Biochemical and Biophysical Research Communications xxx (2016) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

USP15 stabilizes the transcription factor Nrf1 in the nucleus, promoting the proteasome gene expression

Kousuke Fukagai [a], Tsuyoshi Waku [a], A.M. Masudul Azad Chowdhury [a, 1], Kaori Kubo [a, 1], Mariko Matsumoto [a, 1], Hiroki Kato [a], Tohru Natsume [b], Fuminori Tsuruta [c], Tomoki Chiba [c], Hiroaki Taniguchi [a], Akira Kobayashi [a, *]

[a] Laboratory for Genetic Code, Graduate School of Life and Medical Sciences, Doshisha University, Kyotanabe, Kyoto, Japan
[b] National Institutes of Advanced Industrial Science and Technology, Biological Information Research Center (JBIRC), Tokyo, Japan
[c] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

ARTICLE INFO

Article history:
Received 29 June 2016
Accepted 8 July 2016
Available online xxx

Keywords:
Ubiquitination
Deubiquitination
Proteasomal degradation
Transcription factor
Gene regulation

ABSTRACT

The transcriptional factor Nrf1 (NF-E2-related factor 1) sustains protein homeostasis (proteostasis) by regulating the expression of proteasome genes. Under physiological conditions, the transcriptional activity of Nrf1 is repressed by its sequestration into the endoplasmic reticulum (ER) and furthermore by two independent ubiquitin-proteasome pathways, comprising Hrd1 and β-TrCP in the cytoplasm and nucleus, respectively. However, the molecular mechanisms underlying Nrf1 activation remain unclear. Here, we report that USP15 (Ubiquitin-Specific Protease 15) activates Nrf1 in the nucleus by stabilizing it through deubiquitination. We first identified USP15 as an Nrf1-associated factor through proteome analysis. USP15 physically interacts with Nrf1, and it markedly stabilizes Nrf1 by removing its ubiquitin moieties. USP15 activates the Nrf1-mediated expression of a proteasome gene luciferase reporter and endogenous proteasome activity. The siRNA-mediated knockdown of *USP15* diminishes the Nrf1-induced proteasome gene expression in response to proteasome inhibition. These results uncover a new regulatory mechanism that USP15 activates Nrf1 against the β-TrCP inhibition to maintain proteostasis.

1. Introduction

The transcription factor Nrf1 (NF-E2-related factor 1 or NFE2L1) induces the expression of the proteasome subunit genes under proteasome dysfunction [1,2]. This adaptation for proteasome dysfunction is called “proteasome recovery” to sustain protein homeostasis (proteostasis). Consistently, the deletion of the *Nrf1* gene in the central nervous system of mice causes an abnormal accumulation of ubiquitinated protein aggregates in neurons, and these mice show progressive motor ataxia and severe weight loss [3,4]. These observations strongly suggest that Nrf1 plays important roles in proteostasis.

Accumulated evidence has revealed that the molecular function of Nrf1 is regulated by multiple repression mechanisms [5,6]. Under physiological conditions, Nrf1 is sequestered in the endoplasmic reticulum (ER) through its N-terminal NHB1 domain to prevent nuclear translocation and transcriptional activation [7]. Furthermore, Nrf1 is repressed by proteasomal degradation via two independent E3 ubiquitin (Ub) ligases as follows: the SCF (Skp1-Cul1-F-box protein) ubiquitin ligase that contains the β-TrCP adaptor and the endoplasmic reticulum-associated degradation (ERAD) ubiquitin ligase Hrd1, in the nucleus and cytoplasm, respectively [1,8]. It has also been reported that Fbw7 mediates the nuclear degradation of Nrf1 [9]. These findings imply that Nrf1 is activated by escaping from these proteasomal degradation mechanisms. However, the molecular bases of Nrf1 activation remain unclear.

Ubiquitin-specific protease 15 (USP15), which is a ubiquitously expressed Deubiquitinating enzyme (DUB), regulates the biological function of many substrate proteins for various cellular functions

Abbreviations: ARE, antioxidant response element; CNC, Cap'n'Collar; DUB, deubiquitinating enzyme; ER, endoplasmic reticulum; NHB1, N-terminal homology box 1; Nrf1, NF-E2-related factor 1; RT-qPCR, real time-quantitative PCR; SCF, Skp1-Cul1-F-box protein; SEM, standard error of the mean; USP, ubiquitin-specific protease.

* Corresponding author. Laboratory for Genetic Code, Graduate School of Life and Medical Sciences, Doshisha University, 1-3 Tatara Miyakodani, Kyotanabe 610-0394, Japan.
E-mail address: akobayas@mail.doshisha.ac.jp (A. Kobayashi).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2016.07.045
0006-291X/

[10–12]. For example, USP15 regulates the E3 ubiquitin ligase MDM2 for the tumor suppressor p53 [13] and the TGFβ-induced monoubiquitination of SMAD (R-SMAD) [14]. Furthermore, it has been reported that USP15 represses Nrf1-related factor Nrf2 through activating the Keap1-mediated ubiquitination activity by its deubiquitination [15].

For a comprehensive understanding of the physiological function of Nrf1, deciphering the molecular mechanisms underlying Nrf1 activation from multiple repression mechanisms is indispensable. We found that USP15 stabilizes Nrf1 from the β-TrCP-mediated degradation in the nucleus through its deubiquitination, ameliorating its transcription activity for the gene expression of the proteasome subunits. These results suggest that USP15 plays an important role for the regulation of Nrf1 activity to maintain proteostasis.

2. Materials and methods

2.1. Antibodies

The antibodies utilized in this study were anti-FLAG (M2; Sigma), anti-V5 antibody (46-0705; Invitrogen), anti-α-tubulin (DM1A; Sigma), anti-green fluorescent protein (anti-GFP) (B-2; Santa Cruz), anti-Nrf1 (D5B10; Cell Signaling Technology and H285; Santa Cruz), anti-USP15 (2D5; Santa Cruz), anti-hemagglutinin (anti-HA) (Y-11; Santa Cruz), and anti-Histone H3 (06-755; EMD Millipore).

2.2. Expression plasmids

3 × Flag-Nrf1, ΔNHB1 and ΔbZip have been described previously [8]. 3 × Flag-Nrf3 was generated by subcloning the PCR-amplified mouse Nrf3 cDNA into the p3 × FLAG-CMV™10 vector (Sigma). Flag-Nrf2 and Flag-p45 were kindly provided by Ken Itoh.

2.3. Cell culture and transfection

HEK293T cells and HeLa cells were cultured as described [8]. The transfection of plasmid DNA and short interfering RNA (siRNA) was performed using Lipofectamine Plus and RNAiMAX (Invitrogen), respectively, according to the manufacturer's protocols.

2.4. Cycloheximide chase experiments

HEK293T cells were transfected with the expression vectors containing wild-type or deletion mutants of 3 × Flag-Nrf1 [8], along with a GFP expression vector (Clonetech, pEGFP-N1). At 24 h after transfection, the cells were treated with 10 μg/ml cycloheximide (CHX), and the whole cell extracts were prepared at the indicated time points. Immunoblot analyses were conducted with the indicated antibodies. When USP15 siRNAs were transfected into the cells, the cells were pretreated at 32 h after the transfection with 10 μM MG132 for 8 h, followed by the CHX treatment.

2.5. Immunoprecipitation, immunoblot analysis and ubiquitination assay

The expression vectors for 3 × Flag-Nrf1 and V5-USP15 were transfected into HEK293T cells. At 24 h after transfection, whole cell extracts were prepared using lysis buffer (50 mM Tris-HCl [pH 8.0], 10% glycerol, 100 mM NaF, 50 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.1% NP-40, and 1 × protease inhibitor cocktail [Roche]). The whole cell extracts were subjected to immunoprecipitation with Protein G Sepharose 4 Fast Flow beads (GE Healthcare) and the anti-V5 antibody (2D5; Abcam) at 4 °C for 2 h by rotating. The Protein G beads were washed three times with wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1% NP-40). The immunocomplexes were visualized with immunoblot analysis using the indicated antibodies. Ubiquitination assay was performed as described [8].

2.6. Immunofluorescence staining

HEK293T cells were transfected with wild-type or deletion mutants of 3 × Flag-Nrf1 and/or V5-USP15. At 24 h after transfection, the cells were washed with PBS and fixed with 4% formaldehyde for 15 min at room temperature (RT). After the PBS washing, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at RT, washed with PBS and subsequently blocked with 1% skim milk (Nacalai tesque) for 1 h. A primary antibody treatment (anti-FLAG or anti-V5 antibody) was conducted at RT for 1 h. After washing with PBS, the cells were incubated with the Alexa488 or Alexa546 Fluor secondary antibody (Invitrogen) for 1 h in the dark. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The immunofluorescence was viewed using an Olympus IX7 microscope.

2.7. Luciferase assay

HeLa cells were transfected with ΔNHB1 and V5-USP15, along with a luciferase reporter plasmid containing three tandem copies of the ARE of PSMA4 [2] and pRL-TK (Promega) as an internal control. At 24 h after transfection, the luciferase activities were measured using the PicaGene luciferase assay system (Toyo Ink) and a Berthold Lumat LB9507 luminometer according to the manufacturer's protocols.

2.8. RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was prepared using the ISOGENII (Wako). One μg of total RNA was utilized for cDNA synthesis using random hexamer primers (Takara Bio) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Real-time quantitative PCR was conducted using the FastStart Universal SYBR Green Master Mix (Roche) and the Thermal Cycler Dice Real Time System II (Takara Bio). All target gene expression levels were normalized to 18S rRNA expression. The sequences of the primers are listed in Table 1.

2.9. Proteasome fluorogenic peptidase assay

The *in vitro* measurement of proteasome activities was performed as previously described [16]. HeLa cells were transfected

Table 1
Sequences of siRNA and primers for real time PCR.

Gene	Sense strand sequence (5′–3′)	Antisense strand sequence (5′–3′)
NRF1	gggauucggugaagauuugTT	caaaucuucaccgaaucccTT
USP15-1	uauuuguuccacaaucuggTT	ccagauuguggaacaaauaTT
USP15-2	gguuggaauaaacuugucaTT	ugacaaguuuauuccaaccTT
USP15-3	ccagucacuuaaggaacauTT	auguuccuuaagugacuggTT
Control	uucuccgaacgugucacguTT	acgugacacguucggagaaTT
Gene	**Forward primer (5′–3′)**	**Reverse primer (5′–3′)**
18S rRNA	cgccgctagaggtgaaattc	cgaacctccgactttcgttct
USP15	ggtgctgaagatccctgg	tactggaggcagggacca
NRF1	tggaacagcagtggcaagatctca	ggcactgtacaggatttcacttgc
PSMA4	cattggctgggataagca	atgcatgtggccttccat
PSMC4	ggaagaccatgttggcaaag	aagatgatggcaggtgcatt
PSMB6	ctgatggcgggaatcatc	ccaatggcaaaggactgc

Please cite this article in press as: K. Fukagai, et al., USP15 stabilizes the transcription factor Nrf1 in the nucleus, promoting the proteasome gene expression, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.07.045