Kaposi’s sarcoma-associated herpesvirus (KSHV) encodes four viral interferon regulatory factors (vIRF-1–4). We investigated the mechanism and consequences of vIRF-2-mediated inhibition of interferon-response element signalling following type I interferon (IFN) induction. Western blot and electrophoretic mobility-shift assays identified the interferon-stimulated gene factor-3 (ISGF-3) components STAT1 and IRF-9 as the proximal targets of vIRF-2 activity. The biological significance of vIRF-2 inhibition of ISGF-3 was demonstrated by vIRF-2-mediated rescue of the replication of the IFN-sensitive virus encephalomyocarditis virus. This study provides both a mechanism and evidence for KSHV vIRF-2-mediated suppression of the consequences of type 1 IFN-induced signalling.

The earliest response at the cellular level to virus infection is the establishment of the antiviral state that results from induction of type I interferon (IFN) expression. This contains the virus infection and can eliminate the infected cell by inhibiting cellular proliferation, promoting apoptosis and augmenting adaptive immunological surveillance and responses. Consequently, either avoiding or inhibiting the IFN antiviral response is an important component of the biology of many viruses (reviewed by Randall & Goodbourn, 2008).

Kaposi’s sarcoma-associated herpes virus (KSHV) (Chang et al., 1994), the aetiological agent of Kaposi’s sarcoma and primary effusion lymphoma (Bouvard et al., 2009), encodes a family of four viral interferon regulatory factors (vIRFs) (Cunningham et al., 2003), three of which (vIRF-1, -2 and -3) have anti-IFN activity (Burysek et al., 1999; Fuld et al., 2006; Gao et al., 1997; Lubovy & Pitha, 2000; Rezaee et al., 2006; Wies et al., 2009; Zimring et al., 1998). However, other vIRF functions are also evident; for example, vIRF-1 restricts chromatin remodelling, thereby suppressing cytokine gene expression (Li et al., 2000), and augments p53 degradation by the proteasome (Shin et al., 2006), while vIRF-3 inhibits MHC-II expression (Schmidt et al., 2011).

We have analysed the suppressive effects of vIRF-2 on the ‘delayed’ interferon signalling cascade, which is induced following ligation of the type I IFN receptor and depends on the activity of interferon-stimulated gene factor 3 (ISGF-3).

To investigate the mechanism of vIRF-2 inhibition of type I IFN-induced interferon-response element (ISRE) trans-activation, we derived stable cell lines in which vIRF-2 expression could be induced by doxycycline treatment. To negate clone-specific effects, three independent clonal cell lines (clones 3-9, 20 and 24) were analysed. Briefly, vIRF-2 was subcloned in frame into the doxycycline-inducible expression vector pTRE2-pur-Myc (Clontech) to generate...
viRF-2 inhibition of ISGF-3

A 5′-cMyc-tagged viRF-2 derivative. This plasmid was transfected into HEK293-Tet-On cells (Clontech) that stably express the tetracycline-regulated transactivator rtTA; puromycin-resistant clones were then derived (as described by Aresté et al., 2009). In parallel, counterpart ‘empty vector’ (EV) clonal cell lines lacking viRF-2 were derived (EV1, EV4 and EV5). Western blot analysis determined that viRF-2 gene expression was inducible in clones 3-9, 20 and 24 (data not shown). However, the system was inherently leaky since ‘basal’ viRF-2 expression was demonstrable by Western blot analysis for all three clones, even in the absence of doxycycline treatment (data not shown), particularly with increasing passage. Functional studies of these cell lines, and the empty vector controls, were therefore performed in the presence of doxycycline to ensure maximal viRF-2 expression. When compared with their empty-vector control counterparts, each of the three viRF-2-expressing cell lines demonstrated significant inhibition of recombinant (r) IFN-α induction of recombinant (r) IFN-α expression (Fig. 2a). Western blot analysis verified the presence of IFN-α expression in clones 3-9, 20 and 24 and not in the empty vector clones EV1, EV4, EV5; see Fig. 2d, row 1). These data confirmed our previous studies with transient ectopic expression of viRF-2 (Fuld et al., 2006) and confirmed that these cell lines provided a tool with which to investigate the mechanism of viRF-2 function. The type 1 IFN-induced JAK–STAT signal transduction cascade is understood in detail (Randall & Goodbourn, 2008). It culminates in the formation of the heterotrimeric ISGF-3, which consists of IRF-9 and post-translationally activated STAT1 and STAT-2. ISGF-3 has pleiotropic effects that establish the ‘antiviral state’ through ISRE-containing promoters. Given that viRF-2 reduces the activation of the ISRE promoter (Fig. 1), the abundance of functional amounts of ISGF-3 in one representative pair of clones was measured by electrophoretic mobility assay (EMSA). ISGF-3 EMSA for viRF-2-expressing clone 3-9 compared with the counterpart EV clone 5, which lacks viRF-2, demonstrated a significant reduction in ISRE binding (Fig. 2a, b). Probing for TATA-binding protein (TBP) confirmed equal loading of nuclear lysates in the assay (Fig. 2c). These data are consistent with reduced ISRE reporter activity in the viRF-2-expressing cells (Fig. 1). To investigate the mechanism of viRF-2 reduction of functional levels of ISGF-3 in more detail (Fig. 2a, b), the relative levels of the components of the type 1 IFN-induced JAK–STAT signalling cascade were measured by Western blot in viRF-2-expressing clones 3-9, 20 and 24, compared with their empty-vector control counterparts EV1, EV4 and EV5. To induce the antiviral state, cultures were treated with rIFN-α. Verification that ISRE transactivation was inhibited by viRF-2 at the time of cell harvest for Western blotting was provided by transfecting the cultures with pISRE-luc (and the pRL-SV40-luc control plasmid) and taking parallel aliquots for a dual-luciferase assay (data not shown). Immunoblotting demonstrated the presence of expression of viRF-2 in the relevant clones (Fig. 2d, row 1), and probing for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) confirmed the equal loading of lysates in each lane (Fig. 2d, row 11). As expected, and confirming the induction of the signalling cascade induced by IFN-α binding the IFN receptor complex, Tyk2 phosphorylated at residues Tyr1054 and Tyr1055 [pTyk2 (Tyr1054/1055)] increased in all clones following IFN treatment (Fig. 2d, row 3). Tyk2 is the kinase associated with the IFN-α receptor 1 (IFNAR1) component of the common IFN type 1 receptor (reviewed by Randall & Goodbourn, 2008). There were no consistently detectable differences in the level of IFNAR1 between the viRF-2-expressing clones and those lacking viRF-2 (Fig. 2d, row 2). Tyrosine phosphorylation of Tyk2 at residues 1054 and 1055 is dependent upon the IFNAR2-associated kinase JAK1, following IFN receptor ligation and heterodimerization (Gauzzi et al., 1996). Like Tyk2 and IFNAR1, there was no consistent difference in JAK1 levels between the viRF-2-expressing clones and those lacking viRF-2 (Fig. 2d, row 5). Tyk2 phosphorylates STAT2 at tyrosine residue 689 and the level of this [pSTAT2 (Tyr689)]; Fig. 2d, row 6) increased in all clones following IFN treatment. The total STAT2 levels were not consistently different between viRF-2-expressing clones and those lacking viRF-2 (Fig. 2d, row 7). Taken together, these data strongly suggest that viRF-2 does not modulate these IFN-α receptor-proximal events. However, the levels of STAT1 phosphorylated at residue Tyr701 [pSTAT1 (Tyr701); Fig. 2d, row 8] and total STAT1 (Fig. 2d, row 9) were differentially regulated by the expression of viRF-2. For each pair of clones, total STAT1 levels increased following IFN treatment. However, both the basal level and the IFN-induced level of STAT1 were lower in the viRF-2-expressing clones compared with their counterparts lacking the viral protein. The levels of STAT1 phosphorylated at Tyr701 were reduced concomitantly with the level of this [pSTAT1 (Tyr689)]; Fig. 2d, row 6) increased in all clones following IFN treatment. Furthermore, IRS-9 levels were substantially reduced in those clones expressing viRF-2. These data were confirmed by densitometric analysis of the phosphoSTAT1 (Tyr 701) and IRS-9 immunoblot bands (Supplementary Fig. S1, available in JGV Online). Having established that viRF-2 reduces the level of functional ISGF-3 by targeting its STAT1 and IRF-9 components, the physiological impact of this viral protein on the antiviral state was determined in the representative pair of cell lines, viRF-2 clone 3-9 and EV clone 5. The hypothesis that the yield of encephalomyocarditis virus (EMCV), a type I IFN-sensitive picornavirus (Morrison & Racaniello, 2009), would be inhibited by rIFN-α pretreatment and rescued by viRF-2 expression, was tested. EV5 and clone 3-9 cells were treated with doxycycline and increasing amounts of rIFN-α (up to 300 IU ml⁻¹) for 30 h before infection with EMCV at an m.o.i. of 0.1. After a further 30 h the culture fluid was collected for quantification of viral titre by plaque assay (Fig. 3). In the absence of IFN treatment, the titre of EMCV recovered from
vIRF-2-expressing clone 3-9 and EV clone 5 cells was comparable (a mean of $7.4 \times 10^6$ p.f.u. ml$^{-1}$ for clone 3-9 compared with a mean of $6.6 \times 10^6$ p.f.u. ml$^{-1}$ for EV clone 5). As expected, increasing amounts of rIFN-α decreased the titre of EMCV recovered from EV clone 5 cells by as much as two orders of magnitude (to $7.7 \times 10^4$ p.f.u. ml$^{-1}$ for 300 IU rIFN ml$^{-1}$). In contrast, the titre of EMCV recovered from vIRF-2-expressing clone 3-9 cells was only marginally reduced following IFN treatment, to $2.9 \times 10^6$ p.f.u. ml$^{-1}$ at 300 IU rIFN ml$^{-1}$. These data demonstrate the anti-IFN effects of vIRF-2 are sufficient to rescue IFN-sensitive EMCV.

Thus, in the present study, vIRF-2 was demonstrated to inhibit IFN-α-induced ISRE transactivation (Fig. 1) by inhibiting the accumulation of functional ISGF-3 (Fig. 2a, b). Specifically, vIRF-2 attenuated the accumulation of two components of ISGF-3: IRF-9 and phosphorylated STAT1 (Fig. 2d). The third component, STAT2, was unaffected by vIRF-2, as were the type I IFN receptor-proximal signalling components IFNAR1, Tyk2 and JAK1 (Fig. 2d). The attenuation of functional ISGF-3 was confirmed biologically by vIRF-2 rescue of the replication of the type I IFN-sensitive picornavirus, EMCV. EMCV replication in cells pre-treated with up to 300 IU rIFN-α ml$^{-1}$ was rescued almost to the level of no IFN treatment in the presence of vIRF-2 expression (Fig. 3). Thus the biochemical consequences of vIRF-2 expression (Figs 1 and 2) are reflected in measurable changes in the interferon-induced inhibition of viral replication (Fig. 3).

Taken together with our previous data showing vIRF-2 attenuates early events in the type I IFN response by exploiting an extant cellular mechanism of caspase-3-dependent IRF-3 turnover (Aresté et al., 2009), the data of the present study demonstrate the pleiotropic activity of KSHV vIRF-2 in inhibiting the type I IFN response. This broad functional activity of vIRF-2 is also suggested by the report that the part of this protein encoded by exon 1 can suppress the activity of dsRNA-activated protein kinase (Burýsek & Pitha, 2001). Our data also demonstrate the importance to KSHV of inhibiting ISGF-3, since at least one other KSHV protein (viral IL-6) targets this protein (Chatterjee et al., 2002). KSHV is not alone in suppressing early type I IFN events (IFN enhancesome-dependent) and delayed type I IFN events (ISGF-3-dependent) (reviewed by Aresté & Blackbourn, 2009) since many viruses are able to do so (reviewed by Randall & Goodbourn, 2008). The KSHV vIRF-2 protein is unusual in targeting both early and delayed pathways. What roles might this broad activity of vIRF-2 play in defending KSHV-infected cells from the antiviral effects of the type I IFNs? Expression kinetics studies during de novo infection of primary cells in vitro revealed vIRF-2 to be expressed at very early time points post-infection (2–8 h, depending on the cell type).

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**Fig. 1.** vIRF-2 inhibits IFN-α-induced ISRE transactivation in stable cell lines. Subconfluent cultures of vIRF-2-expressing cells (clones 3-9, 20 and 24) or three counterpart clones generated in parallel and lacking vIRF-2 (EV1, -4 and -5) were co-transfected in six-well plates with 500 ng pISRE-luc (Stratagene) and 1 ng pRLSV-40-luc (Stratagene) for 24 h. Plasmid pRLSV-40-luc constitutively expresses Renilla luciferase, to which pISRE-luc firefly (Photinus) luciferase activity was normalized. Cells were then treated with 1μg doxycycline ml$^{-1}$ with or without 300 IU rIFN-α ml$^{-1}$ for 30 h before analysis of the luciferase activity with a Dual Luciferase Reporter Assay (Promega). The data are presented as normalized firefly luciferase activity for: (a) vIRF-2-expressing clone 3-9 versus its counterpart clone EV5, which lacks vIRF2. The clone 3-9 cell line was described previously (Aresté et al., 2009); (b) vIRF-2-expressing clone 20 versus its counterpart clone EV1, which lacks vIRF2; and (c) vIRF-2-expressing clone 24 versus its counterpart clone EV4, which lacks vIRF2. Data are means ± SEM from three to five independent experiments. *, P<0.05, Student’s paired t-test.
Fig. 2. vIRF-2 inhibits phospho-STAT1 and IRF-9 components of ISGF-3. (a, b) vIRF-2 inhibits functional ISGF-3. vIRF-2 clone 3-9 and EV clone 5 cells were treated with 1 μg doxycycline ml⁻¹ for 30 h and treated or not with 300 IU rIFN-α2b ml⁻¹, as indicated, for 4 h. Nuclear extracts were then prepared by using a NE-PER nuclear extraction kit (Pierce Biotechnology). EMSAs were performed with an ISRE probe (5'-GATCAGAAATAGAAGACTG-3') containing a 5'-IRDye-700-label according to the manufacturer's protocol (LI-COR Biotechnology). Briefly, nuclear lysates (5 μg) were mixed with 50 nM of 5'-IRDye-700-labelled oligonucleotides in LI-COR IRDye EMSA reagents for 30 min at room temperature. Reaction mixtures were resolved on 6% polyacrylamide-TBE gels (Invitrogen) and the bands were visualized by using an Odyssey infrared imaging system (LI-COR Biotechnology). (a) Band intensity was quantified by using the associated Odyssey software. Data are means ± SEM from three independent experiments. **, P<0.009, Student's unpaired t-test. (b) One representative EMSA experiment demonstrating reduced nuclear binding of ISGF-3 complexes in rIFN-α2b-treated nuclear extracts from vIRF-2-expressing clone 3-9 cells compared with EV clone 5 cells. (c) Western blot (WB) analysis of TBP detected with Abcam ab818 antibody at 1:2000 dilution was performed in parallel on the nuclear extracts shown in (b) to confirm equal sample loading for the EMSA. (d) vIRF-2 attenuates STAT1 and IRF-9 components of the IFN-α-induced ISGF-3. The cell clones described in Fig. 1 were cultured as described and lysates harvested for relative quantification of components of the IFN-α-induced JAK–STAT signalling cascade by WB analysis. To ensure vIRF-2 was functional at the time of lysate generation, the cells were transiently transfected with pISRE-luc and pRLSV-40-luc as described in Fig. 1. Aliquots of lysates were then assayed at the time of harvest for dual luciferase activity and inhibition of ISRE activity was confirmed as described in Fig. 1 (data not shown). Lysates (20–40 μg each) were loaded in each lane for immunoblot analysis. Equal loading was confirmed by staining for GAPDH (Sigma-Aldrich cat. no. G8795, 1:2000 dilution). The antibodies for each protein and the dilutions they were used at were: vIRF-2 (c-Myc-epitope tagged) Cancer Research UK 9E10, 1:2000; IFNAR1, Abcam cat. no. ab45172, 1:500; Tyk2 phosphorylated at Tyr1054 and Tyr1055 [pTyk2 (Tyr1054/1055)], Cell Signaling Technology cat. no. 9321, 1:200; Tyk2, Santa Cruz Biotechnology cat. no. SC-169, 1:200; Jak1, Santa Cruz Biotechnology cat. no. SC-277, 1:200; STAT2 phosphorylated at Tyr699 [pSTAT2 (Tyr699)], Millipore cat. no. 07-224, 1:200; STAT2, Santa Cruz Biotechnology cat. no. SC-476, 1:200; STAT1 phosphorylated at Tyr701 [pSTAT1 (Tyr701)], Cell Signaling Technology cat. no. 9167, 1:500; STAT1, Santa Cruz Biotechnology cat. no. SC-346, 1:1000; and IRF-9, Santa Cruz Biotechnology cat. no. SC-10793, 1:300. Secondary antibodies were either polyclonal goat anti-rabbit HRP (P0448; Dako) or polyclonal goat anti-mouse HRP (P0447; Dako). For each pair of cell lines (vIRF-2-expressing and EV clone), each protein was probed on the same membrane; however, as the protein bands for these lysates were not electrophoresed in adjacent lanes they are shown in separate boxes.
Fig. 3. vIRF-2 rescues EMCV replication from recombinant IFN-α. vIRF-2 clone 3-9 and EV clone 5 cells were treated with 1 μg doxycycline ml⁻¹ with or without IFN-α (3, 30 and 300 IU ml⁻¹) for 30 h. The cells were then infected with EMCV at an m.o.i. of 0.1 and incubated for 30 h before the supernatant was collected; EMCV titres were determined by limiting dilution plaque assay on L929 cells. After 72 h monolayers were stained with crystal violet and plaques counted. Data are means ± SEM from three independent experiments.

(Krishnan et al., 2004). Our own studies indicate that vIRF-2 is inducible (Cunningham et al., 2003). Thus, vIRF-2 displays a broad spectrum of expression during the KSHV life cycle, and the vIRF-2 protein inhibits induction of both type I IFN gene expression by targeting IRF-3 (Aresté et al., 2009; Fuld et al., 2006) and downstream type I IFN-responsive genes by targeting ISGF-3 (the present study). Taken together, these data suggest that vIRF-2 represents both an infected-cell-intrinsic factor that resists paracrine IFN-α signalling during primary KSHV infection and an inducible factor that suppresses IFN-α responses during KSHV reactivation from latency.

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