Deletion of Kaposi’s Sarcoma-Associated Herpesvirus FLICE Inhibitory Protein, vFLIP, from the Viral Genome Compromises the Activation of STAT1-Responsive Cellular Genes and Spindle Cell Formation in Endothelial Cells

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Received 1 February 2011/Accepted 7 July 2011

Kaposi’s sarcoma herpesvirus (KSHV) Fas-associated death domain (FADD)-like interleukin-1 beta-converting enzyme (FLICE)-inhibitory protein, vFLIP, has antiapoptotic properties, is a potent activator of the NF-κB pathway, and induces the formation of endothelial spindle cells, the hallmark of Kaposi’s sarcoma, when overexpressed in primary endothelial cells. We used a reverse genetics approach to study several functions of KSHV vFLIP in the context of the whole viral genome. Deletion of the gene encoding vFLIP from a KSHV genome cloned in a bacterial artificial chromosome (BAC) reduced the ability of the virus to persist and induce spindle cell formation in primary human umbilical vein endothelial cells (HUVECs). Only a few, mainly interferon (IFN)-responsive, genes were expressed in wild-type KSHV (KSHV-wt)-infected endothelial cells at levels higher than those in KSHV-ΔFLIP-infected endothelial cells, in contrast to the plethora of cellular genes induced by overexpressed vFLIP. In keeping with this observation, vFLIP induces the phosphorylation of STAT1 and STAT2 in an NF-κB-dependent manner in endothelial cells. vFLIP-dependent phosphorylation of STAT1 and STAT2 could be demonstrated after endothelial cells were infected with KSHV-wt, KSHV-ΔFLIP, and a KSHV-vFLIP revertant virus. These findings document the impact of KSHV vFLIP on the transcriptome of primary endothelial cells during viral persistence and highlight the role of vFLIP in the activation of STAT1/STAT2 and STAT-responsive cellular genes by KSHV.

Kaposi’s sarcoma herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), was first detected in KS patient tissue (14) and is an indispensable factor in the development of this tumor (for a review, see reference 56). KSHV was found also to be associated with two other lymphoproliferative disorders, primary effusion lymphoma (12) and the plasma cell variant of multicentric Castleman’s disease (57). Many groups have shown the ability of KSHV to infect primary endothelial cells in vitro and induce spindle cell formation reminiscent of KS tumor cells (10, 18, 24, 26). Most spindle cells are latently infected with KSHV, and only a small proportion of them undergo spontaneous lytic replication. KSHV-infected endothelial cells exhibit a gene expression profile resembling that of lymphatic endothelial cells, and KSHV can reprogram infected vascular endothelial cells to express a lymphatic endothelial profile and vice versa (32, 67). The reprogrammed gene expression profile includes the upregulated expression of several specific lymphatic endothelial genes, including VEGFR3, podoplanin, LYVE1, and Prox-1, in dermal microvascular endothelial cells upon KSHV infection (11). The K13 latent viral gene (also referred to as open reading frame 71 [orf71]) encodes the Fas-associated death domain (FADD)-like interleukin-1 beta-converting enzyme (FLICE)-inhibitory protein (vFLIP). It has homology to cellular FLIP (cFLIP) (15, 63). Two predominant forms of cFLIP exist. cFLIP L (27) is the short form and contains only two N-terminal death effector domains (DEDs), which are very similar to the prodomains of caspase 8 and 10. The long splice variant of cFLIP (cFLIP S) contains tandem N-terminal DEDs with an altered caspase activity of vFLIP, Sun et al. have shown the ability of vFLIP to protect against growth factor withdrawal-induced apoptosis in the growth factor-dependent TF-1 leukemia cell line but not against tumor necrosis factor alpha (TNF-α)-induced or anticancer drug-induced apoptosis (61). Downregulation of vFLIP
in primary effusion lymphoma cell lines led to induction of apoptosis and cell death (27, 31). In KS lesions, the expression of the vFLIP transcript was found to increase in late-stage lesions and was inversely correlated with apoptosis (58). However, thymocytes from a vFLIP-transgenic mouse showed a rate of cell death similar to that of the negative-control cells when induced with either anti-fibroblast cell line (FS-7)-associated surface antigen antibody (FAS-Ab) or dexamethasone, indicating the failure of vFLIP to block either the intrinsic or the extrinsic apoptosis pathway in this system (17). Recently, the role of vFLIP in the initiation of primary effusion lymphoma (PEL) and multicentric Castleman’s disease, by specifically expressing vFLIP at different stages of B cell differentiation in vivo, was determined. The authors reported that vFLIP can induce B cell transdifferentiation (reprogramming), thereby promoting the emergence of B cell-derived tumors that display Ig gene rearrangements and a downregulated expression of B cell-associated antigens, thus resembling a PEL immunophenotype (3). Inhibition of detachment-induced apoptosis (anoikis) in dermal microvascular endothelial cells by vFLIP may contribute to KS oncogenesis by allowing the detachment and spread of single cells from one lesion (metastasis) (21). Recently, vFLIP was also shown to protect human umbilical vein endothelial cells (HUVECs) from superfused-induced apoptosis by upregulating the expression of the manganese superoxide dismutase gene (MnSOD) (64).

Most of these activities are nuclear factor kappa B (NF-κB) dependent. KSHV vFLIP is unique among other viral FLIPs in its ability to activate both the canonical and the alternative NF-κB pathway (15, 41). It binds to IκB kinase gamma (IκK-γ) on the HLX2 domain and induces conformational changes leading to its autoactivation (2, 23). NF-κB activation seems to be central for vFLIP functions. Other than having the above-mentioned cell survival and antiapoptotic functions, vFLIP also induces cytokine secretions like interleukin-8 (IL-8) and CXCL16 in an NF-κB-dependent manner (60, 70). By an NF-κB-dependent mechanism, vFLIP was also shown to manipulate Notch signaling by upregulation of JAG1 gene expression, leading to the expression of Notch-responsive genes like HEY1 in vFLIP-expressing and neighboring lymphatic endothelial cells (22).

Furthermore, the NF-κB-dependent functions of vFLIP extend toward blocking KSHV lytic reactivation and virus production (72), most probably by inhibiting the replication transcription activator (RTA) promoter’s activity through suppression of the AP-1 pathway (71). It was also demonstrated that vFLIP has a unique ability among other viral FLIPs to transform Rat-1 and BALB/3T3 fibroblast cells and to form tumors in nude mice. This ability was also NF-κB associated (61). In addition, overexpression of vFLIP in primary endothelial cells induces, again in an NF-κB-dependent manner, the formation of spindle cells, the hallmark of KS lesions (29). However, in the KSHV genome, vFLIP is transcribed from the second exon of a bicistronic mRNA that also encodes the viral cyclin homologue, vCyc, and is only weakly expressed at the protein level (38). To what extent the results obtained with overexpressed vFLIP reflect its role in the context of the entire viral genome is thus not clear. We therefore applied a reverse genetics approach to investigate several functions of vFLIP in the context of the whole viral genome. We deleted orfK13, the gene encoding vFLIP, from a KSHV genome cloned into a bacterial artificial chromosome (BAC) (73). We confirm the role of vFLIP in inducing spindle cell formation in primary endothelial cells. The absence of vFLIP from the viral genome affects the expression of only a few STAT-regulated genes during viral persistence, in contrast to the plethora of cellular genes that are modulated by overexpressed vFLIP. In keeping with this observation, we show that vFLIP expression induces the activation and phosphorylation of STAT1 and STAT2 in endothelial cells.

**MATERIALS AND METHODS**

**Cells and transfections.** HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Germany), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Cytogen, Germany). Vero cells were maintained in minimal essential medium (MEM) (Earle’s balanced salt solution [BSS], nonessential amino acids [NEAA], l-glutamine, and 2 g/liter NaHCO3 (Cytogen, Germany). HUVECs were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein as described previously (33). HUVECs were cultured in the low-serum (5%) endothelial cell growth medium 2 for microvascular cells (EGM-2-MV) with its specific supplement (Lonza). An endothelial cell line, HuAR2T-tert, conditionally immortalized with doxycycline-dependent human telomerase reverse transcriptase (hTERT) and simian virus 40 (SV40) T antigen (TAG) transgene expression (43), was kindly provided by D. Wirth. HuAR2T-tert cells were maintained in EGM-2-MV medium in the presence of doxycycline at a concentration of 2 μg/ml. Transfection of HUVECs with small interfering RNA (siRNA) was done using the Neon transfection system according to the manufacturer’s instructions (Invitrogen). One hundred picomoles of siRNA was micropolated into 105 cells, which were then plated in one well of a 24-well plate. All siRNA oligonucleotides (siGENOME SMARTPool for JAK1, JAK2, TYK2, RELA, IKKβ, and nontargeting siRNA pool no. 1 were purchased from Thermo Scientific (catalog no. M-003145-02-0005, M003146-02-0005, M-003182-02-0005, M-003533-02-0005, M-003767-02-0005, and D-001206-13-20, respectively). Sf9 cells were maintained in Grace’s medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Cytogen, Germany) and incubated at 28°C. The generation of the recombinant baculovirus expressing KSHV ORF50/RTA was described previously (66).

**Antibodies.** The following antibodies were used: mouse anti-late-associating nuclear antigen (anti-LANA; 1:50; Novocastra) for the immunofluorescence assay, rat anti-LANA (1:2,000; ABI) for Western blotting, anti-green fluorescent protein (anti-GFP) antibody (Clontech), and anti-actin antibody (Chemicon). Antibody were purchased from Santa Cruz Biotechnology and Roche, respectively. A rat monoclonal antibody to vFLIP (4C1) was kindly provided by E. Kremmer.

**Plasmids.** An E. coli strain (DH10B) containing the KSHV genome cloned in a bacterial artificial chromosome (BAC36) was obtained from S. J. Gao (73). BAC-KSHV-ΔFLIP (KSHV-ΔFLIP) was generated from the BAC-KSHV wild type (KSHV-wt) by a RecE/Rect recombinant proteins cloning strategy (ET cloning) (see below). The pKD46 plasmid expressing the recombination enzymes under the L-arabinose-inducible promoter is described elsewhere (20). The pKD46 plasmid expressing the recombination enzymes under the L-arabinose-inducible promoter (see below). The pKD46 plasmid expressing the recombination enzymes under the L-arabinose-inducible promoter is described elsewhere (20). The RecE/Rect recombinant proteins cloning strategy (ET cloning) is described elsewhere (20).

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ATTACCC, VFLIP KIN FOR, AGTTGTTTAAATACGATACATACATTCTACGCGACAAATTTACGATCTGTGCTATCTGTTGATCGGATAGTGTG, and VFLIP KIN REV, GAAAATAAATTTTCCTTTGTTTTTATCATGACCAGAAGGGCAGCCATGCCCCATTAGGCTAGTTCTC.

To construct a VFLIP-expressing lentiviral vector, the DNA fragment containing the VFLIP open reading frame was amplified from KSHV DNA (BAC36-wt) by PCR with the following primers: VFLIP Ncol, 5'-ATCTCTCATGGGGAACACTTTAGAGGAGGCTTTCTCTTCCTCC-3'; and VFLIP SpeI, 5'-TTCTTGGCAGATCTGGTGGTGGATGGCGGATAGTGGGGA-3'. The T2A element was amplified from pIRK36 (kindly provided by A. Schambach) with the primers BsrGI and SalI sites to generate a lentiviral vFLIP vector. Another vFLIP construct tagged with HA at its C-terminal part (vFLIP-HA vector) was prepared with the primers KSHV IKK REV, GAAAAATAAATTTTCCTTTGTTTACGAGGTTCTCTG, and vFLIP KIN FOR, AGTGTTTATTAAATCAGATACATACATTCTACGCGACAAATTTACGATCTGTGCTATCTGTTGATCGGATAGTGTG.

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thesis of cRNA was performed with the one-color Quick Amp labeling kit (Agilent Technologies) according to the manufacturer’s recommendation. cRNA fragmentation, hybridization, and washing steps were performed exactly as recommended by the manufacturer of One-Color Microarray-Based Gene Expression Analysis, version 5.7 (Agilent), except that 2.5 µg of each labeled cRNA sample was used for hybridization. Slides were scanned on an Agilent microarray scanner, model G2505 B, at two different photomultiplier tube (PMT) settings (100% and 5%) to increase the dynamic range of the measurements. Data extraction was performed with the Feature Extraction software, version 9.5.3.1, by using the recommended default extraction protocol file: GE1-v5_95_Feb07.xml.

Processed intensity values of the green channel (processedSignal or gPS) were globally normalized by linear scaling: all gPS values of one sample were multiplied by an array-specific scaling factor. This factor was calculated by dividing the 75th-percentile value taken from one selected reference array, contained within the series, by the 75th-percentile value of the particular microarray ("array i" in the formula shown below). Accordingly, normalized gPS values for all samples (microarray data sets) were calculated by the following formula: normalized gPS array i = gPS array i / (75th-percentile reference array/75th percentile array i). An appropriate low-intensity threshold was established, based primarily on (i) the intensity distribution at the low-intensity end and (ii) the attribute “g is well above BG” (determined by the Feature Extraction software; g is green processed signal, and BG is background). The established threshold values were 34 (vFLIP overexpression experiments) and 120 (KSHV infection experiments). All of those normalized gPS values that fell below this intensity border were replaced by corresponding surrogate values of 34 and 120, respectively.

qPCR analysis. Total RNA, used in the microarray experiments, was reverse transcribed using 50 U of BioScript low-RNase H reverse transcriptase (BIO-Care, Sweden) and oligo(dT) primer at 42°C for 1 h. Conventional PCR was initiated with Expand reverse transcriptase (Roche) and oligo(dT) primer at 72°C for 10 min at 70°C. Aliquots of generated cDNA samples were used for quantitative reverse transcription PCR (qPCR) with the ABI 7500 Fast qPCR system (Applied Biosystems). The enzyme was finally inactivated for 10 min at 70°C. The following primers, probes, and assays were used: EGFP forward primer 5'-GGACGCGCCCTGAGCAAGA-3', EGFP reverse primer 5'-GGCCGCGCGCACTTAC-3', EGFP probe FAM-CCACCAAGGCG-MGBNFQ, where FAM is 6-carboxyfluorescein. K8 forward primer 5'-AACCCATACCCCGACCTTTGT-3', K8 reverse primer 5'-CATGTCGATAAGCTTCTCTCCTC-3', and K8 probe FAM-CCACCAACACAAGTCGGAC-TAMRA, where TAMRA is tetramethylrhodamine. The average cycle threshold (CT) value for each individual amplification reaction was calculated from duplicate or triplicate measurements by means of the instrument’s software in auto-CT mode (7500 Fast system software, version 1.3(A)). Average CT values calculated for EGFP or K8 were normalized by subtraction of the CT value obtained for GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Reverse transcriptase PCR. Total RNA was extracted from the cells with an RNasey kit (Qiagen) according to the manufacturer’s instructions, followed by DNA-free DNase treatment and inactivation according to the manufacturer’s instructions (Ambion). cDNA was synthesized using the Expand reverse transciptase (Roche) and oligo(dt) primer at 42°C for 1 h. Conventional PCR was then performed to amplify vFLP, vCyC, and GAPDH transcripts using the following primers, respectively: K13 seq for (5'-TTACGAGGTTCTCTGTGAGG-3'), vCYC rev (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC seq rev (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC seq 4 for (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC seq 5 rev (5'-TTACGAGGTTCTCTGTGAGG-3'), vCYC seq 4 for (5'-TTACGAGGTTCTCTGTGAGG-3'), vCYC seq 5 rev (5'-TTACGAGGTTCTCTGTGAGG-3'), vCYC rev (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC seq rev (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC rev (5'-TACGGTCTTCTCTTCGTCAG-3'), vCYC seq rev (5'-TACGGTCTTCTCTTCGTCAG-3'), and GAPDH (5'-ACCACGTCCTGCGATGTC-3').

Immunofluorescence assay. Vero cells infected with KSHV-wt, KSHV-ΔFLIP, or KSHV-FLIP-R and selected with hygromycin were plated on glass coverslips in 6-well plates. Twenty-four hours later, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then neutralized with 10% NH₄Cl for 10 min. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were rinsed with PBS, incubated with 10% FCS in PBS for 20 min, and then rinsed again with PBS. Coverslips were incubated for 1 h at 37°C in a humidified chamber with the primary antibody in PBS containing 1% bovine serum albumin (BSA). Coverslips were washed in PBS three times and incubated with the secondary antibody plus DAPI (4',6-diamidino-2-phenylindole) in 1% BSA in PBS and then rinsed with PBS.

RESULTS

Construction and verification of KSHV-ΔFLIP and KSHV-FLIP-R. To study the role of vFLIP in the context of the viral genome, we took advantage of having the KSHV genome cloned as a bacterial artificial chromosome (BAC) (73). KSHV-ΔFLIP was generated using the ET cloning technique. A cassette encoding kanamycin resistance (psl-neo cassette) flanked by 60-bp sequences bordering the region to be deleted was electroporated into the E. coli strain DH10B harboring the KSHV-wt and the pKD46 plasmid, which encodes the recombination enzymes under the control of an l-arabinose-inducible promoter (Fig. 1A). KSHV-ΔFLIP, as a result of the cassette insertion, lacks the whole vFLIP gene, corresponding to nucleotide position 122959, upstream of the vFLIP start codon, and nucleotide position 122393, downstream of the stop codon (GenBank accession no. AF148805) (Fig. 1A).

vFLIP was reinserted in its original position to generate the KSHV-FLIP-R construct using a two-step markerless mutagenesis strategy termed “en passant mutagenesis” (65). The rpsl-neo cassette in the KSHV-ΔFLIP construct was replaced by a vFLIP-zeocin + homology cassette using an ET cloning strategy. This cassette contains the vFLIP gene with a zeocin resistance-coding DNA sequence inserted in its SacI site and a 50-bp fragment of vFLIP (indicated by black boxes in Fig. 1B) duplicated on both sides of the zeocin gene to create homology for the next step. On the left side of the zeocin gene, an I-SceI restriction site had previously been inserted (Fig. 1B). In the next step, the expression of the I-SceI enzyme was induced by 1% arabinose to cut the unique site between zeocin and the duplicated fragment of vFLIP. Directly afterward, the expression of the recombination enzymes was induced at 42°C to initiate the homologous recombination of the duplicated vFLIP region (Fig. 1B).

After a screening for positive colonies by PCR (data not shown), the DNA of KSHV-wt, KSHV-ΔFLIP, and KSHV-FLIP-R were digested with NruI. The insertion of the rpsl-neo cassette replaced the 566-bp vFLIP fragment with a 1,320-bp fragment, leading to a shift of the 14-kb band in KSHV-ΔFLIP by 754 bp (indicated by the arrow in Fig. 1C). The reinsertion of vFLIP shifted this band to its position in the KSHV-wt genome. For further verification, the three constructs were also digested with several other restriction enzymes (KpnI, EcoRI, SacI [data not shown]). To confirm the specificity of the band shift in the NruI digestion pattern and to exclude the possibility that the rpsl-neo cassette was inserted twice in the genome, the gel was analyzed by Southern blotting. The digested DNA was transferred to a nylon membrane and hybridized with the rpsl-neo probe labeled with alkaline phosphatase, which showed the presence of one specific band only in KSHV-ΔFLIP (Fig. 1C, lanes 5, 6, and 7). The membrane was stripped and rehybridized with a vFLIP PCR fragment labeled with alkaline phosphatase. One specific band was detected in KSHV-wt and KSHV-FLIP-R but not in KSHV-ΔFLIP (Fig. 1C, lanes 8, 9, and 10). The junction regions, as well as the newly inserted vFLIP gene, were also sequenced and proved to be identical to the wt sequence (data not shown).

Deletion of vFLIP does not affect the expression of neighboring viral genes. vFLIP is located in the KSHV genome between LANA and vCyc (upstream) and a cluster of miRNAs.
We checked the expression of these genes to ensure the integrity of our KSHV constructs. Total RNA was extracted from HEK293 cell lines stably transfected with KSHV-wt, KSHV-ΔvFLIP, or KSHV-FLIP-R and treated with DNase I. The cDNA was prepared using the oligo(dT) primer. Specific primers were then used to amplify the vFLIP, vCyc, and GAPDH genes (see Materials and Methods). The vCyc transcript was present in all clones, while vFLIP was absent in the KSHV-ΔvFLIP clone only (Fig. 2A). Empty HEK293 cells showed neither vCyc nor vFLIP bands indicating the specificities of the primers. The same RT-PCR without reverse transcriptase showed no signal for any of the tested genes, excluding the possibility of genomic DNA amplification (data not shown). LANA expression was tested by immunofluorescence assay (IFA) on Vero cell lines stably harboring the KSHV-wt, KSHV-ΔvFLIP, or KSHV-FLIP-R genome. All clones showed the presence of the characteristic LANA speckles in the nucleus (Fig. 2B). The miRNAs are located downstream of vFLIP and are transcribed from the same promoter with vCyc and vFLIP. Nonetheless, deletion of vFLIP did not affect the expression of these miRNAs, as shown by the transcription of the miRNA K12-10 (Fig. 2C).

Contribution of KSHV vFLIP to spindle cell formation in infected primary endothelial cells. Spindle cells are the hallmark of KS lesions and considered to be tumor cells. In vitro, KSHV has been shown to infect lineages of endothelial cells, including those of lymphatic and microvascular origins, and to induce spindling reminiscent of that in KS lesions (10, 18, 24, 26, 32, 67). Overexpression of vFLIP in endothelial cells using retroviral or lentiviral vectors has been shown to induce spindling in an NF-κB-dependent manner (21, 29, 42).

To investigate the contribution of vFLIP to KSHV-induced spindle cell formation in the context of the whole virus, a monolayer of primary human umbilical vein endothelial cells (HUVECs) was infected with KSHV-wt, KSHV-ΔvFLIP, or KSHV-FLIP-R at a multiplicity of infection (MOI) of approximately 0.2 for HUVECs. KSHV-wt, KSHV-ΔvFLIP, and KSHV-FLIP-R stocks were produced in stably infected Vero cells, as described in Materials and Methods. GFP expression, encoded by the recombinant virus (BAC36-KSHV), was used to monitor the infected endothelial cells. Green cells were observed at 1 day postinfection in all infected culture plates.
Cells infected with the wild-type virus or the vFLIP revertant virus showed spindling morphology, in contrast to the cells infected with the vFLIP knockout virus (Fig. 3A), in line with the effects of overexpressed vFLIP on endothelial spindle cell formation (21, 29, 42). However, at a higher MOI (MOI of approximately 1 for HUVECs), HUVECs infected with KSHV-ΔFLIP also showed spindling morphology similar to that in KSHV-wt- or KSHV-FLIP-R-infected cells (Fig. 3B).

**vFLIP enhances the survival of KSHV-infected cells.** During our experiments, we observed a more rapid loss of KSHV-ΔFLIP-infected HUVECs from culture plates than that of KSHV-wt- or KSHV-FLIP-R-infected cells, indicating a role for vFLIP in the maintenance or survival of infected cells. To quantify the effect of vFLIP on the persistence of KSHV-infected cells, HUVECs were plated in 24-well plates and infected the next day at an MOI of approximately 0.1 with KSHV-wt, KSHV-ΔFLIP, or heat-inactivated KSHV-wt. Four days after infection, cells were lysed, and total RNA was ex...
tracted and processed using Agilent standard protocols and hybridized onto whole-genome 4X44K microarrays. Two independent experiments were performed on cells from two different donors using different virus preparations. The level of GFP mRNA in KSHV-ΔFLIP-infected cells was comparable to that in KSHV-wt-infected cells, indicating comparable rates of infection (data not shown).

We compared the cellular gene expression profiles from our microarray experiments (KSHV-wt versus KSHV-ΔFLIP infection) to the expression profile obtained from HUVECs transduced for 72 h with a lentiviral vector for vFLIP (see Materials and Methods) and to those of two other previously published whole-genome microarray studies employing overexpression of vFLIP via a retrovirus (53, 64). In agreement with the two previously published studies, vFLIP overexpression in HUVECs showed a consistent upregulation of a large set of genes (Fig. 5A and B, columns 1 and 2). Among the vFLIP-induced genes were those for several cytokines and their receptors (e.g., IL-1β, IL-7 receptor [IL-7R], and IL-18R), genes for chemokines (IL-8, CXCL10, CCL5, CXCL5, CXCL6, and CCL20), genes involved in antiapoptotic processes (BCL2A1, BIRC3, XAF1, and SOD2 genes), genes with proapoptotic functions (RIPK2 and TNFRSF19 genes), and many antiviral and interferon (IFN)-inducible genes (MX1 and -2, OAS-1-3, viperin, and IFI6 genes). Interestingly, the expression of only a few of the genes induced by overexpressed vFLIP turned out to be significantly upregulated in KSHV-infected HUVECs compared to their expression in ΔFLIP-infected cells (Fig. 5A and B). Only a group of 15 cellular genes that were induced more than 2-fold by vFLIP overexpression in this study and in the studies by Sakakibara et al. (53) and Thurau et al. (64) were also upregulated in KSHV-wt-infected cells, in comparison to KSHV-ΔFLIP-infected cells (Fig. 6). The expression of these 15 differentially regulated genes was also increased in HUVECs treated with either alpha or beta interferon (results not shown; see the summary in Fig. 6), suggesting that, in the context of the viral genome and during viral latency, vFLIP shows the strongest effect on a set of interferon-regulated cellular genes. However, overexpression of vFLIP did not upregulate the expression of type I or type II interferon genes (data not shown), suggesting that the upregulation of interferon-responsive genes in KSHV-wt- versus KSHV-ΔvFLIP-infected cells is not due to an increased expression of type I or II interferons.

vFLIP induces STAT1 and STAT2 phosphorylation. As vFLIP induced the expression of interferon-responsive genes without inducing type I or II interferon genes, we investigated the impact of vFLIP on the signaling cascade downstream of the interferon receptor. We transduced HUVECs with a lentivirus expressing either vFLIP or an IKK-α/binding-deficient mutant (vFLIP-A57L) in the form of GFP-T2A-vFLIP-wt-HA, GFP-T2A-vFLIP-A57L, or the control lentivirus. vFLIP-HA and the IKK-α/binding-deficient mutant are cleaved cotranslationally into a GFP-T2A fusion protein and vFLIP-HA. Cells were lysed 48 h after transduction and tested for JAK1, JAK2, Tyk2, and STAT1 to STAT6 expression and phosphorylation by immunoblotting. vFLIP induced the phosphorylation of STAT1 on both residues tyrosine 701 and serine 727, along with a moderate increase in the STAT1 protein level (Fig. 7A). Additionally, vFLIP induced the upregulation and phosphorylation of STAT2 on the tyrosine 690 residue (Fig. 7A) but not STAT3, STAT5, or STAT6 (Fig. 7A, C, and D). vFLIP also upregulated the expression of JAK2 and slightly that of JAK1 and Tyk2 without inducing an increase in their phosphorylation (Fig. 7B). This upregulation of JAK2/STAT1/STAT2 and the increased phosphorylation of STAT1 and STAT2 are NF-κB dependent because the vFLIP-A57L mutant, which is completely defective in NF-κB activation (2), failed to induce STAT1 and STAT2 phosphorylation (Fig. 7A and B). Furthermore, knockdown of either IKK-γ or RelA (p65) by siRNA prevents vFLIP-mediated STAT1 and STAT2 phosphorylation (Fig. 7E). Knocking down JAK2 expression by siRNA had only a moderate effect on vFLIP-mediated STAT1/STAT2 phosphorylation (Fig. 7E), while knocking down either JAK1 or Tyk2 had no appreciable effect on vFLIP-mediated STAT1/STAT2 phosphorylation (Fig. 7F).

Furthermore, vFLIP seems to phosphorylate STAT1 and STAT2 in a cell type-dependent manner. While vFLIP induces the upregulation and phosphorylation of STAT1 at both the tyrosine 701 and serine 727 residues and STAT2 in primary endothelial cells as well as in HeLa cells (Fig. 7A and 8), it does not have this effect in B cells like BJAB or KSHV-infected primary effusion lymphoma cells (BCBL-1) (Fig. 8). vFLIP also fails to activate or phosphorylate STAT1 and STAT2 in other epithelial cell lines like Vero and HEK293 cells (data not shown).

Furthermore, to determine the role of vFLIP-mediated STAT1 and STAT2 phosphorylation in the context of the entire virus, we analyzed the phosphorylation levels of STAT1/STAT2 in KSHV-wt, KSHV-ΔFLIP-, and KSHV-FLIP-R-infected, conditionally immortalized endothelial cell lines (HuAR2T-tert) (Fig. 9). HuAR2T-tert cells were infected with KSHV-wt, KSHV-ΔFLIP, or KSHV-FLIP-R at an MOI of 1 and selected with hygromycin to generate stable cell lines. All hygromycin-selected lines showed close to 100% GFP expression (data not shown). Stably selected cells were plated in 6-well plates and were lysed 24 h later. The cellular lysates were analyzed for STAT1/STAT2 phosphorylation. KSHV-ΔFLIP-infected cells showed a reduction in the levels of phosphorylated STAT1/STAT2 relative to those in KSHV-wt- and KSHV-FLIP-R-infected cells. As expected, the induction of STAT1/STAT2 phosphorylation in KSHV-wt- and KSHV-FLIP-R-infected cells was less pronounced than in endothelial cells in which vFLIP had been overexpressed by means of a lentiviral vector (Fig. 9). Taken together, our data suggest that vFLIP induces the phosphorylation of STAT1/STAT2 in endothelial cells in the context of the entire virus.

The complete transcriptome profile of KSHV-infected HUVECs or vFLIP-transduced HUVECs is available upon request (contact Oliver Dittrich-Breiholz at dittrich.oliver@mh-hannover.de).

**DISCUSSION**

The aim of this study was to investigate the role of vFLIP in the context of the entire viral genome during viral persistence in endothelial cells and to assess the contribution of vFLIP-induced effects previously found in studies that overexpressed vFLIP in endothelial cells. As it is translated from the second exon of a bicistronic mRNA by means of an internal ribosome...
FIG. 5. Only a subset of cellular genes consistently upregulated by overexpressed vFLIP are differentially expressed in KSHV-wt- and KSHV-ΔvFLIP-infected cells. (A) Microarray analysis of HUVECs infected at an MOI of approximately 1 with either KSHV-wt or KSHV-ΔvFLIP, compared to HUVECs transduced with the vFLIP-expressing vector. Filters were set to exclude all flagged values and poorly annotated or poorly characterized transcripts. Depicted are 281 genes, which showed more than 4-fold upregulation in vFLIP-transduced cells compared to their expression levels in control-vector-transduced cells in one (experiment 1, column 1) out of two experiments performed (columns 1 and 2). Columns 3 and 4 represent the differential regulation of these genes in HUVECs infected with KSHV-wt or KSHV-ΔvFLIP in two independent experiments. Ratio values were calculated from processed signal intensities of KSHV-wt-infected/KSHV-ΔvFLIP-infected HUVEC samples and are color coded.

### Table: Gene Expression

<table>
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<th>Gene</th>
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**Legend:**
- > 32-fold upregulation
- 8-32-fold upregulation
- 2-8-fold upregulation
- no change
- 2-8-fold downregulation
- 8-32-fold downregulation
- > 32-fold downregulation
entry site (IRES) (6, 38), vFLIP protein levels in PEL cells or infected endothelial cells are low (38) (Fig. 9 and data not shown), and effects ascribed to vFLIP on the basis of its overexpression in transfected or transduced cells may therefore be mitigated in the context of the natural infection.

Among these effects is the ability of vFLIP to induce endothelial cell spindling when transduced into primary endothelial cells (21, 29, 42, 53, 64). We found that a vFLIP knockout virus was not able to induce spindling morphology in HUVECs infected at a low MOI, suggesting a role for vFLIP in KSHV-infected cell morphogenesis. However, other viral proteins may contribute to the spindle cell phenotype, since we did observe spindle cell formation in HUVEC infection with KSHV/H9004 FLIP at an MOI of approximately 1. Grossmann et al. and others showed that the inhibition of NF-κB activation prevents vFLIP-induced spindling in HUVECs (21, 29, 42, 53) and that the equine herpesvirus 2 vFLIP, which does not activate NF-κB, could not induce spindle cell formation, suggesting a link between NF-κB formation and KSHV vFLIP-induced spindling (29). It is thus possible that other NF-κB-activating viral genes could contribute to spindle cell formation, like K1, K15, orf75, or mirK1 (8, 36, 37, 54, 68).

There is evidence for the expression of all of these genes, with the exception of orf75, in latently/persistently infected cells (9, 13, 30, 51, 55). However, since infection at a low MOI probably reflects the in vivo situation more closely, vFLIP is likely to be an important player in KSHV-induced spindle cell formation. As an oncogenic virus, KSHV provides infected cells with a survival advantage (69). We observed that endothelial cells infected with KSHV-wt have a moderate growth advantage over KSHV-/H9004 FLIP-infected cells. This function of vFLIP was previously reported also in PEL cells (27, 31). Thus, vFLIP plays a vital role in KSHV pathogenesis in infected endothelial cells as well as B cells by enhancing their survival most likely via NF-κB activation.

Several groups have shown that the ectopic expression of vFLIP in HUVECs leads to the upregulation of hundreds of genes with diverse functions (52, 53, 64), and we could confirm appropriately. (B) Selective representation of the 45 genes upregulated by vFLIP more than 32-fold. Asterisks indicate genes that were induced more than 2-fold in KSHV-wt-infected cells compared to cells mock infected with heat-inactivated virus. The designation "IFN" to the right of the table indicates that this gene was responsive to IFN-α and IFN-β in an experiment conducted on primary endothelial cells (our unpublished results).

FIG. 6. Genes from Fig. 5A, which were induced at least 2-fold by transduced vFLIP in three studies (see below) and which were also upregulated at least 2-fold in KSHV-wt-versus KSHV-ΔFLIP-infected HUVECs. Lane 1, response to retrovirally transduced vFLIP as reported by Thurau et al. (64); lanes 2 and 3, response to transduced vFLIP as reported by Sakakibara et al. (53); lanes 4 and 5, response to lentivirally transduced vFLIP (this study); lanes 6 and 7, cellular genes upregulated at least 2-fold in KSHV-wt-versus KSHV-ΔFLIP-infected HUVECs in two independent experiments. Asterisks indicate genes that were induced more than 2-fold in KSHV-wt-infected cells compared to in cells infected with heat-inactivated KSHV. The designation "IFN" to the right of the table indicates that this gene was responsive to IFN-α and IFN-β in an experiment conducted on primary endothelial cells (our unpublished results).
this in our study. In contrast, only a few cellular genes were differentially upregulated by vFLIP in the context of the whole virus genome. This could be attributed to the low level of vFLIP expression and indicates the importance of studying the effects of vFLIP in the course of natural infection. Surprisingly, all the cellular genes that were consistently and unidirectionally regulated by vFLIP overexpression and by vFLIP in the context of the whole viral genome in infected endothelial cells are known to be interferon inducible. As vFLIP does not induce the expression of genes encoding type I and II interferon while permitting the phosphorylation of STAT1 and STAT2, and since the vFLIP-induced phosphorylation of STAT1 and STAT2 does not depend on JAK1, JAK2, or Tyk2 (Fig. 7), it is likely that vFLIP activates the expression of these interferon-inducible genes directly, i.e., independently of interferon induction.

Activation of STAT1 and STAT2 by vFLIP requires the recruitment of IKK-γ to vFLIP and activation of the NF-κB pathway, since the A57L mutant did not induce STAT1 and STAT2 phosphorylation and knockdown of IKK-γ or p65 prevented this activation of STAT1/STAT2 (Fig. 7E and F). However, this vFLIP-mediated STAT1/STAT2 phosphorylation seems to be cell type dependent (Fig. 8). vFLIP failed to induce STAT1/STAT2 phosphorylation in HEK293 cells, in addition to the cell types shown in Fig. 8, although it strongly activates an NF-κB reporter plasmid in this cell line (data not shown), indicating its ability to activate the NF-κB pathway in these cells and suggesting that activation of the NF-κB pathway alone by vFLIP is not sufficient to induce STAT1/STAT2 phosphorylation. In keeping with our observation that a subset of interferon-inducible cellular genes is differentially expressed between KSHV-wt- and KSHV-ΔvFLIP-infected endothelial cells (Fig. 5 and 6), we could show increased STAT1/STAT2 phosphorylation in KSHV-wt- and KSHV-ΔvFLIP-infected, compared to uninfected and KSHV-ΔvFLIP-infected, endothelial cells (Fig. 9). These differences in STAT1/STAT2 phosphorylation were detectable, although vFLIP expression in the infected endothelial cells was too low to be visible on Western blots (Fig. 9).

Activation of STAT1 and STAT2 by vFLIP may serve the purpose of inducing one or several cellular genes identified in this study as being differentially expressed between KSHV-wt- and KSHV-ΔvFLIP-infected cells. Some of these have other functions that fit with properties previously attributed to

This image contains figures illustrating the expression and phosphorylation of STAT1 and STAT2 in different cell lines after transduction with vFLIP-expressing lentivirus. The figures show the expression of various proteins under different conditions, including untransduced cells and cells transduced with control vectors or vFLIP-expressing vectors. The analysis includes western blots probed with antibodies to GFP and HA to detect the expression of the transfected proteins.
vFLIP, IFI6 (G1P3), for example, localizes to mitochondria and inhibits mitochondrial-mediated apoptosis in human myeloma cells and the gastric cancer cell line (16, 62). Thereby, upregulation of IFI6 by vFLIP, in addition to previously reported SOD2 (64), may protect infected cells from the signature genes in breast cancer metastasizing to lung (46) and was shown to be involved in the self-renewal of myeloma cells and the gastric cancer cell line (16, 62).

Although the mouse Mx1 and -2 as well as the human MxA proteins exhibit antiviral activities, the human Mx2 homolog (MxB), upregulated by vFLIP, shows no antiviral functions (25, 47, 48). King et al. have shown that MxB localizes to the cytoplasmic phase of the nuclear pore and that the expression of either GTP-binding- or GTP hydrolysis-defective mutants disrupts nuclear import and significantly delays the progression of the cell cycle from G0/G1 into S phase (34).

On the other hand, activation of the interferon response pathway by vFLIP may also contribute to the inhibition of lytic reactivation, since a recent report described that the murine herpesvirus 68 (MHV-68) orf50/RTA gene promoter is repressed by gamma interferon and STAT1, implying a role for the interferon response pathway in the control of lytic replication (28). In addition, interferon-independent activation of STAT1 in EBV-infected B cells has been shown to prevent the activation of the lytic replication cycle and to maintain the EBV latency III program in LCL cells (45). Interestingly, the interferon-independent activation of STAT1 in EBV-infected lymphoma or LCL cells occurs in the absence of Tyr 701 phosphorylation (44). Thus, the lack of Tyr 701 phosphorylation in vFLIP-transduced B cells (Fig. 7 and 8) may not preclude an activation of STAT1 by vFLIP in these cells.

A recent study (35) found that the human cytomegalovirus IE1 protein induces STAT1 phosphorylation and elicits a type II interferon-like host cell response involving the selective upregulation of immune-stimulatory genes, including proinflammatory cytokines. Several large DNA viruses may therefore be able to exploit a component of the innate immune response, which is usually known for its antiviral properties, for their own benefit. Further studies are needed to clarify the benefit that activation of a STAT1- and STAT2-dependent pathway by vFLIP affords to KSHV. However, the results reported in this study indicate that the effect of vFLIP on STAT1-dependent cellular genes appears to dominate the difference in cellular gene expression observed between endothelial cells infected with a wild-type and a vFLIP-deleted KSHV.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG; Schu1688-2/3) and EU integrated project INCA (LSHC-CT-2005-018704) to T.F.S. as well as grant DFG-GK1071 and a grant from the Interdisciplinary Center for Clinical Research (IZKF) of the University of Erlangen-Nuremberg to M.S. We thank Axel Schambach and Renata Stripeke for providing us with the plasmids required for the production of the vFLIP lentivirus.

REFERENCES


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