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KSHV vIRF4 enhances BCL6 transcription via downregulation of IRF4 expression

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ABSTRACT

Primary effusion lymphoma (PEL), strongly linked with latent infection of Kaposi's sarcoma-associated herpesvirus (KSHV), constitutively expresses cellular interferon regulatory factor 4 (IRF4) while suppressing the expression of B cell lymphoma 6 (BCL6). Recently, it was shown that IRF4, a key transcriptional repressor of BCL6, might be a pivotal regulator of KSHV for balancing between latency and its reactivation in PEL cells. However, the action of the BCL6-IRF4 transcription factor axis during KSHV's life cycle is not clear. Herein we found that the KSHV lytic protein viral interferon regulatory factor 4 (vIRF4) dramatically enhanced the transcriptional activity of the BCL6 through the inhibition of its negative regulator IRF4. Using a chromatin immunoprecipitation (ChIP) assay, we further showed that vIRF4 bound to the specific promoter region of IRF4, contributing to a dramatic suppression of IRF4 gene expression. Correspondingly, we also found BCL6 expression to be positively and inversely correlated with vIRF4 and IRF4 expression, respectively, during KSHV reactivation. Finally, we observed that these processes require efficient KSHV lytic replication. Thus, our findings suggest a crucial role of the BCL6-IRF4 axis in triggering the transition between KSHV latency and lytic reactivation.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is etiologically linked to Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric castleman's disease (MCD) [1,2]. KSHV has a life cycle with two distinct stages termed the latent and lytic life states. In particular, KSHV-associated lesions are predominantly latent infections, expressing a limited number of latent genes [3]. However, in certain stimulating conditions, latently infected KSHV can be reactivated through expression of RTA, which is the master regulator of the lytic switch [3]. Emerging evidence shows that modulation of key cellular transcription factors, such as c-Myc and cellular interferon regulatory factor 4 (IRF4), by KSHV genes plays a critical role in coordinating its life cycle and pathogenesis [4–7]. In order to maintain KSHV latency, c-Myc transcription factor is deregulated and functionally activated in PEL cells by two KSHV latent proteins, latency-associated nuclear antigen (LANA) and viral interferon regulatory factor (vIRF) 3 [8–10]. On the other hand, in order to establish a favorable environment for its viral lytic replication, KSHV vIRF4 lytic protein downregulates c-Myc expression by targeting the IRF4 function [7]. However, it is not clear how KSHV vIRF4 lytic protein deregulates IRF4 gene expression to facilitate KSHV lytic replication.

IRF4, a lymphoid-restricted member of the IRF family, is a transcription factor that is involved in both positive and negative regulation of several genes [11]. Thus, expression of IRF4 participates in B-cell proliferation and differentiation as well as cellular transformation that contribute to some lymphoproliferative disorders [11,12]. Notably, in the KSHV-infected PEL cells, IRF4 is highly upregulated and RTA expression is functionally suppressed, ultimately contributing to the maintenance of KSHV latency [6]. Interestingly, several gene expression studies have shown that expression levels of IRF4 and B-cell lymphoma 6 (BCL6) is inversely correlated in KSHV-infected PEL cells [13,14]. However, the precise action of the BCL6-IRF4 axis within the KSHV life cycle remains unknown.

Herein, we show that KSHV vIRF4 specifically binds the cellular IRF4 promoter, leading to suppression of IRF4 expression. Ultimately, vIRF4 blocks the IRF4-mediated inhibition of BCL6 transcriptional activity, resulting in increased expression of BCL6 that contributes to efficient induction of KSHV lytic genes. During the KSHV reactivation, we also found that expression of IRF4 and BCL6

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is inversely correlated. Thus, our results indicate that KSHV vIRF4 is a pivotal viral transcription factor that deregulates the key cellular regulator, BCL6-IRF4, which is linked to distinct stages of the KSHV life cycle.

2. Material and methods

2.1. Plasmids, cells, and transfections

V5-tagged wild-type vIRF4 or vIRF4 (ΔDBD) has been described previously [7]. Flag-tagged IRF4, the −1.2 nucleotides of IRF4 promoter plasmid, and 5′ promoter serial deletion of IRF4 promoter plasmids were kindly provided by Dr. John Hiscott from McGill University [15]. BCL6 promoter-containing reporter plasmid (pLA/B9wt) was kindly provided by Dr. Riccardo Dalla-Favera at Columbia University [16]. 293T cells were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin-streptomycin (P/S) and 10% fetal bovine serum (FBS). TRExBCBL-1 vIRF4/Au and TRExBCBL-1 Myc/RTA cell lines were cultured in RPMI 1640 medium supplemented with 10% tetracycline-negative FBS, 100 U/ml P/S, and 200 μg/ml hygromycin B (Invitrogen). Doxycycline (Doxy) and polyethylenimine (PEI) were purchased from Sigma. 1×10^7 TRExBCBL-1 Myc/RTA cells were transfected with 700 pmol of siRNA by electroporation at 250 V, 950 μF using Bio-Rad GenePulser X cell™ electroporator. siBCL6 and negative control siRNA were purchased from Ambion.

2.2. shRNA knockdown

The shRNA target sequence for vIRF4 is 5′-GGTGGTAGCTACGTAGTATGG-3′ and it was cloned into the pLKO.1 lentiviral vector. Lentiviral production and transduction into the target cells have been described previously [7].

2.3. Antibodies for immunoblots

Anti-V5, Flag, Myc, Tubulin, and Actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-Au antibody was from Covance (Princeton, NJ). KSHV vIRF4-specific antibodies have been previously described [7]. Anti-IRF4 was from Cell Signaling.

2.4. Reporter assay

Transfections were performed with the luciferase reporter plasmids, indicated plasmids, and the pRL-SV40 renilla plasmid. Luciferase reporter assay was performed by using a dual luciferase assay kit following the manufacturer's manual (Promega).

2.5. ChIP assay and RT-PCR

Chromatin immunoprecipitation (ChIP) assays were carried out according to a previously published protocol [7]. Subsequently, ChIP DNA was quantified by qPCR and the enrichment of the vIRF4 on specific IRF4 promoter regions was measured as a percentage of the input DNA. The ChIP assay graphs represent the average of at least two independent experiments. The oligonucleotides used for qPCR of immunoprecipitated chromatin fragments are described below: IRF4_P1 (5′- CGGGTGAAAGCTCAGGGG-3′ and 5′-CGAGCCACGAGAATCGC-3′), IRF4_P2 (5′-CCTCCCGGGTTCAAGCGATTC-3′ and 5′-GAGGCCGAGGCGGTGGATC-3′), IRF4_P3 (5′-GCTTTTATACCCCACTGG-3′ and 5′-GGTGAATTGAAGTGACTCAG-3′), IRF4_P4 (5′-GAGACAGTATTTGAATCAAGC-3′ and 5′-CAAAGAGAACTGGAGGTG-3′), IRF4_P5 (5′-CGCACCAGATTCCCGCTAC-3′ and 5′-CGCCGCTTCGGGGACTGTCAC-3′), and IRF4_P6 (5′-GCGGAGGGTCGCCAAGGGCG-3′ and 5′-CGGGGCGCCGAGGCCTGTG-3′). For RT-PCR, total RNA was purified using TRI Reagent™ (Sigma) and reverse-transcription was performed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. RT-qPCR was performed with SYBR green-based detection methods using a CFX96TM real time system (Bio-Rad). Expression of 18S was used for normalization of RT-qPCR results. Results of RT-qPCR were based on the average of at least three independent experiments. The oligonucleotides used are as follows: IRF4 (5′-AGAAGAGCATCTTCCGCATC-3′ and 5′-TGCTCTTGTTCAAAGCGCAC-3′), BCL6 (5′-TTGTTGTGAGCCGTGAGCAGTT-3′ and 5′-CCCGCAAATTGAGCCGAGAT-3′), LANA (5′-GAGTCTGGTGACGACTTGGAG-3′ and 5′-AGGAAGGCCAGACTCTTCAAC-3′), RTA (5′-TTGCCAAGTTTGTACAACTGCT-3′ and 5′-ACCTTGCAAAGACCATTCAGAT-3′), ORF25 (5′-ACAGTTTATGGCACGCATAGTG-3′ and 5′-GGTTCTCTGAATCTCGTCGTGT-3′), ORF45 (5′-CCATACAGCGACCCTGATGA-3′ and 5′-CCGATTCTCTGACTCAATACT-3′), ORF56 (5′-CACAGATTCCCGTCAATACAAA-3′ and 5′-GTATCTTCAGTAGGCGGCAGAG-3′), ORF64 (5′-CTTCCTCGAGGGCATCATATAC-3′ and 5′-TATACGGTGATGGACTTGATGG-3′), and vIRF4 (5′-CCTGCCGGCAGCGATATCCCGCCT-3′ and 5′-CAAATGCATGGTACACCGAATACC-3′).

3. Results and discussion

3.1. KSHV vIRF4 inversely regulates gene expression of IRF4 and BCL6

Expression of IRF4 and BCL6 are inversely regulated in KSHV-latently infected PEL cells [13]. Interestingly, our previous results showed that KSHV lytic vIRF4 protein effectively suppressed IRF4, thus facilitating its replication [7]. Given that IRF4 is a negative regulator of BCL6 [16], we postulated that vIRF4 induced expression of BCL6 via suppressing IRF4. To determine whether vIRF4 inversely deregulated IRF4 and BCL6, we treated TRExBCBL-1 vIRF4/Au cells with or without Doxycycline (Doxy) for 24 h, followed by RT-qPCR analysis with IRF4-specific or BCL6-specific primers. This showed that expression of vIRF4 dramatically increased BCL6 mRNAs, while significantly suppressing IRF4 mRNAs (Fig. 1A left panel). However, when using vIRF4 (ΔDBD), in which the DNA binding domain (DBD) was deleted, vIRF4(ΔDBD) expression did not alter IRF4 or BCL6 mRNAs levels (Fig. 1A right panel). Next, we ectopically overexpressed Flag-IRF4 in the TRExBCBL-1 vIRF4/Au cells, followed by exposing Doxy for the indicated times. Subsequently, we performed RT-qPCR with TRExBCBL-1 vIRF4/Au&Flag-IRF4 cells and TRExBCBL-1 vIRF4/Au cells to compare BCL6 expression kinetics. This showed that ectopic expression of IRF4 suppressed vIRF4-mediated BCL6 induction (Fig. 1B). This result indicates that vIRF4 enhances BCL6 expression in an IRF4-dependent manner.

3.2. KSHV vIRF4 inhibits IRF4-mediated BCL6 suppression

It is known that IRF4 directly binds to the BCL6 promoter and negatively controls BCL6 transcriptional activity [16]. Hence, to examine the effect of the vIRF4 on IRF4-mediated BCL6 transcriptional activity, transient transfection/reporter assay was performed with a reporter plasmid that contained a wild-type BCL6 promoter (pLA/B9wt). Various combinations of a pLA/B9wt-luciferase reporter plasmid, Flag-tagged IRF4, V5-tagged vIRF4, or V5-tagged vIRF4 (ΔDBD) were transfected either individually or in combination into 293T cells. As previously shown [16,17], IRF4 robustly suppressed BCL6 transcriptional activity. In contrast, coexpression of IRF4 and vIRF4 induced BCL6 transcriptional activity in a dose-dependent manner (Fig. 2A). Furthermore, the vIRF4 (ΔDBD)