring sequence but is not influenced by addition of APOBEC-1, suggesting that a different catalytic subunit may be involved in this editing reaction.

RNA editing enzymes involved in specific deamination, the cytidine deaminases and the dsRNA adenosine deaminases, seem to belong to families of proteins whose members have different or overlapping substrate specificities. Because these enzymes are related in their primary amino acid sequence, their enzymatic mechanisms as well as their function are probably very similar. Thus, it is surprising that there exist so many different editing deaminases. A possible explanation could be that RNA editing has to be regulated. This can be achieved either at the RNA substrate level by the binding of auxiliary factors or by controlling the expression of RNA editing enzymes that differ in their specificity of RNA recognition. The degree of editing of a specific site can vary in different tissues and during development. Perhaps it is easier and more direct to regulate the expression of specific editing enzymes than to control the potential of sites in the RNA to be edited by different RNA binding proteins.

Acknowledgments—We thank Silvia Barabino, Sabine Krause, and Liam Keegan for reading this manuscript and Andrea Bürer for preparing HeLa nuclear extracts. We are grateful to P. H. Seeburg, T. Melcher, S. Maas, and M. Higuchi (Heidelberg) for sharing protocols and unpublished results.

Note Added in Proof—At a higher antibody titer, the hRED1 serum displays some cross-reactivity with DRADA.

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Purification of Human Double-stranded RNA-specific Editase 1 (hRED1) Involved in Editing of Brain Glutamate Receptor B Pre-mRNA

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J. Biol. Chem. 1997, 272:473-478.
doi: 10.1074/jbc.272.1.473