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HIPPOCAMPAL CHARACTERISTICS AND INVARIANT SEQUENCE ELEMENTS DISTRIBUTION OF *GLRA2* AND *GLRA3* C-TO-U EDITING

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RUNNING TITLE

Breaking Down Edited *GLRA2* and *GLRA3*

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ABSTRACT

Glycine receptor subunit alpha2 and alpha3 high-affinity variants, of which the subjacent amino acid substitutions issue from C-to-U RNA editing, are thought to influence tonic inhibition and pathophysiology. In light of the detection of *GLRA3* NM_006529:r.1157C>U and *GLRA2* NM_002063:r.1416C>U exchanges in hippocampus explants of temporal lobe epilepsy patients, we now examine the healthy situation, and relate it to the epileptic situation by ascertaining controls in a legitimate re-analysis. The *GLRA2* and *GLRA3* editing events that would ultimately result in a glycine receptor with the increased affinity occur in post-mortem, non-epileptic hippocampus. Most notably, their relative amounts do not significantly differ from those in increasedly damaged hippocampus explants. Whereas curbed relative amounts in epileptic explants without cell loss come out statistically significant. Local sequence alignment reveals invariant sequence stretches consistent in *GLRA2*/*GLRA3* and other edited transcripts that coincide with known *APOB* sequence elements. Concerning the essential *mooring* element, *GLRA2/GLRA3* comply strictly only with the motif's 5' part. While this lack of canonical *mooring* elements, and uncertain action of the famous deaminase APOBEC1, suggest a specific regulation of *GLRA2/GLRA3* editing, its reduction in less-damaged epileptic hippocampus could be attributed to anomalous epileptic neurogenesis.

INTRODUCTION

Epilepsy, a major neurological disorder, is associated with a large number of syndromes all involving unpredictable seizures. About 36 percent of epileptic patients have inborn or acquired resistances to antiepileptic pharmaceuticals. Neurosurgery of an identified epileptic focus can provide remedy from seizures for highly affected patients, but novel medications are still sought for [Mohanraj and Brodie, 2006; Kwan and Brodie, 2000; Wood, 2011].

Typically, epileptogenic activities at the hippocampus would be balanced by its inhibitory neurons. A disruption of hippocampus interneurons can contribute to excitatory-inhibitory imbalance within the epileptic network [Reyes 2006; Fröhlich et al., 2006; Dudek and Sutula, 2007; Nadler, 2003]. The glycine receptor (GlyR) is an inhibitory ligand-gated ion channel, like the GABA(A) receptor, although less abundant. It consists of an extracellular ligand binding domain, a transmembrane chloride pore, formed by

a pentameric setup of ligand-binding alpha subunits (GLRA1, GLRA2, GLRA3, also referred to as: GlyR alpha1, alpha2, alpha3) with the structuring beta subunit, and a rather variable intracellular domain [Langosch et al., 1990; Betz et al., 1999; Chattipakorn and Mc Mahon, 2002; Keck et al., 2008]. GlyR is located inter alia in the hippocampus, in interneurons, at synaptic sites, as well as extrasynaptically [Avila et al., 2013]. Modulation of excitatory postsynaptic potentials and inhibition in GABAergic interneurons via GlyR [Song et al., 2006] contributes to glycinergic tonic inhibition [Zhang et al., 2008; Xu and Gong, 2010]. Previously, a variant cDNA of the glycine receptor alpha3 subunit (*Glra3* NM_053724:r.701C>U) that encodes a certain proline-to-leucine exchange (GLRA3 NP_446176:p.Pro234Leu; colloquially referred to as: GlyR alpha3 P185L) was described. The exchange alters a loop at the bottom of the ligand-binding domain adjacent to the brink of the pore. The variant subunit displays an increased apparent affinity for its ligand, likely enabling a high-affinity GlyR that responds to ambient glycine [Meier et al., 2005; Ogino et al., 2007; Du et al., 2015; Sherwin, 1999]. The corresponding substitution *Glra2* NM_012568:r.1207C>U causes another high-affinity alpha subunit (GLRA2 NP_036700:p.Pro219Leu; colloquially referred to as: GlyR alpha2 P192L). In an analysis of hippocampus explants of pharmacoresistant temporal lobe epilepsy (TLE) patients, it was reported that the relative amounts of the same variations in humans, *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U (referred to as: GlyR alpha2-C575U and GlyR alpha3-C554U), increase under severe sclerotic cell loss and secondary generalized tonic-clonic seizure (grand mal) frequency. However, no information on the non-epileptic state was given. Genomic *GLRA2* DNA of patients carried no corresponding point mutation or single nucleotide polymorphism (SNP) of [Eichler et al., 2008]. This supported the assumption of C-to-U RNA editing being responsible for the variants' origin, deduced from a sequence stretch similar to a *spacer* sequence downstream of *Glra3* NM_053724:r.701C>U [Meier et al., 2005].

In C-to-U-edited apolipoprotein B mRNA (*APOB*), the *spacer* and the *mooring* sequence elements are constituents of a stem loop that exposes a cytidine to enzymatic deamination. Several additional sequence elements, most important for *APOB* editing, flank the deaminated position, from 60 nucleotides upstream to 160 nucleotides downstream [Hersberger et al., 1999; Maris et al., 2005; Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998]. Two

prominent proteins bind the *APOB* transcript: The cytidine deaminase apolipoprotein B editing complex (APOBEC1), and the RNA-binding protein Apobec complementary factor (ACF). In the liver, APOBEC1 deaminates a cytidine residue in the *APOB* transcript to a uridine residue, resulting in two protein variants, both physiologically relevant in energy metabolism [Teng et al., 1993; Lellek et al., 2000; Mehta et al., 2000; Henderson et al., 2001; Chester et al., 2000]. Until recently, few further C-to-U-edited transcripts had been characterized [Skuse et al., 1996; Wang et al., 2004]. Nowadays numerous, murine targets of APO1 have been described, leading to further characterization of the *mooring* motif [Rosenberg et al., 2012].

In our study post-mortem hippocampus material is used to display the healthy situation of C-to-U edited human *GLRA2* and *GLRA3*. By doing this, control groups are generated that augment the study mentioned above on epileptic hippocampus explants [Eichler et al., 2008]. Consequently, these results are presented alongside a legitimate re-analysis of that former series of experiments, adjusting the previous explanatory view on the relation of editing levels and course of disease. In addition, we describe the situation of several sequence elements and features most crucial to RNA editing in this matter.

MATERIALS AND METHODS

Quantitative cloning analysis

Post-mortem hippocampus tissue was obtained at autopsy from three individuals whose deaths had occurred out of hospital and were attributable to different natural and unnatural causes. Pre-existing pathologic conditions of the central nervous system were ruled out by histology, and underlying intoxications were ruled out by toxicological analysis. Specimen were comparable to the TLE neurosurgery explant series in terms of their local origin, age, and fixativeless procession [Eichler et al., 2008], except for their post-mortem delays that exceeded the surgery explant delays for up to five hours. RNA was isolated with Trizol (Thermo Fisher, Waltham, MA). Additionally, a commercially available pooled hippocampus RNA (provided by R. J. Harvey, London) was used [Rees et al., 2006]. Cloning assays to quantify *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U were performed in the identical setup described previously [Meier et al., 2005; Eichler et al., 2008]. Briefly, PCR used

degenerated mismatch primers that introduce restriction sites only into templates with the respective nucleotide exchanges. After restriction endonuclease digestion, insertion into custom vectors, transformation, and determination of confirmed-positive colony numbers, the percentage of clones with a variant insert proportionate to the total amount of control clones reflected the relative amount of edited *GLRA2/GLRA3* mRNA for each sample.

Instead of averaging triplicate measurements for one specimen and grouping the averages, all individual data points of each condition were grouped collectively. In post-mortem *GLRA2* measurements, thirteen out of fifteen individual data points represent the three matched individuals, while two out of fifteen data points represent the twenty pooled individuals. In the post-mortem *GLRA3*, six out of eight data points represent the three matched individuals, while two out of eight data points represent the twenty pooled individuals. The approach allowed re-analysing the dataset from formerly examined series of epilepsy surgery explants from pharmacoresistant TLE [Eichler et al., 2008], including also several specimen that had not yielded triplicate data. For comparability, general data grouping was retained. Seven individuals of the group of patients explants without marked hippocampal cell loss and sclerosis are represented by 29 data points in *GLRA2* measurements and by eighteen data points in *GLRA3*. In the group with increased damages attributable to sclerosis, cell loss, and frequent grand mal, the eighteen individuals are represented by 57 data points in *GLRA2* measurements, and by 53 data points in *GLRA3*. Mann-Whitney testing was performed with OriginPro 8G (OriginLab, Northampton, MA), with a p value less than 0.05 considered as statistically significant.

Database co-expression approach

Conserved domain database (CDD) entries [Marchler-Bauer et al., 2015] with cytidine deaminase domains were examined for co-expression with GlyR in hippocampus. Geneatlas datasets in BioGPS genereports were used to check for human brain expression of any genes of interest [Wu et al. 2009]. Murine in-situ-hybridization data publicly available from the Allen brain atlas [Lein et al., 2007] was used to compare the hippocampal expression patterns of the candidate deaminase *CDADC1* and of *GLRA2* and *GLRA3*. *GLRA4* expression data served as negative control. Of each set of images (antisense probe, sagittal plane, experiment numbers: 70525807, *CDADC1*; 71587739, *GLRA2*; 70723453, *GLRA3*;

70724744, *GLRA4*) consisting of nineteen to twenty sections, approx. the sixth (for the parasagittal plane) and thirteenth section (for the midsagittal plane) were selected, depending on pattern/plane visibility. To visualise the expression data in greyscale, the ISH and expression layers were transferred into the GIMP 2.8 raster graphics editor. Relevant expression domains were cropped. ISH layers were greyscaled, and brightness was set to +50. Expression layers were inverted, colour white was changed into transparency, and all remaining colour information was changed into black. Then both layers were merged.

Sequence comparison

Nucleotide sequences of C-to-U edited transcripts were traced from their respective genebank entries. Sequence conservation not revealed by conventional comparison matrices was exposed via manual alignment with GeneDoc [Nicholas and Nicholas, 1997]. Hyphens indicate introduced gaps. Shading mode was set to conserved, primary conservation (black shading, upper case consensus line) was set to 100 percent, secondary conservation (dark grey shading, lower case consensus line) was set to 75 percent and tertiary conservation (light grey shading, not marked in consensus line) was set to 40 percent.

RESULTS

GLRA2 and GLRA3 editing in hippocampus

Pronounced relative amounts of the C-to-U editing events *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U occur in post-mortem hippocampus RNA (see Fig. 1), detected via quantitative cloning assays targeting the respective cDNA nucleotide exchanges.

Conjoint with equivalently re-analysed epilepsy surgery explants, the post-mortem series serves as non-epileptic controls. These data show comparatively smaller relative amounts of edited *GLRA2/GLRA3* in explants without hippocampal cell loss than in both the post-mortem control group and the group of explants with marked cell loss and grand mal frequency. The relative amounts of the two latter groups do not show significant differences (see ‘n. s.’ in Fig. 1). The low of editing in the former group is statistically significant (asterisks in Fig. 1), attenuated in *GLRA2*, and more intensive in *GLRA3*.

Consistent stretches of invariant nucleotides, shared in GLRA2 and GLRA3 and other C-to-U-edited

transcripts

Using manual alignment of sequence subsections flanking the edited nucleotide, consistent sequence stretches of invariant and highly invariant nucleotides, shared between *GLRA2*/*GLRA3* as well as other C-to-U-edited transcripts, and distributed in an interlaced pattern, were identified (see Fig. 2A): Viewed from 5' to 3', these consistent stretches appear in the "A....ug.....AA......UG" stretch located at marker position 1-23, the "cAa.........UG....a" stretch located at position 48-66, the "Ga.u.a" stretch at position 80-85, and the "C.....a.uu......uga" stretch located around position 101-118 in Fig. 2A), each formed of up to seven shared nucleotides. Another, more weakly shared, stretch, that is not consistent in each inspected transcript, is "c.ga..tata", located at alignment position 63-80. Within the consistent stretches, variant or unique nucleotides appear, up to five or nine in number. Outside of the consistent stretches, variant and unique nucleotides are present more often and in longer succession (see alignment positions 24-47, 86-100, and 130-154 in Fig 2A).

The consistencies agree with sequence elements that have been demonstrated to be highly relevant for *APOB* editing: The "A....ug.....AA......UG" stretch resides directly at the 5' border of the upstream A-rich element determined in *APOB;* the "Ga.u.a" stretch encloses the *regulator* sequence element; the "C.....a.uu......uga" stretch comprises the well-known *spacer* and in parts the *mooring* element. GlyR alpha2 and alpha3 subunit transcript sequences lack most of the 3' and mid parts of the *mooring* motif, and they supplant it at position 120-124. In the 5' part they conform very well to the motif. Downstream of the *mooring*, another A-rich stretch is shared in all inspected transcripts. The 3' untranslated region (UTR) of rat *Glra3* features an almost exact *mooring* sequence, located 1121 nucleotides downstream of the edited cytidine (see Fig. 2B).

Concurrent features of CDADC1

Apparent from database queries, cytidine deaminase domain containing 1 (CDADC1) possesses two cl00269 cytidine deaminase-like superfamily domains that resemble the catalytic domain of APOBEC1 (see Fig. 3A and 3B). In murine hippocampus, there are overlaps in the gene expression patterns of *Cdadc1* in dentate gyrus and in CA1-CA3 with those of *Glra2*, as well as in hilus with *Glra3* (see Fig. 3C).

DISCUSSION

Lately, one could follow a controversial debate over the physiologic extent of RNA editing, highlighting once again the high level of detail necessary for its examination [Li et al., 2011; Chakravarti, 2011; Kleinman and Majewski, 2012; Wang et al., 2014]. Also the case of C-to-U-editing of GlyR alpha subunit RNA deserves thorough interpretation. After the original description, another study added very well to the feasible accuracy of detecting *Glra3* NM_053724:r.701C>U, whereas it was omitted to detect native, edited, *Glra3* transcripts [Meier et al., 2005; Nakae et al., 2008]. Later on, it was assumed that increased levels of editing were a specific attribute of the diseased, epileptic state [Eichler et al., 2008; Winkelmann et al., 2014].

The presented work describes for the first time distinct *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U RNA editing events in healthy hippocampus using heterogenous post-mortem material. This finding then enabled our legitimate re-analysis of the previous series of experiments with the post-mortem material integrated as necessary non-epileptic controls, showing the particular decline of the relative amounts of edited *GLRA2* and *GLRA3* in the lesser-damaged epileptic hippocampus. This is a relevant result and refines that very assessment of how epileptic damages and editing levels are associated in pharmacoresistant TLE.

Ectopic granule neurons or anomalous latency phase neurogenesis [Scharfman and Pierce, 2012; Parent et al., 2006; Parent et al., 1997] might provide one explanation for the pathological decrease in editing, and its different extent in the two subunit transcripts. Newly generated cells in the epileptic hippocampus can arise from more than one source area, and can migrate in varying amounts towards DG and CA1/CA2 (the main expression domains of *Glra2*), as well as towards CA4 [Crespel et al., 2005] (the main region of *Glra3* gene expression). In general, young neurons show less GlyR gene expression [Aroeira et al., 2011], and rodent prenatal hippocampal neurons seldom display pronounced *Glra2/Glra3* C-to-U events (unpublished observation). Accordingly a 'dilution' of hippocampal cells by aberrant, immature cells could be one possible cause of the apparent drop in editing. Sclerotic cell loss [Blümcke et al., 2000; Zhu et al., 1997; Blümcke et al., 2009] in the increased-damage explants group is not accompanied by significant reductions of C-to-U levels, compared to the non-epileptic post-mortem situation, yet they could still play a

role in the very slight reduction visible in this group.

Detecting editing events in PCR amplicons does not directly prove the existence of functional increased-affinity GlyR, but it tracks the activity of its deaminase, whose identity is another open problem. We present properties of CDADC1 that resemble those of the important cytidine deaminase APOBEC1 and of the concerned glycine receptor alpha subunits in hippocampus. In brain, gene expression of *APOBEC1* is relatively meagre (publicly available microarray data) and recent studies reported only very vaguely on its native expression in brain tissue [Gee et al., 2011; Guo a. 2011; Papavasiliou et al., 2014]. Despite this apparent absence, silencing of glycinergic currents following steric inhibition of Apobec cytidine deaminases with a cytidine analogue had been reported [Meier et al., 2005]. Although the drug indeed can affect Apobec-family deaminases, it should be argued that administering the cytidine analogue without sequence context could have influenced channel gating in different ways [Rausch et al., 2009; Wedekind and Smith, 2012; Seifert et al., 2011; Zong et al., 2012].

To determine the extent of the common sequence basis of well-characterized APOBEC1-mediated C-to-U editing with that of *GLRA2*/*GLRA3*, manual alignment of various cDNA sequences with documented C-to-U editing events was performed. The found consistent stretches of invariant nucleotides accord with the upstream A-rich, the *regulator*, and the *spacer* elements, which are very well characterized in *Apob* [Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998; Hersberger et al., 1999]. Discovery of additional, murine targets of APOBEC1 had led to further characterization of the *mooring* element [Rosenberg et al., 2012], but GlyR alpha2 and alpha3 transcript sequences fit this motif only in its 5’ part (it should be noted that the seemingly merged *spacer* and *mooring* elements of *Serinc1* also deviate from the motif). Whether the conventional *mooring* in the 3’ untranslated region (UTR) of rat *Glra3* corresponds to a respective C-to-U event is not known. Many of the additional murine transcripts carry their edited positions and their *mooring* elements in the 3’ UTR [Rosenberg et al., 2012], whereas in transcripts encoding GlyR alpha2 and alpha3 the edited positions and their adjacent 3’ deviant *mooring* elements are present in the open reading frame (ORF). A *mooring* sequence in an UTR can understandably comply to the motif more closely than in an ORF, where

mandatory compliance to codon information restricts sequence variability.

In this regard, two known synonymous SNP's in the inspected sequence section of *GLRA3* should be mentioned: Rs140655344 shifts a glutamate codon from GAG to GAA, making the A-rich region more A-rich. Rs41279513 can appear in the *spacer,* changing a glutamine codon from CAG to CAA, creating a *spacer* element identical to that of *APOB*. While invariant nucleotides could participate in secondary structures that capacitate binding or efficacy of a deaminase complex [Hersberger et al., 1999; Maris et al., 2005], deviations from the established elements could possibly even condition an unusual deaminase setup.

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STATEMENT OF ETHICS

Ethical approval for the conducted experiments was under Charité EA 1/142/05.

CONFLICTS OF INTEREST

The authors declare absence of conflicts of interest.

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FIGURE 1

Occurrence of *GLRA2/GLRA3* C-to-U RNA editing in healthy post-mortem hippocampus is compared with re-analysed, corresponding data obtained from surgical explants of pharmacoresistant epilepsy patients [Eichler et al., 2008]. The relative amounts of edited *GLRA2/GLRA3* (% edited) in healthy post-mortem hippocampus versus the epileptic situation with increased damage (attributable to sclerotic cell loss and frequent grand mal) do not differ significantly. By contrast, the low relative amounts in epileptic explants without hippocampal cell loss and marked sclerosis are significant.

FIGURE 2

A. Invariant nucleotides of *GLRA2/3* shared with other C-to-U edited transcripts agree with known sequence elements. Rectangular bars on top of the alignment indicate consistent stretches of invariant nucleotides. Below, hatched bars indicate weakly shared stretches, which are not consistent with all inspected transcripts (main diagonal hatching for consistencies in *GLRA2/GLRA3* and few other transcripts, but not with *APOB*; anti-diagonal hatching indicates consistencies in *APOB* and other transcripts, but not with *GLRA2/GLRA3*; and chequered hatching for consistencies in *GLRA2/GLRA3* and *APOB*, but not with every single one of the others). *APOB* sequence elements [Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998; Hersberger et al., 1999] are indicated with lines at the bottom; *mooring* elements are indicated with hooked lines. Transcript prefixes indicate species. Deaminated cytidine at alignment position 100.
B. The 3' UTR of rat *Glra3* (genebank entry M55250) contains a sequence nearly identical to the *mooring* element of *Apob*. Motif sequence from [Rosenberg et al., 2012].

FIGURE 3:

A. Domain structure of CDADC1 compared with those of two paradigmatic Apobec family members. CDADC1 contains two domains that match the zinc-binding region of the cl00269 cytidine deaminase-like superfamily, which also the catalytic domain of the C-to-U deaminase APOBEC1 belongs to. Modified after [Marchler-Bauer et al., 2015].

B. Sequence alignment of the similarities between CDADC1 and APOBEC1, within the cl00269 superfamily of cytidine and deoxycytidylate deaminases. The two deaminase domains of CDADC1 are abbreviated as dom1 and dom2. cd01286: Deoxycytidylate (dCMP) deaminase domain; pfam08210: APOBEC-like N-terminal (catalytic) domain; cd01283: Cytidine deaminase zinc-binding domain. Modified after [Marchler-Bauer et al., 2015].

C. Murine hippocampus gene expression patterns of *Cdadc1* notably overlap with those of *Glra2* in pyramidal layer of CA1-3 and in granule cell layer of dentate gyrus, with those of *Glra3* in hilus or polymorph layer of dentate gyrus. *Glra4* as negative control. Marker bars: parasagittal plane 500 µm; midsagittal plane 1000 µm. Modified after [Lein et al., 2007].

Fig.1

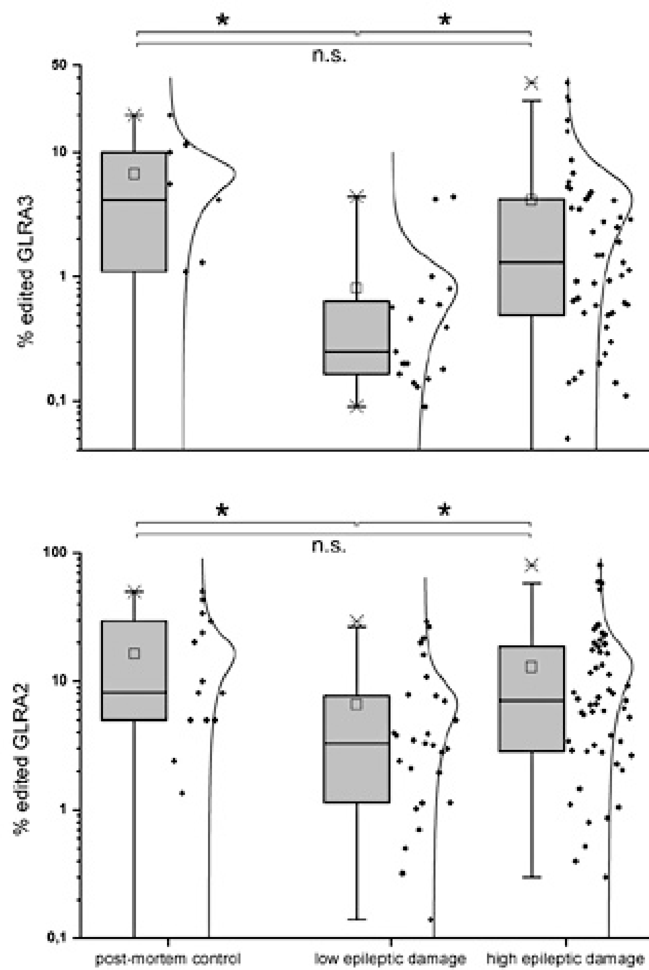
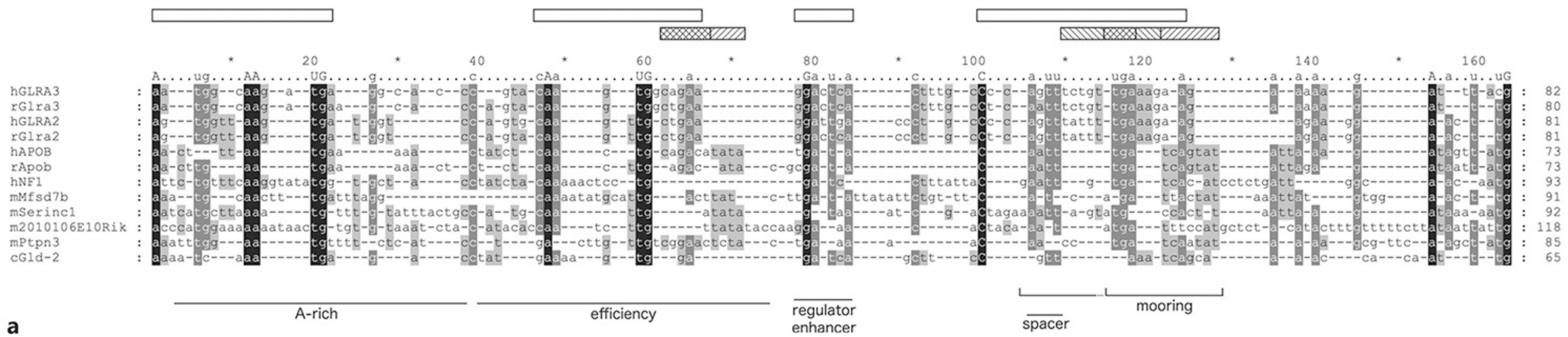
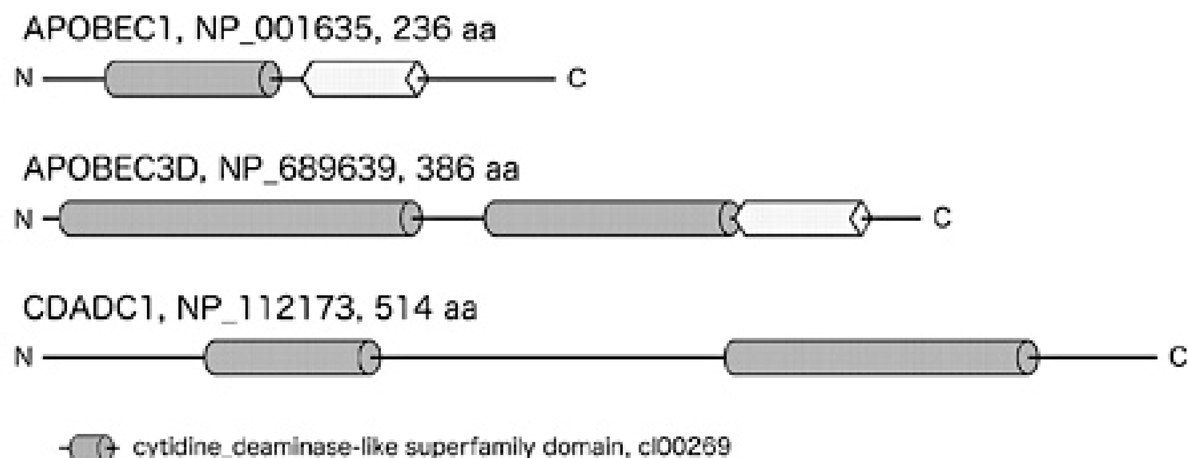


Fig.2



Glra3 3'UTR : TGATGAGTAT
Apob mooring : TGATCAGTAT
mooring motif : WRATYANTAT
b UGAUcAGUAU

Fig.3a + 3b

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CDADC1(dom1) 108 : DLHAGQIALIKH ... KPCSACLKMIVN : 143
CDADC1(dom2) 396 : IIHAEQNALTFR ... CPCDECVPLIKG : 435
cd01286       68 : TVHAEQNAILQA ... FPCIECAKLIIQ : 107
Apobec-1      27 : TNHVEVNFIKKF ... SPCWECSQAIRE : 102
Apobec-3d     76 : ENHAEMCFLSWF ... NPCLPCVVKVTK : 118
pfam08210     43 : SIHAEEQFFRAI ... SPCFDCAEKLAE :  86
cd01283       45 : TLCAERTAIGKA ... SPCGACRQVLAE :  89
                     hae    6          PC  C   6
```

b

Fig.3c