Biochemical and Biophysical Research Communications 403 (2010) 220–224

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Human proteins that specifically bind to 8-oxoguanine-containing RNA and their responses to oxidative stress

Hiroshi Hayakawa [a,*], Aya Fujikane [a], Riyoko Ito [a], Masaki Matsumoto [b], Keiichi I. Nakayama [b], Mutsuo Sekiguchi [a]

[a] Department of Functional Bioscience and Advanced Science Research Center, Fukuoka Dental College, Fukuoka 814-0193, Japan
[b] Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 2 November 2010
Available online 10 November 2010

Keywords:
Oxidative stress
Binding protein
HNRNPD
HNRNPC

ABSTRACT

Exposure of cells to oxygen radicals damage various biologically important molecules. Among the oxidized bases produced in nucleic acids, 8-oxo-7,8-dihydroguanine (8-oxoguanine) is particularly important since it causes base mispairing. To ensure accurate gene expression, organisms must have a mechanism to discriminate 8-oxoguanine-containing RNA from normal transcripts. We searched for proteins that specifically bind to 8-oxoguanine-containing RNA from human HeLa cell extracts, and the candidate proteins were identified using mass spectrometry. Among the identified candidates, splicing isoform 1 of heterogeneous nuclear ribonucleoprotein D0 (HNRNPD) and splicing isoform C1 of heterogeneous nuclear ribonucleoprotein C1/C2 (HNRNPC) exhibited strong abilities to bind to oxidized RNA. The amount of HNRNPD protein rapidly decreased when cells were exposed to hydrogen peroxide, an agent that enhances oxidative stress. Moreover, the suppression of HNRNPD expression by siRNA caused cells to exhibit an increased sensitivity to hydrogen peroxide. The application of siRNA against HNRNPC also caused an increase in sensitivity to hydrogen peroxide. Since no additive effect was observed with a combined addition of siRNAs for HNRNPD and HNRNPC, we concluded that the two proteins may function in the same mechanism for the accurate gene expression.

1. Introduction

Reactive oxygen species are produced through normal cellular metabolism, and the formation of such radicals is further enhanced by exposure of cells to ionizing radiation and certain chemicals [1]. Various modified bases are generated in nucleic acids exposed to oxygen radicals, and more than 20 different types of oxidatively altered purines and pyrimidines have been identified [2,3]. Among them, an oxidized form of guanine base, 8-oxo-7,8-dihydroguanine (8-oxoguanine), appears to be most important with respect to the maintenance and transfer of genetic information. This oxidized base can pair with adenine as well as cytosine during DNA and RNA syntheses, thus causing a high frequency of mutation and alteration in gene expression [4–6].

The oxidation of guanine residues in RNA may occur more frequently than in DNA, since RNA molecules are mostly single-stranded and their bases are less protected by hydrogen bonding. Moreover, the excision repair system would not function for RNA. Therefore, under aerobic conditions a large number of oxidized guanine residues may be formed and persist in RNA, which could lead to a decrease in the fidelity of gene expression [6]. Although most of erroneous proteins thus formed may be inactive, some may exhibit dominant characteristic, which would cause disorders of some cellular functions [7,8]. It is possible that the dysfunction of a single cell, caused by the accumulation of abnormal proteins, may be amplified with increasing age, since many differentiated cells remain in the G0/G1 state and exert their functions via interactions with sophisticated networks of cells. To avoid such a catastrophe, the cells must have a mechanism to prevent 8-oxoguanien-containing RNA from entering into the cellular translation process.

This study initiated a search of proteins that can discriminate 8-oxoguanine-containing RNA form normal transcripts to elucidate this problem. An initial study with *Escherichia coli* found that a 77 kDa protein, subsequently identified as polynucleotide phosphorylase (PNP), has the capacity to bind to RNA carrying 8-oxoguanine [9]. Mutants that lack this protein exhibited unique responses to paraquat, an agent that induces oxidative stress. Although PNP is found in a wide variety of bacteria and plants, no PNP activity had been detected in the extracts of mammalian cells. However, subsequent sequence analyses revealed that the

Abbreviations: 8-oxoguanine, 8-oxo-7,8-dihydroguanine; PNP, polynucleotide phosphorylase; SA-PMP, streptavidin paramagnetic particle.

* Corresponding author. Fax: +81 92 801 4909.
E-mail address: hiroshi@college.fdcnet.ac.jp (H. Hayakawa).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.011

genes coding for PNP exist also in mammalian genomes [10–12]. A GST-PNP fusion protein was produced based on this finding, and antibodies were raised against human PNP [13]. The recombinant protein can bind to RNA containing 8-oxoguanine and the 80 kDa human PNP was purified using the antibodies. Knockdown of PNP protein by siRNA decreased the viability of HeLa cells exposed to hydrogen peroxide, although the effect was small [14].

The present study was performed to identify proteins with the capacity to discriminate oxidized RNA from normal RNA. For this purpose, we developed a procedure for screening proteins that specifically bind to 8-oxoguanine-containing RNA. We herein present the characterization of proteins identified using this procedure, and discuss their possible roles in ensuring correct gene expression under conditions of oxidative stress.

2. Materials and methods

2.1. Chemicals

The complete EDTA-free, protease inhibitor cocktail tablet, was obtained from Roche, and *E. coli* poly(A) polymerase was purchased from TAKARA. Lumigen™ PS-3 detection reagent kit, RPN2132V1&2, was purchased from GE Healthcare, UK. Rabbit polyclonal antibodies to DAZAP1(ab51010), SF3B4(ab66659) and hnRNP D/AUF (ab61193) were obtained from Abcam. Rabbit polyclonal antibodies to ELAVL2 (NB110-10865) and HNRNPC (ARP41037_P050) were purchased from Novus Biologicals and Aviva Systems Biology, respectively. Monoclonal anti-β-action clone AC-15 (A-5441) was purchased from Sigma. Anti-rabbit IgG HRP-linked whole donkey antibodies (NA934) and anti-mouse IgG HRP-linked whole sheep antibodies (NA931), used as the second antibodies, were purchased from Sigma and GE Healthcare, UK respectively. The PureYield™ RNA Midiprep. System IV and PolyA Tract® mRNA isolation System IV were purchased from Promega. The OxiSelect™ Oxidative RNA Damage ELISA kit was purchased from Cell Biolabs Inc., Lipofectamine RNAiMAX and OPTI-MEM were obtained from Invitrogen. The BCA™ protein assay kit was purchased from PIERCE.

2.2. Cell extract

Cell extract (1–2 mg/ml) was prepared by sonication of cells in 20 mM HEPES (pH 7.5)–150 mM NaCl–0.1%(v/v) Triton X-100–10%(w/v) glycerol (HNTG) containing protease inhibitors, followed by centrifugation.

2.3. Preparation of polyA-tailed RNA

Total RNA was prepared from HeLa MR cells using the PureYield™ RNA Midiprep. System IV according to the manufacturer's instructions. The RNA was oxidized as described [6] and re-purified by using the above-mentioned system. Briefly, 0.8 mg of total RNA was incubated at 37 °C for 2 h in 2 ml of a reaction mixture containing 100 mM sodium phosphate (pH 6.8), 30 mM ascorbic acid, and 100 mM H_2O_2 in the dark. The oxidized RNA was immediately purified using the purification kit. The amounts of 8-oxoguanine in the RNA preparations were determined by OxiSelect™ Oxidative RNA Damage ELISA kit according to the manufacturer's instructions. Under the conditions used, the oxidized RNA contains one 8-oxoguanine residue per 25 guanine residues. On the other hand, normal RNA (control) carries one 8-oxoguanine out of 1100 guanine residues. Since the size of the oxidized RNA was reduced during the oxidation reaction, normal RNA, used as control, was partially digested by RNase A to adjust the length equal to that of the oxidized RNA. To the preparations of normal and oxidized RNAs, polyA tails were added by using *E. coli* poly(A) polymerase. Briefly, 30 μg of RNA were pre-heated at 65 °C for 5 min and incubated with poly(A) polymerase (3 U) in 50 mM Tris–HCl (pH 7.9)–10 mM $MgCl_2$–2.5 mM $MnCl_2$–250 mM NaCl–1 mM DTT–0.05% BSA–1 mM ATP at 37 °C overnight. After incubation, the existence of polyA-tail was confirmed by using Promega PolyA Tract® mRNA isolation System IV, followed by gel electrophoresis.

2.4. Comprehensive screening of oxidized RNA-binding proteins

Forty microlitres (0.8 μg) of polyA-tailed normal or oxidized RNA were heated at 65 °C for 5 min, and then incubated with 2 μl of biotinylated–oligo(dT) of Promega PolyA Tract® mRNA isolation System IV at room temperature for 60 min. The annealed RNAs were incubated with 0.1 ml of cell extract and then placed at 4 °C for 1 h. Streptavidin paramagnetic particle (SA-PMP) of Promega PolyA Tract® mRNA isolation System IV was added to the reaction mixture, and further incubated for 1 h. The particle was washed with 0.3 ml of 1× SSC three times, binding proteins were eluted with 30 μl of 2× SDS-sample buffer containing 0.12 M Tris–HCl, 4% (w/w) SDS and 20% glycerol. The materials were applied to SDS–PAGE, and proteins were visualized with silver staining.

2.5. LC–MS/MS analysis and data base searching

Proteins samples derived from SDS–15%PAGE were analyzed by LC–MS/MS. Briefly, the sections of gels were cut from the two types of samples, one eluted from oxidized RNA and the other from normal RNA (control), and applied to an ion-trap mass spectrometer, according to the procedure described by Matsumoto et al. [15]. The semiquantitative estimation of protein abundance was based on the number of identified peptides [16].

2.6. Western blot analysis

Rabbit polyclonal antibodies against each of the proteins were used as the first antibodies and anti-rabbit IgG and horseradish peroxidase-linked whole donkey antibodies as the second antibodies. Antibodies for β-actin were used to provide internal standards. Quantitative analyses were performed with the use of Western Lumigen™ PS-3 detection reagent according to the manufacturer's instructions.

2.7. Competition for binding to oxidized RNAs

A 0.08 μg sample of polyA-tailed oxidized RNA probe was annealed with biotinylated-oligo(dT). Next, 10 μg of non-polyA tailed RNA were added to the annealed RNA as a competitor. Cell extract was added to the RNA mixture and incubated for 1 h at 4 °C. SA-PMP was added to the reaction mixture and further incubated for 1 h. The RNA probe fixed with SA-PMP was intensively washed, and proteins bound to the probe were eluted with SDS-sample buffer. The eluent was analyzed by Western blotting.

2.8. Interference with siRNA

21mers-dsRNAs corresponding to parts of the human HNRNPC (NM_001077442) and HNRNPD cDNA sequence (NM_002138A) and a control dsRNA were obtained from TAKARA Bio. Inc. and were used for the RNAi experiments. Transfection was performed using Lipofectamine RNAiMAX according to the manufacturer's instructions.

3. Results

Two types of RNA probes were used to screen proteins that bind specifically to oxidized RNA. An HeLa cell extract was applied to