FEBS Letters 585 (2011) 2405–2409

FEBS Letters

journal homepage: www.FEBSLetters.org

SUMOylation of Blimp-1 promotes its proteasomal degradation

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ARTICLE INFO

Article history:
Received 1 May 2011
Revised 13 June 2011
Accepted 15 June 2011
Available online 25 June 2011

Edited by Noboru Mizushima

Keywords:
Plasma cell
SENP1
SUMO protease
Proteasome

ABSTRACT

B lymphocyte induced maturation protein-1 (Blimp-1) is a transcription repressor of the Krueppel-like family. Blimp-1 plays important roles in developmental processes, such as of germ cells and hair follicle stem cells. In B lymphocytes Blimp-1 orchestrates the terminal differentiation into plasma cells.

We discovered that Blimp-1 undergoes SUMOylation by SUMO-1. This SUMOylation is modulated by the SUMO protease SENP1. While Blimp-1 is relatively stable in 293T cells, a fusion with SUMO1 rendered it to rapid proteasomal degradation. Increase in SENP1 activity stabilized Blimp-1, while a decrease promoted its degradation. Our data indicate that SUMOylation of Blimp-1 regulates its intracellular stability.

Structured summary of protein interactions:
Blimp1 physically interacts with **SUMO1** by anti tag coimmunoprecipitation (View Interaction 1, 2).
SUMO1 physically interacts with **Blimp1** by anti tag coimmunoprecipitation (View interaction).

1. Introduction

B lymphocyte induced maturation protein-1 (Blimp-1) is a transcriptional repressor with five Krueppel-type zinc fingers [1]. When attached to DNA by virtue of its Zinc fingers, transcriptional repression of the target gene is achieved by recruitment of corepressors of the Groucho family [2], chromatin-modifying enzymes such as histone H3 methyltransferase G9a [3] and histone deacetylases (HDACs) [4].

Blimp-1 exerts diverse biological activities in different cellular contexts. Primarily, it is essential for stemness characteristics of various types of stem cells [5–7] and for the terminal differentiation of B lymphocytes into antibody-secreting plasma cells [1]. In the absence of Blimp-1 little Ig is secreted in response to both T cell-independent and T cell-dependent antigens [8]. In this context, Blimp-1 directly suppresses *Pax5* [9] and *Myc* [10] and thereby interferes with the expression of genes necessary for the B cell identity and proliferation. In fact, Blimp-1 is a critical tumor suppressor in the B cell lineage [11,12]. Importantly, the levels of Blimp-1 are a key for its biological activity. While plasmablasts express low levels of Blimp-1, establishment of mature long-lived plasma cells (PCs) requires increased levels of Blimp-1 expression [13]. Moreover, the continuous expression of Blimp-1 is critical for the maintenance of PCs after full differentiation [14]. Therefore, understanding the various mechanisms that control Blimp-1 levels are of paramount importance.

Post-translational modification of proteins with the small ubiquitin-like molecule (SUMO) is a dynamic and fundamentally important modification, which plays a role in a range of biological processes. Human and mouse genomes encode three functional SUMO isoforms (SUMO-1, SUMO-2 and SUMO-3) and one isoform that may not be processed to its mature form (SUMO-4). SUMO-1 differs significantly in structure from SUMO-2 and SUMO-3, which are very similar, and modifies a distinct yet overlapping group of proteins [15]. SUMOylation is reversible by a family of SUMO isopeptidases called SENPs.

Here, we show that Blimp-1 is SUMOylated by SUMO-1 soon after synthesis. The SUMOylation is reversible primarily by SENP1. While Blimp-1 is stable in 293T HEK cells an N-terminal fusion to SUMO-1 renders it unstable. In other cell types Blimp-1 is turned over more rapidly and its stability can be increased by the expression of SENP1

Abbreviations: Blimp-1, B lymphocyte induced maturation protein-1; KLF, Krueppel-like factor; PC, plasma cells; SENP, SUMO-specific protease; SUMO, small ubiquitin-like modifier

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doi:10.1016/j.febslet.2011.06.022

or decreased by SENP1 knockdown. We conclude that SUMOylation of Blimp-1 serves as a signal for its degradation.

2. Materials and methods

2.1. Cells, transfection and reagents

P3X cells were grown in RPMI medium supplemented with 10% FCS (Hyclone, EU approved), 2 mM glutamine, 50 u/ml penicillin, 50 μM 2-mercaptoethanol and 50 μg/ml of streptomycin. 293T and HeLa cells were maintained in DMEM supplemented with 10% FCS (Hyclone, EU approved), 2 mM glutamine, 50 μ/ml penicillin and 50 μg/ml of streptomycin. Cells were transfected using standard calcium phosphate precipitation protocol. Expression vectors for SENP-1 and SENP-1-DN (a catalytically inactive SENP1) constructs were provided by Dr. Grace Gill (Tufts University, Boston, MA). Expression vector for C-terminal FLAG-tagged Blimp-1 was provided by Dr. Katherine Calame (Columbia University, New-York, NY). Expression vectors for HA-tagged ubiquitin like proteins were provided by Dr. Hidde Ploegh (Whitehead Institute, Cambridge, MA). M2 anti-FLAG antibodies and beads were purchased from Sigma. The hybridoma clone 12CA5 was used to detect the HA epitope. Anti SUMO-1 antibody was purchased from Cell Signaling Technology (C21A7). Endogenous Blimp-1 was detected by two monoclonal antibodies: rat anti-Blimp-1, Santa Cruz, 6D3 and mouse anti-Blimp-1, Novus, 3H2-E8. Secondary HRP-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Retroviral transduction with SENP1 shRNA

Retroviral vectors containing mouse SENP1-specific shRNA targets were purchased from Open Biosystems. Retroviruses were generated in 293T cells by triple transfection with Gag-Pol and Env expressing vectors. Following retroviral transduction and puromycin selection of P3X cells we selected clone V2MM_25105 as the most efficient in knocking down SENP1 (see Fig. S1).

2.3. Pulse-chase analysis and immunoprecipitation

Metabolic labeling was performed after starvation in methionine/cysteine-free Dulbecco's modified Eagle's medium (Biological Industries Beit Haemek, Israel) for 45 min. Then, cells were pulse-labeled with [^{35}S]-methionine/cysteine (7.5 mCi/500 μl) (Amersham, USA) at 37° C for the indicated time. Chase period was initiated by addition of complete cell medium supplemented with a 50-fold excess of non-radiolabeled cystein/methionine. Cells were then lysed in 1% SDS preheated to 70 °C to avoid post-lysis deSUMOylation. Lysates were subsequently diluted by addition of 1.5 ml lysis buffer (Tris pH = 8 50 mM, NaCl 200 mM, $MgCl_2$ 20 mM and 1% NP-40, 3 μl/ml normal rabbit serum, 10 μl/ml 0.1% BSA and protease inhibitors). Non specific protein binding was removed by addition of protein A beads (ADAR BioTech, Israel). Immunoprecipitation was performed using the indicated antibodies. Anti-FLAG immunoprecipitations were performed with anti-FLAG beads (A2220, Sigma). Immunoprecipitates were washed four times in the lysis buffer that contained 0.1% SDS, boiled in sample buffer and analyzed by SDS–PAGE, followed by auto-radiography or Western blotting.

3. Results

3.1. Blimp-1. is reversibly modified by SUMO-1

When examined by immunoblot (rat anti-Blimp-1, Santa Cruz, 6D3) in the plasmacytoma cell line P3X, which endogenously expresses Blimp-1, we noticed that Blimp-1 yields two distinct polypeptides; one that migrated on SDS-PAGE at the expected size (~100 Kd) and an additional polypeptide that was slightly heavier estimated to be 10–15 Kd heavier. To exclude concerns that the second band represents a non-specific interaction with the anti-Blimp-1 antibody, we immunoprecipitated Blimp-1 from a pre-boiled SDS lysate, and immunoblotted with the same antibody (Santa Cruz 6D3, Fig. 1A). Similar results were obtained with a different commercial antibody (Novus, 3H2-E8). We concluded that the upper band represents a modified Blimp-1.

Because the observed modification in Blimp-1 was too large to be accounted for by phosphorylation we have examined whether Blimp-1 was modified by either ubiquitin or an ubiquitin-like protein. To identify the modifier we expressed a C-terminal FLAG-tagged Blimp-1 (Blimp-1-FL) in 293T cells together with a panel of HA-tagged members of the ubiquitin family. Cells were pulse-labeled with ^{35}S-methionine for 30 min and Blimp-1 was recovered by anti-FLAG immunoprecipitation. A portion of the immunoprecipitate was denatured and re-immunoprecipitated with anti-HA. When expressed in 293T cells Blimp-1-FL yielded two polypeptides recovered by anti-FLAG immunoprecipitation, reinforcing our conclusion that Blimp-1 undergoes post-translation modification. The modified Blimp-1 was recovered only when HA-SUMO-1 was coexpressed (Fig. 1B). Thus, we conclude that Blimp-1 is SUMOylated.

To determine whether Blimp-1 exerts a preference to either of the SUMO isotypes we coexpressed Blimp-1-FL with an HA-tagged SUMO-1, 2 or 3 in 293T cells. Fourty-eight hours after transfection, cell lysates were prepared and Blimp-1-FL was immunoprecipitated with anti-FLAG antibodies and subjected to immunostaining with anti-HA and anti-FLAG antibodies. Blimp-1 exerted a strong preference for HA-tagged SUMO-1 (Fig. 1C). Blimp-1 was also modified with endogenous SUMO-1 (Fig. 1D). Taken together we demonstrate for the first time the SUMOylation of Blimp-1 with SUMO-1.

Next, we wanted to compare the turnover of unmodified Blimp-1 to the SUMOylated form (Blimp-1-SUMO). We transfected 293T cells with Blimp-1-FL. Twenty-four hours after transfection the cells were pulse-labeled with ^{35}S-methionine for different time periods. Autoradiography analysis of the immunoprecipitates showed that SUMOylation was clearly discerned after 10 min pulse. Interestingly, as the pulse period increased the level of Blimp-1 increased more than Blimp-1-SUMO, suggesting that the modification is removed (Fig. 1E). To test this possibility we performed a pulse-chase analysis. The unmodified Blimp-1 was relatively stable, while Blimp-1-SUMO decayed during this time (Fig. 1F). Our data show that in 293T cells the modification of Blimp-1 with SUMO-1 occurs rapidly after synthesis.

Blimp-1 contains several sequences of the consensus SUMOylation motifs (for a list of putative SUMOylation sites and their calculated score see Fig. S2). However, in almost half of the proteins SUMOylation occurs on non-motif residues [16]. To map the SUMOylation sites on Blimp-1 we individually mutated to arginine all consensus lysines with high scores. All mutants exhibited SUMOylation (not shown) suggesting that Blimp-1 is most likely SUMOylated on non-canonical sites.

3.2. SENP1 regulates Blimp-1 SUMOylation

Of the different SUMO proteases, only SENP1 is specific to SUMO-1 [17,18]. To test whether SENP1 is able to deSUMOylate Blimp-1 we expressed a FLAG-tagged version of either active SENP1 (FL-SENP1) or a catalytically dead mutant with dominant negative activity (FL-SENP-1-DN). These constructs when overexpressed diminish or increase the total SUMOylation level, respectively (Fig. 2A). SENP1 or its DN version were expressed together with Blimp-1 and HA-SUMO-1. Cells were pulse-labeled and chased for 1 h and lysates thereof were immunoprecipitated with anti-FLAG. The expression