Cytokine 88 (2016) 209–213

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Short communication

Phorbol-12-myristate-13-acetate (PMA) mediated transcriptional regulation of Oncostatin-M

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 8 September 2016
Accepted 13 September 2016
Available online 24 September 2016

Keywords:
C/EBP-β
CHOP
Genistein
CCAAT consensus sequence

ABSTRACT

Oncostatin-M (OSM), an IL-6 family cytokine, exhibits varied roles in different patho-physiological conditions. Differential expression of OSM in response to varying stimuli indicates importance of its regulation of expression. The present study illustrated transcriptional induction of *osm* on treatment with chemical inducer, phorbol-12-myristate-13-acetate (PMA). Following initial hours of PMA treatment, a nuclear protein C/EBP-β binds specifically to the CCAAT consensus sequence at the proximal end of the OSM promoter. Genistein (a specific Tyr phosphorylation inhibitor) leads to the interaction of CHOP (C/EBP Homologous Protein) with C/EBP-β, thus negatively regulating it. Knockdown of C/EBP-β also leads to inhibition of PMA-mediated OSM induction.

1. Introduction

Oncostatin-M is a secreted glycoprotein monomer of 28 kDa [1] first purified from a conditioned media containing U937 monocytic cells treated with phorbol 12-myristate 13-acetate (PMA), which eventually resulted in abrogation of growth of melanoma cells and induction of monocyte-macrophage differentiation [2]. A differentiating cell undergoes new gene transcription resulting in the expression of several proteins thus confirming a significant role of transcription factors in this context [3,4]. Functional characterization of the promoter region of *osm* revealed the presence of several putative transcription factor binding sites including GC-rich element and CCAAT box [5]. The latter is known to be the binding site for C/EBP (CCAAT-enhancer binding proteins), which belong to a family of the basic region–leucine zipper (bZip) class of transcription factors recognizing the consensus DNA-binding sequence 5′-ATTGCGCAAT-3′ or putative CCAAT box in the regulatory regions of target genes [6]. C/EBP family proteins (C/EBP-α to ζ) bind as either homodimers or heterodimers and are expressed in a wide variety of tissues [7]. Of these, C/EBP-β is dramatically induced during monocyte-macrophage differentiation [8] and C/EBP-binding motifs are found in the functional regulatory regions of genes specifically induced in activated macrophages [8–10]. However, the DNA binding and transcriptional activity of C/EBP-β during adipocyte differentiation was reported to be blocked by tyrosine kinase inhibitor, Genistein, by promotion of the expression of CHOP (C/EBP homologous protein), a dominant-negative member of the C/EBP family [11].

In the present study, we intend to show that PMA mediates transcriptional upregulation of Oncostatin-M by binding of the transcription factor, C/EBP-β to the CCAAT-consensus sequence present in *osm*-promoter.

2. Materials and methods

2.1. Reagents

The reagents are listed in supplementary material (S1).

2.2. Cell culture and treatment with PMA and Genistein

Human histiocytic lymphoma cell line, U937 was cultured and treated with PMA (32 nM) according to [12]. Genistein (100 μM), a tyrosine kinase inhibitor, was co-treated with PMA.

2.3. RNA extraction and RT-PCR

RNA was isolated from U937 (2×10^6) cells with TRIzol according to the manufacturers' instruction, which were reverse transcribed and PCR amplified semi-quantitatively or quantitatively [12]. Primers used are provided in supplementary material (S1).

Abbreviations: PMA, phorbol 12-myristate 13-acetate; OSM, Oncostatin-M; IL-6, interleukin 6; C/EBP, CCAAT-enhancer binding protein; CHOP, C/EBP homologous protein; hnRNA, hetero nuclear RNA.

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http://dx.doi.org/10.1016/j.cyto.2016.09.006
1043-4666/

2.4. Cloning of reporter plasmids

Full length (FL) promoter of *osm* gene (960 bp) and CCAAT-containing region (CCAAT) were PCR amplified from human genomic DNA (5 ng) of U937 cells using Fwd-1/Rev-2 and Fwd-CEBP/Rev-2 primer pairs respectively (S1). PCR products were cloned in pTZ-57-R/T vector. The CCAAT was then sub-cloned into mammalian expression vector, pEGFP-1 yielding pE-CCAAT.

2.5. Transfection of plasmids and siRNAs

U937 cells were transfected using jetPRIME plasmid/siRNA transfection reagent (Polyplus-transfections, Illkirch, France) according to manufacturer's protocol, followed by treatment with Genistein and/or PMA after 48 h of transfection. For siRNA transfections, 50 nM of control siRNA or C/EBP-β duplex RNA (Eurogentec, Belgium) were used (S1).

2.6. Preparation of cell-extracts

U937 cells (10×10^6 cells each) were treated without or with PMA for different time periods. Nuclear extracts were prepared using standardized protocols [13].

2.7. Western blot

Western blot was performed with nuclear extracts of untreated and treated U937 cells with anti-C/EBP β, anti-CHOP, anti-histone H2A and (1:1000 overnight) following standardized protocol [14].

2.8. Preparations of radiolabelled oligomer

5′-end labeling of oligonucleotides (mentioned in S1) were done with [γ-^{32}P] ATP using T4 polynucleotide kinase. After cleaning, the labelled oligomers (20 nM each) and their complementary strands (1:1) were mixed and heated at 95 °C for 5 min and gradually cooled down to RT.

2.9. EMSA, competition assay and antibody supershift assay

For EMSA, 1.5 μg of PMA-treated nuclear proteins were incubated with [γ-^{32}P]-labelled double stranded oligonucleotides (CCAAT region of *osm* promoter) in buffer-A [13] for 15 min in ice. Specificity of binding was checked by competition assay using unlabelled heterologous and homologous DNA. For supershift assay, 1 μg of anti-C/EBP-β and anti β-actin (non-specific) antibodies were pre-incubated with 0.5 μg of nuclear extract for 30 min at 4 °C prior to addition of [γ-^{32}P]-labelled DNA. The reaction mixtures were separated on 6% TBE-polyacrylamide gel, dried and exposed to Phosphor-imager.

2.10. Chromatin immunoprecipitation

Formaldehyde-cross-linked PGE_2 treated U937 cells were taken in RIPA buffer [12] and sonicated (5 pulses) supernatant was subjected to preclearance with protein A/G Sepharose beads. Immunoprecipitation of precleared supernatant was then performed overnight with anti-C/EBP β and normal IgG (control). Next day, the washed beads were reverse cross-linked in buffer D [12] at 65 °C, chloroform extracted and precipitated. Semi-quantitative PCR was performed with Fwd C/EBP and Rev 7 (S1).

2.11. Protein-protein co-immunoprecipitation

Nuclear extract of PMA treated U937 cells (4×10^7) were prepared as mentioned in [13]. Protein-A/G-Sepharose bead (20 μl) was washed in RIPA buffer [12] and pre-incubated with monoclonal anti-CHOP (1:50) or mouse IgG (0.5 μg/μl) antibodies for 4 h with mild shaking at 4 °C followed by gentle washing with RIPA buffer for five times. Equal amount of pre-cleared nuclear extract was added to the antibodies and incubated overnight under mild shaking followed by 4–5 times washing with RIPA buffer. The precipitate was subjected to western blot.

2.12. Statistical analysis

All graphs were generated in Microsoft Office Excel 2007 (Microsoft Corporation, Washington) and data are represented as mean (± standard deviation or SD) of at least three independent experiments.

3. Results and discussion

3.1. PMA induces Oncostatin-M expression transcriptionally

U937 cells, on treatment with PMA, displayed an initial burst (~14 folds within 30 min) of *osm* mRNA level (normalized to β-actin) as measured by qPCR, which decreased to normal level by 2 h (Fig. 1A). The levels of nascent mRNA (hnRNA) that denotes active transcription followed similar pattern of elevation (~52 folds within 30 min) indicating transcriptional induction of OSM.

3.2. CCAAT box present in OSM proximal promoter region is a PMA-responsive element

The proximal promoter region of *osm* containing consensus CCAAT sequence (-48) was cloned upstream of GFP reporter of a promoter-less vector, pEGFP-1. U937 cells were transfected with this vector (pE-CCAAT) and expression of GFP (normalized to Neomycin) was measured by qPCR after PMA induction. Results show instantaneous increase in GFP expression (~5 folds within 30 min) by PMA, which decreased gradually (Fig. 1B), indicating CCAAT region of *osm* promoter to be a PMA-responsive *cis*-regulatory element.

3.3. Specific binding of proteins of PMA-treated nuclear extract with CCAAT-containing basal promoter sequences

Fig. 1C shows interaction of nuclear proteins present in PMA treated nuclear extracts of U937 cells with radiolabelled oligomer containing CCAAT region of *osm* promoter by EMSA. Although proteins of untreated cells displayed binding, it increased with time, peaking at 10 min post-treatment, and then decreasing to the basal levels within 1 h. The specificity of binding was evident by competition assay (Fig. 1D) using 2X and 5X molar excess of homologous (unlabelled CCAAT) and heterologous (unlabelled non-CCAAT) oligonucleotides. EMSA with GC-rich region of the proximal *osm* promoter (Fig. 1E) showed binding of nuclear protein with no changes on PMA treatment, and thus was not considered as a PMA inducible element. Thus, the above results signify that PMA-treated nuclear proteins (initial 2 h) of U937 cells specifically bind to the CCAAT-box consensus sequence of *osm* promoter.

3.4. The presence of C/EBP-β in the PMA-treated DNA-protein complex and direct association with CCAAT box

C/EBP-β, a protein often involved in differentiation and induction of IL-6 family cytokines, could be the *trans*-acting factor responsible for PMA-induced transcription, as it has binding affinity towards CCAAT-box. Fig. 1F shows that DNA-protein complexes got super-shifted in presence of C/EBP-β antibody, while