International Journal of Biological Macromolecules 65 (2014) 41–50

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Interferon regulatory factor-1 (IRF-1) interacts with regulated in development and DNA damage response 2 (REDD2) in the cytoplasm of mouse bone marrow cells

Manish Gupta, Pramod C. Rath *

Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, 110067, India

ARTICLE INFO

Article history:
Received 14 September 2013
Received in revised form
12 December 2013
Accepted 2 January 2014
Available online 9 January 2014

Keywords:
Interferon regulatory factor-1
Regulated in development and DNA damage response 2
Protein-protein interaction
Bone marrow cells

ABSTRACT

IRF-1 is a critical hematopoietic transcription factor, which regulates cell growth, development of immune cells, immune response, tumor suppression, apoptosis and autophagy in mammalian cells. Protein-protein interactions of IRF-1 in mouse bone marrow cells (BMCs) by GST-IRF-1 pull-down followed by mass spectrometry, coimmunoprecipitation, immunoblotting and colocalization show that regulated in development and DNA damage response 2 (REDD2) is an IRF-1-interacting protein. REDD2 is a highly conserved mammalian regulatory protein of the TSC2/mTOR pathway. It is structurally similar to REDD1 but has a distinct loop region. Cellular IRF-1 and REDD2 complex is present in the cytoplasm of BMCs as distinct speckles in punctate pattern. In vitro interaction of recombinant IRF-1 and REDD2 shows their physical interaction. Taken together, our results suggest that IRF-1 physically interacts with REDD2 in the large cytoplasmic protein complex, which may function as cellular signaling proteins for 'cross-talk' of mTOR and cytokine pathways during regulation of cell growth/proliferation, apoptosis and autophagy of mammalian bone marrow cells during health and disease.

1. Introduction

Mammalian bone marrow is a major source of adult stem cells and the site for production of blood and immune cells. The immune cells produce cytokines like interferons (IFNs) upon exposure to viral and bacterial pathogens as well as tumor cells [1]. Type I IFN (IFN-α/β) acts on the dormant hematopoietic stem cells (HSCs) and increase their proliferation and turnover into the progenitor cells in the bone marrow [2,3]. IRF-1 regulates expression of type I and type II IFNs and its own expression is regulated by both type I and type II IFNs [4]. IRF-1 is a critical transcription factor involved in differentiation and maturation of immune cells and blood cells [5]. IRF-1$^{-/-}$ mice exhibited an increased number of immature granulocytic precursor cells, reduction of TCRαβ^{+}CD4^{-}CD8^{+} T cells with defects in thymocyte development [6]. Clonogenetic analyses in IRF-1$^{-/-}$ mice revealed a reduced number of CFU (colony forming units)-G, CFU-M and CFU-GM colonies, while the number of BFU-E/CFU-E colonies (G, granulocytic; M, monocytic; GM, granulo-monocytic; BFU, burst forming unit; E, erythroid) remained unchanged [7]. IRF-1 also participates in establishment of the microenvironment for NK-cell development through secretion of IL-15 from the bone marrow stromal cells [8].

IRF-1 activates transcription of genes involved in immune response, cell growth regulation, tumor suppression, autophagy and apoptosis [9–12] and its activity is regulated by association with other proteins and post-translational modifications [13,14]. IRF-1 interacts with ICSBP/IRF-8 in DNA-dependent and independent manners [15], which result in inhibition of IRF-1-stimulated effects on gene expression [16]. IFN-γ induces IRF-1, which is activated via interaction with MyD88 and it rapidly migrates to nucleus of HEK-293 T cells [17]. IRF-1 makes complex(es) with ICSBP along with PU.1 [18,19], CBP [20] and NF-κB [21] to stimulate gene expression for host-defense response. Binding of IRF-1 to coactivator p300 stabilizes the association of p300 with transactivation domain of p53 and thus enhancing acetylation and activity of p53 and increased p21^{WAF1} expression [22]. IRF-1 associated with unphosphorylated STAT1 and occupied ICS-2/GAS element of the LMP2 promoter [23].

In addition to its well-studied transcriptional function, IRF-1 may also regulate MAPK-signaling by protein-protein interaction with a signaling complex containing c-Cbl [24]. IRF-1 has a short half-life of 20–40 min [25] and several partners are engaged in its post-translational regulation by covalent amino acid modifications,

Abbreviations: CBP, CREB-binding protein; CHIP, C terminus of Hsc (heat-shock cognate) 70-interacting protein; GAS, γ interferon-activated sequence; Hsp, heat shock protein; ICS, interferon consensus sequence; ICSBP, interferon consensus sequence-binding protein; LMP2, low molecular mass polypeptide 2; PIAS3, protein inhibitor of activated STAT3; SUMO-1, small ubiquitin-related modifier-1; Ubc9, ubiquitin-conjugating enzyme 9.

* Corresponding author. Tel.: +911126704525; fax: +911126742558.
E-mail addresses: pcrath@mail.jnu.ac.in, rathpuri@yahoo.co.in (P.C. Rath).

0141-8130/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijbiomac.2014.01.005

proteolysis and stabilization. Hsp70/Hsp90 and CHIP or MDM2 bind to multifunctional 1 (Mf1) (301–325 aa) and Mf2 (106–140 aa) intrinsically disorder domains of IRF-1, respectively and regulate its steady-state level, localization, transcriptional activity and turnover [26–28]. CHIP and MDM2 are E3 ligase components of the ubiquitin machinery, which mediates ubiquitination of IRF-1 DNA binding domain only when unbound to cognate DNA sequence [28]. PIAS3 enhances SUMOylation of IRF-1, which leads to suppression of its transcriptional activity and increases stability. The tumor cells have high level of SUMOylated IRF-1 level, which prevents IRF-1-mediated cell death [29,30]. Moreover, casein kinase II phosphorylates IRF-1 at two different amino acid clusters located at N- and C-terminal. Loss of C-terminal phosphorylation significantly decreased the transactivation by IRF-1 [14]. In patients with myelodysplastic syndrome (MDS) or leukemia, IRF-1 mRNA lacks exon 2, includes AUG initiation codon and exon 3. The exon-skipped form of IRF-1 is devoid of its DNA binding- and transactivation activities [31]. Thus protein-protein interactions of IRF-1 may regulate its functions.

We show that IRF-1 interacts with REDD2 in the mouse bone marrow cells. Both IRF-1, REDD2 are colocalized in the cytoplasm of cultured bone marrow cells as distinct speckles in punctate foci, which may represent large protein complexes as cell signaling hubs. IRF-1 physically interacts with REDD2 *in vitro*. In melanoma, REDD2 expression is silenced by promoter hypermethylation [32]. Moreover, REED2 is a negative regulator of mTOR pathway and it increases susceptibility for cell death [33,34]. Hence, we propose that interaction of IRF-1 and REDD2 may be involved in regulation of cell growth/proliferation, apoptosis and autophagy of mammalian bone marrow cells.

2. Materials and methods

2.1. Materials

C57Bl6/J mouse (4–6 weeks old) was obtained from the animal house facility and used for the study as per the guidelines of the Institutional Animal Ethics Committee of the University. Plasmids: pGEX2TK vector, pGEX-IRF-1[35], pCDNA3.1 + Zeo-3HA-REDD2 [36], pET28a(+) vector, pET-REDD2; Oligonucleotides: REDD2forward = 5′GGGAATTCCATATGGTTGCAACGGGC3′, REDD2reverse = 5′CGCGGATCCTTAGCACTCTTCAATGACTG3′; Antibodies: rabbit polyclonal anti-IRF-1 and goat polyclonal anti-REDD2, normal goat IgG, normal rabbit IgG (Santacruz), rabbit-anti-GST, goat-anti-rabbit IgG(HRP) and rabbit-anti-mouse IgG(HRP) (Sigma), goat-anti-rabbit IgG(TRITC), rabbit-anti-goat IgG(FITC), rabbit-anti-goat IgG(HRP) (Bangalore Genei); DMEM culture medium, FBS, antibiotics + antimycotic, tissue culture items and glutathione-agarose, Ni-NTA-agarose, protein A/G-sepharose, aprotinin, leupeptin, benzamidine, IPTG (Sigma); Vectasheild-DAPI (Vector Labs) and molecular biology reagents (Sigma/Merck) were used for the study. Molecular biology methods were followed as described in [37] with certain modifications.

2.2. Expression, purification and immunoblotting of GST-IRF-1

Recombinant GST-IRF-1 was expressed as described previously with some modifications [35]. The pGEX-2TK and pGEX-IRF-1 plasmids in *E. coli* BL21 cells were grown in LB-medium with 100 μg/ml ampicilin for 2–3 h at 37 °C up to 0.5–0.6 OD600 nm and induced by IPTG (100 μM) at 18 °C for 4 h. Cells were lysed in lysis buffer (50 mM Tris-Cl, 140 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 μg/ml each aprotinin and leupeptin, 250 μg/ml benzamidine and 1 mM PMSF). The cell extract was incubated with glutathione-agarose beads on ice for 4 h, washed three times, 10 min each with lysis buffer on ice. Bead bound protein was eluted in elution buffer (50 mM Tris-Cl, 140 mM, NaCl, 20 mM reduced glutathione and protease inhibitors as per lysis buffer) or used directly in further experiment(s). The affinity purified GST-IRF-1 protein was resolved in 10% SDS-PAGE and blotted onto nitrocellulose membrane, blocked with 5% non-fat dry milk for 2 h at RT and incubated with polyclonal IRF-1-antibody (Santacruz) at 1:1000 dilution for 2 h at RT. The blot was washed by PBST for 3 × 10 min and incubated with goat anti-rabbit-HRP antibody (Sigma) at 1:10,000 dilution for 2 h. The blot was washed with PBST and developed with 3,3′-diaminobenzidine (DAB) staining.

2.3. GST-IRF-1 pull-down assay

Bone marrow cells (BMCs) from the fore and hind limbs were aseptically flushed out by ice cold 1X PBS (pH 7.4) and collected by centrifugation. The cellular protein extract was prepared in cell extract buffer I (20 mM Tris-Cl, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% Triton X-100, 1 mM DTT, 10 μg/ml each aprotinin and leupeptin, 250 μg/ml benzamidine and 2 mM PMSF). Total protein (500 μg) extract was precleared with 150 nM of bead bound GST for 1 h on ice and centrifuged. Precleared supernatant extract was divided into aliquots and incubated with equimolar (150 nM) amounts of GST and GST-IRF-1 on ice for 3 h. Beads were collected by centrifugation and washed three times with five bed-volume of cell extract buffer for 10 min each on ice. Equal volume of 1X SDS-loading dye was added to the beads and boiled. Samples were resolved on 10% SDS-PAGE and proteins in the gel were stained with Coomassie Brilliant Blue R-250 (CBB).

2.4. Enzymatic in-gel digestion and MALDI mass spectrometry

The distinct and specific protein bands were excised from SDS-PAGE and minced into small size pieces of 1 mm^3. Minced band was destained with destaining solution [40% 100 mM ammonium bicarbonate (ABC) and 60% acetonitrile] for 30 min at 37 °C. The dehydration (2:1 of acetonitrile:50 mM ABC) and rehydration (50 mM ABC) step was carried out three times at RT for 5 min each. After dehydration, samples were reduced by addition of 20 mM DTT for 1 h at 60 °C, followed by alkylation with 10 mM idoacetamide for 20 min in dark at RT. Gel pieces were rinsed three times with rehydration and dehydration buffer, and vacuum dried. Trypsin (250 ng/μg in 50 mM ABC) was added to the dried-gel pieces and incubated at 37 °C for overnight. Peptide extraction was carried out three times with freshly prepared 0.5% trifluoro acetic acid (TFA) in 50% acetonitrile for 20 min at 37 °C. Extracted peptides were pooled and vacuum dried. For MALDI-TOF/MS analysis, peptides were dissolved in 0.1% TFA and mixed with equal volume of α-cyano-4-hydroxycinnamic acid matrix (Bruker). Mass spectra were measured on Autoflex™ III TOF/TOF of Bruker Daltonics. Peptide mass fingerprinting (PMF) spectra was acquired in the mass range of 600–3500 Da. The peak lists were searched using MASCOT search engine against mass spectrometry protein sequence database (MSDB) or National Center for Biotechnology Information non-reduntant (NCBInr) database with the following search setting: peptide tolerance from 100–200 ppm, missed cleavage site value set to one, variable carbamidomethylation of cysteine, oxidation on methonine and protein N-terminal acetylation. No restriction on peptide mass and pI value were applied.

2.5. Immunoprecipitation and immunoblotting

BMCs were isolated and lysed in cell extract buffer II (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5% NP-40, 0.1% Triton X-100, 5 mM NaF, 20 mM sodium orthovanadate, 2 mM PMSF, 10 μg/ml each aprotinin and leupeptin, 250 μg/ml