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EZH2 phosphorylation regulates Tat-induced HIV-1 transactivation via ROS/Akt signaling pathway

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ABSTRACT

EZH2 plays a major role in HIV-1 latency, however, the molecular linkage between Tat-induced HIV-1 transactivation and EZH2 activity is not fully understood. It was shown Tat induced HIV-1 transactivation through inhibiting EZH2 activity. Tat decreased the levels of H3K27me3 and EZH2 occupy at the long terminal repeat (LTR) of HIV-1. We further showed for the first time that transfected with Tat construct resulted in an increase in phosphorylated EZH2 (p-EZH2), mediated by active Akt. ROS/Akt-dependent p-EZH2 was correlated with Tat-induced transactivation. Our study reveals that novel mechanisms allow Tat-induced HIV-1 transactivation by ROS/Akt-dependent downregulating the EZH2 epigenetic silencing machinery.
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1. Introduction

Despite the great success of the highly active anti-retroviral therapy (HAART) in treatment of HIV-1 infection and acquired immunodeficiency syndrome (AIDS), HAART fails to completely eradicate the virus in AIDS [1–2]. In addition to reverse transcriptase, protease, and gp41 as HIV-1 targets, HIV-1 transcriptional regulation seems to be another important target [3]. The comprehensive understanding of the molecular control of HIV-1 transcriptional regulation should inform the development of optimal reactivation strategies that are intended to purge the latent viral reservoir.

Chromatin remodeling plays an important role in the transcriptional activation of HIV-1 [4–6]. During HIV-1 activation, transcription cofactors including chromatin-modifying enzymes and chromatin-remodeling complexes are recruited to host chromatin-associated proteins [7]. In the contrary, multiple epigenetic regulatory mechanisms including repressive histone methylation and histone deacetylation are involved in mediating latent HIV-1 genomes silenced [8–10]. The recruitment of polycomb repressive complex-2 (PRC2) which mediates histone methylation becomes the target for additional silencing via deacetylated proviral chromatin [11].

Enhancer of zeste homolog 2 (EZH2) as an important histone methyltranferase, functions to trimethylate lysine 27 on histone H3 (H3K27me3). In resting $CD4^+$ cells and HIV-1 latent cells, the increase of EZH2 expression has been linked to silencing HIV-1 gene expression [12]. EZH2 activity is regulated in a number of ways including transcription factors, microRNAs, signaling pathways, post-translational modifications, and reactive oxygen species (ROS) [13]. In addition, phosphorylation of EZH2 protein by activation of AKT signaling pathway can inhibit EZH2 activity and hence release gene silencing [14]. Although some reports suggest that EZH2 plays an important role in HIV-1 postintegration latency [15], the molecular and signaling pathway linkage between Tat-induced HIV-1 transactivation and EZH2 activity is not fully elucidated.

In this study, we silenced EZH2 and treated with EZH2 inhibitor DZNep, which potentiated Tat-induced HIV-1 transactivation. We also explored the underlying mechanism and related pathway that modulates HIV-1 transcription. Exploration of the molecular mechanism of the effect of EZH2 on Tat-induced HIV-1 transactivation will help us develop novel approaches for the diagnosis, treatment, and prevention of HIV-1 infection.

2. Materials and methods

2.1. Materials

The PI3K inhibitor LY294002 was obtained from Calbiochem (San Diego, CA). 3-Deazaneplanocin A (DZNep) (Cayman Chemical Company, MI) was dissolved in dimethyl sulfoxide (DMSO) at the

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final concentration of 10 mM. The siRNA sequence targeting EZH2, Akt, control siRNA were chemically synthesized by GenePharma (Shanghai, China). The antibody against Akt was purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibodies against β-actin, GAPDH, and Lamin B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against H3K27 trimethylation, EZH2, p-EZH2 (S21) were obtained from Abcam (Cambridge, UK). The secondary antibodies IR700 anti-rabbit and IR800 anti-mouse were obtained from LI-COR Biosciences (Lincoln, Nebraska, USA). Opti-MEM medium, and Lipofectmanine 2000 transfection reagent were obtained from Invitrogen (Grand Island, NY). The pCI-Tat plasmid was kindly provided by Prof. Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing). All other chemicals were of the highest commercial grade available.

2.2. Cloning and site-directed mutagenesis

Human Flag-EZH2 and Flag-EZH2-H689A plasmids were obtained from Addgene. EZH2/S21A and EZH2/S21D mutants were generated using the QuikChange II Site Directed Mutagenesis Kit (Stratagene) as per the manufacturer's protocol. S21 in EZH2 was replaced with either alanine or aspartic acid, using the following primers: S21A, 5′-TGT CTC AGT CGC ATG TAC TCA GCT TTT ACA CGC TTC CGC CAA C; S21D, 5′-TGT CTC AGT CGC ATG TAC TCA TCT TTT ACA CGC TTC CGC CAA C. All products of PCR mutagenesis were sequenced and confirmed its correction.

2.3. Cell culture and cell transfection

TZM-bl cells were HeLa cells which contain both an integrated LTR-Luc reporter gene and an integrated LTR-β-Gal gene [16]. TZM-bl cells were cultured in high glucose-containing DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. For all transfections, TZM-bl cells were transfected with 100 nM of Tat plasmid by using Genejuice transfection reagent as we previously described [17]. Cell extracts were prepared 48 h after transfection.

2.4. siRNA Transfection

TZM-bl cells were plated in six-well plates at a density of 2×10^5 cells per well and grown overnight until they were 50–60% confluent to obtain maximum transfection efficiency. siRNA transfection was preformed as previously described [17].

2.5. Luciferase assay

Forty-eight hours post transfection, luciferase activity of the firefly luciferase of the TZM-bl cells was measured with the Dual-Glo Luciferase Assay (Promega). Luciferase assays were conducted with the Dual luciferase Reporter Kit (Promega). The pRL vector constitutively expressing Renilla luciferase was used to normalize for transfection efficiency [18].

2.6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

QRT-PCR was preformed as previously described [20]. mRNA levels were normalized to GAPDH, which was used as an internal control. All data were obtained from three independent experiments [19].

2.7. Preparation of nuclear, cytoplasmic extracts and whole cell lysate

Nuclear and cytoplasmic protein extracts were prepared as previously described [20].

2.8. Western blot

The nuclear and cytoplasmic extracts and whole cell lysate were fractionated in 10% SDS/polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The blots were incubated overnight at room temperature with primary antibodies and then washed six times in Tris-buffered saline/0.1% Tween 20 prior to 1 h of incubation and visualized with secondary antibodies using Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). All immunoblots were visualized using the Odyssey infrared imaging system (LI-COR Biosciences) [21].

2.9. Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to the manufacturer's instructions. The precipitated DNA was analyzed by q-PCR and primers for HIV-1 LTR (−176 to −61; forward, 5′-CGA GAC CTG CAT CCG GAG TA-3′; reverse, 5′-AGT TTT ATT GAG GCT TAA GC-3′). The primers for GAPDH (forward, 5′-TAC TAG CGG TTT TAC GGG CG-3′; reverse, 5′-TCG AAC AGG AGG AGC AGA GAG CGA-3′). For each reaction, 10% of the recovered DNA was used as an input control [22].

2.10. Statistical analysis

Data were obtained from at least three independent experiments and presented as means ± SD. The significance of differences was calculated with SPSS using one-way ANOVA. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Depletion of EZH2 led to increase in Tat-mediated HIV-1 transactivation

We plated cells in 96-well plate and allowed them to grow overnight before transfecting both Tat construct and the relevant siRNAs. The confirmation RT-PCR and Western blot of the knockdown of EZH2 was shown in Fig. 1A and B. It was shown that the knockdown of EZH2 in these cells resulted in significantly increased Tat-mediated transcription of the HIV-1 LTR (Fig. 1C). Treatment with DZNep for 48 h significantly increased Tat-mediated transcription of the HIV-1 LTR in a dose-dependent manner (Fig. 1C). Collectively, these results implied that reduced EZH2 levels in a cell results in greater activation of the LTR.

3.2. Tat decreased the levels of H3K27me3 and EZH2 occupy at the LTR of HIV-1

Importantly, both H3K27me3 and EZH2 are found occupied at the HIV-1 LTR prior to activation [12]. We performed ChIP assay to examine whether the activation of HIV-1 LTR promoter after Tat construct treatment was related to the occupation of H3K27me3 and EZH2. Following Tat construct transfection, there was a significant decrease in the EZH2 levels detected at the HIV-1 LTR, suggesting that EZH2 was important for maintaining proviral silencing (Fig. 2). The levels of H3K27me3 detected at the HIV-1 LTR followed a pattern similar to EZH2 after Tat construct treatment.

3.3. Tat-induced EZH2 phosphorylation and cytoplasmic translocation of the EZH2 in TZM-bl cells

To investigate whether Tat construct affects EZH2 expression in TZM-bl cells, RT-PCR and Western blotting were used to measure

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