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

Osamu Ikeda, a,b Sumiihi Togi, a,b Shinya Kamitani, a Ryuta Muromoto, a Yuichi Sekine, a
Asuka Nanbo, b Masahiro Fujimuro, b and Tadashi Matsuda a,∗

a Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University; Kita-ku Kita 12 Nishi 6, Sapporo 060–0812, Japan; and b Department of Molecular Cell Biology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi; Chuo 409–3898, Japan.

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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 corepressor of STAT3 by influencing the SMRT corepressor complex.

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

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. Expression vector for epitope-tagged-EBNA2 was previously described. 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-hemaglutinin 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. Three or more independent experiments were carried out for each assay. HeLa cells were treated with 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. siRNA targeting human SMRT used in this study was follows: siSMRT, 5’-UCAGUGAGGUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAU
(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'-CACCAGAACAGCTATGACC-3' (sense), 5'-GTTTGTAAGGAATTGCGTG-3' (antisense).

**Immunoprecipitation, Immunoblotting and DNA Binding Assay**

Immunoprecipitation and Western blotting were performed as previously described.15 Recently, it has been demonstrated that SMRT interacts with activated STAT3 and subsequently decreases STAT3 transcriptional activity in multiple myeloma cells.16 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 α2-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 a previous report.10 Importantly, coexpression of EBNA2 with SMRT 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 immunoprecipitation 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 above results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

**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-Jκ/CBF1.15,16 Recently, it has been demonstrated that SMRT interacts with activated STAT3 and subsequently decreases STAT3 transcriptional activity in multiple myeloma cells.16 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 α2-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 co-transfected 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 a previous report.10 Importantly, coexpression of EBNA2 with SMRT 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.

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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/PPARγ, 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. On the other hand, transcription is negatively regulated by coressor complexes composed of SMRT, mSin3A/B, c-Ski and histone deacetylases. SMRT was originally identified as a corepressor protein bound to unliganded retinoic acid receptors or thyroid hormone receptors. 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. SMRT has also been shown to interact with STAT3, STAT4 and STAT5 in vitro and act as a corepressor of STATs. 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 (PPARγ) and its agonists 15-deoxy-Δ12,14-prostaglandin J2 and troglitazone completely suppresses IL-6/STAT3-mediated transcriptional activation in multiple myeloma cells. Moreover, overexpression of SMRT potently augmented the inhibitory effects of troglitazone-activated PPARγ, 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 PPARγ 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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REFERENCES