Neuroscience Research 73 (2012) 42–48

Contents lists available at SciVerse ScienceDirect

Neuroscience Research

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

RNA editing of the Q/R site of GluA2 in different cultured cell lines that constitutively express different levels of RNA editing enzyme ADAR2

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ARTICLE INFO

Article history:
Received 1 December 2011
Received in revised form 2 February 2012
Accepted 3 February 2012
Available online 18 February 2012

Keywords:
AMPA receptor
GluA2
RNA editing
Adenosine deaminase acting on RNA type 2 (ADAR2)
Splicing
Amyotrophic lateral sclerosis (ALS)

ABSTRACT

Adenosine deaminase acting on RNA 2 (ADAR2) catalyzes RNA editing at the glutamine/arginine (Q/R) site of GluA2, and an ADAR2 deficiency may play a role in the death of motor neurons in ALS patients. The expression level of ADAR2 mRNA is a determinant of the editing activity at the GluA2 Q/R site in human brain but not in cultured cells. Therefore, we investigated the extent of Q/R site-editing in the GluA2 mRNA and pre-mRNA as well as the ADAR2 mRNA and GluA2 mRNA and pre-mRNA levels in various cultured cell lines. The extent of the GluA2 mRNA editing was 100% except in SH-SY5Y cells, which have a much lower level of ADAR2 than the other cell lines examined. The ADAR2 activity at the GluA2 pre-mRNA Q/R site correlated with the ADAR2 mRNA level relative to the GluA2 pre-mRNA. SH-SY5Y cells expressed higher level of the GluA2 mRNA in the cytoplasm compared with other cell lines. These results suggest that the ADAR2 expression level reflects editing activity at the GluA2 Q/R site and that although the edited GluA2 pre-mRNA is readily spliced, the unedited GluA2 pre-mRNA is also spliced and transported to the cytoplasm when ADAR2 expression is low.

1. Introduction

Adenosine deaminase acting on RNA 2 (ADAR2) belongs to a family of mammalian RNA-editing enzymes (ADAR1–3) that have a catalytic domain responsible for the conversion of adenosine to inosine (A-to-I conversion) and two or three double-stranded RNA-binding domains (Kim et al., 1994; O'Connell et al., 1995, 1997; Melcher et al., 1996a,b; Chen et al., 2000). ADAR2 is expressed ubiquitously in mammalian organs and localizes predominantly in the nucleus (Desterro et al., 2003; Sansam et al., 2003; Aizawa et al., 2010). A-to-I conversions occur most abundantly in transcripts that are expressed predominantly in the central nervous system (CNS) of humans and other mammals, and ADAR2 plays a key role in regulating neuronal function by converting A into I in several pre-mRNAs (Kwak et al., 2008; Nishimoto et al., 2008; Riedmann et al., 2008; Sakurai et al., 2010), including the glutamine/arginine (Q/R) site in GluA2, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor (Brusa et al., 1995; Higuchi et al., 2000). In both mammalian and human CNSs, RNA editing at the GluA2 Q/R site occurs in neurons with virtually 100% efficiency throughout life beginning at the embryonic stage (Burnashev et al., 1992; Paschen and Djuricic, 1995; Carlson et al., 2000; Kawahara et al., 2003, 2004a,b). This conversion regulates the biologically indispensable properties of AMPA receptors, including permeability to Ca^{2+} and receptor trafficking (Hume et al., 1991; Burnashev et al., 1992; Lomeli et al., 1994). AMPA receptors containing an unedited GluA2 are permeable to Ca^{2+}, and ADAR2-null mice exhibit fatal epilepsy (Higuchi et al., 2000). In conditional ADAR2 knockout mice, motor neurons lacking ADAR2 slowly die as a result of the lack of GluA2 Q/R site-editing (Hideyama et al., 2010). Notably, motor neurons in patients with sporadic amyotrophic lateral sclerosis (ALS) express abundant unedited GluA2 mRNA (Takuma et al., 1999; Kawahara et al., 2004a; Kwak and Kawahara, 2005; Kwak et al., 2010), indicating that abnormal regulation of ADAR2 may play a role in the pathogenesis of sporadic ALS. Therefore, elucidation of the regulatory mechanisms underlying the ADAR2-mediated RNA editing of the GluA2 Q/R site is necessary.

In contrast to the neurons in the mammalian CNS, ADAR2-mediated GluA2 Q/R site-editing occurs with less than 100% efficiency in cultured cells (Rueter et al., 1995; Maas et al., 2001). Splicing and RNA editing are both post-transcriptional events that

Abbreviations: ADAR, adenosine deaminase acting on RNA; dsRNA, double-stranded RNA; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; GAPDH, glyceraldehyde-3 phosphate dehydrogenase.

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doi:10.1016/j.neures.2012.02.002

modify pre-mRNA. The double strand RNA structure required for ADAR2-mediated GluA2 Q/R site-editing is formed by an exon sequence encoding the Q/R site and the exon complementary sequence located in the downstream intron of GluA2 pre-mRNA. It is common that sites of selective editing are in the proximity of splice sites and that the editing event is dependent on intron sequence. In these cases, the complex of both editing and splicing might corroborate proper transcription (Bass, 2002; Nishikura, 2004). Several lines of evidence showed that the editing and splicing of the GluA2 pre-mRNA influence each other and the A-to-I conversion at the Q/R site has a positive effect on splicing (Gan et al., 2006; Ryman et al., 2007; Schoft et al., 2007).

In this study, we investigated the extent of Q/R site-editing of the GluA2 mRNA and pre-mRNA and the expression levels of the ADAR2 mRNA, GluA2 pre-mRNA and mRNA in several cultured cell lines. We found that the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA was correlated with the extent of RNA editing at the Q/R site of the GluA2 pre-mRNA. We also found that although the edited GluA2 pre-mRNA was more readily spliced than unedited pre-mRNA, the unedited GluA2 mRNA was expressed when the ADAR2 level was low and that both the edited and unedited free GluA2 mRNA were transported to the cytoplasm.

2. Materials and methods

2.1. Cell culture

Tet-on-HeLa cells (TAKARA, Tokyo, Japan) were cultured in MEM-α medium (WAKO, Tokyo, Japan) supplemented with 10% Tet System Approved fetal bovine serum (TAKARA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) with incubation condition at 37 °C with 5% CO_2. TetHeLaG2m cells (Nishimoto et al., 2008; Sawada et al., 2009) were cultured in the same condition except adding 0.75 μg/ml puromycin (TAKARA) in the medium. Rat INS1-D cells were cultured in RPMI-1640 (Invitrogen) with 11.1 mmol/l D-glucose (WAKO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES (WAKO), 2 mmol/l L-glutamine (WAKO), 1 mmol/l sodium pyruvate (WAKO), and 50 μmol/l mercaptoethanol with incubation condition at 37 °C with 5% CO_2. Human neuroblastoma SH-SY5Y cells and human glioma U87MG and T98G cells were cultured in DMEM HAM F-12 (Invitrogen) and DMEM high glucose (WAKO), respectively, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 at 37 °C. The culture medium was changed once after 24 h and then every 2 days. The cells were grown in 6-well plates at the density of 3.5×10^4 cell/cm^2 and were cultured for 24 h and 48 h, and then were harvested. In some experiments, we harvested cells after 72 h in culture (for Fig. 3).

2.2. Protein extraction and Western blot analysis

Nuclear and cytoplasmic fractions were separated with PARIS Protein and RNA Isolation System (Life Technologies) according to the manufacturer's instructions. The cultured cells (approximately 10^7 cells) were washed with cold PBS. The harvested cells were resuspended in 300 μl of ice-cold cell fractionation buffer of PARIS protein and RNA isolation system (Life Technologies), gently agitated, then incubated on ice for 10 min. The incubated cells were centrifuged at 500 × *g* at 4 °C for 5 min. The resultant supernatant was used as the cytoplasmic fraction and the pellet as the nuclear fraction. Aliquots of the nuclear and cytoplasmic fractions were boiled with 300 μl of 2× SDS gel loading buffer, and subjected to SDS-PAGE. After electrophoresis, proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) and immunoblotting for histon protein (MAB052 (CHEMICON, Temecula, CA, USA) (1:1000), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (MAB374 (CHEMICON) (1:600) and ADAR2 (E-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000) was conducted. For secondary antibodies, goat anti-mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA) (1: 5000), mouse and goat anti-mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA) (1:5000) and donkey anti-goat IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000) were used for respective primary antibodies. Visualization was carried out using ECL plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ, USA). Specific bands were detected with a LAS 3000 system (Fujifilm, Tokyo).

2.3. RNA extraction and reverse transcription

RNA was extracted from their cells in each well using an RNAspin mini (GE Healthcare Bioscience) kit or PARIS Protein and RNA Isolation System (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA extracted from nuclear and cytoplasmic fractions after treatment with *DNaseI* (Invitrogen) using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bioscience) and 50 ng random primer (Invitrogen) in a final volume of 33 μl according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed under the condition as indicated in Table 1. The PCR amplification began with a 2 min denaturation step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 10 s, annealing at 66 °C for 30 s and extension at 68 °C for 40 s. Nested PCR reaction was initiated at 95 °C for 2 min and amplification of templates was performed by 30 cycles of denaturation at 95 °C for 10 s annealing and extension at 68 °C for 30 s.

2.4. Analyses for extents of A-to-I editing sites

Extents of RNA editing at the Q/R site in GluA2 mRNA and pre-mRNA were expressed as the proportion (%) of edited transcripts in total transcripts calculated by quantitative analyses of the restriction digests of PCR products with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) (Kawahara et al., 2003, 2004b; Bhalla et al., 2004). The PCR products for GluA2 mRNA and GluA2 pre-mRNA were cleaved by *BbvI* (New England Biolabs, Ipswich, MA, USA).

Digestion of The PCR products with *BbvI* yields two bands at 129 and 71 bp when they were originated from edited GluA2 mRNA, whereas three bands at 91, 38 and 71 bp when they were originated from unedited GluA2 mRNA. The 71-bp band was originated from both edited and unedited GluA2 mRNA, while the 129-bp band was originated from only edited mRNA, hence the extent of editing was calculated as the molarity of the former to that of the latter for each sample.

2.5. Preparation of standard cDNA for quantitative polymerase chain reaction

Using the primers shown in Table 1, 2 μl of cDNA extracted from HeLa cells (human control) was subjected to PCR with 1 μl of advantage 2 polymerase mix (BD Biosciences Clontech, Palo Alto, CA, USA). After gel purification, PCR products were subcloned using the TOPO TA cloning kit (Invitrogen), and clones containing inserts were sequenced with an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The concentration of each standard plasmid was measured spectrophotometrically at 260 nm (Nano Drop™ ND-1000; Nano Drop Technologies, Wilmington,