Kaposi’s sarcoma-associated herpesvirus (KSHV), also termed human herpesvirus-8 belongs to the herpesvirus family. KSHV was initially discovered in AIDS-associated Kaposi’s sarcoma, a solid tumor (1). It is now clear that KSHV is invariably found in all epidemiological forms of KS (for reviews, see Refs. 2 and 3). In addition, persistent KSHV infection is associated with two malignant lymphoproliferative disorders: primary effusion lymphoma (PEL) (4) and certain forms of multicentric Castleman disease (5). B-lymphoid cell lines established from PEL can be continuously propagated in culture. Even upon prolonged passage of PEL cells in culture, the latent genome is not lost from the cells, pointing to an essential role of KSHV genes for the continuous proliferation of these cells.

There is a surprisingly long list of KSHV genes thought to be involved in lymphomagenesis. Among them are K1 (6), vIL-6 (7), the viral interferon regulatory factors-1 (vIRF-1) (8) and -3 (vIRF-3) (9), K12/Kaposin (10), vFLIP (11), vCyclin (12), LANA (13), and K15 (reviewed in Ref. 14). Recently, we were able to show that knockdown of vIRF-3 by RNA interference results in reduced proliferation and induction of apoptosis in cultured PEL cells (15).

vIRF-3, also termed latency-associated nuclear antigen 2 (LANA2) or K10.5, is one of four KSHV genes with homology to the family of interferon regulatory factors. Notably, vIRF-3 is among the few viral genes expressed in essentially all latently infected PEL cells (9, 15). IRFs constitute a family of related transcription factors initially identified as mediators of the interferon response. The interferon (IFN) system is an early and important defense system of the cell against viral infections. Manipulation of this effective antiviral mechanism by encoding antagonistic viral IRFs is thus obviously of benefit for KSHV and likely to contribute to pathogenesis. It has been shown in the past that vIRF-3 is able to counteract the interferon response by interfering with cellular IRF-7 and -3 (16, 17) as well as PKR (18). Although inhibition of the interferon response is certainly important for pathogenesis, it is most likely not sufficient to explain the dependence of cultured PEL cells on vIRF-3, since class I interferons are not produced by these cells at detectable quantities. Like certain cellular IRFs (reviewed in Refs. 19 and 20), vIRF-3 is most likely not only involved in the innate immune response; it is also involved in pathways of apoptosis and cell growth regulation, which are not directly linked to the interferon response. An example is IRF-4, which is required for proliferation and differentiation of lymphocytes (21). In good agreement with this hypothesis, several functions not directly associated with the interferon system have been described for vIRF-3. These include the repression of p53 (9) and nuclear factor kappa B (NFκB) (22). Recently, vIRF-3 has been shown to stimulate Myc-dependent transcription (23).
**KSHV vIRF-3 Inhibits IRF-5**

The aim of this study was to identify cellular binding partners of vIRF-3 and thus pathways potentially deregulated by vIRF-3. We report here that vIRF-3 interacts with cellular IRF-5. Co-expression of vIRF-3 inhibited binding of IRF-5 to DNA and thereby the activation of IRF-5-regulated promoters. A central putative double α-helix motif in vIRF-3 was sufficient for this interaction. As shown previously, IRF-5 is not only a central mediator of the TLR-7 (Toll-like receptor-7) signaling pathway (24) and a main stimulatory transcription factor for both IFNα (25) and IFNβ (26) expression. Apart from its interferon-regulatory function, IRF-5 has also been described as a proapoptotic protein with tumor suppressor properties (27–29). Consistent with these findings, we report here that 1) vIRF-3 was able to inhibit IRF-5-dependent p21 promoter activation, 2) knockdown of vIRF-3 rendered PEL cells more sensitive to interferon treatment, and 3) release of IRF-5 from vIRF-3–mediated repression led to an enhanced transcription of anti-proliferative IRF-5 target genes. Thus, inhibition of IRF-5 DNA-binding and activity by vIRF-3 is likely to be a mechanism contributing to the continuous proliferation of PEL cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—KSHV–positive B-cells BC-3 (30), JSC-1 (31) and KSHV–negative B-cells (Akata, BJAB) were maintained as described previously (15). An EBV–negative variant of the Burkitt’s lymphoma cell line Akata was used (T. Grimm, Arndt Kieser; GSF, Munich). HEK 293T and p53–negative H1299 cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

**Plasmids and Cloning**—To construct IRF-5 expression plasmids, the IRF-5 coding sequence was amplified from cDNA of unstimulated or IFNβ–stimulated (1000 units/ml; 12 h) BC-3 cells using oligonucleotides IRF-5-EcoRI (ATA-GAATTCGTCAAGCTTGCACTCAGCAGCGCGCATCAGCGGCTGTAATTTG) and IRF-5-XbaI (ATAATCTAGATTGCTGCGACCGGTGTTACCTCACTGAGG). The PCR product was ligated into pcDNA4-Myc-his (Invitrogen). Clones were verified by DNA sequencing, and protein expression was confirmed by Western blot. The clones for IRF5 variant 1 displayed one silent point mutation at position 549 compared with the reference sequence gi38683857 (GenBank™). IRF5 variant 2 showed one silent point mutation at position 1473 (reference sequence: gi38114631). The cloned variants 3 and 4 turned out to be identical with the sequences published in GenBank™ (gi40792575 and gi40792578, respectively). Relative to its GenBank™ entry (gi51890957), variant 5 displayed three mutations at positions 213 (silent), 549 (silent), and 899 (resulting in a mutation from glutamate to glycine). Construction of V5–tagged and glutathione S–transferase (GST)–tagged vIRF-3 constructs has been described elsewhere (17). Briefly, amino acids 220–290 were deleted in vIRF-3ddV5. Aspartic acids and glutamic acids in both helix motifs of vIRF-3-HmBV5 were replaced with asparagines and glutamines, respectively. Whereas plasmid vIRF-3wtGST expressed full-length vIRF-3 fused to GST, only the putative double helix motif corresponding to amino acids 240–280 of vIRF-3 is expressed in fusion with GST from plasmid vIRF-3DH40GST (17). The ISRE–luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA). The IFNβ–luciferase promoter construct was provided by Prof. Jae Jung. Plasmid pCK10.5 was used for expression of vIRF-3 in mammalian cells. Briefly, the cDNA encoding vIRF-3 was amplified from BCBL-1 cells using oligonucleotides K10.1Bam (GAT-CTGGATCAACTGCGGGACGAGCTTACCTGGAGTTTC) and K10.1-Xba (GATCTCTAGATATTGATCATCAGATACTGAAAC). The amplified DNA fragment was cloned into pcDNA3.1+ via BamHI and XbaI restriction sites (in italic type). The expression construct for transglutaminase 2 was a kind gift from Detlef Schuppan (Universitätsklinikum Erlangen). The p21–luciferase reporter construct was kindly provided by Dr. R. Wessely (Klinikum Rechts der Isar, Munich) (32).

**Yeast Two–hybrid Screening**—Yeast two–hybrid screening was performed using the Matchmaker library construction and screening kit (Clontech, Palo Alto, CA), following the manufacturer’s instructions. Since vIRF-3 amino acids 242–279 constitute a strong transcriptional transactivation domain, a vIRF-3 mutant lacking the corresponding nucleotide stretch (nucleotides 727–837) was cloned into vector pGBK17 (Clontech) via BamHI and NotI restriction sites and used as bait. A cDNA library obtained from EBV–transformed B-lymphoblasts (33) and ligated into pGAD77Rec (Clontech) served as prey. Transformation into Saccharomyces cerevisiae strains AH109 (library) or Y187 (bait) was carried out following the manufacturer’s instructions. Mating experiments were carried out by picking one colony of each selective yeast strain and incubation in YPAD medium overnight at 30 °C. Aliquots were spread on appropriate minimal medium plates the next day.

**Separation of Nuclear and Cytoplasmic Proteins, Western Blot Analyses, and Antibodies**—Extraction of nuclear proteins was carried out using a nuclear extraction kit (Biovista Inc., Mountain View, CA) according to the manufacturer’s instructions. Nuclear and cytoplasmic extracts were loaded on polyacrylamide gels and checked for purity by Western blot with anti–tubulin and anti–SP1 antibody. Western blot analysis was performed as described elsewhere (34). Primary antibodies were obtained from Serotec (V5 antibody), Acris (IRF-5 antibody), Sigma (tubulin antibody), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (SP1 antibody), and Biozol (GST antibody). Rat monoclonal vIRF-3 antibody was produced in cooperation with E. Kremmer as described previously (15). Myc antibody was purified by protein A affinity chromatography from supernatant of 9E10 mouse hybridoma (ATCC) cells. Secondary antibodies conjugated with horseradish peroxidase were obtained from DAKO (Dako Deutschland GmbH, Hamburg, Germany). Peroxidase activity was detected by enhanced chemiluminescence using an ImageReader LAS-1000 (Fujifilm, Tokyo, Japan) CCD camera and software.

**Pull–down Experiments**—For pull–down experiments, “bait” proteins tagged with either an epitope (Myc or V5) or GST were recombinantly expressed in transfected HEK 293T cells and adsorbed to either antibody–loaded protein G–Sepharose (GE Healthcare) or glutathione–Sepharose 4B (GE Healthcare), respectively. For this purpose, five 10-cm cell culture dishes were transfected with 10 μg of DNA of different expression
Plasmids. vIRF-3 proteins were either Myc-, V5-, or GST-tagged. Constructs expressing IRF-5 or transglutaminase 2 (TGM2) encoded Myc-tagged proteins. Cells were harvested after 2 days and lysed in 1% Nonidet P-40, 500 mM NaCl, 25 mM HEPES with 2.5 mM EDTA/EGTA and 1 mM phenylmethylsulfonyl fluoride (high salt lysis buffer). Lysates were cleared by centrifugation for 1 h at 20,000 × g and then incubated for 4 h with either antibodies (anti-Myc or anti-V5, 10 µg in 10 µl) immobilized on protein G-Sepharose beads or glutathione-Sepharose. Precipitates were washed twice with high salt lysis buffer and once with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM HEPES, 2.5 mM EDTA/EGTA, 1 mM phenylmethylsulfonyl fluoride). Proteins coupled to Sepharose beads via either antibodies or glutathione where then used for the second round of pull-down. For this purpose, either HEK 293T cells or JSC-1 PEL cells were lysed in lysis buffer, followed by removal of insoluble components by centrifugation for 1 h at 20,000 × g. One ml of the lysate (corresponding to ~30 million cells) was then incubated overnight with either one of the previously precipitated proteins (vIRF-3 (Myc-, V5-, or GST-tagged), Myc-tagged IRF-5 or Myc-tagged TGM2 as a control) or the respective antibody only. The precipitates were washed three times with lysis buffer and then subjected to Western blot analysis.

Chromatin Immunoprecipitation (ChIP)—10-cm cell culture dishes (~20 million cells) of HEK 293T cells were transfected with Lipofectamine and Plus reagent (Invitrogen) with 8 µg of expression plasmid for IRF-5 (variant 1, c-terminal Myc epitope), vIRF-3 (no epitope tag), or both. Empty vector was used to make up for equal DNA amounts. Two days after transfection, the cells were fixed with 9 ml of 1% formaldehyde in cell culture medium for 30 min directly in the culture dishes at room temperature. Formaldehyde was quenched by the addition of 1 ml of 1 mM glycine in phosphate-buffered saline. The cells were washed three times in phosphate-buffered saline and then lysed for 20 min on ice in 1 ml of hypotonic lysis buffer (0.5% Nonidet P-40, 50 mM KCl, 2.5 mM EDTA/EGTA, 25 mM HEPES). The cells were then washed once in hypotonic lysis buffer, and the resulting nuclear pellet was dissolved in 1 ml of ChIP lysis buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mM NaCl, 2.5 mM EDTA/EGTA, 25 mM HEPES). The nuclear lysates were then sonified in a Branson cup sonifier at maximum output and 80% duty cycle for 20 min, resulting in DNA fragments of an average length of ~2000 bp. 10 µg of pcDNA4a plasmid DNA was added to each lysate to block non-specific binding. The lysates were then cleared by centrifugation for 1 h at 20,000 × g. Input samples were taken aside, and 500 µl of the supernatants were incubated overnight with 10 µg of 9E10 anti-Myc mouse monoclonal antibody or 10 µg of anti-V5 mouse monoclonal antibody as a control. The antibodies were precipitated for 30 min with salmon sperm blocked protein G beads (Millipore). Washing of the precipitates was performed once with ChIP lysis buffer, once with hypotonic lysis buffer, and twice with ChIP lysis buffer supplemented with 500 mM NaCl. The precipitates were then eluted by two rounds of incubation with 200 µl of elution buffer (1% SDS, 100 mM NaHCO₃) for 10 min at 65 °C. Salt concentration was adjusted to 200 mM NaCl, and proteinase K was added at 50 µg/ml. The eluates and equally treated input samples were digested and decross-linked at 65 °C overnight. All samples were extracted with phenol/chloroform/isoamyl alcohol, ethanol-precipitated with the addition of 30 µg of glycogen and 0.3 M NaOAc, dried, and reconstituted in 200 µl of TE buffer. The samples were then analyzed by standard semiquantitative PCR with primer pairs specific for the promoter regions of IFNβ (IFNβ-P-forward, TCAAGTTGTTTGGTTTGC; IFNβ-P-reverse, GAGAATTTTCTCTTCTC), of JunB (JunB-P-forward, CCTCTCGGAT-TACAGTTCT; JunB-P-reverse, AAATACCAGGAGTGTATC-CAGC), of IFIT1 (IFIT1-P-forward, GACAGTGTTCCATGATTGAGAC; IFIT1-P-reverse, GGGAAATAGCAACCAACTGAGCT), and of glyceraldehyde-3-phosphate dehydrogenase (GAPDH-P-forward, CCAACTTCTCCGCCTCTC; GAPDH-P-reverse, CAGCCGCCTGTTCAACTG).

Semiquantitative RT-PCR—RNA isolation of ~1 million PEL cells was carried out using the Nucleospin RNAII RNA isolation kit (Machery & Nagel, Düren, Germany) according to the manufacturer’s instructions. Semiquantitative RT-PCR was performed by a two-step protocol. After cDNA synthesis with reverse transcriptase Superscript II and random hexamers (Bioline GmBH) as primers, amplification of target genes was achieved using oligonucleotide pairs for IFIT1 (IFIT1-forward, CGGCTGCTATTATTTACAGCAAC; IFIT1-reverse, GGCATTTCATGTACATTAAG), for JunB (JunB-forward, GTGCACTAAATGGGAACGC; JunB-reverse, CAGGCTCGGTTTCAAGGATT), and for β-actin (β-actin-forward, CCA-TCTACGGGGGATGCC; β-actin-reverse, CGTGGCCA-TCTCTTGTG). For each primer pair, the number of cycles required for optimal amplification in the linear range was used. After an initial denaturation step (95 °C, 2 min), the PCR was performed using denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 40 s.

Immunofluorescence—Immunofluorescence was performed as described previously (15). As first antibodies, rat monoclonal antibody 3G7 directed against vIRF-3 and goat polyclonal anti-IRF-5 or rabbit polyclonal anti-IRF-1 (Santa Cruz Biotechnology) were used. For detection of primary antibodies, cells were then incubated with an anti-rat Alexa-fluor-488 conjugate (Invitrogen) and an anti-goat (IRF-5) or anti-rabbit (IRF-1) Alexa-fluor-555 antibody (Invitrogen). Cells were visualized using a Zeiss Axioplan microscope with fluorescent lamp (Carl Zeiss Microimaging, Göttingen, Germany). The objective lens magnification was ×40 with total final magnification of ×400. Images were taken using a Spot Diagnostic Imaging camera and software (Diagnostic Instruments, Burroughs, MI). Laser scanning images were taken on a Leica TCS SP5 confocal microscope (Leica Microsystems, Westlar, Germany). Co-localization was quantified with the Leica software co-localization tool.

Luciferase Reporter Assay—HEK 293T cells were seeded 12–16 h prior to transfection onto 12-well cell culture plates. The cells were transfected at 70% density with luciferase reporter constructs and the respective expression plasmids using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer’s instructions. Samples were normalized for DNA content with empty vector control. Two days after transfection, cells were harvested by centrifugation and resuspended in luciferase lysis buffer (50 mM Tris, pH 7.8, 0.5 mM dithiothreitol, 1% Triton X-100, 20% glycerol). Luciferase activity was
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determined in duplicates with a microplate luminometer (Berthold Detection Systems, Pforzheim, Germany). Luciferase counts were normalized to total protein content of the sample as measured with the Bio-Rad assay.

siRNA Transfection and Apoptosis Assay—siRNA transfection of BC-3 cells was carried out as described previously (15). For knockdown of vIRF-3, a synthetic siRNA targeting the sequence AAGGCCAUUGUGGUGAAA was used. A “nonsense” siRNA targeted at the sequence AGAGCUACCGUUGAUAGUAG, which does not occur in KSHV or any sequence currently present in GenBank™, served as negative control. For simultaneous determination of caspase 3 and caspase 7 activities in the cells, the Caspase-GloTM 3/7 Assay (Promega, Madison, WI) was used according to the manufacturer’s instructions. Cells treated with 10 μM etoposide (Sigma) were used as positive control for caspase activation.

Promoter Pull-down—For promoter pull-down assays, 1 μg of vIRF-3 expression plasmid or 1 μg of IRF-5 expression plasmid or both were transfected into HEK 293T cells. pcDNA3.1 was co-transfected to make up for equal amounts of DNA. Cells were harvested 2 days after transfection, and nuclear extracts were prepared as described above and adjusted to a protein concentration of 2 mg/ml by the addition of nuclear extraction buffer. Biotinylated probes for the IFNβ promoter were generated by PCR using either IFNβ reporter plasmid or pcDNA4 (Invitrogen) as template and biotinylated oligonucleotides IFNβ-forward (TCAGGTCGT-TCTGTTCCCC-TTG) and IFNβ-reverse (GAGGAATTC-CCACGCTTACTTTC) or control-oligo-forward (TATTGCAGCTTATAATGGTTACAAATAAAAAGC) and control-oligo-reverse (CCGGCTCTGATGTTGTTGTTG), respectively. Oligonucleotides were obtained from Biomers.net GmbH (Ulm, Germany). ISRE probes were generated by annealing 5’-biotin-labeled oligonucleotides ISRE-for (TAGTTTCACTTT-CAC) and ISRE-rev (GGGAAGTGAACCAACTA) or ISREmut-for (TGTACCTATCTTCC) and ISREmut-rev (GGAGAGAT-AGATACA), respectively, at 50 μM each. 20 μl (5 μl for ISRE pull-down) corresponding to 40 μg (10 μg for ISRE pull-down) of nuclear extract were incubated for 30 min on ice with 160 ng of nuclear extract were incubated for 30 min on ice with 160 ng of biotinylated IFNβ promoter or control probe in a total volume of 200 μl. For ISRE pull-down, 50 pmol of the probe and 500 pmol of unlabeled competitor probe were used. Reaction conditions were adjusted to 0.5% Nonidet P-40, 50 mM KCl, 50 mM Tris, pH 7.5, supplemented with 50 μg/ml oligo(dI-dC) (Amersham Biosciences, Little Chelfont, UK) and 1 mM dithiothreitol. 20 μl of the protein solution were then taken aside as input control, and 500 μl of band shift buffer (0.5% Nonidet P-40, 50 mM KCl, 50 mM Tris, pH 7.5) was added to the reaction. The whole sample was then transferred to a fresh tube containing streptavidin-agarose beads (Amersham Biosciences). The samples were incubated for 30 min at 4°C with gentle agitation. Samples were then washed three times with 1 ml of band shift buffer and subjected to Western blot analysis. For promoter pull-down from knockdown PEL cells, an equal number of cells was harvested and lysed in 0.5% Nonidet P-40, 400 mM NaCl, 2.5 mM EDTA/EGTA, 25 mM HEPES with 2 min of sonification to release nuclear proteins. The lysates were cleared by centrifugation, and 50 μl (corresponding to 4 million cells) were incubated for 1 h in a total volume of 500 μl with 250 pmol of biotinylated probe in band shift buffer. 200 μl of band shift buffer with streptavidin-agarose was added, and the reaction was precipitated for 1 h with agitation. Detection was carried out as above. For each experiment, the amount of IRF-5 input and the amount precipitated by ISRE-biotin was quantified densitometrically, and the ratio (precipitate/input) was calculated for siNonsense and sivIRF-3 samples. The value for siNonsense was set to 100% in each experiment.

RESULTS

Identification of IRF-5 as a Protein Interacting with vIRF-3 by Yeast Two-hybrid Screening—vIRF-3 contains an unusually strong transactivation domain. A fusion protein consisting of the DNA binding domain of the yeast transcription factor GAL4 (amino acids 1–147) fused to full-length vIRF-3 most potently transactivated a reporter construct containing five GAL4 binding sites both in mammalian and in yeast cells (data not shown). We were able to map this transactivation domain to amino acids 242–280 of vIRF-3 (data not shown). A vIRF-3 deletion mutant lacking amino acids 242–280 was not self-transactivating and could thus be used as bait protein in yeast two-hybrid screening. A cDNA library derived from EBV-transformed B-lymphoblasts (33) served as prey. Of 91 clones growing on selection medium lacking Trp, Leu, His, and Ade, 47 were also β-galactosidase-positive and contained a DNA fragment from one of 30 different cellular genes. Only three of these putative interactions could be confirmed in remating experiments. One of them contained splice variant 1 of the cellular IRF-5 (interferon regulatory factor 5) (25).

Interaction of vIRF-3 with IRF-5 in Vitro—Pull-down experiments using Myc-tagged IRF-5 bound to Sepharose beads were done to confirm the interaction with endogenously expressed vIRF-3 (Fig. 1A). Endogenous vIRF-3 was readily detectable in the PEL cell line JSC-1 (Fig. 1A, upper panel, lane 4). Immobilized IRF-5, but not immobilized TGM2, clearly precipitated endogenous vIRF-3 from JSC-1 cell lysates (Fig. 1A, lanes 2 and 1, respectively). Expression of Myc-tagged proteins was confirmed by Western blot (Fig. 1A, lower panel, lanes 1 and 2). A reciprocal pull-down experiment using Myc-tagged vIRF-3 is shown in Fig. 1B. To avoid competition with endogenous vIRF-3, protein lysates from HEK 293T cells were used here (Fig. 1B, lane 4). Endogenous IRF-5 was pulled down with Myc-tagged vIRF-3 (Fig. 1B, upper panel, lane 2). Negative controls with immobilized TGM2 protein or antibody only are shown in lanes 1 and 3, respectively.

Interaction of vIRF-3 and IRF-5 in PEL Cells—We next performed co-immunoprecipitation experiments using JSC-1 cells and an anti-IRF-5 antibody coupled to protein G-Sepharose. The result is shown in Fig. 1C. Endogenous vIRF-3 co-precipitated with endogenous IRF-5 from PEL cell lysates (lane 2, upper panel). Neither vIRF-3 nor IRF-5 was detectable in the precipitate when an antibody of different specificity (CD-87) from the same species was coupled to protein G-Sepharose (lane 3). Fig. 1C also shows that, in comparison with IRF-5, vIRF-3 is abundantly expressed in PEL cells (lane 1, input control).
Localization of vIRF-3 and IRF-5 in PEL Cells—There is general agreement in the literature that IRF-5 is predominantly located in the cytoplasm unless the interferon system is induced (e.g. by viral infection) (26, 28, 35, 36). However, we and others showed that vIRF-3 is primarily present in the nuclei of latently infected PEL cells. We therefore examined the subcellular localization of vIRF-3 and IRF-5 in PEL cells. Nuclear and cellular proteins were fractionated and separated by PAGE, and the relative abundance of vIRF-3, IRF-5, tubulin, and SP1 was determined by Western blot. As expected, tubulin was mainly present in the cytoplasmic fraction, whereas the transcription factor SP1 was almost exclusively located in the nucleus (Fig. 2A, bottom panels, lanes labeled C and N, respectively), documenting the efficiency of the separation procedure. Both vIRF-3 and IRF-5 were present in the nuclear compartment of PEL cells (Fig. 2A, upper panels, lanes labeled N). Interestingly, a higher molecular weight form of IRF-5 was almost exclusively present in the nuclei, whereas an IRF-5 with lower molecular weight was predominantly located in the cytoplasm. Standard immunofluorescence analysis (Fig. 2B) confirmed the localization pattern of endogenous vIRF-3 and IRF-5 in PEL cells; vIRF-3 was predominantly nuclear (Fig. 2B, left), whereas IRF-5 was present in both compartments of the cell (Fig. 2B, middle). In addition, laser-scanning microscopy revealed that IRF-5 and vIRF-3 were largely co-localized (Fig. 2C). Using the Leica software co-localization tool, 10 microphotographs were subjected to co-localization analyses. This revealed a mean co-localization rate for both proteins of 88% (range 65–96%) with a mean Pearson’s coefficient of 0.81. Although present in the nucleus, cellular IRF-1 did not co-localize with vIRF-3 (Fig. 2C, bottom). Quantitative co-localization analysis of 10 IRF-1/vIRF-3 laser-scanning microscopy images yielded a mean co-localization rate of only 57% with a mean Pearson’s coefficient of 0.61.

A Double α-Helix Motif of vIRF-3 Is Sufficient for the Interaction with IRF-5—As a basis for further studies on the functional importance of the vIRF-3/IRF-5 interaction, we defined the part of vIRF-3 required for binding to IRF-5. As recently described, amino acids 240–280 of vIRF-3 are both necessary and sufficient for the interaction of vIRF-3 with cellular IRF-7 (17). The epitope-tagged vIRF-3 proteins used in the latter study were used here for pull-down experiments. Full-length vIRF-3 protein was able to pull down endogenously expressed IRF-5 (Fig. 3A, lane 2, upper panel). The specificity of this interaction was confirmed by the fact that two other cellular IRFs did not bind to vIRF-3; although both IRF-1 and IRF-3 were clearly expressed in HEK 293T cells (Fig. 2A, lane 3), they did not precipitate with full-length vIRF-3 (Fig. 3A, middle panel). IRF-1 and IRF-3 were clearly expressed in HEK 293T cells (Fig. 3A, lane 2, upper panel). The specificity of this interaction was confirmed by the fact that two other cellular IRFs did not bind to vIRF-3; although both IRF-1 and IRF-3 were clearly expressed in HEK 293T cells (Fig. 2A, lane 3), they did not precipitate with full-length vIRF-3 (Fig. 3A, middle panel).
panels, lane 2). Precipitation of IRF-5 was essentially impossible with a vIRF-3 deletion mutant lacking amino acids 240–280 (Fig. 3A, upper panel, lane 3 (vIRF-3dd2V5)). Residual binding to the higher Mr form of IRF-5 was seen when a vIRF-3 variant was used that carried several mutations in this area (vIRF-3HmBV5; Fig. 3A, lane 4, asterisk). Comparable expression of both “wild-type” vIRF-3 and the mutants could be confirmed by Western blot (Fig. 3A, lanes 2–4, lower panel). To corroborate this observation, similar experiments were performed using GST-tagged proteins to

FIGURE 2. Subcellular localization of vIRF-3 and IRF-5. A, separation of nuclear and cytoplasmic proteins was carried out with one million KSHV-positive cells (BC-3 and JSC-1) as well as KSHV-negative B-cells (Akata and BJAB). IRF-5 was detectable in both cytoplasmic (C) and nuclear (N) fractions. The larger form of IRF-5 was predominantly located in the nucleus. An anti-tubulin antibody was used to confirm the quality of the separations. As a nuclear marker SP1 was used. B and C, light and laser-scanning microscopic immunofluorescence analysis of paraformaldehyde fixed, Triton-permeabilized KSHV-positive BC-3 cells and KSHV-negative Akata cells. Intrinsic vIRF-3 expression was detected using rat monoclonal anti-vIRF-3 antibody (3G7) and Alexa-fluor-488-coupled anti-rat antibody (green fluorescence). Intrinsic IRF-5 protein was visualized by anti-IRF-5 antibody and secondary Alexa-fluor-555-labeled anti-goat antibody (red fluorescence). Intrinsic IRF-1 protein was detected using rabbit-anti-IRF-1 antibody and Alexa-fluor-555-labeled anti-rabbit antibody for secondary detection.

FIGURE 3. A double α-helix motif in vIRF-3 is sufficient for binding of vIRF-3 to IRF-5. A, V5-tagged vIRF-3 constructs were expressed in HEK 293T cells and precipitated with anti-V5-loaded protein G-Sepharose beads. Cells transfected with empty V5-expression vector were used as control (lane 1). These beads were incubated with a lysate of HEK 293T cells to pull down endogenously expressed proteins. The same membrane was consecutively probed with antibodies against IRF-5, IRF-1, and IRF-3. It was finally reprobed with anti-V5 to check for expression of V5-tagged proteins (equivalent to vIRF-3 constructs). V5-tagged full-length vIRF-3 (vIRF-3wtV5; lane 2) bound to both forms of IRF-5. Deletion of the central double α-helix motif of vIRF-3 (vIRF-3dd2V5; lane 3) almost completely abolished binding to IRF-5. vIRF-3HmBV5 is a construct where acidic residues aspartic acid and glutamic acid in the double α-helix motif of vIRF-3 were replaced by asparagine and glutamine, respectively (lane 4). Residual binding to IRF-5 was still detectable (asterisk). Antibody heavy (hc) and light chains (lc) were detectable at comparable levels in all pull-down reactions, confirming uniform loading of the gel. B, in a GST pull-down experiment, the wild-type vIRF-3 GST fusion protein (lane 2), in contrast to GST alone (lane 1), was able to interact with intrinsic IRF-5 and IRF-3. A GST fusion protein comprising the central 40 amino acids of the double α-helix motif of vIRF-3 also pulled down IRF-5 and -3 but not IRF-1. Proteins from HEK 293T cells were used. Membranes were consecutively probed with antibodies against IRF-5, IRF-1, and GST. WB, Western blot.
HEK 293T cells were transfected with an ISRE-luciferase reporter construct and the indicated amounts of IRF-5, vIRF-3, and K3 expression constructs. Luciferase assays were performed on day 2 after transfection. Values are given as -fold inductions relative to the activity of reporter construct and empty expression plasmid only. Bars represent arithmetic mean and S.E. from seven independent experiments. Western blot analysis of one representative experiment is shown below. Endogenous IRF-5 was detectable in cells transfected with vIRF-3 only.

Inhibition of IRF-5-mediated Promoter Activation by vIRF-3—IRF-5 activates interferon class I genes via ISRE elements (25). In a first series of experiments, we examined whether vIRF-3 was able to modulate the transactivation of ISRE-containing promoters by IRF-5. Reporter construct pISRE-luciferase contains five ISRE elements (derived from the ISG54 promoter) upstream of a luciferase reporter gene. It was co-transfected into HEK 293T cells with an IRF-5 (splice variant 1) expression construct and either empty vector, vIRF-3, or a K3 expression plasmid, respectively. Expression of IRF-5 alone resulted in an ~80-fold activation of the ISRE reporter construct (Fig. 4). However, co-expression of vIRF-3 almost completely abolished this activation, whereas co-expression of the KSHV gene K3 had no effect on the activation of the ISRE reporter construct by IRF-5.

Inhibition of IRF-5 activity by vIRF-3 in these co-transfection experiments was dose-dependent and specific for vIRF-3 expression (Fig. 5); activation of the ISRE reporter construct by IRF-5 decreased with increasing amounts of vIRF-3. Knockdown of vIRF-3 expression by RNA interference abolished this effect (Fig. 5, right half). The specificity of this siRNA (si463) for knockdown of vIRF-3 in PEL cells has been shown before (15).

IFNβ is the major inducer of the type I interferon response in nucleated cells. A reporter construct comprising the complete IFNβ promoter upstream of a luciferase reporter gene was co-transfected with an expression construct for splice variant 1 of IRF-5 and a vIRF-3 expression plasmid. IRF-5 alone strongly activated the IFNβ promoter (Fig. 6A, second bar). This activation could be fully abolished by simultaneous expression of vIRF-3 (Fig. 6A, fourth bar). In contrast to vIRF-3, co-expression of the KSHV latent nuclear antigen 1 (LANA) had no effect on the IFNβ promoter (Fig 6A, third bar).

Twelve splice variants of IRF-5 have been described so far. We were able to detect expression of variants 1, 2, 3/4, and 5 in BC-3 cells. All four variants detectable in BC-3 cells clearly transactivated an IFNβ promoter construct to about the same extent. They were also all highly susceptible to repression by vIRF-3 (Fig. 6B).

IRF-5 alone strongly activated the IFNβ promoter (Fig. 7A, second bar). This was completely abolished by co-transfection of an expression construct for full-length vIRF-3 (Fig. 7A, third bar). However, a vIRF-3 mutant lacking the complete double helix domain (amino acids 220–290) was entirely inactive (Fig. 7A, vIRF-3-3dd2, fifth bar), and only residual inhibition of IRF-5 was seen when a vIRF-3 variant with mutations in both helix

KSHV vIRF-3 Inhibits IRF-5

nucleases
KSHV vIRF-3 Inhibits IRF-5

**A**

![Graph showing inhibition of IRF-5-mediated activation of the IFNβ promoter by vIRF-3.](image)

**B**

![Graph comparing reporter activity in lanes 1 versus lane 5.](image)

domains was used (Fig. 7A, vIRF-3HmB, fourth bar). It is important to stress that deletion or mutation of the double helix domain of vIRF-3 did not interfere with nuclear localization of the protein. Both mutants retained the ability to reside in the nucleus (supplemental Fig. 1). Although data presented in Fig. 7A demonstrate that the vIRF-3 double helix motif is required for blocking the transactivation of the IFNβ promoter by two splice variants of IRF-5, data in Fig. 7B show that this region is sufficient. Co-expression of a GST fusion protein containing only amino acids 240–280 of vIRF-3 fused to GST completely blocked IFNβ promoter activation by IRF-5. In summary, the central acidic double helix motif of vIRF-3 is both necessary and sufficient for the inhibition of IRF-5-mediated promoter transactivation.

IRF-5 has recently been shown to be involved in mechanisms of tumor suppression not related to induction of apoptosis or the “classical” interferon pathway. For example, it induces expression of the cyclin D inhibitor p21 independently from p53 (27). Co-transfection experiments were performed to ascertain whether vIRF-3 interferes with this function of IRF-5 too. p53-negative H1299 cells were co-transfected with a p21 reporter construct, expression plasmids for IRF-5, and vIRF-3 or its variants. In accordance with other reports, IRF-5 stimulated the p21 promoter 2–3-fold (Fig. 8). However, activity of the p21 promoter construct returned to basal levels upon the addition of wild-type vIRF-3. Although the vIRF-3 deletion mutant lacking the 40-amino acid putative double helix motif (vIRF-3dd2) was not able to inhibit activation of the p21 promoter by IRF-5, the mutated variant vIRF-3 (vIRF-3HmB) had intermediate activity. vIRF-3 itself did not reduce the basal activity of the p21 promoter (data not shown). This suggests that repression of the p21 promoter by vIRF-3 in the presence of IRF-5 is due to interference of vIRF-3 with IRF-5 and not with the p21 promoter directly.

**vIRF-3 Abrogates Binding of IRF-5 to DNA**—Since inhibition of IRF-5 DNA binding by vIRF-3 is a likely explanation for the functional interference we observed, promoter pull-down experiments were performed. HEK 293T cells were transfected with expression plasmids for IRF-5 and vIRF-3. Nuclear extracts were prepared 2 days later. In an initial series of experiments, the nuclear extracts were first incubated with double-stranded, biotinylated synthetic oligonucleotides corresponding to the ISG54 ISRE element or a scrambled sequence (ISREmut). Oligonucleotide-DNA complexes were then precipitated with streptavidin-Sepharose beads and analyzed via SDS-PAGE and Western blot. Biotinylated ISRE oligonucleotides, but not mutated ISRE oligonucleotides, associated with IRF-5 when the latter was expressed in the absence of vIRF-3 (Fig. 9A, lanes 3 and 4, respectively). This association could be competed out by the addition of an excess of unbiotinylated ISRE, but not ISREmut oligonucleotides (Fig. 9A, lanes 7 and 8). vIRF-3 did not bind to either ISRE or ISREmut oligonucleotides (Fig. 9A, top). However, when both IRF-5 and vIRF-3 were expressed in co-transfected cells, binding of IRF-5 to ISRE oligonucleotides was greatly diminished (Fig. 9A, lane 1). Similar data were obtained when the complete IFNβ promoter was used instead of short ISRE oligonucleotides (Fig. 9B). When IRF-5 was expressed alone, it was clearly associated with the IFNβ promoter fragment but not an unrelated control DNA (Fig. 9B, lanes 4 and 3, respectively). Although vIRF-3 expressed at comparatively low levels (Fig. 9B, lane 7), it completely prevented binding of IRF-5 to the IFNβ promoter fragment (Fig. 9B, lane 2).

Next, oligonucleotide pull-down experiments were performed in order to assess whether endogenous vIRF-3 inhibits DNA binding of endogenous IRF-5 in PEL cells. As published previously by our group (15), vIRF-3 is required for the sustained proliferation of PEL cells. Since efficient knockdown of vIRF-3 expression would result in growth arrest and cell death, we adjusted siRNA transfection conditions for the experiments shown in Fig. 10 to yield an only moderate knockdown of vIRF-3 expression (~30–40%; Fig. 10A, lane 6 versus lane 5). Binding of IRF-5 to an ISRE oligonucleotide was still detectable at rather low levels in lysates from PEL cells treated with a nonsense siRNA (Fig. 10A, lane 1). However, moderate reduction of vIRF-3 expression by RNA interference resulted in markedly enhanced binding of IRF-5 to an ISRE oligonucleotide but not to a scrambled control DNA (Fig. 10A, lanes 3 and 4, respectively). Three independent experiments were per-
formed, and IRF-5 binding to the ISRE oligonucleotide was quantified by image analysis using the AIDA software package. As shown in Fig. 10, the only moderate reduction of vIRF-3 expression by RNA interference led to a marked increase (mean = 75%) of IRF-5 binding to the ISRE oligonucleotide.

vIRF-3 Abrogates Association of IRF-5 with Promoters in Vivo—In a study published in 2004, systematic examination using RT-PCR and microarray analyses revealed that IRF-5 directly controls the expression of several cellular genes in addition to the aforementioned type I interferon (26). Among them are the interferon-stimulated gene IFIT1 and the transcription factor JunB. We performed ChIP experiments to study the interference of vIRF-3 with IRF-5 promoter binding in vivo. Since we were unable to identify a ChIP-grade IRF-5 antibody, the experiments were performed in HEK 293T cells expressing either Myc-tagged IRF-5 or co-expressing both Myc-tagged IRF-5 and untagged vIRF-3. In good agreement with data published by others (26), ChIP revealed that IRF-5 is associated with the promoter regions of all three genes examined, namely interferon β, IFIT1, and JunB (Fig. 11A, lane 6), but not with the promoter of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Fig. 11A, bottom row). Negative control precipitations using an isotype antibody (anti-V5) are shown in lanes 2, 5, and 8 of Fig. 11A. However, co-expression of vIRF-3 with IRF-5 clearly altered the binding pattern of IRF-5 to the three intrinsic promoters examined (Fig. 11A, lane 9). Association of IRF-5 with both the IFIT1 and the JunB promoter was completely

3DH40GST), respectively. Bars represent arithmetic mean and S.E. of relative luciferase activities from four independent experiments. Expression of proteins was controlled by Western blot analyses with either anti-GST antibody or anti-Myc antibody for detection of GST constructs or IRF-5, respectively.
FIGURE 9. Inhibition of IRF-5 binding by vIRF-3, A, inhibition of binding to an ISRE element. Oligonucleotide pull-down was carried out with a biotinylated ISRE element as probe. Probe (lanes 1, 3, 5, 7, and 8) or mutated probe (lanes 2, 4, and 6) were incubated with nuclear extracts of transfected HEK 293T cells. Unbiotinylated oligonucleotides were added in competition experiments (lane 7, ISRE; lane 8, mutated ISRE). Streptavidin-agarose beads were used for precipitation. Western blot analyses of the precipitates were performed with antibodies against vIRF-3 (top) or IRF-5 (bottom). Binding of IRF-5 to the ISRE element is specific and could be competed with unbiotinylated ISRE probe (lane 7) but not with mutated unbiotinylated probe (lane 8). Expression of vIRF-3 strongly impaired IRF-5 binding to the ISRE probe (lane 1). 10% of the reaction were loaded (lanes 9 and 10 as input control and detected with antibodies against vIRF-3 (top) or IRF-5 (bottom), respectively. B, inhibition of binding to the IFNβ promoter. Biotinylated IFNβ promoter probe (lanes 2, 4, and 6) or biotinylated control probe (lanes 1, 3, and 5) was incubated with nuclear extracts from transfected HEK 293T cells. Pull-down was carried out with streptavidin-agarose beads and subjected to Western blot analysis. Membranes were probed for vIRF-3 (top) and IRF-5 (bottom). 10% of the reaction was loaded as input control and probed for the respective proteins (lanes 7–9, top and bottom).

abolished. IRF-5 binding to the IFNβ promoter was strongly diminished, especially when compared with the isotype control (lane 8). As mentioned above, these assays were performed in transfected HEK 293T cells due to the lack of an IRF-5 antibody suitable for ChIP. We thus wanted to investigate whether our findings apply for KSHV-transformed PEL cells too. For this purpose, we compared mock-, siNonsense-, and sivIRF-3-treated PEL cells for the expression of IFIT1 and JunB by semiquantitative RT-PCR. Expression of the typical interferon target gene IFIT1 was almost undetectable in mock or siNonsense RNA-transfected PEL cells (Fig. 11B, lanes 2 and 3). However, IFIT1 expression was markedly up-regulated (22-fold as compared with siN) in vIRF-3 knockdown cells (lane 4). As expected, IFIT1 expression was clearly induced by interferon treatment even in PEL cells expressing normal levels of vIRF-3 (mock- or siNonsense-transfected; Fig. 11B, lanes 5 and 6, respectively). However, the induction by interferon was still weaker than the induction seen as a consequence of vIRF-3 knockdown only. The combination of interferon treatment and thus result in increased interferon sensitivity. To address this question, JSC-1 cells were transfected with amounts of synthetic siRNAs adjusted to yield a moderate knockdown of vIRF-3 (30%; Fig. 12A). Under these circumstances, the initial induction of caspase activity by vIRF-3 knockdown was barely detectable (Fig. 12B, bars labeled unstimulated). Cells were then treated with IFNγ at concentrations of 100 or 1000 units/ml, respectively. This resulted in a pronounced increase of caspase 3/7 activity in cells treated with sivIRF-3 (Fig. 12B). The increase in caspase activity induced by vIRF-3 silencing was significant for treatment with 100 units of IFNγ (p = 0.017) and highly significant when cells were treated with 1000 units of the interferon (p = 0.0003). In contrast, untreated cells or cells transfected with nonsense siRNA (siNonsense) were fairly resistant to IFNγ. To exclude unspecific effects of the siRNA itself on caspase activation, the experiment was repeated once using dually EBV- and KSHV-negative Akata cells. As shown in Fig. 12C, these cells were highly resistant to interferon treatment. Functionality of the caspase assay was controlled by vIRF-3 silencing led to a further induction of IFIT1 transcription (lane 7; IFIT1 up-regulation 1.4-fold as compared with siN). In contrast to IFIT1, expression of JunB was scarcely regulated by interferon β. Nevertheless, JunB transcription was clearly induced upon knockdown of vIRF-3 expression in PEL cells, especially when the cells were sensitized using interferon β (Fig. 11B, 2.4-fold increase in cells treated with sivIRF-3 (lane 7) compared with cells transfected with a nonsense siRNA (lane 6). Actin levels remained unchanged irrespective of interferon β treatment or siRNA transfection. An EBV-negative variant of the KSHV-negative Burkitt lymphoma cell line Akata was used as control in order to exclude nonspecific or direct interferon stimulatory effects of the siRNA. As can be seen in the lower part of Fig. 11B, expression of both IFIT1 and JunB was not altered by siRNA treatment in Akata cells. This confirms that the induction of IFIT1 and JunB, two genes known to be regulated by IRF-5, was indeed due to the RNA interference-mediated reduction of vIRF-3 expression in PEL cells.

Increased Interferon Sensitivity by vIRF-3 Knockdown—IRF-5 is a major inducer of apoptosis in response to class I interferons (27, 37). Derepression of IRF-5 activity by knockdown of vIRF-3 should
inducing apoptosis in both JSC-1 and Akata cells with 10 μM etoposide (Fig. 12, B and C, right columns). Akata cells were highly sensitive to apoptosis induction by etoposide but not by the siRNAs. We thus conclude that the siRNAs used here activated caspases 3/7 neither directly nor via off-target effects.

**DISCUSSION**

We show in this study that vIRF-3 is associated with IRF-5 both in vitro (Fig. 1, A and B) and in latently KSHV infected PEL cells (Figs. 1C and 2C). As a result of this interaction, IRF-5 was no longer able to bind to DNA (Figs. 9, 10, and 11A). Consequently, vIRF-3 blocked IRF-5-mediated activation of reporter constructs containing ISRE sequences (Figs. 4 and 5), the complete IFNβ promoter (Fig. 6), or the cyclin-dependent kinase inhibitor p21 (Fig. 8). In addition, in vivo binding of IRF-5 to relevant target gene promoters and their activation was reduced in the presence of vIRF-3 (Fig. 11). A short stretch of about 40 amino acids of vIRF-3 was sufficient for both the physical interaction with (Fig. 3) and the functional inhibition of IRF-5 (Fig. 7). We have shown recently that vIRF-3 is required for the survival of cultured PEL cells; vIRF-3 silencing by RNA interference results in induction of apoptosis and reduced proliferation (15). Two established IRF-5 targets that are likely to play a role in this phenotype are IFIT1 and JunB. We could confirm association of IRF-5 with the promoters of both genes in vivo. Most notably, binding of IRF-5 to its promoters was clearly diminished in the presence of vIRF-3 protein. Vice versa, up-regulation of IFIT1 and JunB transcription was seen upon knockdown of vIRF-3 expression in PEL cells. vIRF-3 knockdown resulted in a vigorous increase of IFIT1 mRNA levels (Fig. 11B). IFIT1 has been shown to inhibit protein synthesis and thus cell proliferation and cell activation by binding to the translation initiation factor complex eIF3 (38). In addition, overexpression of eIF3 subunits has been implicated in the malignant transformation of cells (39). In comparison with IFIT1, JunB was not significantly up-regulated by mere type I interferon treatment of PEL cells (Fig. 11B). JunB is known to inhibit proliferation and oncogenic transformation of B-lymphocytes. It acts rather antagonistically to the proto-oncogene c-Jun (40, 41). Inhibition of IRF-5-mediated JunB activation by vIRF-3 might thus contribute to securing the continuous proliferation of latently KSHV infected B-cells. As reported here, an only moderate reduction of vIRF-3 expression sensitizes PEL cells to interferon. These effects of vIRF-3 silencing are possibly due to the release and reactivation of IRF-5 from inactive complexes with vIRF-3.

The interferon system is the first line of defense against viral infections. To successfully replicate or persist in host cells, viruses have to avoid or down-regulate the interferon response. Key factors in induction and amplification of the IFN response are the interferon regulatory factors, a family of homologous cellular transcription factors. IRF-3 and -7, two other proteins binding to vIRF-3 (17, 42), are important for induction and amplification of the IFN system in response to double-stranded RNA or lipopolysaccharide signaling (e.g. via Toll-like receptors). In contrast, IRF-5 is not only involved in induction of type I IFNs and several cytokines in response to pathogen-associated molecular patterns. It is also involved in induction of apoptosis and control of the cell cycle (28, 43–45). Thus, IRF-5 is one of several points where antiviral and antioncogenic pathways converge. Inhibition of the IFN system and oncogenesis can be seen as two sides of the same coin (46).

Like several other IRFs, IRF-5 is widely expressed in cells prior to interferon stimulation but resides in the cytoplasm as an inactive protein. Upon induction of the innate immune response, posttranslational modifications of IRF-5 like phosphorylation or ubiquitination cause its translocation to the nucleus (47, 48). Although transcription of IFNα or IFNβ genes was not detectable in cultured PEL cells (data not shown), a considerable fraction of IRF-5 was present in the nucleus (Fig. 2). Interestingly, the nuclear IRF-5 was of higher molecular weight than the cytoplasmic fraction. This finding is compatible with activation of IRF-5 in PEL cells by posttranslational modification. The type of the latter remains to be identified. In summary, the IFN system seems to be abortively activated in PEL, and inhibitors of the early steps of the IFN induction cascade must be present. Since vIRF-3 is constitutively expressed in latently infected PEL cells (9, 15, 42, 49), our data point at a pathway regulating both immune escape and induction of sustained cell proliferation.

Initially, we identified IRF-5 as a novel vIRF-3 interaction partner by using the yeast two-hybrid system. The specificity of
the vIRF-3/IRF-5 interaction was then confirmed by in vitro pull-down using recombinantly expressed proteins (Fig. 1) as well as by co-immunoprecipitation of endogenous PEL cell proteins (Fig. 3). Interestingly, whereas vIRF-3 readily precipitated IRF-5 and vice versa (Fig. 1), V5-tagged vIRF-3 did not associate with either cellular IRF-1 or IRF-3 (Fig. 4A). However, vIRF-3 was able to pull down IRF-3 (but still not IRF-1) when fused to GST (Fig. 4B). This is in good agreement with a previous report showing that IRF-3 interacts with vIRF-3 GST fusion protein (42). However, we did not see an association of IRF-3 with native vIRF-3. It is not surprising that GST fusion proteins exhibit higher avidity, since they readily dimerize. The fact that vIRF-3 dimerization is apparently required for binding to IRF-3 but not to IRF-5 points at higher affinity of vIRF-3 to the latter protein.

Remarkably, the same central 40-amino acid region (residues 240–280) of vIRF-3 that has been shown recently to be responsible for the interaction of vIRF-3 with IRF-7 (17) is also sufficient and required for efficient binding to IRF-5. Secondary structure prediction revealed that it contains a double helix motif (17). Deletion of this area completely abolished inhibition of IRF-5 activity by vIRF-3. Interestingly, the higher molecular weight, nuclear form of IRF-5 was still bound by a vIRF-3 mutant with six point mutations in the 40-amino acid double helix region. Although the central helix-turn-helix motif is functionally required and sufficient for binding, additional domains weakly interacting with IRF-5 must exist in vIRF-3. At least in yeast cells, a vIRF-3 mutant lacking this central double helix was still able to interact with IRF-5.

Helix-turn-helix structures have been shown to mediate both DNA binding and protein-protein interaction. It was thus not very surprising to see that the same 40-amino acid stretch 1) also represents a strong transcriptional transactivation domain when fused to the GAL4 DNA binding area (data not shown) and 2) is the part of vIRF-3 that is recognized by our monoclonal antibody 3G7 (data not shown). This provides a plausible explanation for the inability to co-precipitate IRF-5 with vIRF-3 from PEL cells, whereas the reciprocal co-IP was possible (Fig. 1C); the central interaction domain of vIRF-3 was occupied by IRF-5. The monoclonal antibody 3G7 was therefore not able to bind to vIRF-3 when it was complexed with IRF-5. In contrast, pull-down experiments using C-terminally tagged proteins were successful in both directions (Fig. 1), proving the specificity of the vIRF-3/IRF-5 interaction reported here.

Although we provide evidence that vIRF-3 interacts and functionally interferes with cellular IRF-5, this is certainly not the only mode of action of vIRF-3. It is clear that vIRF-3 also inhibits another key player of the early IFN response, IRF-7 (17). However, in contrast to IRF-5, IRF-7 is not generally seen as proapoptotic and antioncogenic (19). IRF-3 is another protein binding to vIRF-3 (42). However, binding of vIRF-3 to IRF-3 was not detectable without marked induction of the interferon system. In addition, the interaction with vIRF-3...
resulted in further induction of IRF-3 activity. Thus, the described interaction with IRF-3 is unlikely to explain the effects observed in PEL cells upon vIRF-3 knockdown. In addition, vIRF-3 has been shown to inhibit p53-mediated transcription in vitro (9). It remains to be shown whether the two proteins interact in vivo. Other possible mechanisms of oncogenesis by vIRF-3 include induction of c-Myc transcriptional activity (23) as well as inhibition of the cell cycle arrest induced by overexpression of 14-3-3 proteins, another interaction partner of vIRF-3 (50).

The importance of cellular IRFs for herpesviral oncogenesis is well established for EBV. IRF-7 is a key mediator of LMP-1-mediated signal transduction (51). It is activated by LMP-1 and has oncopgenic properties (52). Knockdown of IRF-4 expression inhibits the growth of EBV-transformed lymphocytes (53). Whereas IRF-4 and -7 are activated in EBV-transformed cells and promote lymphomagenesis, recent work showed that IRF-5 is inhibited (54). EBV does not encode genes with homology to the IRF family of transcription factors. However, EBV infection induces the expression of a novel splice variant of IRF-5, V12. This V12 acts as a dominant negative form of IRF-5 in several reporter assays and blocks the TLR-7-mediated activation of the interferon response (54). Thus, both EBV and KSHV evolved to use rather different genes to fulfill the same purpose: dominant negative inhibition of IRF-5 transcriptional activity by either altering the cellular splicing machinery (EBV) or encoding a dominant negative homolog (KSHV).

Oncogenesis is a multistep process requiring both the inactivation of tumor suppressors like p53 and the activation of at least one oncogene. Especially in virus-induced oncogenesis, the inhibition of the antiviral response is also required. vIRF-3 is a multifunctional, latently expressed protein that is required for the sustained proliferation of PEL cells. vIRF-3 influences pathways of the IFN response as well as apoptosis control and/or cell cycle. It is able to activate a proto-oncogene (c-myc), inhibit the tumor suppressor IRF-5, and block the interferon response (IRF-5 and IRF-7). Currently available data indicate that it fulfills its role by interacting with a surprising variety of host cell proteins, whose relative importance is difficult to judge at the moment. It is likely, however, that more than just one of these interactions contributes to oncogenesis. Where characterized (IRF-5, IRF-7, and HIF-1), one central vIRF-3 region of only 40 amino acids is both required and sufficient for these interactions, making this short motif a promising target for therapeutic intervention.

FIGURE 12. Knockdown of vIRF-3 renders PEL cells more sensitive to interferon treatment. Cells were either mock-transfected (mock; dark gray), transfected with nonsense siRNA (siN; light gray), or siRNA against vIRF-3 (sivIRF-3; white). Eleven independent experiments were performed, and one representative Western blot of vIRF-3, IRF-5, and tubulin expression is shown in A. One day after transfection, cells were stimulated with the indicated amounts of interferon alpha or left untreated. The activity of effector caspases 3 and 7 was measured on day 2 after transfection. B, error bars represent S.E. from 11 independent experiments. A two-sided t test gives a significant increase in caspase sensitivity of vIRF-3 knockdown cells when treated with 100 units of IFNa (p = 0.017) and a highly significant increase when treated with 1000 units of IFNa (p = 0.0003). As control for the functionality of the caspase assay, PEL cells treated with the strong apoptosis inducer etoposide were included in the assay (Etop). Error bars of the etoposide-treated cells represent S.E. of five different experiments. C, the experiment was done once with KSHV (and thus vIRF-3)-negative Akata cells.
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