Virology 458-459 (2014) 4–10

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Brief Communication

Downregulation of IRF4 induces lytic reactivation of KSHV in primary effusion lymphoma cells ☆

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ARTICLE INFO

Article history:
Received 7 December 2013
Returned to author for revisions
7 January 2014
Accepted 13 April 2014

Keywords:
Primary effusion lymphoma
Kaposi's sarcoma-associated herpesvirus
Interferon regulatory factor 4

ABSTRACT

Primary effusion lymphoma (PEL), associated with the latent infection by KSHV, constitutively expresses interferon-regulatory factor 4 (IRF4). We recently showed that IRF4 differentially regulates expression of cellular interferon-stimulated genes (ISGs) and viral genes (Forero et al., 2013). Here, using inducible IRF4 knockdown, we demonstrate that IRF4 silencing results in enhanced transcription of KSHV replication transactivator RTA. As a result viral transcription is increased leading to virus reactivation. Taken together, our results show that IRF4 helps maintain the balance between latency and KSHV reactivation in PEL cells.

Introduction

The interferon regulatory factors (IRFs) are critical in the regulation of innate and adaptive immune response (Tamura et al., 2008). In contrast with other ubiquitously expressed IRFs, IRF4 is restricted to immune cells and is required for the maturation of lymphocytes (Mittrucker et al., 1997). IRF4 expression has been linked to cellular transformation, increased proliferation, and decreased apoptotic responses in diseases like multiple myeloma (Iida et al., 1997), human T-lymphotropic virus 1 (HTLV-1) infected adult T-cell leukemia (ATL) (Mamane et al., 2002; Sharma et al., 2000), Epstein–Barr Virus (EBV) transformation of B cells (Banerjee et al., 2013; Izumiya et al., 2009; Wang et al., 2011; Xu et al., 2008), and primary effusion lymphoma (PEL) (Carbone et al., 2000). Furthermore, IRF4 is involved in autoimmune diseases (reviewed in Xu et al. (2012)) and diet-induced inflammation (Eguchi et al., 2011, 2013) indicating the need to improve our understanding of IRF4 function in the context of specific disease. However, the role of IRF4 in Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8, HHV-8) maintenance and promoting PEL is yet to be clarified.

PEL is a B cell neoplasm, common amongst immunocompromised individuals (Dotti et al., 1999; Jaffe, 1996). It is characterized by a plasma cell-like phenotype and is associated with latent infection by KSHV. In PEL cells, KSHV persists as a naked episome with expression of a subset of viral genes (latency-associated genes) (Cesarman et al., 1995; Dresang et al., 2011; Sarid et al., 1998; Zhong et al., 1996). The transition from latency to lytic replication is controlled by the KSHV replication transactivator (RTA), which is necessary and sufficient to initiate lytic gene transcription, virion formation, and cell death. The role of RTA in driving lytic replication has been extensively studied and factors mediating RTA function have been well described (Guito and Lukac, 2012). However, the signaling pathways and cellular factors that control the transcriptional induction of RTA and their effects on downstream gene expression remain elusive.

We have recently reported that IRF4 can directly induce a specific subset of IFN-stimulated genes (ISGs) in a type I IFN-independent manner, and can negatively regulate KSHV RTA induction (Forero et al., 2013). Therefore, we hypothesized that downregulation of IRF4 would result in the derepression of RTA expression in PEL cells and induction of the lytic gene cascade. In this study, we have taken a reverse genetics approach to examine the effects of IRF4 on ISG induction and the maintenance of KSHV latency. Our results show that IRF4 downregulation does not result in a loss of ISG expression, but rather an increase in RTA transcription and translation accompanied by a subsequent lytic gene expression and increased virus production. These results

☆This work was supported in part by AI082673 from National Institute of Allergy and Infectious Diseases Grant (SNS) and AIDS-related malignancy supplement from UPCI Cancer Center Support Grant. This project used the UPCI core facilities and was supported in part by award P30CA047904.

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http://dx.doi.org/10.1016/j.virol.2014.04.020
0042-6822/

indicate a complex interplay between RTA-responsive element (RRE) and interferon-sensitive response element (ISRE) regulated gene expression and establish IRF4 as a key regulator of KSHV reactivation.

Results

Downregulation of IRF4 leads to induction of specific ISG expression in PEL cells

The roles of IRF4 in regulating B-cell specific transcription programs, by cooperating with various transcription factors, such as PU.1, have been well documented (Eisenbeis et al., 1995; van der Stoep et al., 2004). However, our previous results showed that IRF4 alone can directly bind to ISRE elements and is capable of upregulating ISG transcription in various cell types, including PEL cells, in the absence of PU.1 expression. This phenomenon is further influenced by the KSHV-latency associated gene v-FLIP (viral FLICE inhibitory protein) through its ability to activate NF-κB (Forero et al., 2013). To investigate whether the ISG upregulation is solely mediated through IRF4 expression, we attempted IRF4 silencing in PEL cells. Given that we were unable to obtain stable PEL cell lines constitutively expressing IRF4-targeted shRNA, we engineered BCBL-1 cells with doxycycline (Dox) inducible expression of either control or two separate IRF4-targeting shRNAs (sh-IRF4, and sh-IRF4b) using strategies previously described (Shaffer et al., 2008). Treatment of cells with Dox (100 ng/ml) resulted in appreciable reduction in IRF4 protein (Fig. 1A) and mRNA (Fig. 1B) in sh-IRF4 expressing cells, but not in scramble control (sh-CTRL) or sh-IRF4b expressing cells. To verify the specificity for IRF4 knockdown and exclude any off-target effect by shIRF4, we analyzed the expression of IRF3 and IRF9, which showed no detectable changes (Fig.1C). As we have previously shown that IRF4 acts as a positive regulator of ISRE-mediated expression of ISG60 and Cig5, we evaluated the effect of IRF4 knockdown on the expression of these genes. Unexpectedly, transcription of ISG60 and Cig5 was increased after IRF4 depletion, 4-fold and 6-fold respectively (Fig. 1D). This was accompanied by an increase in ISG60 protein synthesis after Dox treatment of BCBL-1 cells (Fig. 1E).

We have reported that ectopic expression of IRF4 resulted in the inhibition of TPA-stimulated RTA (encoded by ORF50) transcription in PEL cells (Forero et al., 2013). To determine whether depletion of IRF4 affects RTA expression in PEL cells, we examined RTA protein synthesis in Dox (100 ng/ml) treated BCBL-1 sh-IRF4 and observed an overall increase in RTA protein expression (Fig. 2A), and nuclear accumulation (Fig. 2B). RTA is a sequence-specific DNA binding protein that recognizes and binds to RRE containing viral gene promoters, as well as ISRE and ISRE-like sequences found in the promoter regulatory regions of cellular ISGs (Zhang et al., 2005). Indeed, ectopic expression of RTA resulted in an almost 5-fold increase in the activity of the IFNβ reporter relative to vector transfected cells (Fig. 2C). Furthermore, ectopic RTA expression resulted in a 5-fold increase in the induction of ISG60 mRNA transcription (Fig. 2D) accompanied by an increase in ISG60 protein synthesis (Fig. 2E). It is likely then that the unexpected ISG induction observed after IRF4 silencing is mediated by the upregulation of RTA (Zhang et al., 2005). Taken together, these results indicate that IRF4 is involved in maintaining a fine-tuned equilibrium between the expression levels of ISGs and RTA in PEL cells.

IRF4 inhibits KSHV lytic gene expression

RTA plays a critical role in initiating the switch from latency to the lytic reactivation of KSHV (Guito and Lukac, 2012; Lukac et al., 1999, 1998). In order to define the mechanism of RTA protein expression followed by IRF4 depletion, we treated BCBL-1 sh-CTRL and sh-IRF4 cells with Dox for 72 h followed by stimulation with 15 ng/ml TPA for 12 h. Treatment with Dox resulted in significantly decreased IRF4 protein levels in BCBL-1 sh-IRF4 cells accompanied with an increase in RTA protein synthesis (Fig. 3A, lanes 10–12).

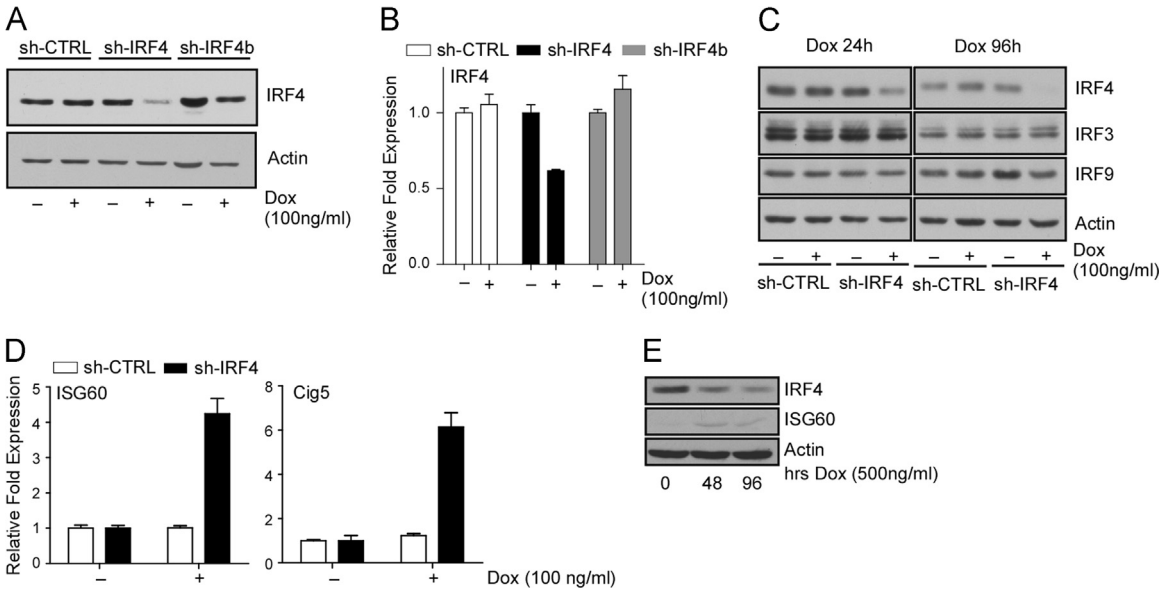

Fig. 1. Downregulation of IRF4 results in the induction of ISG expression in PEL cells. (A) Loss of IRF4 protein expression in cells expressing an inducible IRF4 targeting shRNA. Whole cell lysates prepared from shRNA expressing BCBL-1 cells after 72 h treatment with 100 ng/ml Dox were immunoblotted with antibodies against IRF4 and actin. (B) Quantitative RT-PCR analysis of IRF4 mRNA levels in shRNA expressing BCBL-1 cells after 72 h treatment with 100 ng/ml dox. Samples were normalized to the housekeeping gene, RPL32, and expressed as fold change with respect to untreated cells (value 1). (C) Specificity of IRF4 targeting by the shRNA 24 h (left) and 96 h (right) after stimulation with Dox. Whole cell lysates prepared from shRNA expressing BCBL-1 cells after 72 h Dox treatment were immunoblotted with antibodies against IRF4, IRF3, IRF9 and Actin. (D) Quantitative RT-PCR analysis of ISG60 and Cig5 mRNA expression in shRNA expressing BCBL-1 cells after 72 h Dox treatment. Samples were normalized to RPL32 and expressed as fold change with respect to untreated cells (value 1). (E) Analysis of ISG60 protein induction levels in whole cell lysates prepared from sh-IRF4 expressing BCBL-1 cells after 72 h treatment with Dox.

Please cite this article as: Forero, A., et al., Downregulation of IRF4 induces lytic reactivation of KSHV in primary effusion lymphoma cells. Virology (2014), http://dx.doi.org/10.1016/j.virol.2014.04.020