Biochemical and Biophysical Research Communications 501 (2018) 779–785

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

PIM1-catalyzed CBX8 phosphorylation promotes the oncogene-induced senescence of human diploid fibroblast

Xiangwen Zhan [a], Jianming Yang [b], Zebin Mao [a], Wenhua Yu [a, *]

[a] Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, 100191, People's Republic of China
[b] Department of Immunology, School of Basic Medical Science, Tianjing Medical University, People's Republic of China

ARTICLE INFO

Article history:
Received 8 May 2018
Accepted 11 May 2018

Keywords:
PIM1
CBX8
Phosphorylation
Senescence

ABSTRACT

The proto-oncogene *PIM1* encodes Ser/Thr kinase and regulates cell growth, differentiation and apoptosis. However, more and more studies including ours have found that PIM1 can induce senescence in normal human diploid fibroblasts and behave as a tumor suppressor. But the relevant molecular mechanism of this process is not yet clear. It has been reported that Chromobox homolog 8 (CBX8) binds directly to INK4A as a transcriptional repressor, thereby suppressing stress-induced senescence. Here we report that PIM1 can phosphorylate CBX8 to promote its degradation, thereby up-regulating p16, during PIM1-induced cell senescence. Overexpression of CBX8 can inhibit PIM1-induced cell senescence. These data suggest that to promote CBX8 degradation may be an important molecular mechanism of PIM1-induced cell senescence.

1. Introduction

Cellular senescence is a permanent cell cycle arrest triggered by various stimuli including oncogene activation, telomere shortening, genetoxic insults and other detrimental growth conditions. About two decades ago, Serrano et al. observed normal fibroblasts with ectopic expression of RAS entered senescence different from replicative senescence without telomere shortening [1], termed oncogene-induced senescence (OIS) [2]. Subsequent research demonstrated OIS acted as a barrier preventing tumor formation. OIS was associated with signs of DNA replication stress including prematurely terminated DNA replication forks and DNA double-strand breaks [3]. But the following molecular events involved in induction of senescence have not been elucidated very clearly.

PIM1 gene was originally identified as an oncogene in murine leukemia virus-induced lymphomas, which was activated transcriptionally by the proviral insertion, encoding a constitutively active serine/threonine protein kinase. Plentiful research demonstrated PIM1 promoted tumor progression through regulation of proliferation, differentiation, survival and apoptosis cooperating with other oncogenes such as Myc [4], Bcl2 [5] and GFI-1 [6]. Interestingly, more and more studies have recently reported that PIM1 can induce cell senescence and inhibit proliferation, thus showing tumor suppressor properties. In 2008, Hogan et al. reported that overexpression of PIM1 in normal fibroblasts can induce senescence rather than enhance growth in a p53-dependent manner [7]. Our lab also reported later that PIM1 induced cellular senescence through phosphorylating UHRF1 and HP1γ, which led to DNA methylation [8] and chromatin remodeling [9], respectively. Furthermore, overexpression of PIM1 can inhibit cell and tumor growth specifically in 22Rv1 human prostate cells by inducing marked increases in cellular senescence [10]. These data indicated PIM1's role in a certain cell may depend on its environment context.

CBX8 is chromobox homolog 8 (CBX8) protein belonging to Polycomb Group (PcG) proteins which were originally discovered as a crucial regulator of development in Drosophila [11]. The PcG proteins form two distinct multimeric protein complexes named as polycomb repressive complexes 1 and 2 (PRC1 and PCRC2), which play significant roles in a wide variety of biological processes, including pluripotency, differentiation, senescence, carcinogenesis and so on [12,13]. Chromobox homolog 8 (CBX8) has been identified as a transcriptional repressor that functionally associate with RING1, BMI1 and MLL-ENL oncoprotein [14,15]. CBX8 is closely related to tumorigenesis and tumor progression, while the understanding of its role in cellular senescense is fewer than that in

* Corresponding author. Department of Biochemistry and Molecular Biology Peking University Health Science Center, 38 Xue Yuan Road, Beijing, 100191, China.
E-mail address: yuwenhua@hsc.pku.edu.cn (W. Yu).

https://doi.org/10.1016/j.bbrc.2018.05.070
0006-291X/

tumors. A classic research reported that CBX8 can directly bind to and repress the *INK4A-ARF* locus and therefore bypassing cellular senescence in fibroblasts [16]. In addition, the relationship between CBX8 and senescence has also been reported in the inhibition of Sirtinol-induced premature aging in certain tumor cells [17,18].

Our study characterizes CBX8 as a new substrate of PIM1 kinase. CBX8 phosphorylation by PIM1 can lead to its destabilization and degradation, thereby promoting p16 expression during oncogene-induced cell senescence. Overexpression of CBX8 shows counteractive effect on PIM1-induced cell senescence. These results indicate that CBX8 is involved in PIM1-induced cell senescence and his down-regulation is an important molecular mechanism in the process of cell senescence.

2. Materials and methods

2.1. Cell culture and reagent

HEK293T cells and Human diploid fibroblasts 2BS cells were obtained from the National Institute of Biological Products, Beijing, China. These cells were cultured in complete Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml), penicillin (100 U/ml), and maintained in a humidified atmosphere of 5% CO2 at 37 °C. MG132 (M8699; Sigma) was dissolved in dimethyl sulfoxide (DMSO; C6295; Sigma) and added to the culture medium at 20 μM for the indicated time periods. A corresponding volume of DMSO was added to untreated control cells.

2.2. Western blot analysis

Western blot analysis was performed using standard method. Specific primary antibodies against PIM1 (3247, Cell Signaling), CBX8 (A300-882 A, Bethyl), Phosphoserine (ab9332, Abcam), p16 (sc-468, Santa Cruz), β-actin (PM053; MBL).

2.3. Immunoprecipitation, silver staining and mass spectrometry

Cell lysate enriched overnight with M2 beads at 4 °C. Beads were washed five times with lysis buffer and resolved on NuPAGE 4–12% Bis-Tris gel (Invitrogen), silver stained using Pierce silver stain kit (Thermo) and subjected to LC–MS/MS analysis.

2.4. Co-immunoprecipitation and in vivo phosphorylation

Endogenous PIM1 or CBX8 was immunoprecipitated with anti-PIM1 or anti-CBX8 and protein A-Sepharose beads. After extensive washing and heating in the sample buffer, the complexes were subjected to immunoblot [19]. Cell lysates were subjected to immunoprecipitation (IP) with CBX8 antibody (Endogenous) or gst-specific beads (Exogenous transfection, GST-CBX8). Aliquots of immunoprecipitation were subjected to Western blotting using p-Ser antibody.

2.5. GST pull-down and in vitro kinase assay

GST and GST-CBX8 or His-PIM1 or His-mPIM1^{K67M} were purified from Transetta (DE3) cells using glutathione Sepharose or Ni Sepharose. Co-incubate equal amounts of GST or GST-CBX8 (200 ng/each) with His-PIM1 (200 ng) in BC100 buffer overnight at 4 °C, adding Glutathione-sepharose beads for 2 h. The mixture (resin and binding proteins) was washed three times by centrifugation at 500 × g for 3 min to remove the unbound proteins and was analyzed by western blotting using anti-GST and anti-His. For *in vitro* kinase assay, Co-incubate purified GST-CBX8 with purchased full-length PIM1 or purified mPIM^{K67M}, and added 10 μM ATP in kinase buffer (Cell Signaling) for 30 min at 30 °C. Western blot analysis was performed using p-Ser antibody.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as previously described [20]. The antibodies used was purchased from Bethyl, while the immunoprecipitated DNA was quantified by real-time PCR. ChIP primers were designed in the region 200bp after first exon of *IN4A* and primer pairs only amplify one amplicon. Sequences was followed: 5′-GCCAAGGAAGAGGAATGAGGAG-3′; 5′-CCTTCAGATCTTCTCA GCATTCG-3′.

2.7. SA-β-gal staining

SA-β-gal activity staining was performed as described previously [21]. All cells were examined using fluorescence microscopy with the appropriate filters.

2.8. Cell proliferation assay

The CCK-8, colony formation and EdU incorporation assays were adopted to evaluate the cell proliferation.

For the CCK-8 assay, CCK8 kit (CCK-8; Dojindo Laboratories, Japan) was used according to the manufacturer's instructions. 3 × 103 cells were planted into 96-well dish with three replicates. Then, 100 μl of serum-free cell culture medium containing 10 μl WST-8 reagent was added into each well every 24 h and the plates were incubated at standard conditions for 1 h. Optical absorbance of each well at 450 nm and 630 nm were measured with a microplate reader (Bio-Rad Laboratories, USA). Three independent experiments were performed for quantification.

For colony formation assay, cells were plated in 6-well plates in triplicates (400 cells per well). After 10 days, the cells were washed with PBS, fixed with 4% formaldehyde for 10min and stained with crystal violet staining solution (Sigma-Aldrich) for 15min. The colonies were counted and photographed.

For the EdU incorporation assay, EdU Apollo®567 In Vitro Imaging Kit (Ribo Bio, China) was used according to the manufacturer's instructions except that cell nuclei were stained with DAPI instead of Hoechst for 30 min. Five fields of each well were randomly chosen and observed under fluorescence microscopy. All images were processed using Image J software and the proportion of EdU incorporated cells was calculated. Three independent experiments were performed for quantification.

3. Result

3.1. PIM1 interacts with CBX8

To explore the mechanism by which PIM1 induces cell senescence, we employed immunopurification and mass spectrometry to identify proteins that are potentially associated with PIM1 in 2BS cells (human embryonic lung fibroblasts, a cell model commonly used in studies of replicative senescence [22,23]). To this end, FLAG-PIM1 was transiently expressed in 2BS cells. Cellular extracts were prepared and subjected to affinity purification using anti-FLAG affinity gels. The eluted protein complex was then resolved on SDS-PAGE, silver stained, and subjected to LC-MS/MS analysis (Fig. 1A). The results showed that PIM1 was co-purified with a number of proteins, including CBX8, as well as other known interacting proteins of PIM1 such as SND1 and HSP90 [24,25].

To confirm that CBX8 is physically associated with PIM1 in cultured cells, we co-transfected 293 T cells with GST-CBX8 and