Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Smurf2 regulates the degradation of YY1

Hyung Min Jeong [a], Sung Ho Lee [a], Jinah Yum [b], Chang-Yeol Yeo [b], Kwang Youl Lee [a,*]

[a] College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, South Korea
[b] Department of Life Science and Global Top5 Research Program, Ewha Womans University, Seoul 120-750, South Korea

ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 23 April 2014
Accepted 28 April 2014
Available online xxxx

Keywords:
YY1
Smurf2
Ubiquitination
Protein stability

ABSTRACT

Transcription factor YY1 plays important roles in cell proliferation and differentiation. For example, YY1 represses the expression of muscle-specific genes and the degradation of YY1 is required for myocyte differentiation. The activity of YY1 can be regulated by various post-translational modifications; however, little is known about the regulatory mechanisms for YY1 degradation. In this report, we attempted to identify potential E3 ubiquitin ligases for YY1, and found that Smurf2 E3 ubiquitin ligase can negatively regulate YY1 protein level, but not mRNA level. Smurf2 interacted with YY1, induced the poly-ubiquitination of YY1 and shortened the half-life of YY1 protein. Conversely, an E3 ubiquitin ligase-defective mutant form of Smurf2 or knockdown of *Smurf2* increased YY1 protein level. PPxY motif is a typical target recognition site for Smurf2, and the PPxY motif in YY1 was important for Smurf2 interaction and Smurf2-induced degradation of YY1 protein. In addition, Smurf2 reduced the YY1-mediated activation of a YY1-responsive reporter whereas *Smurf2* knockdown increased it. Finally, Smurf2 relieved the suppression of p53 activity by YY1. Taken together, our results suggest a novel regulatory mechanism for YY1 function by Smurf2 in which the protein stability and transcriptional activity of YY1 are regulated by Smurf2 through the ubiquitin-proteasome-mediated degradation of YY1.

1. Introduction

The transcription factor Yin Yang 1 (YY1) plays important roles in cell proliferation and differentiation, and it is highly conserved from insects to mammals [1–6]. YY1 directly or indirectly regulates the expression of target genes, and YY1 can induce or repress target gene expression depending on the cofactors it recruits [6–9]. In addition, YY1 can control gene expression by promoting the post-translational modifications of cofactors. The activity of YY1 itself is also regulated by various post-translational modifications. Acetylation and deacetylation of YY1 by p300 and histone deacetylases modulate the transcriptional activity of YY1 [9–11]. Phosphorylation of YY1 affects its DNA binding ability [12], and sumoylation can alter the specificity of target promoters [13]. YY1 mono-ubiquitination enhances its interaction with C-terminal binding protein (CtBP) and HDAC3, resulting in the formation of a transcription repressor complex that suppresses the expression of *Mmp9* [14].

YY1 represses the expression of muscle-specific genes [15,16], and YY1 degradation is a prerequisite for myocyte differentiation [17]. However, little is known about the regulatory mechanism for YY1 degradation and the enzymes that control this process. In ubiquitin-proteasome-mediated degradation, proteins are targeted for degradation by covalent poly-ubiquitination at lysine residues. This requires a coordinated action of three different types of enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase [18]. In this study, we attempted to identify potential E3 ubiquitin ligases for YY1. Among the E3 ligases tested, Smurf2 (Smad ubiquitination regulatory factor 2) affected YY1 protein level significantly. Smurf2 interacted with YY1 and promoted the poly-ubiquitination of YY1. In addition, Smurf2 decreased the protein half-life and transcriptional activity of YY1. Conversely, knockdown of *Smurf2* increased the protein level and transcriptional activity of YY1. Smurf2 binding to YY1 and Smurf2-induced degradation of YY1 required the PPxY motif of YY1. PPxY motif is the typical target recognition site of Smurf2. Finally, Smurf2 relieved the suppressive effect of YY1 on p53 activity. Taken together, our results indicate that Smurf2 can act as an E3 ubiquitin ligase for YY1.

2. Materials and methods

2.1. Cell culture

293 human embryonic kidney cells were maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, antibiotics and antimycotics. All culture media and supplements were purchased from Life Technologies.

* Corresponding author. Tel.: +82 62 530 2939; fax: +82 62 530 2949.
E-mail address: kwanglee@chonnam.ac.kr (K.Y. Lee).

http://dx.doi.org/10.1016/j.bbamcr.2014.04.023
0167-4889/

2.2. Plasmids, antibodies and reagents

Expression plasmids for N-terminal epitope-tagged human YY1, Smurf2 and p53 were constructed in a CMV promoter-derived mammalian expression vector (pCS4). Deletion mutants of YY1 were generated by PCR-based mutagenesis and confirmed by DNA sequencing. Plasmid for Smurf2 (C716G) mutant was generously provided by Dr. Eek-Hoon Jho (The University of Seoul). YY1-Luc and p53-Luc were generated by inserting 3 tandem repeats of a consensus binding sequence for YY1 (5′-CGC CAT TTT-3′) or p53 to pGL3-Basic. For knockdown of *Smurf2*, oligonucleotides targeting following sense sequences were synthesized: si-*Smurf2* #1, 5′-CCT TCT GTG TTG AAC ATA A-3′; si-*Smurf2* #2, 5′-GAC CAA CAG CAA CAG CAA G-3′. Sense and antisense oligonucleotides were annealed and ligated into pSuper-retro vector (Oligoengine). Retroviruses were produced according to the manufacturer's instruction. The following antibodies were used: anti-Flag (M2) from Sigma-Aldrich; anti-GFP (B-2), anti-YY1 (H-414) and anti-Myc (9E10) from Santa Cruz Biotechnology; anti-HA (12CA5) from Roche Applied Science; anti-Smurf2 (2078-1) from Epitomics; and anti-α-tubulin (DM1A) from Cell Signaling Technology.

2.3. DNA transfection and reporter assays

Transient transfection was performed using the calcium phosphate-mediated method or the polyethyleneimine (Polysciences, Inc.)-mediated method. Unless otherwise specified, cells were analyzed 48 h after transfection. For luciferase assays, cells were transfected with indicated plasmids along with pCMV-β-Gal. 36 h later, luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-galactosidase activities.

2.4. Immunoblotting and immunoprecipitation

Cells were lysed in an ice-cold lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Lysates were cleared by centrifugation. For immunoblotting, α-tubulin was used as a loading control. For immunoprecipitation, the supernatants were incubated with appropriate antibodies and protein A or G-sepharose beads. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and visualized using appropriate antibodies and chemiluminescence Western blotting reagent (GE Healthcare).

2.5. RNA preparation and semi-quantitative RT-PCR

Total cellular RNA was prepared using TRIzol reagent (Life Technologies) according to the manufacturer's instruction. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). The following conditions were used for PCR amplification of cDNA: initial denaturation at 94 °C for 1 min; 22–30 cycles of denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The following PCR primers were used: *YY1*, forward 5′-ATG GCC TCG GGG GAC ACC-3′ and reverse 5′-TCA CTG GTT GTT TTT GGC-3′; *p21*, forward 5′-GGG GAA GGG ACA CAC AAG AAG A-3′ and reverse 5′-AAT GAA CTG GGG AGG GAT GG-3′; *BAX*, forward 5′-TTT GCT TCA GGG TTT CAT CC-3′ and reverse 5′-CAG TTG AAG TTG CCG TCA GA-3′; *NOXA*, forward 5′-CTG GAA GTC GAG TGT GCT ACT-3′ and reverse 5′-TCA GGT TCC TGA GCA GAA GAG-3′; *GAPDH*, forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′. *GAPDH* was used as a loading control.

2.6. GST pull down assay

Aliquots of cell lysates corresponding to approximately 10^7 cells were incubated with glutathione-Sepharose beads carrying 10 μg of GST-fusion protein (GST-YY1 or GST-Smurf2) for 5 h at 4 °C. Bound proteins were analyzed by immunoblotting.

3. Results

3.1. Smurf2 can down-regulate YY1

In order to identify potential E3 ubiquitin ligases for YY1, we examined the effects of various E3 ligases on YY1 protein level. Among the E3 ligases tested, two types of E3 ligases affected YY1 reproducibly (data not shown). One was the HECT domain type E3 ligase family that includes Smurf1/2 and Itch, and the other was the RING finger type E3 ligase family that includes Cbl-b and c-Cbl. The HECT domain type and the RING finger type E3 ligases differ in their ubiquitin ligation chemistry [19]. We investigated the ability of these two types of E3 ligases for modulating YY1 protein level, and found that YY1 protein level is reduced significantly by the HECT domain type E3 ligases (Fig. 1 A). We decided to investigate the function of Smurf proteins further, since YY1 contains the PPxY motif which is the typical target recognition and binding site of Smurf1/2.

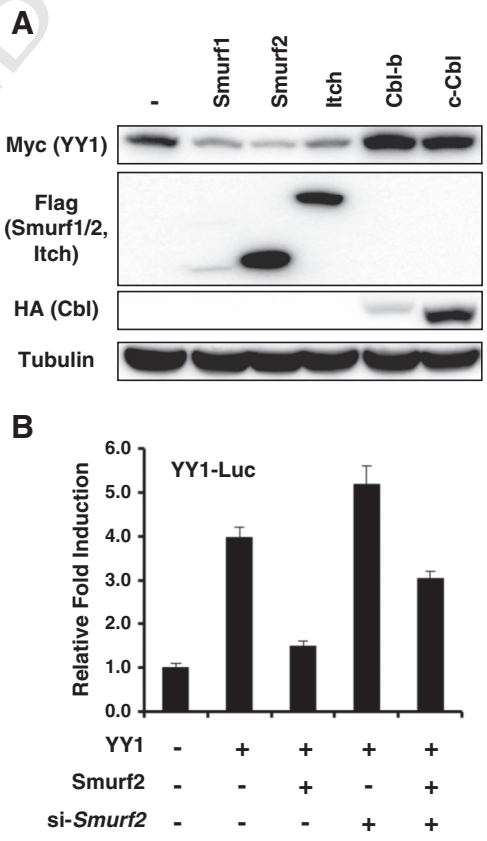

Fig. 1. Smurf2 down-regulates YY1. (A) 293 cells were transfected with Myc-tagged YY1 and indicated E3 ubiquitin ligases. Levels of overexpressed YY1 protein are compared by anti-Myc immunoblotting [Myc (YY1)]. Levels of overexpressed E3 ligases are also compared by anti-Flag [Flag (Smurf1/2, Itch)] and anti-HA [HA (Cbl)] immunoblotting. Tubulin is used as a loading control. (B) 293 cells were transfected with a YY1-responsive luciferase reporter (YY1-Luc) and indicated combinations of YY1, Smurf2, or *Smurf2* siRNA #1 (si-*Smurf2*). 36 h later, cells were assayed for luciferase activities. Results were analyzed using Student's *t*-test, with $p < 0.05$. Experiments were performed in triplicate and repeated three times. The averages and standard deviations of a representative experiment are shown.

Please cite this article as: H.M. Jeong, et al., Smurf2 regulates the degradation of YY1, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamcr.2014.04.023