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Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor Regulates Enhanced Activation of Signal Transducer and Activator of Transcription 3 by Epstein–Barr Virus-Derived Epstein–Barr Nuclear Antigen 2

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The Epstein–Barr virus (EBV)-encoded latency protein Epstein–Barr nuclear antigen 2 (EBNA2) is a nuclear transcriptional activator that is essential for EBV-induced cellular transformation. In a previous study, we demonstrated that EBNA2 interacts with signal transducer and activator of transcription 3 (STAT3), a signal transducer for an interleukin (IL)-6 family cytokine, and enhances its transcriptional activity. Here, we show that overexpression of a corepressor, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), decreases the EBNA2-mediated enhanced STAT3 activation. Furthermore, small-interfering RNA-mediated reduction of endogenous SMRT expression augments the EBNA2-mediated enhanced STAT3 activation. Importantly, EBNA2 reduces interactions between STAT3 and SMRT. These data demonstrate that EBNA2 acts as a transcriptional coactivator of STAT3 by influencing the SMRT corepressor complex.

Key words Epstein–Barr virus; Epstein–Barr nuclear antigen 2; SMRT; STAT3

STAT3 is a member of the signal transducer and activator of transcription (STAT) family of proteins, and mainly activated by members of the interleukin (IL)-6 family of cytokines including leukemia inhibitory factor (LIF) and leptin.^{1,2)} Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jaks, and subsequently forms a dimer and translocates into the nucleus to activate its target genes. Activated STAT3 can mediate cellular transformation and is found in numerous cancers, including prostate cancer.^{3,4)} Furthermore, STAT3 has recently been shown to act as an oncoprotein.⁵⁾

Epstein–Barr nuclear antigen 2 (EBNA2), one of the six viral nuclear proteins expressed in latently infected B cells, is essential for immortalization of B cells by the Epstein–Barr virus (EBV).⁶⁾ EBNA2 is also known to promote transcriptional transactivation of viral and cellular genes by acting as an adapter molecule that binds to cellular sequence-specific DNA-binding proteins, namely J κ recombination signal-binding protein (RBP-J κ /CBF1) and PU.1, and engages multiple members of the RNA polymerase II transcription complex.^{7–9)} Recently, we showed that EBNA2 interacts with STAT3 and enhances its DNA-binding activity.¹⁰⁾ However, the molecular mechanisms of the enhanced STAT3 activation by EBNA2 remain unknown.

In the present study, we show that overexpression of a corepressor, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), decreases EBNA2-mediated enhanced STAT3 activation. In contrast, small-interfering RNA (siRNA)-mediated reduction of SMRT expression augments EBNA2-mediated enhanced STAT3 activation. Importantly, EBNA2 relieves STAT3 from SMRT–STAT3 complex formation. These results indicate that EBNA2 and SMRT regulate STAT3-mediated transcriptional activity by influencing their physical protein–protein interactions.

MATERIALS AND METHODS

Reagents and Antibodies Recombinant human LIF was purchased from INTERGEN (Purchase, NY, U.S.A.). Expression vectors, epitope-tagged STAT3, STAT3-LUC and SMRT were provided by Dr. T. Hirano (Osaka University, Osaka, Japan), Drs. H. Nakajima and J. N. Ihle (St. Jude CRH, Memphis, TN, U.S.A.), respectively.^{11,12)} Expression vector for epitope-tagged-EBNA2 was previously described.^{10,13,14)} Anti-EBNA2 monoclonal antibody (mAb) was purchased from Advanced Biotechnologies Inc. (Maryland, U.S.A.). Anti-Myc and anti-STAT3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-FLAG mAb (M2) and anti-hemagglutinin epitope (HA) antibodies were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.).

Cell Culture, Transfections, Luciferase Assays, siRNA and Reverse Transcription (RT)-polymerase chain reaction (PCR) Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HeLa cells were transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. 293T cells were transfected with the standard calcium precipitation protocol. Luciferase assay was performed as described.¹⁵⁾ Three or more independent experiments were carried out for each assay. HeLa cells were treated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FBS as previously described.¹⁶⁾ siRNA targeting human SMRT used in this study was follows: siSMRT, 5'-UCAGUGAGGUCAUCACACATT-3'. Total RNAs were prepared by using Iso-Gen

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(Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high-Plus-Kit (TOYOBO, Tokyo, Japan). Primers used for RT-PCR were: SMRT: 5'-CACCGGAACAGGCCTTATGACC-3' (sense), 5'-GGTTGTAGGGGAATGGCGTGG-3' (antisense).

Immunoprecipitation, Immunoblotting and DNA Binding Assay Immunoprecipitation and Western blotting were performed as previously described.¹⁷ The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each of aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to an Immobilon filter (Millipore; Bedford, MA, U.S.A.), which was then immunoblotted with antibodies.

To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 consensus oligonucleotide-sepharose conjugate (Santa Cruz) as described previously.¹⁰ The precipitates were subjected to Western blot analysis using anti-STAT3 antibody.

RESULTS AND DISCUSSION

SMRT Regulates EBNA2-Mediated Enhanced STAT3

Activation EBNA2 has been shown to act as a transcriptional activator by competing with the SMRT corepressor complex for contacts on SKIP and RBP-J κ /CBF1.^{13,14} Recently, it has been demonstrated that SMRT interacts with activated STAT3 and subsequently decreases STAT3 transcriptional activity in multiple myeloma cells.¹⁸ Indeed, overexpression of SMRT decreased LIF-induced STAT3 transcriptional activation (Figs. 1A,B). The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the α 2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. Using this system, we examined whether SMRT affects EBNA2-mediated enhanced STAT3 transcriptional activation. To this end, we coexpressed SMRT with EBNA2 in HeLa cells. When cells were cotransfected with SMRT, the enhanced transcriptional activation of STAT3-LUC by EBNA2 was decreased (Fig. 1C). To further examine whether endogenous SMRT is involved in EBNA2-mediated enhancement of LIF-induced STAT3-LUC activation, we examined the effects of SMRT knockdown in HeLa cells. A specific siRNA for SMRT or a control siRNA was transfected into HeLa cells. Total RNA isolated from the transfected cells was subjected to RT-PCR analysis, which confirmed a reduction in SMRT mRNA expression. As shown in Fig. 1D, a reduction in the level of SMRT expression in HeLa cells resulted in further enhancement of the LIF-induced STAT3-LUC activation by EBNA2. Importantly, reduction of SMRT enhanced LIF-induced STAT3-LUC activation in the absence of EBNA2, suggesting that endogenous SMRT regulates the transcriptional activity of STAT3. Therefore, reduction of SMRT in HeLa cells showed an effective enhanced STAT3 activation by EBNA2. These results indicate that SMRT is involved in the regulation of EBNA2-mediated enhanced STAT3 transcriptional activation in HeLa cells. We further examined whether SMRT affects the enhanced DNA-binding activity of STAT3 by EBNA2. As shown in Fig. 2A, the LIF-induced DNA-binding activity of STAT3 was enhanced by EBNA2 expression, consistent with

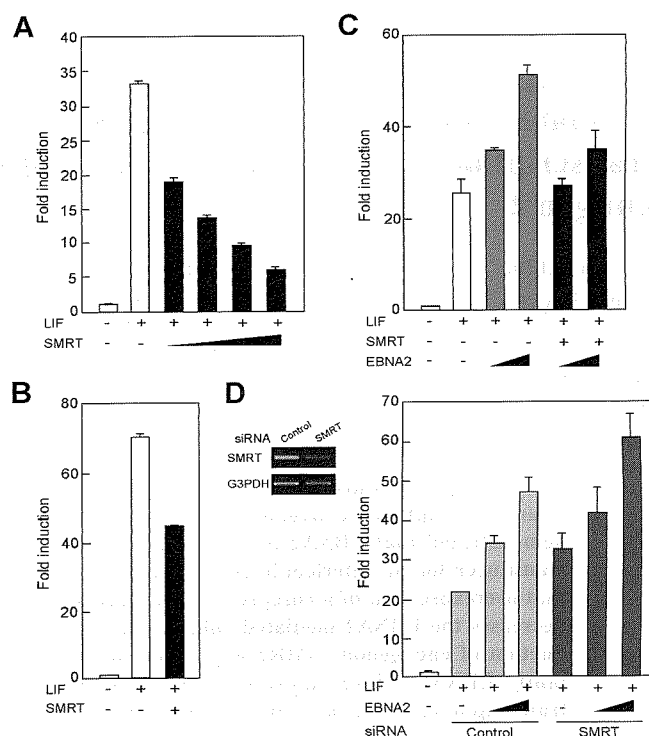


Fig. 1. SMRT Regulates EBNA2-Mediated Enhanced STAT3 Activation

(A) 293T cells in a 24-well plate were transfected with STAT3-LUC (200 ng) and/or expression vector for SMRT (10, 20, 40, 80 ng). At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured. (B) HeLa cells in a 24-well plate were transfected with STAT3-LUC (200 ng) and/or empty vector, expression vector for SMRT (500 ng). At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured. (C) HeLa cells in a 24-well plate were transfected with STAT3-LUC (200 ng) and/or empty vector, expression vector for SMRT (500 ng) and/or increasing amount of EBNA2 (100, 300 ng). At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured. (D) HeLa cells in a 24-well plate were transfected with control siRNA or siRNA targeting human SMRT using Lipofectamine 2000. The cells were then transfected with STAT3-LUC (200 ng) together with EBNA2 (100, 300 ng) using jetPEI. At 36 h after transfection, cells were treated with LIF (50 ng/ml) for an additional 8 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SMRT or G3PDH primers. The above results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

a previous report.¹⁰ Importantly, coexpression of SMRT with EBNA2 resulted in a reduction of the enhanced DNA-binding activity of STAT3. Taken together, these results show that SMRT regulates enhanced STAT3-mediated transcriptional activation by EBNA2.

EBNA2 Relieves STAT3 from STAT3-SMRT Complex Formation To further delineate the molecular mechanisms of how EBNA2 can overcome the STAT3-mediated transcriptional activation by SMRT blockade, we performed coimmunoprecipitation experiments by introducing expression vectors into 293T cells. Expression vectors encoding HA-tagged STAT3 and FLAG-tagged SMRT were transiently transfected into 293T cells. The cells were then lysed and subjected to immunoprecipitation with an anti-HA antibody. The immunoprecipitates were subjected to Western blot analysis with an anti-FLAG antibody. As shown in Fig. 2B, STAT3 interacted with SMRT. Interestingly, when we coexpressed Myc-tagged EBNA2, the amount of STAT3-bound SMRT protein was remarkably decreased. These results show that EBNA2 relieves STAT3 from STAT3-SMRT complex

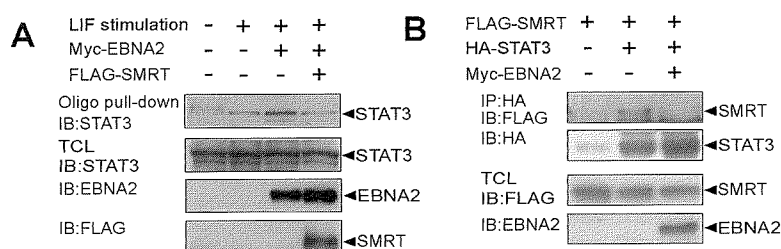


Fig. 2. Molecular Interactions among STAT3, EBNA2 and SMRT

(A) HeLa cells (1×10^7 cells) were transfected with empty vector, expression vector for Myc-EBNA2 (10 μ g) and/or FLAG-SMRT (10 μ g). At 48 h after transfection, cells were stimulated with LIF (50 ng/ml) for 30 min, and lysed. To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 consensus oligonucleotide-Sepharose conjugate. The precipitates were subjected to Western blot analysis using anti-STAT3 antibody. An aliquot of total cell lysates (TCL) was blotted with anti-STAT3, anti-EBNA2 and anti-FLAG antibodies. Data shown are representative of similar results obtained in at least three independent experiments. (B) 293T cells (1×10^7 cells) were transfected with FLAG-SMRT (10 μ g), HA-STAT3 (8 μ g) together with or without Myc-EBNA2 (10 μ g). At 48 h after transfection, cells were stimulated with LIF (50 ng/ml) for 30 min, and lysed, immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG (upper panel) or anti-HA antibody (middle panel). An aliquot of TCL (1%) were blotted with anti-FLAG and anti-EBNA2 antibodies (lower panels).

formation by influencing their physical protein-protein interactions.

Concluding Remarks Transcriptional activation is up-regulated in part through chromatin remodeling by histone acetylation, which is mediated by CREB-binding protein/p300, PCAF and PCIP. These proteins possess histone acetyltransferase activity, and have been shown to associate with various transcription factors and act as general integrators of the transcription machinery.¹⁹ On the other hand, transcription is negatively regulated by corepressor complexes composed of SMRT, mSin3A/B, c-Ski and histone deacetylases.²⁰ SMRT was originally identified as a corepressor protein bound to unliganded retinoic acid receptors or thyroid hormone receptors.²¹ Recent studies have revealed that SMRT interacts with not only nuclear receptors but also other transcription factors or nuclear proteins, such as Bcl-6, and leukemic fusion protein partners PLZF and ETO.²² SMRT has also been shown to interact with STAT3, STAT4 and STAT5 *in vitro* and act as a corepressor of STAT5.¹² However, it was unclear whether SMRT affects the transcriptional activity of STAT3. As shown in Fig. 1D, our data suggest that endogenous SMRT regulates the transcriptional activity of STAT3. It has recently been shown that expression of peroxisome proliferator-activated receptor γ (PPAR γ) and its agonists 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 and troglitazone completely suppresses IL-6/STAT3-mediated transcriptional activation in multiple myeloma cells.¹⁸ Moreover, overexpression of SMRT potentially augmented the inhibitory effects of troglitazone-activated PPAR γ , suggesting that SMRT, as a corepressor, is able to attenuate the transcriptional activity of STAT3 in its target gene promoters. Indeed, troglitazone treatment redistributed the corepressor SMRT from PPAR γ to activated STAT3, thereby transrepressing STAT3 transcriptional activation.

In this study, we have proposed novel interactions among STAT3, SMRT and EBV-derived EBNA2. EBNA2 augmented the transcriptional activity of STAT3 by influencing its DNA-binding activity. Overexpression of SMRT affected EBNA2-mediated enhanced STAT3 transcriptional activation and the DNA-binding activity of STAT3. Importantly, EBNA2 relieved STAT3 from SMRT-STAT3 complex formation. Thus further understanding of the detailed molecular interactions among STAT3, EBNA2 and SMRT may provide a novel therapeutic strategy for EBV-associated diseases.

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