Minireview

Editing of messenger RNA precursors and of tRNAs by adenosine to inosine conversion

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Abstract The double-stranded RNA-specific adenosine deaminases ADAR1 and ADAR2 convert adenosine (A) residues to inosine (I) in messenger RNA precursors (pre-mRNA). Their main physiological substrates are pre-mRNAs encoding subunits of ionotropic glutamate receptors or serotonin receptors in the brain. ADAR1 and ADAR2 have similar sequence features, including double-stranded RNA binding domains (dsRBDs) and a deaminase domain. The tRNA-specific adenosine deaminases Tad1p and Tad2p/Tad3p modify A 37 in tRNA-Ala$_1$ of eukaryotes and the first nucleotide of the anticodon (A 34) of several bacterial and eukaryotic tRNAs, respectively. Tad1p is related to ADAR1 and ADAR2 throughout its sequence but lacks dsRBDs. Tad1p could be the ancestor of ADAR1 and ADAR2. The deaminase domains of ADAR1, ADAR2 and Tad1p are very similar and resemble the active site domains of cytosine/cytidine deaminases.

Key words: Messenger RNA editing; tRNA modification; RNA-specific adenosine deaminase; Cytosine/cytidine deaminase; Inosine

1. Introduction

RNA editing can be defined as cotranscriptional or post-transcriptional RNA processing reactions other than capping, splicing or 3′-end formation that change the nucleotide sequence of the RNA product. RNA editing reactions occur in many organisms and operate by different molecular mechanisms. Major editing types are the insertion or deletion of nucleotides and the substitution of bases by modification (for reviews see [1,2]). Here we discuss the reactions that lead to the conversion of adenosine (A) residues to inosine (I) in mammalian messenger RNA precursors (pre-mRNA) and in eukaryotic and prokaryotic tRNAs.

2. Double-stranded RNA-specific adenosine deaminases and pre-mRNA editing

Results accumulated in the last 10 years strongly suggest that one type of pre-mRNA editing in mammals is catalyzed by a group of enzymes originally called double-stranded RNA-specific adenosine deaminases (dsRAD) or DRADA (for ds RNA adenosine deaminase; reviewed in [3]). More recently, the enzymes have been renamed and are now called ADARs, for adenosine deaminases that act on RNA [4]. ADARs catalyze the hydrolytic deamination at the C-6 atom of adenosine, thus converting the nucleoside within the RNA chain to inosine (reviewed in [5–7]). In extended double-stranded RNA (dsRNA) in vitro, any adenosine can be deaminated unspecifically, although with some preference for C or U 5′ to the target A [8]. Such 'hypermutation type' of editing has also been observed in the RNAs encoding the matrix protein of measles virus and other negative-strand viruses and in polyoma virus. Non-viral examples are pre-mRNA of the *Drosophila 4f-rnp* gene, which encodes an RNA binding protein of unknown function and in transcripts encoding a voltage-dependent potassium channel in the squid *Loligo peali* (reviewed in [5,7,9]).

Inosine base-pairs with cytidine during reverse transcription when cDNA is made from an edited RNA. Thus, the editing event will appear as a conversion of adenosine to guanosine upon sequencing the cDNA. Since inosine is also read as guanosine by the translation apparatus [10], A to I editing in nuclear pre-mRNA can alter codon specificities in the mature mRNA and lead to changes of single amino acids within a protein. The first physiological substrates identified for this site-selective mode of RNA editing were pre-mRNAs encoding subunits of ionotropic glutamate-gated cation channels (GluRs) in the mammalian brain [11] (reviewed in [12,13]). Some of the resulting changes of amino acids alter the functional properties of GluRs formed by the assembly of edited and unedited subunits. The physiologically most important editing event controls the Ca^{2+} permeability of the AMPA-type GluRs which mediate fast excitatory synaptic transmission in central neurons. This site, designated the Q/R (glutamate/arginine) site according to the amino acid encoded by the unedited and edited RNA, respectively, is nearly 100% edited in mammalian AMPA receptor subunit GluR-B mRNA. The Q/R site is located in the second membrane segment (M2) of the GluR-B subunit and editing results in reduced permeability to Ca^{2+} ions of AMPA receptors containing this subunit. GluR-B pre-mRNA contains a second editing site (the arginine/glycine or R/G site) at the beginning of a small alternatively spliced exon that is located in front of the fourth membrane segment (M4). The extent of R/G site editing is regulated during the development of the mammalian brain and editing controls the kinetic properties of the AMPA receptor and leads to faster recovery rates from receptor desensitization [13].

In five of the approximately 18 mRNAs encoding sequence-related subunits for ionotropic GluRs, up to three codons per transcript can be altered by adenosine deamination [12,13]. In all cases investigated, this type of editing requires dsRNA

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structures, which are formed by intramolecular base-pairing between exonic nucleotides around the editing site and a short sequence that is complementary to the editing site, located in the downstream intron and termed ECS for 'editing site complementary sequence'. Mutations in the ECS that prevent base-pairing and thus change the structure of the RNA abolish its potential to be edited [14]. Deletion of the ECS from the nuclear gene leads to the complete loss of edited mRNA in mice and causes an early lethal phenotype [15]. This shows that the RNA structure is necessary for GluR-B pre-mRNA editing and underscores the physiological importance of the codon change at the Q/R site in vivo.

In addition to pre-mRNAs encoding GluRs other pre-mRNA substrates have been identified which are site-selectively edited by adenosine to inosine conversion. Examples are serotonin receptor 5HT2C pre-mRNA in the brain [16]) and hepatitis delta virus (HDV) antigenomic RNA [17]. Serotonin receptors transmit signals by coupling to G-proteins and stimulation of phospholipase C. They have diverse functions, including the regulation of synaptic transmission. Pre-mRNA encoding the 5HT2C receptor subtype is edited at four major sites within the second intracellular loop of the protein. Differential editing generates seven receptor isoforms which differ in their efficiency of signal transduction. Editing in the HDV antigenomic RNA converts a single adenosine residue to inosine and causes the change of a stop codon (UAG) to a tryptophan codon (UIG) in the viral RNA encoding hepatitis delta antigen. The shorter form of the protein participates in the transcription of HDV RNA; the extended protein derived from the edited RNA represses RNA replication and is involved in packaging the viral RNA.

In vitro systems have been developed with which the site-specific editing of the AMPA receptor GluR-B pre-mRNA could be reproduced by incubating synthetic RNA substrates in nuclear extract from HeLa cells [18–20]. The Q/R site and the R/G site of GluR-B pre-mRNA can be edited in vitro. Moreover, analysis of mutant substrates, nucleotide analysis, sequencing and mapping of fragments after nuclease digestion showed that editing occurred by deamination of adenosine residues and that in vitro editing depends on a double-stranded RNA configuration around the editing sites. These results suggested that the editing of GluR-B pre-mRNA was catalyzed by dsRNA-specific adenosine deaminase 1 (ADAR1), an enzyme that had been purified and cloned from various sources [21–25]. Indeed, purified or recombinant ADAR1 was able to specifically edit certain sites in synthetic pre-mRNAs. However, not all potential editing sites could be converted by the enzyme [26]. For example, hADAR1 could edit the R/G site of GluR-B pre-mRNA and the so-called hotspot 1 site within the adjacent intron but was inactive on substrates carrying the Q/R site. Fractionation of HeLa cell nuclear extracts showed that hADAR1 could be separated from a second activity which was able to edit the Q/R site [27,28]. The second activity corresponds to the human homologue of rat RED1 (RNA-specific editase 1, now called hADAR2 [29,30]). Like ADAR1, ADAR2 can also deaminate the adenosine at the R/G site. Thus, the two enzymes have distinct but overlapping substrate specificities (Fig. 1, right panel). In transfection experiments and in vitro, both ADAR1 and ADAR2 are required to catalyze the editing of the different sites in serotonin receptor pre-mRNA and of the glutamate receptor subtypes GluR-C, GluR-D, GluR-5 and GluR-6 [6,7].

3. Domain organization of ADARs

All ADARs consist of a single polypeptide with a modular domain organization (Fig. 1, left panel). A variable N-terminal region is followed by two or three dsRNA binding domains (dsRBD). dsRBDs are structures found in many proteins that interact with dsRNAs and are responsible for the recognition of the secondary and tertiary structure of double-

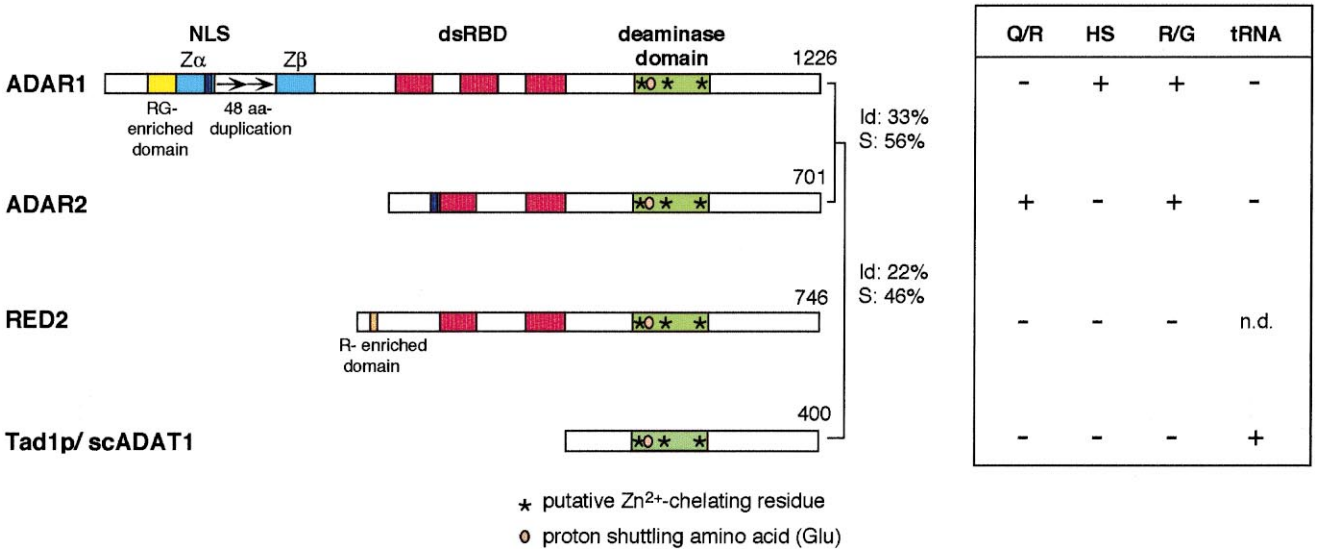

Fig. 1. Protein domain structure of mammalian adenosine deaminases that act on RNA (ADARs) and the *S. cerevisiae* protein Tad1p/scADAT1. ADAR1 and ADAR2 contain bipartite nuclear localization signals (NLS, dark blue), double-stranded RNAbinding domains (dsRBDs, red) and a catalytic deaminase domain (green box). Human ADAR1 contains an RG-enriched domain (yellow box) and a duplication of 48 amino acids (marked by arrows). The two Z-DNA binding domains are indicated as light blue boxes. The size of the proteins is indicated at the C-terminus by their number of amino acids. The aa sequence relation is indicated as percentage identity (Id) and similarity (S). The right panel depicts the ability of the RNA deaminases to modify selected sites in diverse substrates in vitro.

stranded regions within RNA molecules [31]. The active site is represented by a deaminase domain containing three conserved deaminase motifs [6,7]. The deaminase motifs are distantly related to those of cytosine deaminase, cytidine/deoxycytidine deaminases and to the catalytic subunit (APOBEC-1) of the mammalian apolipoprotein B (apoB) mRNA editing complex involved in C to U conversion [1,32] (see below).

The extended N-terminal portion of ADAR1 contains a potential nuclear localization signal and two Z-DNA binding motifs [33], the function of which is not known. Human ADAR1 has a perfect duplication of 48 amino acids and six Arg/Gly repeats within a stretch of 40 amino acids [24]. This RG-enriched domain may be a target for posttranslational modification by arginine dimethylation and may mediate the shuttling of the protein between the nucleus and the cytoplasm [34]. ADAR1 and ADAR2 occur as multiple isoforms generated by alternative splicing. These variants differ in enzyme activity and in their preference for editing different sites in pre-mRNA substrates (reviewed in [7]).

With the exception of RED2, which is expressed predominantly in the brain and for which no RNA substrate has been identified yet [35], ADARs are found in most mammalian tissues [6,7]. For this reason, A to I-type editing of messenger RNA precursors has been postulated to be a widespread phenomenon and the existence of many other target pre-mRNAs outside the central nervous system has been predicted. However, except for the few examples mentioned above, no additional physiological substrates have been found until now.

4. Adenosine deaminases acting on tRNA

The sequence information obtained from the cloning of ADARs was used to search for homologous proteins in the yeast genome and revealed an open reading frame (ORF) with sequence homology to the deaminase motifs in the mammalian RNA editing enzymes ADAR1 and ADAR2 [36] (Fig. 1). The yeast sequence contains the three putative zinc-chelating amino acids and a proton-transferring amino acid typical of cytidine deaminases (see below). However, the predicted protein lacks dsRDBs that are hallmarks of ADARs acting in the editing of messenger RNA precursors and on extended dsRNA substrates. Accordingly, in vitro assays with yeast extracts and dsRNA substrates did not reveal any such activity. Because modification of adenosine to inosine is also occurring in some tRNAs, we considered tRNAs potential substrates for the yeast enzyme. It is known that in all eukaryotes, inosine is present at position 37 (3′ adjacent to the anticodon) of tRNA-Ala$_1$ and at position 34 (the first base of the anticodon) of seven or eight different tRNA species in yeast and higher eukaryotes, respectively [37] (Fig. 2B). Inosine at position 34 plays a crucial role in protein synthesis by allowing alternative pairing with U, C, or A in the third position of codons [38]. The physiological significance of inosine 37, which is present in some eukaryotic tRNA-Ala isoacceptors and is secondarily converted to N^1-methylinosine by a SAM-dependent methyltransferase [39] (H. Grosjean, personal communication), is not known.

We have cloned the candidate yeast gene, expressed and purified the recombinant protein, and tested it with different tRNA substrates [36]. The protein is indeed a tRNA modification enzyme, which specifically deaminates adenosine 37 of alanine tRNA. We named the enzyme scADAT1 (for S. cerevisiae adenosine deaminase acting on tRNA 1) and its gene *TAD1*, for tRNA adenosine deaminase 1.

Adenosine or inosine at position 34 is not required for the modification of A 37 by Tad1p, as shown by testing mutant tRNA-Ala where A 34 was changed to guanosine. Removing one base from the anticodon loop abolished the reaction. Mutations affecting the three-dimensional structure of

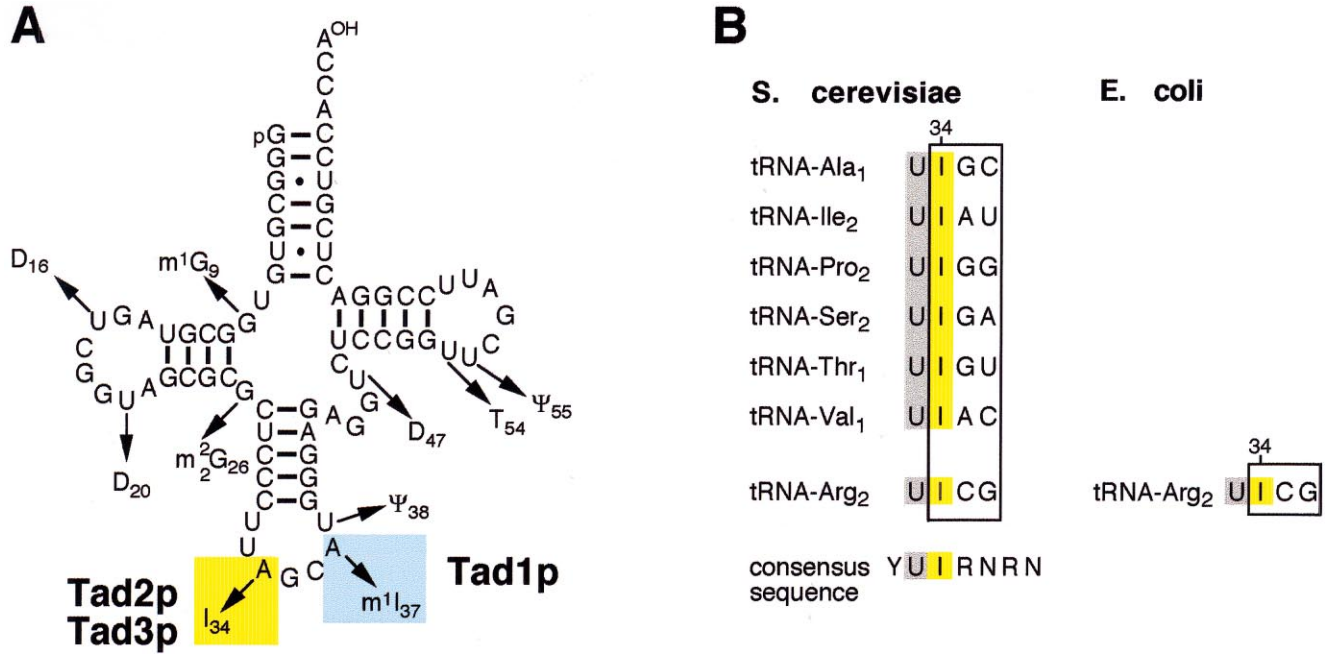

Fig. 2. A: Modifications in yeast tRNA-Ala. Adenosine at position 34 is converted to inosine by the tRNA-specific adenosine deaminase Tad2/Tad3 (yellow). Adenosine at position 37 is deaminated by Tad1p/ADAT1 (blue box) and further methylated to N^1-methylinosine. B: *S. cerevisiae* and *E. coli* tRNAs containing inosine (yellow) at the wobble position of the anticodon (boxed).

tRNA-Ala also prevent the formation of I 37 by Tad1p. Thus, the reaction requires the correct folding of the tRNA and is absolutely specific for tRNA-Ala.

The *TAD1* gene is not essential and a mutant strain (*tad1-1*) grows as efficiently as wild-type controls. The in vivo modification state of tRNA-Ala was monitored by RT-PCR amplification and sequence analysis. A 37 remained unmodified in the mutant cells whereas A 34 was edited normally. These results indicate that the modifications of A 37 and of A 34 are catalyzed by two different enzymes and that the activity converting A 34 to I 34 is not affected by the disruption of the *TAD1* gene.

A search of the yeast genome for the presence of an ORF related to *TAD1* revealed two additional yeast genes and their protein products (termed *TAD2*/scADAT2 and *TAD3*/scADAT3) that are distantly related to Tad1p (A. Gerber and W. Keller, unpublished results). Tad2p and Tad3p form a two-subunit heteromeric enzyme which specifically deaminates A 34 to I 34 in the wobble position of several tRNAs. In contrast to the *TAD1* gene, both *TAD2* and *TAD3* are essential genes. This underscores the importance of the inosine in the anticodon of the affected tRNAs to function as wobble base in codon-anticodon pairing.

We have recently also identified homologues of *TAD2* in *Escherichia coli* and in the human genome (J. Wolf, A. Gerber and W. Keller, unpublished results). *E. coli* Tad2p is involved in deaminating adenosine at position 34 of tRNA-Arg_2, the only bacterial tRNA containing inosine [39,40].

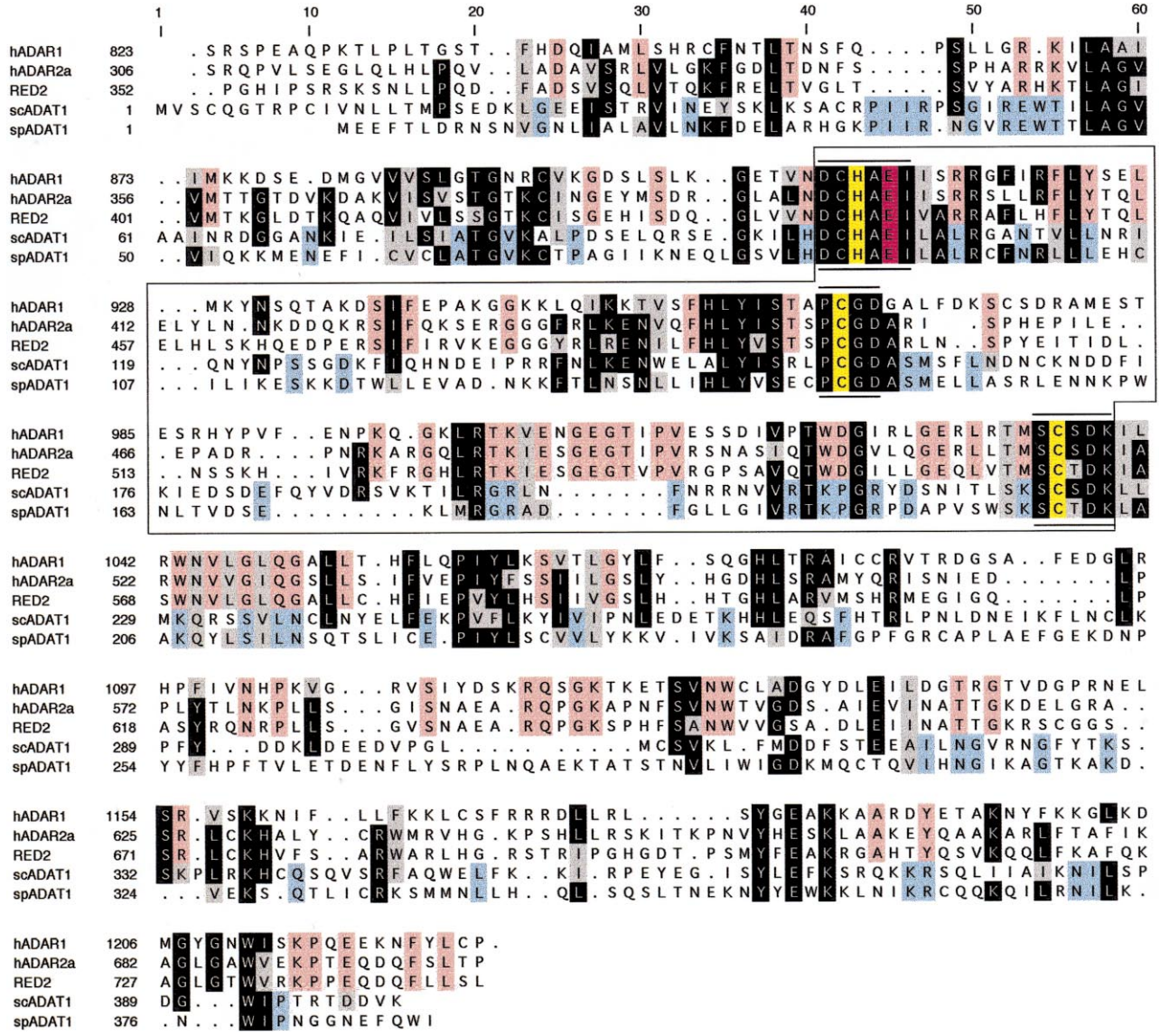

Fig. 3. Multiple sequence alignment of members of the ADAR family, scADAT1 and the putative *S. pombe* homolog spADAT1. Conserved amino acids (>60%) occurring in both types of enzymes are depicted within a black frame, similar amino acids which are conserved in at least four proteins are boxed in gray. Amino acids only conserved in ADARs are boxed light red and residues conserved in ADATs/Tad1p are marked blue. The deaminase domain (boxed) is characterized by conserved motifs (underlined) containing three putative Zn^{2+}-chelating residues (yellow) and a proton-transferring amino acid (red). The alignment was generated with the GCG software programs LINEUP (manual alignment) and PILEUP (computer alignment) with a gap weight of 3.0 [44]. The carboxy-terminal parts of yeast Tad1ps were further aligned manually to the other three sequences.

5. Tad1p, an ancestor of ADARs?

Tad1p is 22% identical and 46% similar to the C-terminal part of mammalian ADARs Fig. 1). Surprisingly, the sequence homology between Tad1p and ADAR1/2 extends through the entire protein sequence of Tad1p (Fig. 3) and is not limited to the characteristic deaminase domain found in all ADAR enzymes (see below). This suggests that the two classes of proteins, the mammalian pre-mRNA editing enzymes and the yeast tRNA modification enzyme, did not obtain catalytic capacity by independent evolution of a similar catalytic center, but more likely, the enzymes are directly related to each other. Because Tad1p has no dsRBD or any other recognizable RNA binding motif, it must recognize its tRNA substrate by some other means. Perhaps, the enzyme binds to RNA via residues localized within its deaminase domain in a manner similar to APOBEC-1 [41]). The sequence conservation between Tad1p and the mammalian RNA editing enzymes ADAR1 and ADAR2 and the similarities in their biochemical properties suggest a possible evolutionary relationship between the two classes of enzymes. Whereas the ADAR1 proteins contain three and ADAR2 and RED2 two dsRBDs, only one dsRBD was predicted for putative deaminases from *Caenorhabditis elegans*. The different numbers of dsRBDs may reflect a progressive acquisition of dsRBDs in the amino-terminal region upstream of the deaminase domain during evolution. In this scenario, the ancestral gene did not have dsRBDs, as is the case for the yeast gene *TAD1*. Alternatively, it is possible that Tad1p has lost its dsRBDs during evolution to gain the new function of a tRNA-specific adenosine deaminase. We favor the first scenario in which the dsRBD building blocks may have fused to the amino-terminus of an ancestral gene after the animal/fungal divergence.

The common ancestor of the deaminase domain of Tad1p and the other members of the ADAR family is not known. *TAD1* represents the only gene in the genomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* containing an ADAR-like deaminase domain. In contrast, no ADAR-related domain is present in the genome of *E. coli* K-12 [42]. This correlates with the observation that I 37 is only found in tRNA-Ala of eukaryotes.

6. Relationships between deaminase domains

The active site domains of enzymes catalyzing base conversions by hydrolytic deamination are shown in Fig. 4. As has been pointed out before [1,6,22,24,32,43], ADARs have a deaminase domain that resembles that of other nucleoside/nucleotide deaminases, including the active site domain of the subunit (APOBEC-1) which converts C to U in mammalian apolipoprotein B pre-mRNA. Remarkably, all of these enzymes show little resemblance to adenosine deaminases (ADAs, Fig. 4, bottom). Instead, they share distinctive sequence signatures containing three key amino acids (His, Cys, Cys) involved in zinc coordination (marked yellow in Fig. 4) and a glutamine known to be responsible for transition state stabilization and proton shuttling in the prototype enzyme cytidine deaminase of *E. coli* (ECCD; marked red in Fig. 4). These critical residues are embedded within short conserved sequence motifs (designated I, II and III in Fig. 4).

The deaminase domains of ADARs and Tad1p differ from the other proteins in the deaminase family by an extended spacing between the second and third cysteine. In addition,

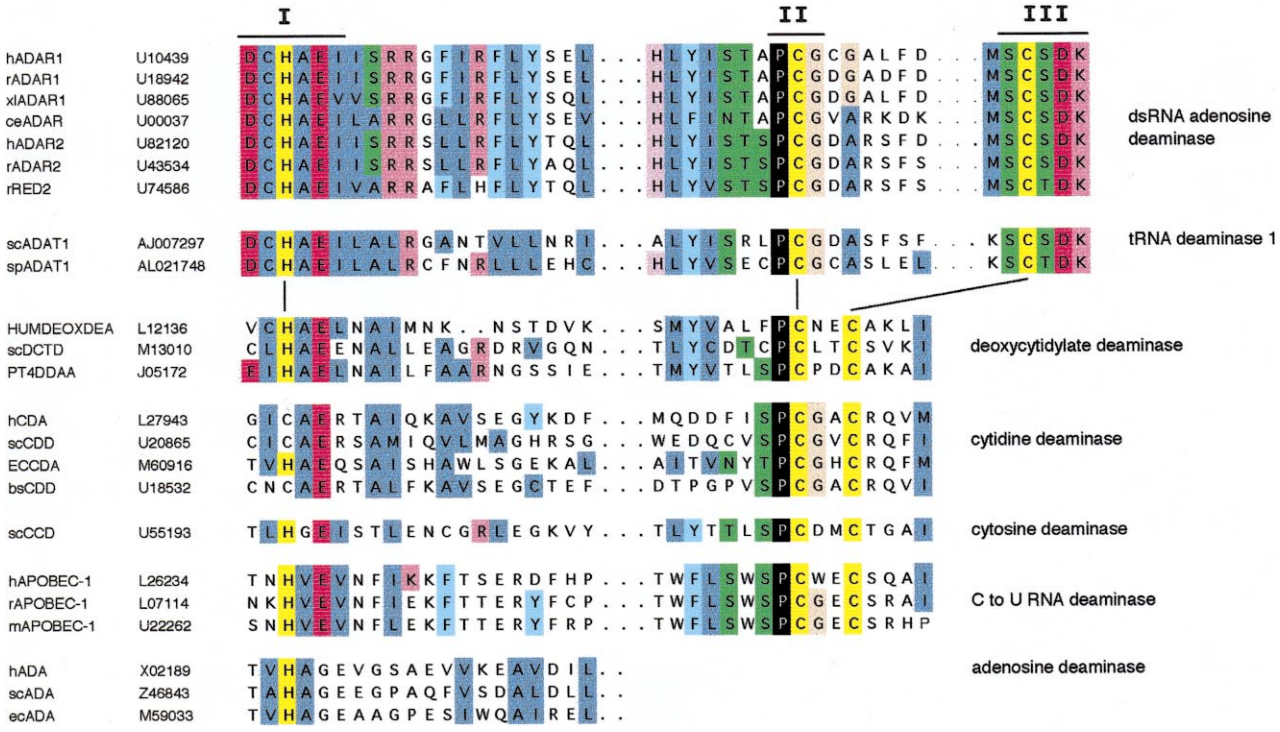

Fig. 4. Multiple sequence alignment of deaminase domains. Putative Zn^{2+}-chelating residues are yellow. Acidic amino acids are boxed in red, basic ones are in purple, hydrophilic in green, hydrophobic in blue, aromatic in light blue, the neutral glycine is brown. The highly conserved ADAR-like deaminase motifs I, II and III are overlined. The GenBank accession numbers are indicated in a column next to the names of the enzymes.

the third cysteine of ADARs and Tad1p is surrounded by four highly conserved amino acids (motif III).

Comparison of the sequence organization of the newly discovered tRNA-specific adenosine deminases Tad2p and Tad3p with the other modifying enzymes will hopefully help to understand how the diverse proteins of the deaminase family have evolved.

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