Kaposi’s Sarcoma-associated Herpesvirus-encoded vIRF-3 Stimulates the Transcriptional Activity of Cellular IRF-3 and IRF-7*

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Kaposi’s sarcoma-associated herpesvirus has been linked to Kaposi’s sarcoma, body cavity-based lymphoma, and Castleman’s disease. The Kaposi’s sarcoma-associated herpesvirus genome contains a cluster of open reading frames encoding proteins (vIRFs) with homology to the cellular transcription factors of the interferon regulatory factor family. vIRF-3, also called LANA2, is a latently expressed nuclear protein. Here we demonstrate that vIRF-3 directly interacts with cellular interferon regulatory factor (IRF) IRF-3, IRF-7, and the transcriptional co-activator CBP/p300. The mapping of the vIRF-3 binding domain revealed that vIRF-3 associates with both IRF-3 and IRF-7 through its C-terminal region. The p300 domain, which interacts with vIRF-3, is distinct from the previously identified IBiD domain, to which vIRF-1 and IRF-9 bind. Thus, in contrast to vIRF-1, vIRF-3 neither blocks the interaction between IRF-3 and p300 nor inhibits the histone acetylation. Although vIRF-3 is not a DNA-binding protein, it is recruited to the IFNA promoters via its interaction with IRF-3 and IRF-7. The presence of vIRF-3 in the enhancerosome assembled on the IFNA promoters increases binding of IRF-3, IRF-7, and acetylated histone H3 to this promoter region. Consequently, vIRF-3 stimulates the IRF-3- and IRF-7-mediated activation of type I interferon (IFNA and IFNB) genes and the synthesis of biologically active type I interferons in infected B cells. These studies illustrate that vIRF-3 and vIRF-1 have clearly distinct functions. In addition to its transcriptional activity, vIRF-3 can also act as a transcriptional activator on genes controlled by cellular IRF-3 and IRF-7.

The interferon regulatory factor (IRF) genes encode DNA-binding proteins that play a critical role in the innate response to viral infection as well as in differentiation of lymphoid cells and apoptosis. Perturbation of these latter functions results in tumorigenicity. To date, nine cellular IRF genes, IRF-1, IRF-2, IRF-3, IRF-4/Pip1/ICSAT, IRF-5, IRF-6, IRF-7, ICSBP/IRF-8, and ISGF3γ/p48/IRF-9, have been identified (1, 2). These factors can function as transcriptional activators (e.g. IRF-1, IRF-4, and IRF-9), repressors (e.g. IRF-8), or both (e.g. IRF-2, IRF-4, IRF-5, and IRF-7). They all share significant homology in the N-terminal 115 amino acids, which comprise the DNA-binding domain, characterized by five tryptophan repeats. Three of these repeats contact DNA with specific recognition of the GAAA and AANNNGAA sequence (3). However, the unique function of a particular IRF is accounted for by a cell type-specific expression, its intrinsic trans-activation potential, and an ability to interact with the other members of IRF family or transcription factors and co-factors (4).

Three IRFs (IRF-3, IRF-5, and IRF-7) function as direct transducers of virus-mediated signaling and play a crucial role in the expression of type I interferon (IFN) genes and some chemokine genes, including RANTES (5–7). Whereas IRF-3 is constitutively expressed in all cell types (8), constitutive expression of IRF-7 can be detected predominantly in cells of lymphoid origin and can be further stimulated by type I IFN. The expression of IRF-5 seems to be restricted to dendritic cells and B cells (9, 10). In monocytes, and particularly in the precursors of dendritic cells (PDC2), which are high producers of IFNa, both IRF-5 and IRF-7 are expressed constitutively (11). In uninfected cells, IRF-3 and IRF-7 reside predominantly in the cytoplasm, but upon virus-induced phosphorylation of C-terminal serine residues, mediated by IKKe and TBK1 (12, 13), they translocate to the nucleus where they associate with other transcription factors and histone acetyl transferases, CBP/p300, forming a ternary complex, enhancerosome, binding to the promoters of IFNA and IFNB genes (5, 14). Whereas IRF-3 expression is sufficient for the expression of IFNb (15), RANTES (16), and some interferon-stimulated genes (ISGs) such as ISG56 (17), IRF-7 has a critical role in the induction of IFNa. Reconstitution of IRF-7 expression in human cells that can express only IFNb gene resulted in virus-mediated induction of IFNA genes (18). These results demonstrate both the essential and distinct roles of IRF-3 and IRF-7, which together control the transcriptional activation of diverse IFNaβ genes in the antiviral response.

The importance of IRFs in the innate antiviral response is further supported by the findings that an increasing number of viruses have been found to encode proteins that target the function of these factors to overcome the antiviral immune response (19). The Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) (20) contains a cluster of four genes (vIRFs) that encode proteins with homology to the cellular transcription factors of the IRF family. Three of these vIRFs (vIRF-1, vIRF-2, and vIRF-3/LANA2) have been cloned and characterized. They all show homology in the N-terminal region to the DNA-binding domain of cellular IRFs; however, the vIRFs lack several of the tryptophan residues that are essential for the DNA binding and thus, in contrast to cellular IRFs, vIRFs are...
not able to directly bind to DNA. The expression of vIRFs in the KSHV life cycle is distinct. vIRF-1 (ORF K9) is expressed during the KSHV replication cycle (21), and its expression is activated by the KSHV-encoded trans-activator, ORF50, and by an auto-activation of its promoter (22). In contrast, vIRF-2 and vIRF-3 are expressed during KSHV latency (23, 24).

Several groups have extensively studied vIRF-1. In a transient transfection assay, vIRF-1 was shown to inhibit both the virus-mediated induction of type I IFN genes and IFN-induced genes (ISGs) (25–27). In addition, vIRF-1 overexpression in NIH/3T3 cells confers tumorigenicity when injected into nude mice (28–30). These data, together with the observation that vIRF-1 binds to p53 and inhibits apoptosis, suggested that vIRF-1 might have an oncogenic potential (25, 31–33). However, targeted expression of vIRF-1 to B cells or endothelial cells failed to induce tumor formation in transgenic mice (34).

Moreover, it was shown that vIRF-1 binds not only to cellular IRFs but also to CBP/p300 and inhibits its acetyltransferase activity (25, 35), which results in a global inhibition of acetylation of histones H3 and H4 (26).

vIRF-2 (ORF K11.1) encodes a small nuclear protein (163 aa) that is constitutively expressed in primary effusion lymphoma (PEL) cells and specifically associates with several cellular IRFs and p300 (23). In addition, vIRF-2 also binds double-stranded RNA-activated protein kinase, PKR, inhibits its kinase activity, and blocks the phosphorylation of the PKR substrate, eukaryotic translation initiation factor 2

Plasmids and Antibodies—Full-length vIRF-3 tagged with Myc (vIRF-3-myc; aa 1–566), vIRF-3-N (aa 1–254), vIRF-3-C (aa 254–566) (38), IRF-3, IRF-7, vIRF-1 expression plasmids (8, 25, 40), IRF-3 (aa 1–115), IRF-3 (1–231), IRF-3 (aa 145–427) (15), IRF-3/FL, IRF-7-N (aa 1–257), IRF-7-C (aa 257–314) (9), p300-N, p300-C, p300-C-A, p300-C-B, p300-C-C (25), IRF-3-ribosome, pU1/IRF-3 (41), and reporter plasmids, HuIFNB, A1, and A2 secreted alkaline phosphatase (SAP) (18), were described previously. GST-vIRF-3-FL, GST-vIRF-3-N, and GST-vIRF-3-C were cloned by amplification of the vIRF-3 cDNA (aa 1–566, 1–254, and 254–566, respectively) from the vIRF-3-myc cDNA plasmid and subcloned into pGEX-4T vector (Amersham Biosciences). The IRF-3 (aa 145–231) T7 was cloned by amplification of the IRF-3 expression plasmid as a template. The 5′ primer carried a T7 tag sequence that was in-frame with IRF-3 open reading frame. The PCR product was sub-cloned into pCNA3.1 vector (Invitrogen). The vIRF-3 antibodies were raised in rabbits against two peptides (n-VLLEKRRRRPPRPVYEC-c and n-CWDDGPRRHERPTTR-c) and purified by protein A chromatography. Polyclonal IRF-3, IRF-7, p300, CBP, Mhc, actin, and Sp1 antibodies were obtained from Santa Cruz Biotechnology. The M2 anti-FLAG monoclonal antibodies were purchased from Sigma; T7 antibodies were from Novagen, and acetyl-H5 and acetyl-H4 antibodies were obtained from Abcam Biotechnology Inc.

Transfections and SAP Assays—In transient transfection assays, 2 × 106 cells were transfected with 10 μg of DNA by using Superfect (Qiagen). For SAP assays, equal amounts (2 μg) of reporter plasmid and IRF- or vIRF-expressing plasmids were co-transfected with β-galactosidase-expressing plasmid. The transfected cells were divided 24 h later and infected with Sendai virus for 16 h or left uninfected. The SAP assay was performed as described previously (42, 43). Each experiment was repeated three times. The β-galactosidase expression levels were used to normalize the difference in transfection efficiency.

Immunoprecipitation and Western Blot Analysis—293 cells co-transfected with various vIRF-3, IRF-3, and IRF-7 expression plasmids were lysed in lysis buffer (20 mm HEPES (pH 7.9), 50 mm NaCl, 5 mm EDTA, 2 mm EGTA, 0.1% Nonidet P-40, 10% glycerol, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, and 0.2 μg protease inhibitor mixture (Sigma)). The protein extracts (40 μg) were then incubated with the respective antibodies. After extensive washing with co-precipitation buffer, precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot.

Oligonucleotide Pull-down Assay—The DNA pull-down assay was done as described previously (5). Briefly, double-stranded oligomers corresponding to the huIFNA1 VRE region (bp −110 to −53) were synthesized and biotin-labeled at the 5′ end of the antisense strand and coupled with streptavidin magnetic beads (Dynal, Inc.). Whole cell lysates were then incubated with the DNA bound to magnetic beads for 3 h at 4 °C. After extensive washing, the bound proteins were resolved by SDS-PAGE and analyzed by Western blot.

**vIRF-3 Stimulates Transcriptional Activity of IRF-3 and -7**

Sendai virus was purchased from Specific Pathogen-free Avian Supply (Dulbecco’s, CT), and Newcastle disease virus (NDV) was purchased from ATCC (VR-699).

Although vIRF-3 does not bind DNA, in virus-infected cells, vIRF-3 is tethered to the IFNA promoter by its association with IRF-3 and IRF-7. Finally, we provide evidence that the overexpression of vIRF-3 stimulates IRF-3 and IRF-7 transcriptional activity of IFNA and IFNB promoters and thus enhances virus-mediated induction of type I interferons in B cells. Our study reveals that in addition to its co-repressor function, vIRF-3 can also act as a transcriptional activator.

**EXPERIMENTAL PROCEDURES**

**Cells and Virus**—293GH and p2.1 (kindly provided by Dr. G. Stark), HeLa, and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. BJAB, BCBL-1, and BC-3 cells were grown in RPMI 1640 supplemented with 10% or 20% fetal bovine serum (for BC-3 cells). BJAB cells constitutively expressing vIRF-3 or vIRF-1 (BJAB/vIRF-3, BJAB/vIRF-1) were generated by transfection of BJAB cells with Myc-tagged vIRF-3 or vIRF-1-expressing plasmids, and transfected cells were selected by growth in G418.

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B. Lubysova and P. M. Pitha, unpublished observations.
technology), acetyl-H3 (Upstate Biotechnology, Inc.), and vIRF-3. Immunoprecipitation with p65 antibodies was used as a negative control. Immunocomplexes were extensively washed, and the DNA was recovered by phenol/chloroform extraction. For exogenous IFNA promoter studies, the DNA template was amplified with universal primers corresponding to the regions of human endogenous IFNA genes that are conserved in all subtypes (18).

Reverse Transcription-PCR Analysis and Antiviral Assay—1 μg of total RNA isolated by the Trizol method (Invitrogen) was reverse-transcribed to cDNA with oligo(dT) primers in a 30-μl reaction volume. From this mixture of cDNAs, IFNA, IFNB, RANTES, and β-actin were amplified by PCR as described previously (18). For the amplification of studied genes, the following primer sets were used: (i) huIFNA sense 5′-GTACTGAGAATCTCTCCTTTCTCCTG-3′, antisense 5′-GTGTCTAGATCTGACAACTCTCCAGGCACA-3′; (ii) huIFNB sense 5′-TTGTGCTTCTCCACTAGC-3′, antisense 5′-CTGTAAGTCTGTTAATGAA-3′; (iii) huRANTES sense 5′-AGGTCCTCGCGGCACGCTGGC-3′, antisense 5′-CACAAGGTTGATGTACTCCCG-3′; (iv) huβ-actin sense 5′-ACATGAGCTGCTGGTGGCT-3′, antisense 5′-TACGGCCACAGTCTGCTTGA-3′. The levels of biologically active interferons in the medium were determined by the antiviral assay on human fibroblast cells, as detected with polyclonal vIRF-3 antibodies, has shown the presence of vIRF-3 transcripts in the KSHV-infected primary effusion lymphoma cells, and its levels are not modulated by TPA treatment. A, BCBL-1 and BC-3 cells were treated with TPA (10 ng/ml) for the indicated times. The whole cell lysates (50 μg) were separated by the SDS-PAGE and analyzed by Western blot using polyclonal vIRF-3 antibodies. The levels of vIRF-3 expression in the stable BJAB/vIRF-3 cell line are shown for comparison. Protein lysates isolated from BJAB/vIRF-1 and Daudi cells were used as a control for specificity of vIRF-3 antibodies. The bottom panels show levels of actin that were used as the loading control. B, nuclear (N) and cytoplasmic (C) extracts were isolated from KSHV-negative Daudi and KSHV-positive BCBL-1 and BC-3 cells and stable BJAB/vIRF-3 cell lines and analyzed by Western blot with vIRF-3 antibodies. The distribution of nuclear Sp1 protein was used as a marker for integrity of cytoplasmic and nuclear fractions.

RESULTS

Expression and Localization of the vIRF-3 Protein in PEL Cell Lines—Our previous data, as well as the data of Jenner et al. (21), have shown the presence of vIRF-3 transcripts in the KSHV-latently infected primary effusion lymphoma cells, BCBL-1, that could be moderately increased upon TPA treatment that stimulates productive KSHV infection (38). In contrast to our data, Rivas et al. (24) and Fakhari and Dittmer (45) characterized vIRF-3 as a constitutively expressed nuclear antigen. By using polyclonal vIRF-3 antiserum, which specifically recognizes vIRF-3 but not IFN-β, we now show that vIRF-3 is expressed both in untreated and TPA-treated BCBL-1 and BC-3 cells (Fig. 1A). The vIRF-3 detected in PEL cells has the mobility of ~70 kDa that is identical to the mobility of vIRF-3 encoded by the ectopic vIRF-3 cDNA in the permanently transfected BJAB cells. No increase in the relative levels of the vIRF-3 protein was observed upon TPA treatment, which correlates with the published data of Moore and co-workers (24) that detected the expression of vIRF-3 in the nucleus of PEL cells and therefore named it latency-associated nuclear antigen 2 (LANA2). The analysis of nuclear and cytoplasmic extracts isolated from BCBL-1 cells has shown that vIRF-3 is present both in the nucleus and the cytoplasm (Fig. 1B), whereas in BC-3 cells the majority of vIRF-3 can be detected in the nucleus. To examine the function of vIRF-3 in B cells, we established a human B lymphoma cell line, BJAB, stably expressing vIRF-3 tagged with Myc epitope. Expression of vIRF-3 in these cells, as detected with polyclonal vIRF-3 antibodies, has shown that the ectopic vIRF-3 is present both in the nucleus and the cytoplasm. Expression of vIRF-3 in BJAB cells results in neither morphologic changes nor the alteration of the growth rate. In addition, the vIRF-3 expression in BJAB cells did not modulate their ability to grow in soft agar (data not shown).

Interaction of vIRF-3 with Cellular IRFs—To address the potential role of vIRF-3 in the antiviral innate response, we have examined the interactions between vIRF-3 and the transcription factors of the IRF family, namely IRF-3 and IRF-7, that play a critical role in the induction of the type I IFN genes, interferon-stimulated genes (ISGs), and chemokines (6, 17). To determine whether vIRF-3 interacts with these two factors in infected and uninfected cells, 293 cells that constitutively expressed IRF-3 were transfected with the Myc-tagged vIRF-3-expressing plasmid and then infected with Sendai virus for 6 h. Whole cell extracts from virus-infected and uninfected cells were immunoprecipitated with anti-Myc antibodies. vIRF-3 co-precipitated with IRF-3 in the infected but not in the uninfected cells (Fig. 2A), indicating that vIRF-3 can interact only with activated IRF-3 that is transported to the nucleus. As previously reported (46), the relative levels of IRF-3 were significantly lower in infected cells, because of the higher rate of degradation of the phosphorylated IRF-3. Nevertheless, the co-precipitation between vIRF-3 and IRF-3 could not be detected in uninfected cells where the levels of IRF-3 were significantly higher. Although 293 cells are transformed by adenovirus E1A, which was shown to compete with IRF-3 for binding to p300 (15), E1A protein did not interfere with the complex formation between vIRF-3 and IRF-3.

To determine the region within IRF-3 that interacts with vIRF-3, p2.1 cells (47), which express very low levels of inactive IRF-3, were co-transfected with vIRF-3 and different IRF-3 deletion mutants (Fig. 2B). All of these transfected mutants were well expressed, and their relative levels were not affected by viral infection. The results of the co-precipitation assay revealed that whereas the N-terminal polypeptide of IRF-3 (aa 1–115) did not interact with vIRF-3, the extended N-terminal region (aa 1–231) and the C-terminal part of IRF-3 (aa 145–427) strongly associated with vIRF-3 (Fig. 2C, lanes 4 and 6). Therefore, we hypothesized that vIRF-3 interacts with the IRF-3 domain located in the region between amino acids 145 and 231, which contains the nuclear export signal, the proline-rich domain, and a proximal part of the IAD domain (Fig. 2B). To confirm our hypothesis, we constructed the IRF-3 (aa 145–231) deletion mutant and analyzed its ability to bind to vIRF-3 in the GST pull-down assay. The results in Fig. 2D showed that in vitro translated IRF-3 (aa 145–231) mutant bound strongly to the full-length (FL) and the C-terminal half of GST-vIRF-3 fusion proteins, whereas it did not interact with either N-terminal part of vIRF-3 or GST alone. These data suggest that interaction between vIRF-3 and IRF-3 is direct and that the
Fig. 2. vIRF-3 associates with cellular IRF-3. A, vIRF-3 protein forms complexes with IRF-3 in the virus-infected cells. 293 cells were transfected either with empty vector or full-length (FL) vIRF-3 tagged with Myc epitope. 24 h post-transfection, cells were infected with Sendai virus for 6 h (lanes 2 and 4) or left uninfected (lanes 1 and 3). The whole cell lysates were immunoprecipitated (IP) with Myc antibodies, and IRF-3 was detected in immunoprecipitated complexes after Western blotting (WB) with IRF-3 antibodies. The relative levels of endogenous IRF-3 and transfected vIRF-3 proteins are shown for comparison (two bottom panels). B, structure of the IRF-3 protein and schematic representation of IRF-3 deletion mutants. DBD, DNA-binding domain; NES, nuclear export signal; PRO, proline-rich domain; IAD, IRF-association domain. C, the p2.1 cells were transfected with full-length vIRF-3, tagged with Myc epitope, together with the IRF-3 deletion constructs, IRF-3 (aa 1–115 (lanes 1 and 2), IRF-3 (aa 1–231) (lanes 3 and 4), and IRF-3 (aa 145–427) (lanes 5 and 6). 24 h post-transfection, the cells were infected with Sendai virus for 6 h (lanes 2, 4, and 6) or left uninfected (lanes 1, 3, and 5). The co-immunoprecipitation experiment was performed as outlined in A. The vIRF-3 protein specifically interacted with endogenous IRF-3 protein (marked by the asterisks) and with two transfected deletion mutants, IRF-3 (aa 1–231) and IRF-3 (aa 145–427) in virus-infected cells. The relative levels of endogenous IRF-3 and transfected vIRF-3 and IRF-3 deletion mutants are shown for comparison. D, vIRF-3 interacts with IRF-3 (aa 145–231). In vitro translated IRF-3 (aa 145–231) tagged with T7 epitope was incubated with GST-vIRF-3-FL, -N, and -C recombinant proteins (lanes 2–4, respectively) immobilized on glutathione-Sepharose beads. The bound proteins were eluted and resolved on 12% SDS-PAGE followed by Western blotting with T7 antibodies. 10% of IRF-3 (aa 145–231) protein input is shown in lane 1, and binding to GST beads represents a negative control (lane 5).

region from amino acid 145–231 of IRF-3 protein is the primary domain interacting with vIRF-3. Interestingly, Lin et al. (27) have mapped the binding of vIRF-1 to amino acids 197–240 of the IRF-3 protein. In order to determine whether vIRF-1 and vIRF-3 interact with distinct regions of IRF-3, a more detailed analysis of the IRF-3/vIRF-3 interaction is under investigation.

The interaction between vIRF-3 and IRF-7 was examined in BJAB/vIRF-3 cells by co-immunoprecipitation. To increase the relative levels of IRF-7, cells were pre-treated with IFNβ (200 units/ml) for 16 h (9). As a control, we used a BJAB cell line that was stably transfected with an empty vector. Because IRF-7 is phosphorylated in infected cells (48), cells were also infected with NDV to determine whether the phosphorylation of IRF-7 is required for the interaction with vIRF-3. As shown in Fig. 3A, vIRF-3 co-precipitated with IRF-7 regardless of virus infection, indicating that vIRF-3 can bind to both the phosphorylated and unphosphorylated forms of IRF-7.

To localize the region within IRF-7 that interacts with vIRF-3, two different IRF-7 deletion mutants were tested for interaction with vIRF-3-myc. As shown in Fig. 3B, the C-terminal region of IRF-7 associated with vIRF-3, because IRF-7 (aa 237–514) co-precipitated with vIRF-3, whereas no interaction could be detected with the N-terminal part of IRF-7 (aa 1–237). When the deletion plasmids of vIRF-3, expressing the N- or C-terminal parts of vIRF-3, were co-transfected together with the IRF-7-expressing plasmid to 293 cells, only the full-length and the C-terminal part (aa 254–566) of vIRF-3 could be immunoprecipitated together with IRF-7 (Fig. 3C). Altogether, these data indicate that although the vIRF-3/IRF-3 interaction occurs only in the virus-infected cells, association between vIRF-3 and IRF-7 does not require phosphorylation of IRF-7 and occurs also in uninfected cells.

Interaction between vIRF-3 and the Transcriptional Co-activator CBP/p300——Interaction between IRF-3, IRF-7, and the transcriptional co-activator p300 has been shown to stimulate transcriptional activity of IRFs (5, 15, 46, 49). Inhibition of this interaction by adenovirus-encoded E1A or KSHV-encoded vIRF-1 resulted in the inhibition of the IRF-3-mediated antiviral response (27, 50). The association between ectopic vIRF-3 and endogenous CBP/p300 was therefore tested in vIRF-3- transfected 293 cells by co-immunoprecipitation. vIRF-3 interacts with CBP/p300 both in infected (data not shown) and uninfected cells (Fig. 4A). In contrast to vIRF-3, cellular IRF-3 interacts with CBP/p300 only in the infected cells (46). When the plasmids encoding the N- and C-terminal parts of vIRF-3 were transfected into 293 cells, only the C-terminal part of vIRF-3 co-precipitated with CBP/p300, whereas no binding between N-terminal part of vIRF-3 and CBP/p300 could be detected (Fig. 4A).

It was shown previously that the C-terminal part of vIRF-1 also interacts with p300 (25, 27) and in a transient transfection assay inhibits the virus-mediated activation of the murine IFNA4 promoter. Furthermore, both vIRF-1 and IRF-3 factors were found to bind to the identical domain located in the C terminus of CBP/p300 and thus vIRF-1 competed with IRF-3 for binding to CBP/p300 (27). Therefore, we have mapped the region within the p300 polypeptide that interacts with vIRF-3. Different p300 deletion mutants (Fig. 4B) were synthesized in vitro as 35S-labeled proteins and incubated with the GST-vIRF-3 fusion protein immobilized on glutathione-Sepharose
vIRF-3 and IRF-7 deletion mutants in 40 deletion mutants in the immunoprecipitated complexes were detected by Western blotting with FLAG antibodies. The relative levels of transfected marked by an asterisk.

3 to fragments C similar approach, we have shown previously that vIRF-1 binds IBiD (aa 2067–2112) which is distinct from the vIRF-3-binding domain on p300. Therefore, we examined whether binding of vIRF-1 to CBP/p300 nor inhibits the histone acetyltransferase activity of CBP/p300 or possibly other acetyltransferases.

**Analysis of the IFNA Enhanceosome-like Complex in Infected vIRF-3-expressing Cells**—We and others (5, 14) have shown previously that transcriptional complexes assembled on the promoters of IFNA and IFNB genes in infected cells include IRF-3 and IRF-7. We have therefore examined whether the binding of IRF-3 and IRF-7 to IFNA virus-responsive element (VRE) is altered in the cells expressing vIRF-3. To analyze the DNA binding, we have used a DNA pull-down assay in which oligonucleotides corresponding to the IFNA1 VRE were biotinylated and coupled to streptavidin-coated magnetic beads (5). The DNA-containing beads were then incubated with extracts.
from infected and uninfected 2fTGH cells transfected either with an empty vector, IRF-7, vIRF-3 or a combination of IRF-7 and vIRF-3-expressing plasmids. We have shown previously (18) that the 2fTGH cells express IRF-3 but do not express IRF-7. The bound IRFs and vIRF-3 were then detected by Western blot. As shown in Fig. 6, IRF-3 bound to IFNA1 VRE only in infected cells, and no binding was detected in uninfected cells. However, the presence of IRF-7 or IRF-7 and vIRF-3 increased the ability of IRF-3 to bind to the VRE both in infected and uninfected cells. The enhanced binding of IRF-3 to IFNA1 VRE in the presence of IRF-7 was observed previously (5). The IRF-7 protein bound effectively to the IFNA1 VRE both in the lysates from infected and uninfected cells, and this binding was not affected by the presence of either IRF-3 or vIRF-3. The ability of IRF-7 to bind to DNA from the lysates of uninfected cells was due to the fact that whole cell extracts were analyzed in this assay, whereas when nuclear extracts were used in the DNA pull-down assay, very little binding of IRF-7 could be detected in uninfected cells (5). However, our results suggest that activation of IRF-7 by C-terminal phosphorylation is not required for its DNA binding capacity. Interestingly, the association of the vIRF-3 protein with the IFNA1 VRE was dependent on the binding of IRF-3 and/or IRF-7. Whereas no vIRF-3 was detected at the IFNA1 VRE in the absence of IRF-3 binding in the lysates from uninfected cells (Fig. 6, lane 3), binding of both IRF-3 and vIRF-3 was demonstrated in the lysates from infected cells (Fig. 6, lane 4). Furthermore, in the presence of IRF-7, binding of vIRF-3 to the IFNA1 VRE was significantly increased (Fig. 6, lanes 7 and 8). These results suggest that although vIRF-3 is not a DNA-binding protein, it is recruited to the IFNA1 VRE via its association with IRF-3 and/or IRF-7. The observed differences in the binding of cellular IRFs or vIRF-3 to the IFNA1 VRE were not a consequence of different levels of their expression in the analyzed protein extracts. As shown in Fig. 6 (bottom panels), the relative levels of IRF-3 in the input lysates were comparable in the uninfected cells and only slightly lower in the infected cells. Also both vIRF-3 and IRF-7 were expressed effectively in transfected cells, and their relative levels were almost identical.

The transcriptional activation of IFNA and IFNB genes in infected cells is mediated by a multicomponent complex, enhanceosome assembled on the IFNA or IFNB VREs (5, 51). To analyze further how the expression of vIRF-3 modulates the assembly of IRF-3, IRF-7, and acetylated histone H3 on the IFNA promoters in infected cells, we used the chromatin immunoprecipitation assay. Because the endogenous IFNA promoters show a high degree of homology and are difficult to distinguish, we first analyzed the assembly of IRF-3 and acetylated H3 on the IFNA1 VRE of the transfected reporter construct. We (18) and others have shown previously that IRF-3 binds to this promoter, but its activation requires both IRF-3 and IRF-7. HeLa cells were co-transfected with the reporter plasmid, IFNA1-SAP, containing the IFNA1 promoter, together with vIRF-1 or vIRF-3 expression plasmids and infected with NDV for 6 h or left uninfected. The proteins were then cross-linked to DNA, and the DNA-protein complexes were precipitated with antibodies against IRF-3 and acetylated H3. The DNA in the precipitates was then amplified by PCR with universal primers to the IFNA1 VRE-containing reporter plasmid (5, 6). As shown in Fig. 7A, the IFNA1 VRE was amplified from the DNA before immunoprecipitation (inputs, bottom panel), and the relative levels of the amplified fragments were almost identical in all the samples, indicating that the amount of IFNA1 DNA input used for immunoprecipitation was comparable in all samples tested. After precipitation with IRF-3 antibodies, the IFNA1 VRE was amplified from the DNA of infected cells transfected either with an empty vector or the vIRF-3-expressing plasmid. However, very little of IFNA1 VRE DNA was amplified from the cells that expressed vIRF-1. In-
Interestingly, the relative levels of the amplified DNA were higher from the cells that expressed vIRF-3 than from the cells that were transfected with an empty vector. Thus, these results suggest that in the presence of vIRF-3, the DNA-binding affinity of IRF-3 is increased. In contrast to vIRF-3, the vIRF-1 expression strongly inhibits IRF-3 binding to the IFNA1 promoter. It should also be noted that the relative levels of vIRF-1 and vIRF-3 proteins expressed in the transfected cells were almost the same as detected by Western blot with Myc antibodies (data not shown). Furthermore, the immunoprecipitation procedure was performed with antibodies against IRF-3 and acetylated histone H3 and H4.

**Fig. 5.** vIRF-3 neither blocks the IRF-3-CBP-p300 complex formation nor inhibits histone acetylation. A, the control BJAB/vector, BJAB/vIRF-3, or BJAB/vIRF-1 cells were infected with SeV for 6 h or left uninfected. Whole cell extracts (400 μg) were immunoprecipitated (IP) with anti-CBP antibody. Immunoprecipitated complexes (upper panel) or 40 μg of whole cell extracts (lower panel) were separated by SDS-PAGE and subsequently probed with an IRF-3 antibody. WB, Western blot. B, equal amounts of protein (40 μg) from the control BJAB/vector (lane 1) and BJAB/vIRF-3 (lane 2) cells, treated with butyric acid (5 mM) overnight, were used for immunoblotting analysis with antibodies specific for the acetylated histone H3 and H4. The bottom panel shows levels of actin which were used as the loading control.

**Fig. 6.** IRF-3 and IRF-7 recruit vIRF-3 to the IFNA1 promoter. Binding of cellular IRF-3, IRF-7, and vIRF-3 from virus-infected and uninfected 2TGH cells to the IFNA1 VRE was analyzed by a DNA pull-down assay. 2TGH cells were transfected with IRF-7 or Myc-tagged vIRF-3, and 24 h post-transfection, cells were infected with NDV for 6 h. Whole cell extracts (350 μg) were then incubated with IFNA1 VRE oligonucleotides coupled to magnetic beads as described under “Experimental Procedures.” The IRFs and vIRF-3 pulled down by DNA were identified by Western blot (WB) with specific antibodies. The relative levels of endogenous IRF-3 and transfected IRF-7 and vIRF-3 in 35 μg of cell extracts were estimated by Western blot (inputs).
and BJAB/vIRF-3 cells were infected with NDV, and 6 h post-infection the chromatin immunoprecipitation analysis was performed as the assembly of the enhanceosome on the endogenous IFNA promoters in the infected BJAB/vector and BJAB/vIRF-3 cells. The histone acetyltransferases, CBP/p300, are recruited to IRF-3 to the IFNA1 VRE.

IRF-3 to the IFNA1 VRE. As a negative control, we used p65 antibodies that did not result in any amplification product (data not shown), indicating that the precipitation and amplification of IFNA1 VRE DNA was specific.

Enhancement of IRF-3- and IRF-7 Transcriptional Activity by vIRF-3—To determine the functional consequences of the vIRF-3 interaction with IRF-3 or IRF-7, as well as with the co-activators CBP/p300, we have examined the effect of vIRF-3 on the transcriptional activation of IRF-targeted promoters of the IFNA, IFNB, and RANTES genes. The role of IRF-3 and IRF-7 in the activation of IFNA, IFNB, and RANTES genes has been well documented (7, 9, 15, 16, 53). The activation of IRF-3 in infected cells was also identified as a primary response to viral infection that induces secondary response and amplification of the antiviral defense (54). Hence, we have used the transient transfection assay to examine the effect of vIRF-3 on IRF-3- and IRF-7-mediated stimulation of type I IFN gene promoters. The reporter plasmids in which the expression of SAP was under the control of IFNA1 or IFNB promoters (42) were co-transfected with IRF-3, IRF-7, vIRF-1, or vIRF-3 into HeLa cells that express endogenous IRF-3 and low levels of IRF-7. As shown in Fig. 6A, co-transfection of IFNB-SAP with IRF-3 or IRF-7 activated transcriptional activity of this promoter in both uninfected and infected cells. In uninfected cells, transfection of IRF-3, IRF-7, or vIRF-3 stimulated the IFN promoter activity by ~4-fold. However, the co-transfection of IRF-7 and vIRF-3 further stimulated the IRF-7-mediated activation of the IFNB promoter by 2-fold. Similar results were obtained in virus-infected cells. Transfection of IRF-3- or IRF-7-expressing plasmids increased virus-mediated activation of IFNB promoter by ~3-fold. Transfection of vIRF-3 also moderately stimulated the activity of the IFNB promoter. Moreover, co-transfection of vIRF-3 and IRF-3 or IRF-7 enhanced the IRF-3- and IRF-7-stimulated activation of the IFNB promoter in infected cells (1.7- and 2.2-fold, respectively). In contrast to vIRF-3, expression of vIRF-1 blocked the IRF-3-mediated activation of the IFNB promoter in both uninfected and virus-infected cells (2- and 3-fold, respectively), whereas it had a very marginal effect on IRF-7 transcriptional activity.

Similar data were obtained when activity of IFNA1 promoter was tested under the same conditions (Fig. 8B). In uninfected cells, expression of IRF-3, IRF-7, or vIRF-3 slightly stimulated IFNA1 promoter activity, whereas co-expression of IRF-7 and vIRF-3 further enhanced the IRF-7 transcriptional activity (2.6-fold). In Sendai virus-infected cells, IRF-3 and IRF-7 increased the virus-mediated stimulation of IFNA1 promoter by ~2-fold. The expression of vIRF-3 also resulted in a slight activation of the reporter construct. Co-transfection of vIRF-3 with IRF-3 further enhanced the IRF-3-mediated activation of the IFNA1 promoter in infected cells by about 2-fold. However, a stronger activation of IFNA1 promoter was detected in virus-infected cells after co-transfection of both vIRF-3 and IRF-7 (3.3-fold enhancement). In agreement with the previous results (25), vIRF-1 inhibited IRF-3-mediated activation of IFNA1 promoter in infected cells by ~2-fold. As we observed for IFNB activation, vIRF-1 did not exhibit a pronounced effect on IRF-7 transcriptional activity. To support these data further, we have also studied the activation of the RANTES gene promoter. In co-transfection assay with RANTES reporter construct, vIRF-3 increased the IRF-3- and IRF-7-mediated activation of this promoter (data not shown). Altogether, these findings were rather unexpected, because both vIRF-1 and vIRF-3 were previously shown to inhibit the virus-mediated activation of murine IFNA4 promoter as well as type I IFN-mediated activation of ISG15 ISRE in mouse fibroblasts (25, 26, 29, 38, 55).

To determine whether vIRF-3 functions as a nonspecific transcriptional activator, like HSV-1-encoded ICP0, we examined whether vIRF-3 enhances activity of the minimal TK promoter. However, neither vIRF-3 nor IRF-7 regulated the activity of this promoter (Fig. 8C). In addition, vIRF-3 inhibited the IFNγ-induced, STAT1-mediated activation of the GAS element-containing promoter (data not shown). These data suggest that the association of vIRF-3 with IRF-3- and IRF-7-containing enhanceosome specifically increases transcriptional activity of IRF-3 and/or IRF-7.

To determine whether the enhancing effect of vIRF-3 is me-
Fig. 8. Distinct effects of vIRF-3 and vIRF-1 on the IRF-3- and IRF-7-mediated transcriptional activation of type I interferon genes. A, vIRF-3 increases the IRF-3- and IRF-7-mediated activation of the IFNB promoter. Human IFNB-SAP reporter and β-galactosidase plasmids were co-transfected into HeLa cells with either empty vector (con) or respective IRFs and vIRFs expressing plasmids as described under "Experimental Procedures." 24 h after transfection, the cells were infected with Sendai virus for 16 h, and the culture medium was analyzed for SAP activity. Error bars show standard errors for triplicate experiments. B, vIRF-3 increases IRF-3- and IRF-7-mediated activation of the IFNA1 promoter. HeLa cells were co-transfected with human IFNA1-SAP-reporter and β-galactosidase plasmids with either empty vector (con) or respective IRF- or vIRF-expressing plasmids. 24 h after transfection, cells were infected with Sendai virus for 16 h, and the culture medium collected from cells was subsequently analyzed for SAP activity. Error bars represent standard errors for three experiments. C, vIRF-3 does not modulate the expression of minimal TK promoter. HeLa cells were co-transfected with the pGL3 promoter-luciferase reporter (Promega) and β-galactosidase plasmids with either empty vector (con) or vIRF-3 and IRF-7 expression plasmids. 24 h after transfection, protein extracts were isolated and analyzed for luciferase activity. Error bars represent standard errors for three experiments.

vIRF-3 Stimulates Transcriptional Activity of IRF-3 and IRF-7

The enhancement of interferon production in BJAB/vIRF-3-expressing cells was also detected on the protein level (Table I). Although none or low levels of IFNA or IFNB mRNAs were detected in uninfected BJAB/vector or BJAB/vIRF-3 cells, the relative levels of IFNA, IFNB, and RAN-TES transcripts were determined by a semi-quantitative RT-PCR (Fig. 10). The expression of IFNA genes was analyzed with universal primers, which can detect a majority of the IFNA subtypes. Although none or low levels of IFNA or IFNB mRNAs were detected in uninfected BJAB/vector or BJAB/vIRF-3 cells, the virus infection more effectively stimulated the expression of type I interferon genes in BJAB/vIRF-3 cells than in BJAB/vector cells (Fig. 10, lanes 4 and 2, respectively). However, vIRF-3 was not able to stimulate expression of IFNA or IFNB genes in uninfected cells. Although RANTES gene was expressed constitutively in these cells, it was more effectively induced in infected BJAB/vIRF-3 than BJAB/vector cells.

The enhancement of interferon production in BJAB/vIRF-3-expressing cells was also detected on the protein level (Table I). The levels of biologically active IFNa/β and IFNa synthesized...
vIRF-3 Stimulates Transcriptional Activity of IRF-3 and -7

**Fig. 9.** vIRF-3 stimulates the transcriptional activity of both IRF-3 and IRF-7. A. effect of IRF-3 ribozyme on vIRF-3-mediated activation of IFNB promoter. HeLa cells were co-transfected with human IFNB-SAP reporter plasmid together with either empty vector, IRF-3 ribozyme (pU1/IRF-3), IRF-3, or vIRF-3-expressing plasmids. 24 h after transfection, the cells were infected with Sendai virus for 16 h, and the culture medium was subsequently analyzed for SAP activity. Error bars represent standard errors for three experiments. B. effect of vIRF-3 on the IRF-7-mediated activation of IFNA2 promoter in 2fTGH cells. Human IFNA2-SAP reporter plasmid was co-transfected into 2fTGH cells together with either empty vector or IRF-7 and vIRF-3-expressing plasmids. The Sendai virus infection and SAP analysis were carried out as outlined in A. Error bars represent standard errors for three experiments.

**Fig. 10.** Activation of endogenous type I IFN genes and RANTES by vIRF-3. BJAB/vector or BJAB/vIRF-3 cells were infected with NDV for 6 h (lanes 2 and 4) or left uninfected (lanes 1 and 3). RT-PCR for IFNA, IFNB, RANTES, and β-actin was performed as described under “Experimental Procedures.”

by virus-infected BJAB/vector and BJAB/vIRF-3 cells (10^6) were determined by an antiviral assay as described under “Experimental Procedures.”

The initial studies have indicated that the KSHV-encoded vIRFs antagonize the innate antiviral response and inhibit the transcriptional activation of the promoters of type I IFN and interferon-stimulated genes (ISGs). In this report, we have shown that vIRF-3, when overexpressed in KSHV-negative B cells, activates the promoters of type I IFN genes and enhances virus-induced expression of endogenous IFNA and IFNB genes. In vIRF-3 transfection experiments, the activation of IFNA and IFNB promoters by vIRF-3 is dependent on the binding of IRF-3 or IRF-7 to the VREs of these promoters, whereas vIRF-3 alone neither binds nor activates these promoters. The ability of vIRF-3 to stimulate the transcriptional activity of IRF-3 and IRF-7 has not been observed before. In fact, we have reported previously (38) that in a co-transfection experiment in NIH/3T3 cells, vIRF-3 inhibited the transcriptional activation of the murine IFNA4 promoter. We believe that the discrepancy between these results may be due to the selection of the transient transfection systems used. Murine IFNA4 promoter can be activated by IRF-3 alone, whereas for the activation of human IFNA promoters, IRF-7 is a critical factor. In addition, previously published studies were done in mouse fibroblast cells that may not manifest the activation of the same pathways as the human cells used in this study. Moreover, our transient transfection results from the vIRF-3 stimulation of IRF-3- and IRF7-mediated activation of type I IFN promoters were supported by the observation that infected vIRF-3-overexpressing B cells produced higher levels of biologically active type I IFNs than the infected parental B cells.

Addressing the molecular mechanism of vIRF-3-mediated stimulation, we have shown that the effect is specific for IRF-3- and/or IRF-7-targeted promoters. vIRF-3 did not enhance the basal transcriptional activity of the minimal promoter of TK gene or the STAT1-mediated activation of the GAS element-containing promoter. Although the N-terminal domain of vIRF-3 shows some homology with the N-terminal DNA-binding domain of cellular IRFs, vIRF-3 does not bind IFNA or IFNB VREs but exerts its effects by directly binding to IRF-3 and IRF-7. While the association between vIRF-3 and IRF-3 can be detected only in infected cells, binding to IRF-7 occurs also in the absence of viral infection. The C-terminal half of the vIRF-3 protein associates with both IRF-3 and IRF-7. The domains through which IRF-3 interacts with vIRF-1 and vIRF-3 are partially overlapping; therefore, additional experiments are required to determine whether vIRF-1 and vIRF-3 interact with distinct regions of IRF-3.

Previously, it was demonstrated that the IRF proteins associate with acetyltransferases, CBP/p300, PCAF, and GCN5 (46,
The transcriptional activation of IFNA and IFNB promoters in infected cells is associated with the assembly of a multiple-component nucleoprotein complex enhanceosome on the VREs of the respective IFN promoters. Both IRF-3 and IRF-7 are the components of these enhanceosomes that also contain CBP/p300 (5, 14). Here we show by DNA pull-down and chromatin immunoprecipitation assays that vIRF-3 is recruited to the IFNA promoter in infected cells via its interaction with IRF-3 and IRF-7. There was no recruitment of vIRF-3 to the IFNA VRE in the absence of IRF-3 or IRF-7 binding. The IFNA enhanceosome also contains histone acetyltransferases as demonstrated by the association of acetylated histone H3 with the IFNA promoter in infected cells. In agreement with previous observations (26, 27), vIRF-1 decreased the binding of both IRF-3 and acetylated H3 to the IFNA1 VRE. Interestingly, vIRF-1 can also activate transcription when it is directed to the DNA by the GAL4 DNA-binding domain (65).

The molecular mechanism of the vIRF-3-mediated stimulation is not yet clear. It has been shown that IRF-3 homodimers, which are formed in infected cells, could activate the IFNB promoter (57), whereas the IRF-3/IRF-7 heterodimers were the major inducers of IFNA promoters (5). The binding of the IRF-3/IRF-7 complex to the IFNA and IFNB promoters, as well as the recruitment of IRF-1 and CBP/p300 acetyltransferase to these promoters, was also observed in infected human cells (5, 52). In this study, we have shown that vIRF-3 can bind to IRF-3 and IRF-7, and thus vIRF-3 may directly associate with the IRF3/IRF-7 heterodimer and increase its stability or the DNA-binding capacity. Notably, in infected 2TGH cells, the vIRF-3 enhancement of transcriptional activity of the IFNA2 promoter, which is mediated by the IRF-3/IRF-7 heterodimers (5), was more efficient (6-fold) than the enhancement of the IFNB promoter mediated by IRF-3 homodimers (2.5-fold). Alternatively, vIRF-3 association with IRF-3 and IRF-7 may enhance the recruitment of CBP/p300 or another acetyltransferase to the enhanceosome. Further studies will seek the additional components of the IFNA and IFNB enhanceosomes and establish their role in vIRF-3-mediated stimulation of type I IFN genes expression.

The significance of vIRF-3-mediated enhancement of the transcriptional activity of IRF-3 and IRF-7 may extend beyond the activation of type I IFN and chemokine genes. Like vIRF-1 (25), vIRF-3 associates with a number of cellular IRFs, including IRF-1 (data not shown), that play a role in apoptosis (66), tumorigenicity (67), and the immune response (68). It remains to be determined whether any of these functions are modulated by vIRF-3. Also the identification of additional IRF target genes whose expression is provoked by vIRF-3, deserves further evaluation. Although the aim of this study has been to determine the function of vIRF-3 out of the context of KSHV-infection, the role of vIRF-3 in the KSHV replication cycle needs to be addressed. It is unlikely that KSHV captured vIRF-3 to enhance antiviral response that would block its replication. Interestingly, several KSHV-encoded genes, expressed during the lytic KSHV replication cycle, target the functions of IRF-3 and/or IRF-7 or induce their degradation thus eliminating the induction of an antiviral response (25, 27, 69). Moreover, it needs to be kept in mind that vIRF-3 is a latently expressed nuclear antigen, and thus its primary role may be to facilitate the KSHV latency. The growth of PEL cells in vitro depends on an autocline production of vIL-6 that can be expressed in PEL cells during KSHV latency, but its expression is substantially increased during lytic replication by KSHV-encoded transcription activator, ORF50 (Rta) (70). Recently, it was shown that the promotor of vIL-6 contains two ISRE-like elements that can be activated by type I IFNs (71). Whether vIRF-3 and IRF-3 or IRF-7 participates in the transcriptional activation of vIL-6 in latently infected cells remains to be examined. Finally, vIRF-3, which is unable to bind to DNA, can be tethered to the IFNA enhanceosome and increase its transcriptional activity through the interaction with IRF-3, IRF-7, and CBP/p300. This indicates that the spliced forms, which were identified for IRF-3 and IRF-7 (72–74), may function not only as dominant negative mutants of the respective IRFs but could potentially stimulate the transcriptional activity of IRF-containing enhanceosomes. Future studies will determine the molecular mechanism of vIRF-3-mediated enhanced activation of IRF-3 and IRF-7 transcriptional activity as well as address the role of vIRF-3 in KSHV latency.

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