Analytical Biochemistry 399 (2010) 230–236

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A mammalian reporter system for fast and quantitative detection of intracellular A-to-I RNA editing levels

Willemijn M. Gommans, Jill McCane, Gregory S. Nacarelli, Stefan Maas *

Department of Biological Sciences, 111 Research Drive, Iacocca Hall D226, Bethlehem, PA 18015-4732, USA

ARTICLE INFO

Article history:
Received 9 October 2009
Received in revised form 22 December 2009
Accepted 31 December 2009
Available online 4 January 2010

Keywords:
RNA editing
Adar
Inosine
Adenosine deamination

ABSTRACT

An important molecular mechanism to create protein diversity from a limited set of genes is A-to-I RNA editing. RNA editing converts single adenosines into inosines in pre-mRNA. These single base conversions can have a wide variety of consequences. Editing can lead to codon changes and, consequently, altered protein function. Moreover, editing can alter splice sites and influences miRNA biogenesis and target recognition. The two enzymes responsible for editing in mammals are adenosine deaminase acting on RNA (ADAR) 1 and 2. However, it is currently largely unknown how the activity of these enzymes is regulated in vivo. Editing activity does not always correlate with ADAR expression levels, suggesting posttranscriptional or posttranslational mechanisms for controlling activity. To investigate how editing is regulated in mammalian cells, we have developed a straightforward quantitative reporter system to detect editing levels. By employing luciferase activity as a readout, we could easily detect different levels of editing in a cellular context. In addition, increased levels of ADAR2 correlated with increased levels of luciferase activity. This reporter system therefore sets the stage for the effective screening of cDNA libraries or small molecules for strong modulators of intracellular editing to ultimately elucidate how A-to-I editing is regulated in vivo.

Posttranscriptional processing of RNA molecules is essential for creating RNA and protein diversity from a limited set of genes. One important mechanism to increase transcriptome variety is A-to-I RNA editing in which single adenosines are converted into inosines within pre-mRNA [1,2]. The consequences of these single base conversions can be numerous. Inosine is read as guanosine by the translational machinery; thus when editing occurs within the coding region of pre-mRNA molecules, codons can be altered. Currently, there are 14 genes known in which editing results in nonsynonymous codon changes [2–4]. The amino acid substitution often alters the function of the protein underscoring the potential impact of editing by single A-to-I modifications.

A-to-I editing can also occur within the noncoding regions of pre-mRNA molecules. Especially, high levels of editing are observed within primate-specific Alu repeats [5–7]. The consequences of editing within Alu repeats are still largely unknown. Recently, it has been suggested that editing within Alu elements might serve as a nuclear retention signal [8], although this has been contradicted by other reports [9]. Also, editing in pre-mRNA can result in the alteration or creation of a splice site [10,11], generating a diverse set of mRNA and protein molecules.

Besides the editing of pre-mRNA, A-to-I editing also occurs in noncoding RNA molecules. Recently, several groups have demonstrated editing of (pri-)miRNA sequences, causing alterations in miRNA processing or target recognition [12–17]. Currently, it is estimated that approximately 16% of all pri-miRNAs are a substrate for the A-to-I editing machinery [18].

Editing is mediated by the family of adenosine deaminases acting on RNA (ADARs).[1] Of the three known family members (ADAR1–3), only ADAR1 and 2 have demonstrated editing activity to date [1,19,20]. Both enzymes bind to double-stranded (ds-)RNA, which is a prerequisite for editing substrates. However, besides the formation of an intramolecular fold-back structure and certain 5′ and 3′ neighbor preferences, the substrate specificity of ADAR1 and 2 is still poorly understood.

At this moment it is unclear how editing activity is regulated in vivo. We and others demonstrated previously that the observed editing extent does not always correlate with the expression levels of ADAR1 and 2, pointing towards a posttranscriptional or posttranslational regulatory mechanism [21–23]. For example, inositol hexaphosphate (IP_6) has been shown to be deeply buried within the catalytic domain of ADAR2, suggesting a role for this molecule in modulating ADAR2 editing activity [24]. However, the regulatory functionality has not yet been proven in an appropriate intracellular system.

* Corresponding author. Fax: +1 610 758 4004.
E-mail address: swm3@lehigh.edu (S. Maas).

[1] *Abbreviations used:* ADAR, adenosine deaminase acting on RNA; PCR, polymerase chain reaction.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.12.037

Currently, screening for alterations in editing activity within a cellular environment is a laborious procedure. It requires the subcloning and sequencing of individual cDNA molecules to determine the degree of A to G substitution within a certain substrate. Therefore, in this study we generated a straightforward system to detect sudden changes in intracellular editing activity. We constructed a mammalian reporter system in which the translational start codon of the *Renilla* luciferase open-reading frame is followed by the stop codon UAG, which is located within an editing substrate [25]. On editing, the stop codon is converted into the codon for tryptophan (UIG), allowing for the synthesis of the *Renilla* luciferase protein. We demonstrate that by employing *Renilla* luciferase activity as a readout, we are able to screen for changes in editing activity within a natural cellular environment. This readout system thus generates a straightforward approach for identifying novel regulators of A-to-I editing within a cellular context.

Materials and methods

Cell culture

The human embryonic cervical cancer cell line HeLa (ATCC CCL-2) was cultured at 37 °C 5% CO_2 in minimum essential medium Eagle containing 2 mM glutamine (Cellgro Mediatech Inc., Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), and an antibiotic–antimycotic mixture (Gibco Invitrogen corporation, Grand Island, NY).

Constructs

To obtain the different *Renilla* luciferase open-reading frames with methionines substituted for either leucine or serine, site-directed mutagenesis was performed using the commercial vector siCheck-2 (Promega, Madison, WI) as the template. All primers used for the site-directed mutagenesis are listed in Table 1A. Similarly, site-directed mutagenesis was also performed to insert Kozak sequences at different positions within the *Renilla* luciferase open-reading frame. The primers for the site-directed mutagenesis are listed in Table 1B.

The final plasmid containing the hairpin and intronic sequences was generated as follows: First, the hairpin according to Beal and co-workers [25] was introduced upstream of the *Renilla* luciferase open-reading frame. To this end, siCheck-M1k1 was restricted with NheI and treated with mungbean nuclease to remove the overhanging single-stranded ends. The hairpin was introduced by filling in the overlapping oligonucleotides 5′-ACCATGGCCCATCACCATCACGTTTAGGTGGGTGGAATAGTATACCATTCGTGGTATAGTATCCCACCTACCCAGACGGGCGGCAGCGGC-3′ and 5′-TCAGGCTGCCGCCGCTGCCGCCGCTGCCGCCCGTCTGGGTAGGTGGGATACTATACCACGAA-3′. With these oligonucleotides, the hairpin is preceded by a Kozak sequence and a his tag, and followed by a glycine (GGS)3 linker. Subsequently, we introduced the 5′-splice site within the loop of the hairpin structure by site-directed mutagenesis with the primers 5′-CGTTTAGGTGGGTGGAATAGTATACCATTCAGGTAAGTGGTATAGTATCCCACC-3′ and 5′-GGTGGGATACTATACCACTTACCTGAATGGTATACTATTCCACCCACCTAAACG-3′. For the subsequent insertion of the 3′-splice site, we first introduced a NheI site in the plasmid by site-directed mutagenesis with the primers 5′-CCTACCCAGACGGGCGGCTAGCGGCGGCAGCGGC-3′ and 5′-GCCGCTGCCGCCGCTAGCCGCCCGTCTGGGTAGG-3′. The resulting vector was restricted with NheI and treated with mungbean nuclease. Finally, the intronic sequences together with the 3′-splice site were introduced by inserting the PCR product generated using siCheck-2 (Promega) as the template and with the primers 5′-ATCAAGGTTACAAGACAGG-3′ and 5′-CTGCCGCCTGTGGAGAGAAAGGCAAAGTGG-3′.

For generating the preedited hairpin structure, the to-be-edited A was altered into a G by site-directed mutagenesis with the primers 5′-CCATCACCATCACGTTTGGGTGGGTGGAATAGTATACC-3′ and 5′-GGTATACTATTCCACCCACCCAAACGTGATGGTGATGG-3′.

Table 1A
Primer sequences for substituting methionines for leucine in the Renilla luciferase ORF.

Substituted methionine ATG to CTG	Primer (5′–3′)
M1	CGACTCACTATAGGCTAGCCACCCTGGCTTCCAAGGTGTACG
M1	CGTACACCTTGGAAGCCAGGGTGGCTAGCCTATAGTGAGTCG
M2	CGAGCAACGCAAACGCCTGATCACTGGGCCTCAGTGG
M2	CCACTGAGGCCCAGTGATCAGGCGTTTGCGTTGCTCG
M3	GCTCGCTGCAAGCAACTGAACGTGCTGGACTCC
M3	GGAGTCCAGCACGTTCAGTTGCTTGCAGCGAGC
M4	CCTGATCTGATCGGACTGGGTAAGTCCGGCAAGAGC
M4	GCTCTTGCCGGACTTACCCAGTCCGATCAGATCAGG
M6	GCTTGAGAATAACTTCTTCGTCGAGACCCTGCTCCCAAGCAAGATCATGC
M6	GCATGATCTTGCTTGGGAGCAGGGTCTCGACGAAGAAGTTATTCTCAAGC
ATG to AGC	
M4	CCTGATCTGATCGGAAGCGGTAAGTCCGGCAAGAGC
M4	GCTCTTGCCGGACTTACCGCTTCCGATCAGATCAGG

Table 1B
Primer sequences for inserting decoy kozak sequences in the Renilla luciferase ORF.

Position insertion Kozak sequence	Primer (5′–3′)
1	CTCACTATAGGCTAGCCACCATGGCTTACCATGGGGTGTACGACCCCGAGCAACGC
1	GCGTTGCTCGGGGTCGTACACCCCATGGTAAGCCATGGTGGCTAGCCTATAGTGAG
1 + M1	CTCACTATAGGCTAGCCACCCTGGCTTACCATGGGGTGTACGACCCCGAGCAACGC
1 + M1	GCGTTGCTCGGGGTCGTACACCCCATGGTAAGCCAGGGTGGCTAGCCTATAGTGAG
2	CCTCAGTGGTGGGCTCGCTGCAAGCAACCATGGGTGCTGGACTCCTTCATCAACT
2	AGTTGATGAAGGAGTCCAGCACCCATGGTTGCTTGCAGCGAGCCCACCACTGAGG
3	CCTCAGTGGTGGGCTCGCTGCAAGCAACCATGGAACGTGCTGGACTCCTTCATCAACT
3	AGTTGATGAAGGAGTCCAGCACGTTCCATGGTTGCTTGCAGCGAGCCCACCACTGAGG
4	GCTGGACTCCTTCATCAACTACCATGGTTCCGAGAAGCACGCCGAGAACG
4	CGTTCTCGGCGTGCTTCTCGGAACCATGGTAGTTGATGAAGGAGTCCAGC
5	CCTTCATCAACTACTATGATTCCGACCATGGCGCCGAGAACGCCGTGATTTTTCTGC
5	GCAGAAAAATCACGGCGTTCTCGGCGCCATGGTCGGAATCATAGTAGTTGATGAAGG
6	CGAGAACGCCGTGATTTTTCACCATGGTAACGCTGCCTCCAGC
6	GCTGGAGGCAGCGTTACCATGGTGAAAAATCACGGCGTTCTCG

Transient transfections

To determine the functionality of the different *Renilla* luciferase constructs, transient transfections were performed in HeLa cells. All transfections were done in 24-wells plates (Becton Dickinson and Company, Franklin Lakes, NJ) using Superfect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed with cell culture lysis buffer (Promega, Catalogue Number E1960) and luciferase activity was measured using a fluorometer (Thermo Fisher Scientific, Waltham, MA). All constructs constitutively ex-