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Determination of editors at the novel A-to-I editing positions

Yoshinori Nishimoto [a,b,1], Takenari Yamashita [a,1], Takuto Hideyama [a], Shoji Tsuji [a], Norihiro Suzuki [b], Shin Kwak [a,*]

[a] Department of Neurology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Department of Neurology, Graduate School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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Abstract

A-to-I RNA editing modifies a variety of biologically important mRNAs, and is specifically catalyzed by either adenosine deaminase acting on RNA type 1 (ADAR1) or type 2 (ADAR2) in mammals including human. Recently several novel A-to-I editing sites were identified in mRNAs abundantly expressed in mammalian organs by means of computational genomic analysis, but which enzyme catalyzes these editing sites has not been determined. Using RNA interference (RNAi) knockdowns, we found that cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) mRNA had an ADAR2-mediated editing position and bladder cancer associated protein (BLCAP) mRNA had an ADAR1-mediated editing position. In addition, we found that ADAR2 forms a complex with mRNAs with ADAR2-mediated editing positions including GluR2, kv1.1 and CYFIP2 mRNAs, particularly when the editing sites were edited in human cerebellum by means of immunoprecipitation (IP) method. CYFIP2 mRNA was ubiquitously expressed in human tissues with variable extents of K/E site editing. Because ADAR2 underactivity may be a causative molecular change of death of motor neurons in sporadic amyotrophic lateral sclerosis (ALS), this newly identified ADAR2-mediated editing position may become a useful tool for ALS research.

Keywords: RNA editing; Adenosine deaminase acting on RNA (ADAR); Immunoprecipitation (IP); RNA interference (RNAi); Cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2); Bladder cancer associated protein (BLCAP)

1. Introduction

Adenosine deaminases acting on RNA (ADARs) catalyze A-to-I RNA editing in a wide range of organisms including human. Among three structurally related ADARs (Keegan et al., 2001; Bass, 2002; Maas et al., 2003), ADAR1 is indispensable for normal development (Wang et al., 2000) and ADAR2 plays a key role in the regulation of neuronal excitability in mice (Brusa et al., 1995; Higuchi et al., 2000), and presumably in the pathogenesis of sporadic amyotrophic lateral sclerosis (ALS) in humans, by specifically editing the Q/R site of GluR2, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Takuma et al., 1999; Kawahara et al., 2004; Kwak and Kawahara, 2005). Extensive A-to-I conversion occurs in the large numbers of mRNAs (Burns et al., 1997; Higuchi et al., 2000; Wang et al., 2000; Bhalla et al., 2004), and studies using a computational genomic approach have recently demonstrated several novel A-to-I editing sites in mRNAs abundantly expressed in peripheral as well as neuronal tissues (E.Y. Levanon et al., 2005). Using immunoprecipitation (IP) and the RNA interference (RNAi) knockdown system *in vitro*, we investigated whether the recently reported A-to-I editing sites in cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2), filamin A (FLNA), bladder cancer associated protein (BLCAP), and insulin-like growth factor binding protein 7 (IGFBP7) mRNAs (E.Y. Levanon et al., 2005) are the substrates of ADAR1 or ADAR2 in humans. Furthermore, we also investigated whether these mRNAs in humans form complex with ADAR2 by means of ADAR2-immunoprecipitation method on nuclear extracts of human cerebellum.

* Corresponding author.
E-mail address: kwak-tky@umin.ac.jp (S. Kwak).
[1] Both these authors equally contributed to this study.

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2. Materials and methods

2.1. Isolation of RNA–protein complexes

The nuclear pellet was extracted from 6 g of frozen human cerebella. Briefly, after homogenizing the tissue in ten-volumes of cold 0.25 M sucrose in buffer A (Tris–saline-HCl buffer (pH 7.5) containing 25 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol), the nuclear pellet was obtained by centrifuging the P1-homogenate in 1.6 M sucrose in buffer A at 130,000 × *g* for 1 h. The RNA–protein complex was isolated from the nuclear pellet according to previously described methods (Ohlson et al., 2005). Briefly, the nuclear pellet was sonicated in 8 ml of ice-cold buffer solution containing 0.1% sodium dodecylsulphate (SDS), 0.5% sodium deoxycholate, 0.5% Igepal CA-630 (Sigma Chemicals, St. Louis, MO) and 1 mM ribonucleoside vanadyl complex (Sigma), and then treated with 800 units of DNase I (Sigma). The resultant solution was centrifuged at 10,000 × *g*, 4 °C for 20 min, and RNA–protein complexes were obtained in the supernatant. All studies were carried out in accordance with the Declaration of Helsinki and the Ethics Committee of the University of Tokyo has approved the experimental procedures used.

2.2. Immunoprecipitation of ADAR2-RNA complexes

Stock Sepharose G was prepared by suspending Protein G Sepharose 4 Fast Flow beads (swollen Sepharose G beads; GE healthcare Bioscience, Piscataway, NJ) treated with tRNA (1 mg/ml) and bovine serum albumin (1 mg/ml) in two volumes of phosphate buffered saline (PBS) containing 0.05% of NaN_3. First, after pre-clearing once with 50 μl of the untreated Sepharose G suspension in PBS, recombinant ADAR2a and FLAG-ADAR2a proteins which were prepared with TNT T7 Quick for PCR DNA kit (Promega, Madison, WI) were incubated with 50 μl of the stock Sepharose G at 4 °C for 2 h in the presence of 2 μg of either E-20 or C-15 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody–bead complex was collected by centrifugation and eluted with PBS containing 1% SDS at 65 °C for 10 min. Presence of recombinant ADAR2a and FLAG-ADAR2a proteins in the eluate were verified with western blotting.

Because E-20 more effectively bound to recombinant ADAR2a and Flag-ADAR2a proteins than C-15 (Fig. 1a), we used only E-20 for the immunoprecipitation of nuclear eluate. One ml of the nuclear eluate obtained from 0.75 g of human cerebellum was incubated at 4 °C for 2 h in the presence of 2 μg of E-20 or control goat anti-human IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) after pre-clearing once with 50 μl of the untreated Sepharose G suspension in PBS, and then for another h with additional 50 μl of stock Sepharose G beads. The nuclear eluate–antibody–bead complex was collected by centrifugation and eluted with 50 μl of PBS containing 1% SDS at 65 °C for 10 min and then the eluate was treated with proteinase-K at 37 °C for 60 min. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA).

2.3. RNAi of ADAR1 and ADAR2

HeLa cells were cultured in MEMα (Wako, Osaka, Japan), and then in Opti-MEM I Reduced-Serum Medium (GIBCO, Langley, OK) without fetal bovine serum (FBS) or antibiotics, containing 30 nM of one of the small interference RNA (siRNA) listed in Supplementary Table S1 (Qiagen HP GenomeWide siRNAs; Qiagen, Valencia, CA) and Lipofectamine RNAiMAX (1:600; Invitrogen) (Forward Transfection). The following siRNAs were used: siR1a and siR1b were used to target human ADAR1, and siR2a and siR2b to target human ADAR2. Cells cultured in Opti-MEM containing 30 nM ALLStars Negative Control siRNA (siR n/c; Qiagen) were used as the negative control. After 5 h of incubation, the medium was switched back to the original MEMα. Total RNA was extracted 96 h after the administration of siRNAs using an RNA spin Mini RNA Isolation kit (Qiagen), and reverse transcription (RT)-polymerase chain reactions (PCRs) were carried out (Supplementary Tables S1 and S2).

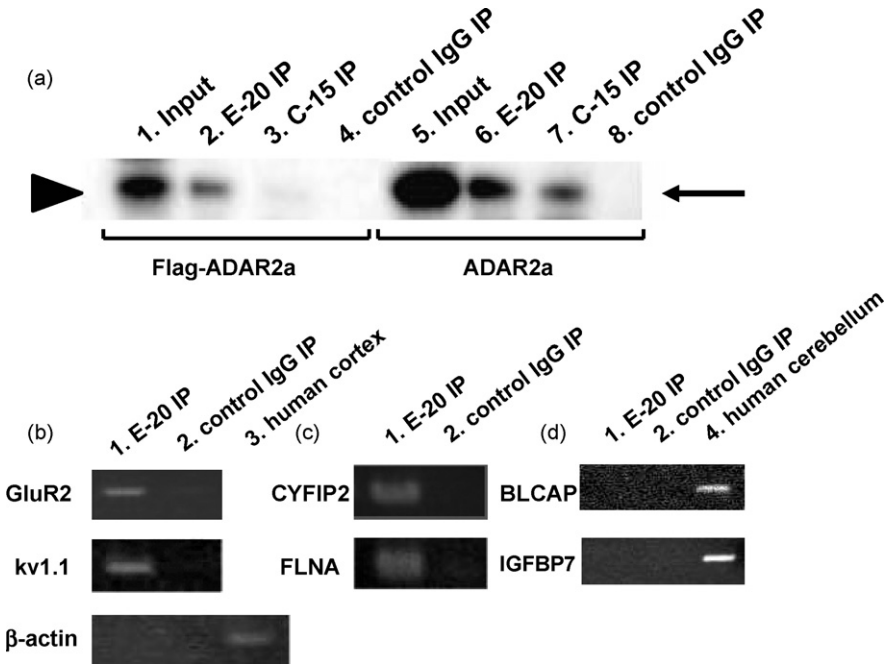

Fig. 1. Immunoprecipitated ADAR2 protein complex specifically contains substrates with selective editing sites. (a) Western blot analysis of eluates immunoprecipitated with anti-human ADAR2 polyclonal antibodies, E-20 (lanes 2 and 6) or C-15 (lanes 3 and 7), containing recombinant Flag-ADAR2a (lanes 2–4) or ADAR2a proteins (lanes 6–8) synthesized *in vitro*. Goat anti-human IgG (H + L) was used as a precipitating control (lanes 4 and 8). Untreated recombinant Flag-ADAR2a (lane 1) and ADAR2a proteins (lane 5) are also shown. Flag-ADAR2a (arrowhead) and ADAR2a proteins (arrow) were immunoprecipitated more effectively by E-20 than by C-15 or control goat anti-human IgG. Total RNA was extracted from the eluate of immunoprecipitate (IP) with E-20 and that of control IgG. (b–d) RT-PCR conducted on these eluates demonstrated mRNAs of GluR2 and kv1.1 (b) (known to have ADAR2-mediated editing sites) as well as those of CYFIP2 (cytoplasmic fragile X mental retardation protein interacting protein 2) and FLNA (filamin A) (c) in the IP with E-20 (lane 1) but not that with control goat anti-human IgG (lane 2). The mRNA of β-actin demonstrated in total RNA extracted from the human cortex (b, lane 3) was not detected in either of the eluates (b). Moreover, both the mRNAs of BLCAP (bladder cancer-associated protein) and IGFBP7 (insulin-like growth factor binding protein 7) demonstrated in total RNA extracted from the human cerebellum (d, lane 4) were not detected in either of the eluates (d).