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Short communication

Chromatin remodeling protein SMAR1 regulates NF-κB dependent Interleukin-8 transcription in breast cancer

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ABSTRACT

Interleukin-8 (IL-8) is a pleiotropic chemokine involved in metastasis and angiogenesis of breast tumors. The expression of IL-8 is deregulated in metastatic breast carcinomas owing to aberrant NF-κB activity, which is known to positively regulate IL-8 transcription. Earlier, we have shown that tumor suppressor SMAR1 suppresses NF-κB transcriptional activity by modulating IκBα function. Here, we show that NF-κB target gene IL-8, is a direct transcriptional target of SMAR1. Using chromatin immunoprecipitation and reporter assays, we demonstrate that SMAR1 binds to IL-8 promoter MAR (matrix attachment region) and recruits HDAC1 dependent co-repressor complex. Further, we also show that SMAR1 antagonizes p300-mediated acetylation of RelA/p65, a post-translational modification indispensable for IL-8 transactivation. Thus, we decipher a new role of SMAR1 in NF-κB dependent transcriptional regulation of pro-angiogenic chemokine IL-8.

1. Introduction

Interleukin-8 (IL-8) is a pleiotropic chemokine involved in variety of pathophysiological processes. It has been shown to play an important role in human cancers by modulating metastasis and angiogenesis (Xie, 2001; Ali and Lazennec, 2007). A wealth of evidence suggests that IL-8 is aberrantly expressed in number of cancers including breast cancer (Freund et al., 2003; Yao et al., 2007). An increased serum IL-8 level has been reported in metastatic breast cancers, which correlates with early dissemination and survival (Benoy et al., 2004). The highly metastatic breast carcinoma cells lacking estrogen receptor-α, produce more IL-8 and the expression of this chemokine is differentially regulated in metastatic and non-metastatic breast cancer cells (De Larco et al., 2001; Freund et al., 2004). This is partially attributed to the differential function of inducible transcription factors, which govern IL-8 transcription (Hoffmann et al., 2002). NF-κB transcription factors play a major role in constitutive expression of IL-8 in metastatic breast cancer cells (Freund et al., 2004). Activity of NF-κB is itself regulated by several important post-translational modifications, which dictate stimulus dependent and independent regulation of its target genes. A number of post-translational modifications such as phosphorylation, ubiquitination or acetylation of RelA/p65 has been documented (Perkins, 2006; Oeckinghaus and Ghosh, 2009). Acetylation of RelA/p65 is the most important post-translational modification required for its transactivation function and this is achieved by its interaction with co-activators such as CBP (CREB-binding protein)/p300 (Zhong et al., 2002). On the other hand, deacetylation of RelA/p65 by different histone deacetylases (HDACs) such as HDAC3 and SIRT1 is also reported to modulate its transactivation potential (Chen and Greene, 2004; Yeung et al., 2004). Therefore, the acetylation and deacetylation switch governs the activator or repressor function of RelA/p65. The proteins that can modulate acetylation and deacetylation dynamics of RelA/p65, play a pivotal role in regulation of its target genes.

We previously identified a subset of NF-κB target genes, the expression of which altered upon knockdown and overexpression of SMAR1 (Singh et al., 2009). One such candidate gene was Interleukin-8 (IL-8), a bonafide NF-κB target gene. In this report, we elaborate on molecular mechanisms that confer differential expression/regulation of IL-8 in metastatic and non-metastatic breast

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cancer cells by SMAR1. The chromatin remodeling protein SMAR1 is a putative tumor suppressor (Badhwar et al., 2007; Rampalli et al., 2005), located on 16q24.3 locus, the loss of heterozygosity (LOH) of which has been reported in breast cancers (Kouvaraki et al., 2001). SMAR1 expression is dramatically reduced in metastatic breast cancer cell lines and invasive ductal carcinomas of breast (Rampalli et al., 2005; Singh et al., 2007). Being a nuclear matrix protein, SMAR1 interacts with *cis* regulatory elements known as MARs (matrix attachment regions) present on promoter regions of genes involved in diverse cellular processes (Malonia et al., 2011). Here, we present a dual mechanism by which SMAR1 regulates NF-κB dependent IL-8 transcription in breast cancer cells.

2. Materials and methods

2.1. Cell lines, plasmids and reagents

Breast cancer lines MCF-7, SKBR-3, MDAMB-231 and MDAMB-435 were obtained from NCCS repository. Cells were cultured in media recommended by ATCC supplemented with 10% fetal bovine serum. Flag-SMAR1, pBKCMV-SMAR1, GST-SMAR1, SMAR1-shRNA constructs and recombinant SMAR1 adenovirus were used as described previously (Sinha et al., 2010), p300 expression plasmid was provided by Xuan Liu (University of California, USA). TNF-α (Sigma) was used at concentration (20 ng/ml, 3 h) or as stated in figure legends. All transfections were done using Lipofectamine 2000 (Invitrogen).

2.2. ELISA

Breast cancer cell lines were either transduced with control (Ad-V) or recombinant SMAR1 adenovirus SMAR1 (Ad-SMAR1). Forty-eight hours post-transduction, the vector and SMAR1 expressing cells were seeded at density of 1×10^5 cells per well in a 12 well plate and cultured for additional 24 h. IL-8 concentration in culture supernatants was determined using ELISA kit (BD Biosciences) as per manufacturer instructions.

2.3. Reverse transcription PCR (RT-PCR)

Total RNA was extracted using TRIZol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription was performed using Superscript II kit (Invitrogen) followed by semi quantitative and quantitative PCR with SYBR green Supermix (Invitrogen) on Mastercycler gradient (Eppendorf) and ABI prism 7500 platform (Applied Bio systems) using primers given in Table S1. *GAPDH* was used as an internal control. Quantification of mRNA was done by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.09.008.

2.4. Luciferase reporter assays

IL-8 promoter (-1482 to +44 bp) luciferase construct (xp2-IL8) as described (Freund et al., 2004) was co-transfected with Flag-SMAR1 or SMAR1-shRNA along with pCMV-GFP plasmid. Twenty-four hours post-transfection cells were analyzed for luciferase activity using Luclite substrate (Perkin Elmer, USA). Transfection efficiencies were normalized to GFP fluorescence using Fluoroskan Luminometer (Lab Systems). All assays were done in triplicates.

2.5. Co-immunoprecipitation and immunoblot analysis

The whole cell extracts were prepared by lysing cells in TNN buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA and 1 mM DTT) supplemented with complete protease-inhibitor cocktail (Roche) and subjected to co-immunoprecipitation and immunoblot analysis as described previously (Sinha et al., 2012). The following antibodies were used, Ac-p65 Lys310 (Cell Signaling), RelA/p65, p300, actin, tubulin (Santa-Cruz) and SMAR1 (Bethyl Laboratories).

2.6. ChIP assays

Chromatin immunoprecipitation assays were performed using ChIP assay kit (Upstate) following manufacturer's instructions. Sequential ChIP was performed as described previously (Sinha et al., 2010). Briefly, the sonicated chromatin was immunoprecipitated with SMAR1 antibody, eluted DNA-protein complexes were diluted two fold in ChIP dilution buffer and incubated with SMAR, p65 and HDAC-1 antibodies. IgG was used as a control. Immunoprecipitated DNA was further subjected to semi-quantitative or quantitative PCR using the primers given in Table S1. Site-specific relative fold enrichment was calculated by comparing the amplification threshold (Ct) value of a given ChIP sample at IL-8 promoter with the amplification Ct of IgG control at the same target locus. For enrichment background was subtracted by normalizing over a β-actin promoter.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described (Rampalli et al., 2005). Briefly, a 200 bp IL-8 MAR probe was PCR amplified using human genomic DNA, labeled with [α-^{32}P] dCTP and purified by Probe quant G-50 columns (Amersham). Binding reactions were performed using recombinant GST-SMAR1 at room temperature for 30 min and complexes were resolved by 8% Native-PAGE. Vacuum dried gels were processed for autoradiography. Primer sequences used for amplifying the probe are given in Table S1.

2.8. Statistical analysis

All experiments were performed in triplicates and the quantitative data expressed as ±standard error of mean (SEM) or standard deviation (SD). Differences between groups were determined using two tailed Student's *t* test or one-way ANOVA, using Prism GraphPad Software. Significant differences were considered when $P \leq 0.05$ ($^{*}P < 0.05$ and $^{**}P < 0.01$).

3. Results

3.1. SMAR1 regulates IL-8 expression at transcriptional level

Previous studies have shown a strong correlation between metastatic potential of breast carcinomas and IL-8 expression; the undifferentiated metastatic cells secrete high amount of IL-8 compared to their non-metastatic counterparts (De Larco et al., 2001; Freund et al., 2003). Since, SMAR1 expression is lost in metastatic breast carcinomas (Singh et al., 2007), we investigated if the loss of SMAR1 correlates with IL-8 expression. Analysis of transcript by RT-PCR showed a high level of IL-8 mRNA in metastatic breast cancer cells MDAMB-231 and MDAMB-435, which have relatively low levels of SMAR1 (Fig. 1A, lanes 1 and 2). On the other hand, IL-8 transcript was completely absent in non-metastatic MCF-7 and SKBR-3 cells, which have high expression of SMAR1 (Fig. 1A, lanes 3 and 4). Quantification of IL-8 by ELISA in culture supernatants also demonstrated that IL-8 secretion is high in metastatic MDAMB-231 and MDAMB-435 cells, which do not express SMAR1, compared to non-metastatic MCF-7 and SKBR-3 cells (Fig. 1B). This inverse correlation between SMAR1 and IL-8 expression led us to hypothesize that SMAR1 might regulate IL-8 production. Ectopic