The Human Herpes Virus 8-Encoded Viral FLICE-inhibitory Protein Induces Cellular Transformation via NF-κB Activation

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Infection with human herpes virus 8 (HHV8) has been associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. HHV8 encodes for a viral FLICE-inhibitory protein (vFLIP), designated K13, which resembles the prodomain of caspase-8 in structure and has been shown to protect cells against death receptor-induced apoptosis in vitro and in vivo. In this report, we present evidence that HHV8 vFLIP also possesses the unique ability of transforming Rat-1 and BALB/3T3 fibroblast cells, which is not shared by other vFLIPs. Rat-1 cells expressing HHV8 vFLIP form colonies in soft agar and form tumors in nude mice. The transforming ability of HHV8 vFLIP is associated with the activation of the NF-κB pathway and is blocked by molecular and chemical inhibitors of this pathway. Our results suggest that vFLIP K13 has activity beyond its role as an inhibitor of death receptor signaling and may play a causative role in the pathogenesis of HHV8-associated malignancies. Furthermore, inhibitors of the NF-κB pathway may have a role in the treatment of malignancies linked to HHV8 infection.

Kaposi’s sarcoma (KS) is a mesenchymal tumor of blood and lymphatic vessels, which is the most common malignancy found in the patients with human immunodeficiency virus infection. The isolation of a novel herpes virus, designated human herpes virus 8 (HHV8), as a potential etiologic agent for KS was a major step in understanding the pathogenesis of KS (1). HHV8 genomes have also been consistently found in patients with primary effusion lymphoma (PEL), also known as body cavity-associated lymphoma, a rare form of B-cell lymphoma characterized by malignant pleural, pericardial, or peritoneal effusion in the absence of a tumor mass (2). In addition to KS and PEL, HHV8 genome has been detected in multicentric Castleman’s disease, angioimmunoblastic lymphadenopathy, and some cases of reactive lymphadenopathies (3–5).

Despite the increasing evidence linking the presence of HHV8 with KS and lymphoproliferative disorders, the mechanism by which this virus leads to a transformed phenotype is not entirely clear. Although HHV8 is known to encode for homologs of several cytokines and their receptors, none of them is expressed in latently infected PEL cell lines or KS spindle cells (6). HHV8 is also known to encode for a viral FLICE-inhibitory protein (vFLIP), encoded by the open reading frame K13 (orf-K13; also called orf71). HHV8 vFLIP is one of the few viral proteins to be expressed in latently infected KS spindle cells and PEL cell lines (6–9) and, therefore, is a prime candidate for cellular transformation associated with HHV8 infection. HHV8 vFLIP resembles the prodomain of caspase-8 (also called FLICE) in structure and, like it, contains two homologous copies of a death effector domain (10–12). Similar vFLIPs have been discovered in other viruses (10–12). These include MC159L and MC160 from the molluscum contagiosum virus and E8 from equine herpes virus 2. We have previously demonstrated that the HHV8 vFLIP possesses the unique ability of activating the NF-κB pathway in both solid tumor and lymphoid cell lines, which is not shared by the E8 and MC159L vFLIPs (13, 14). NF-κB activation by vFLIP K13 was recently independently confirmed by several investigators (15, 16).

Since the abnormal activation of the NF-κB pathway has been previously implicated in the cellular transformation induced by several viruses (17), in this study we have investigated the ability of HHV8 vFLIP to induce cellular transformation and analyzed the contribution of the NF-κB to this process.

MATERIALS AND METHODS

Cell Lines and Culture—Rat-1 cells were obtained from Dr. Robert Ilaria (University of Texas Southwestern Medical Center), and BALB/3T3 clone A31 was purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s medium supplemented with 10% calf serum. 293T cells were obtained from Dr. David Han (University of Washington, Seattle, WA). Phenytoin oxide, arsenic trioxide, and aspirin were purchased from Sigma. Lactacystin was purchased from Biomol (Plymouth Meeting, PA). Retrovirus and Adenovirus Constructs—Retrovirus constructs containing C-terminal FLAG epitope-tagged HHV8 vFLIP (K13-FLAG) and EHV2 vFLIP (E8-FLAG) and molluscum contagiosum virus vFLIP (MC159L-FLAG) were generated in MSCV neo-based retroviral vector, and amphotropic viruses were generated and used for infection as described previously (14). Cells were selected in the presence of 1000 μg/ml of G418 (Invitrogen). Adenoviral vectors encoding β-galactosidase and IκB superrepressor (DN-IκBα) were kindly provided by Dr. Richard Gaynor (University of Texas Southwestern Medical Center).

Western Blot Analysis—Western blot analysis was performed essentially as described previously (14). Primary antibody dilutions used in these experiments were FLAG (sc-807, 1:5000; Santa Cruz Biotechnology), α-IκB (SC-371, 1:2000; Santa Cruz Biotechnology), α-LacZ (SC-371, 1:2000; Santa Cruz Biotechnology), and p-IκBα (92418, 1:1000; Cell Signaling).

Soft Agar Assays—Rat-1 cells expressing an empty vector or vFLIP K13 were overlaid as a single cell suspension of 1500 cells in 1 ml of 0.4% Bacto-agar onto a 3.5-cm tissue culture dish containing a 0.6% agar base. All agar media were made with Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, penicillin/streptomycin. Triplicate plates were prepared for each tested cell line and inspected.
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RESULTS

Generation of Rat-1 Cells with Stable Expression of vFLIPs—In order to study the effect of HHV8 vFLIP on cellular transformation, we used retrovirus-mediated gene transfer to generate a polyclonal population of Rat-1 cells with stable expression of FLAG epitope-tagged HHV8 vFLIP (K13-FLAG) or an empty vector. Following infection with the respective retroviruses, colonies were selected with G418 (Geneticin) and pooled to generate a polyclonal population of Rat-1 cells.

Equine herpes virus 2-encoded vFLIP E8 and molluscum contagiosum virus-encoded vFLIP MC159L served as controls. Expression of the transduced proteins was confirmed by Western blot analysis with the FLAG antibody (Fig. 1).

Southern Blot Analysis—Genomic DNA was isolated using the DNeasy tissue kit (Qiagen, Valencia, CA) and digested with EcoRI, which cuts once within the retroviral vector. Southern blot analysis was performed using ExpressHyb hybridization solution (BD Biosciences Clontech, Palo Alto, CA). The blot was hybridized with 32P-labeled, full-length K13 probe according to the manufacturer’s instructions.

For colony formation after incubation at 37 °C for 14 days. To test the effect of inhibitors of the NF-κB pathway on anchorage-independent growth, Rat-1 vFLIP K13 cells were plated at a density of 1500 cells per 3.5-cm plate in soft agar in the presence of inhibitor or Me2SO alone, and colony number was scored on day 14.

Tumorigenicity Assays—Rat-1 cells expressing the empty vector and vFLIP K13 were trypsinized, washed with phosphate-buffered saline, and resuspended in phosphate-buffered saline. 5 × 10⁶ (200 μl) cells were injected in the flanks of 8–10-week-old female nude mice (NCr/nu, Taconic Farm, Germantown, NY). Mice were monitored for 6 weeks following injection, at which time they were sacrificed, and the tumors were resected for histological examination.

Luciferase Reporter Assay—Luciferase reporter assay was performed as described previously (14). Rat-1 cells stably expressing empty vector or different vFLIP were transiently transfected with an NF-κB luciferase reporter construct and a synthetic Renilla luciferase reporter vector (pGL3-TK, Promega, Madison, WI) by using LipofectAMINE PLUS™ reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase activity was normalized relative to the Renilla luciferase activity to control for the difference in the transfection efficiency.

Southern blot analysis with the FLAG antibody confirmed expression of the transduced proteins. Expression of the transduced proteins was confirmed by Western blot analysis with a rabbit polyclonal antibody against the FLAG epitope tag. We have consistently observed that vFLIP E8 is expressed at a relatively low level as compared with K13 and MC159L, respectively. N.S., nonspecific band.

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growth-inhibitory stimuli. As such, we tested whether expres-
sion of vFLIP K13 confers on Rat-1 cells the ability to overcome
contact-mediated block to cellular proliferation. As shown in
Fig. 3A, Rat-1 cells expressing empty vector and vFLIP K13. Focus formation is readily apparent in a monolayer culture of vFLIP K13-expressing
Rat-1 cells after 10 days. B–D, soft agar colony formation. B, representative photomicrographs of colony formation following a 2-week incubation of Rat-1 empty vector and vFLIP K13 cells in soft agar. C, the number of colonies in soft agar per 1000 cells was counted following a 2-week incubation of Rat-1 cells stably expressing an empty vector or the indicated vFLIPs. D, Rat-1 cells were infected with retroviruses encoding an empty vector, vFLIP K13, or vFLIP E8, and 24 h postinfection they were plated in soft agar without prior drug selection. The values shown represent the number of colonies in soft agar per 1000 cells following a 2-week incubation. E, Southern blot analysis. The genomic DNA was isolated from Rat-1 cells expressing an empty vector, mass population of Rat-1 K13 cells, three independent subclones isolated from soft agar colonies, and tumor-derived Rat-1 K13 cells. DNA was digested with EcoRI, which cleaves once within the retrovirus vector. The blot was hybridized with 32P-labeled full-length K13 probe according to the manufacturer's instruction (Clontech). N.S., nonspecific band.

Anchorage-independent growth is another characteristic fea-
ture of the transformed cells. We used a soft agar colony for-
mation assay to test the ability of various vFLIPs to confer
anchorage-independent growth. As shown in Fig. 3, B and C,
Rat-1 cells transduced with the empty vector failed to form any
colony in soft agar, whereas those expressing HHV8 vFLIP K13
formed large colonies with high frequency. Rat-1 cells expressing
E8 and MC159L vFLIPs failed to form any colony in soft
agar (Fig. 3C), suggesting once again that the transforming
property is unique to the HHV8-encoded vFLIP.

**Fig. 3.** HHV8 vFLIP K13 induces transformation of Rat-1 cells. A, shown are phase-contrast micrographs (original magnification, ×100) of Rat-1 cells expressing an empty vector and vFLIP K13. Focus formation is readily apparent in a monolayer culture of vFLIP K13-expressing
Rat-1 cells after 10 days. B–D, soft agar colony formation. B, representative photomicrographs of colony formation following a 2-week incubation of Rat-1 empty vector and vFLIP K13 cells in soft agar. C, the number of colonies in soft agar per 1000 cells was counted following a 2-week incubation of Rat-1 cells stably expressing an empty vector or the indicated vFLIPs. D, Rat-1 cells were infected with retroviruses encoding an empty vector, vFLIP K13, or vFLIP E8, and 24 h postinfection they were plated in soft agar without prior drug selection. The values shown represent the number of colonies in soft agar per 1000 cells following a 2-week incubation. E, Southern blot analysis. The genomic DNA was isolated from Rat-1 cells expressing an empty vector, mass population of Rat-1 K13 cells, three independent subclones isolated from soft agar colonies, and tumor-derived Rat-1 K13 cells. DNA was digested with EcoRI, which cleaves once within the retrovirus vector. The blot was hybridized with 32P-labeled full-length K13 probe according to the manufacturer's instruction (Clontech). N.S., nonspecific band.
In order to rule out the possibility that the transformed phenotype of vFLIP K13 expressing Rat-1 cells is due to inser-
tional mutagenesis/secondary mutation during the selection of
a stable population of cells, we infected Rat-1 cells with retro-
viruses encoding an empty vector, vFLIP K13, or vFLIP E8 and
after 24 h plated them in a soft agar assay without prior drug
selection. As shown in Fig. 3
D
, Rat-1 cells infected with vFLIP
K13 readily formed colonies in soft agar, whereas those in-
fected with empty vector or vFLIP E8 failed to do so. In order
to provide additional evidence that the transformed phenotype
of Rat-1 K13 cells is not due to insertional mutagenesis and
overgrowth of a mutant clone, we used Southern blot to analyze
the site(s) of proviral integration in the mass population of
Rat-1 cells. Furthermore, the three independent soft agar col-
ones demonstrated distinct sites of provirus integration, indicat-
ing that they arose from distinct clones. Taken together, the
above results strongly argue against the possibility that trans-
formed phenotype of Rat-1 K13 cells is due to insertional mu-
tagenesis/secondary mutation followed by selection and over-
growth of a mutant clone.

Tumorigenic Potential of Rat-1 Cells Expressing HHV8
vFLIP K13—After confirming the in vitro transforming ability
of HHV8 vFLIP, we were interested in testing whether expres-
sion of this protein confers tumorigenic potential on cells in
vivo. For this purpose, Rat-1 cells expressing an empty vector
or HHV8 vFLIP K13 were injected subcutaneously into nude
mice, and their ability to form tumors was analyzed. We ob-
served tumor formation in all five mice injected with Rat-1
vFLIP K13 cells, whereas none of the mice injected with the
Rat-1 vector cells developed tumors (Fig. 4,
A
 and
B
). Expres-
sion of vFLIP K13 was readily detected in the freshly dissected
tumors (Fig. 4
C
). The tumors had histomorphological features
of fibrosarcoma and were composed of plump spindle cells with
relatively abundant eosinophilic cytoplasm (Fig. 4,
D
 and
E
).
Cells had atypical morphology with vesicular nuclei, prominent
nucleoli, and abundant mitotic figures. Taken together with
the in vitro studies, the above results demonstrate that HHV8
vFLIP is an oncogene that triggers intracellular signaling
pathways leading to cell transformation and tumorigenicity.

It is conceivable that the tumorigenic potential of vFLIP
K13-expressing Rat-1 cells is due to their ability to block death
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periment, we compared the tumorigenic potential of Rat-1
vFLIP K13 cells with those expressing vFLIP E8, which is
known to block death receptor-induced apoptosis (10–12). As
shown in Table I, whereas eight of nine animals injected with
Rat-1 vFLIP K13 cells developed tumors, none of the animals
injected with Rat-1 cells expressing an empty vector or vFLIP
E8 did so.

In order to rule out the possibility that the tumorigenic
potential of Rat-1 K13 cells in nude mice is due to insertional
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**Fig. 5. Activation of the NF-κB activity in vFLIP K13-transformed cells.**

A. Electrophoretic mobility shift assay demonstrating persistent NF-κB activation in Rat-1 cells expressing vFLIP K13. The position of the induced NF-κB complex is marked by an arrow. The specificity of the induced complex is demonstrated by competition with excess cold NF-κB oligonucleotide or nonspecific (N.S.) oligonucleotides.

B. Rat-1 cells expressing different vFLIPs or an empty vector were transiently transfected with an NF-κB-responsive luciferase reporter construct along with a Renilla luciferase construct. Thirty-six hours after transfection, cells were harvested and assayed for luciferase activities. NF-κB reporter activity was normalized relative to Renilla luciferase activity to control for the difference in transfection efficiency. The values shown are averages (mean ± S.E.) of a representative of three independent experiments in which each transfection was performed in duplicate. C. Western blot analysis, demonstrating increased phosphorylation (top panel) and a decrease in the total IκBα protein in two subclones of K13-expressing Rat-1 cells generated from soft agar colonies (Fig. 5C).

**HHV8 vFLIP Activates the NF-κB Pathway in Rat-1 Cells**—We and others (14, 19) have previously demonstrated constitutive NF-κB activation in HHV8-infected PEL cell lines. We have also demonstrated that HHV8-encoded vFLIP K13 can activate the NF-κB pathway, whereas the E8 and MC159L vFLIPs lack this property (13, 14). We were therefore interested in checking whether the differential ability of these vFLIPs to induce cellular transformation could be attributed to their differential ability to activate the NF-κB pathway in Rat-1 cells. As shown in Fig. 5A, an electrophoretic mobility shift assay demonstrated significant NF-κB-binding activity in Rat-1 cells expressing vFLIP K13 as compared with those expressing an empty vector or vFLIPs E8 and MC159L. Constitutive NF-κB activation in vFLIP K13-expressing Rat-1 cells was further confirmed by transfection of an NF-κB luciferase reporter construct. As shown in Fig. 5B, this assay demonstrated significant NF-κB transcription activity in Rat-1 cells expressing HHV8 vFLIP as compared with those expressing empty vector or vFLIPs E8 and MC159L. NF-κB is usually present in the cytoplasm of cells in association with a family of inhibitory proteins, called IκB (20, 21). Cytokine-inducible phosphorylation of the IκB proteins leads to their rapid ubiquitination and proteasome-mediated degradation, which releases NF-κB from their inhibitory influence (20, 21). Consistent with the increased NF-κB-binding activity in vFLIP K13-expressing Rat-1 cells, we also observed an increase in the phosphorylated IκBα and a decrease in the total IκBα protein in these cells as compared with those expressing empty vector and E8 (Fig. 5C). In addition, we observed an increase in phosphorylated IκBα and a decrease in total IκBα in two subclones of K13-expressing Rat-1 cells generated from soft agar colonies (Fig. 5C).

**HHV8 vFLIP Induces Cellular Transformation via the NF-κB Pathway**—In order to test the hypothesis that vFLIP K13 induces cellular transformation via the activation of the NF-κB pathway, we took advantage of known inhibitors of this pathway. As shown in Fig. 6A, infection of Rat-1 vFLIP K13-expressing cells with an adenovirus encoding a phosphorylation-resistant dominant-negative form of IκBα (IκBα superrepressor), which is known to block NF-κB activation via diverse stimuli (22, 23), led to significant inhibition of soft agar colony formation. We also studied the effect of various chemical inhibitors of the NF-κB pathway on cellular transformation induced by vFLIP K13. Lactacystin is a proteasome inhibitor that blocks NF-κB activation by blocking the degradation of IκB (24), three independent experiments in which each transfection was performed in duplicate. C. Western blot analysis, demonstrating increased phosphorylation (top panel) and a decrease in the total IκBα protein (middle panel) in a polyclonal population of Rat-1 cells expressing vFLIP K13 and two subclones (C1 and C2) generated from soft agar colonies. Blot was reprobed with a polyclonal antibody against actin (bottom panel) to show equal loading of all lanes.
whereas aspirin, phenylarsine oxide, and arsenic trioxide are believed to block NF-κB activation by blocking the IKK complex (25). As shown in Fig. 6B, significant inhibition of colony formation was seen upon treatment with all of the above compounds. Furthermore, treatment with phenylarsine oxide significantly blocked the focus formation and transformed phenotype of Rat-1 K13 cells grown in monolayer culture (Fig. 6C). Taken together with the inhibitory effect of DN-IκBα, the above results support an essential role of the NF-κB pathway in cellular transformation induced by vFLIP K13.

Transforming Ability of HHV8 vFLIP Correlates with Its Ability to Activate the NF-κB Pathway—We used site-directed mutagenesis to mutate several amino acids in the first death effector domain of vFLIP K13 that are conserved among the various death effector domain-containing proteins (Fig. 7A). The amino acid residues 58–60 and 67–69 were replaced with alanine residues in the constructs 58ECL/AAA and 67DLL/AAA, respectively (Fig. 7A). Transient transfection of these constructs into 293 cells along with an NF-κB reporter construct revealed that mutant 67DLL/AAA retained ~20% of the NF-κB activity of the wild-type protein, whereas mutant 58ECL/AAA has completely lost this activity (Fig. 7B). We next compared the ability of the wild-type and mutant K13 constructs to induce cellular transformation of Rat-1 cells using the soft agar colony formation assay. For this purpose, Rat-1 cells were infected with retroviruses encoding the various constructs and subsequently plated in soft agar. As shown in Fig. 7, C and D, Rat-1 cells infected with the wild-type K13 virus gave rise to a large number of colonies, most of which consisted of more than 100 cells. In contrast, an ~10-fold decrease in colony number was seen in cells infected with retroviruses encoding the 67DLL/AAA mutant protein (Fig. 7C). These colonies were also small in size as compared with those formed by the wild-type K13 protein (Fig. 7D). Finally, no significant soft agar colony formation was detected in cells infected with the mutant 58ECL/AAA, which lacks NF-κB activity (Fig. 7, C and D). Collectively, the above results demonstrate a strong correlation between the transforming ability of vFLIP K13 and its mutants with their ability to activate the NF-κB pathway and further support the hypothesis that NF-κB pathway may play an essential role in cellular transformation induced by this protein.

HHV8 vFLIP K13 Transforms Balb/3T3 Cells—We were next interested in finding out whether the transforming ability of vFLIP K13 was limited to Rat-1 cells. In order to test whether vFLIP K13 can transform additional cell lines, we used retrovirus-mediated gene transfer to generate a mass population of mouse Balb/3T3 cells with stable expression of an empty vector or vFLIP K13 (Fig. 8A). As shown in Fig. 8, B and C, Balb/3T3 cells expressing vFLIP K13 could form colonies in soft agar, whereas those an expressing empty vector failed to do so. Consistent with our previous results with Rat-1 cells, expression of vFLIP K13 in Balb/3T3 cells was also associated with constitutive activation of the NF-κB pathway as determined by an electrophoretic mobility shift assay (Fig. 8D).

**DISCUSSION**

In this report, we present evidence that HHV8 vFLIP K13 possesses several characteristics of oncogenes, such as the ability to promote increased cellular proliferation, loss of contact...
inhibition, anchorage-independent growth, and formation of tumors in nude mice. In addition to vFLIP, the HHV8 genome is known to encode for several transformation-related genes, such as K1, K9, K12, LANA, vIRF1, and G-protein-coupled receptor (vGPCR) (6). HHV8-encoded K1 protein is known to immortalize primary T cells and induce lymphoproliferative disease in a transgenic mouse model (26). Similarly, vGPCR has been shown to stimulate both cellular proliferation and angiogenesis in vitro and in transgenic mice (27, 28). Although HHV8 possesses a number of potential oncogenes, the transforming activity of the complete virus is relatively modest. A possible explanation for the above discrepancy may lie in the fact that a majority of HHV8-encoded oncogenes are not expressed in the cells latently infected with this virus (6). Importantly, vFLIP K13 is expressed in latently infected PEL cell lines and KS spindle cells, making it a good candidate for the transforming ability of HHV8 (6–8). However, it is conceivable that vFLIP K13 cooperates with the viral oncogenes expressed during the lytic phase in the pathogenesis of Kaposi’s sarcoma and lymphoproliferative syndromes observed in patients infected with HHV8.

We and others (14, 19) have previously demonstrated that

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**Fig. 7.** Transforming ability of HHV8 vFLIP mutants correlate with their ability to activate the NF-κB pathway. A, sequence alignment of various death effector domain-containing cellular and viral proteins. Identical amino acid residues are shaded dark, and homologous residues are shaded gray. Sites of point mutations generated in the vFLIP K13 are shown by asterisks. B, NF-κB activation by mutants of vFLIP K13. 293T cells were transfected with the empty vector or indicated constructs (100 ng/well) along with an NF-κB/luciferase reporter construct (75 ng/well) and an RSV/LacZ (β-galactosidase) reporter construct (75 ng/well). The values shown are averages (mean ± S.E.) of one representative experiment of three in which each transfection was performed in duplicate. C and D, soft agar colony formation by vFLIP K13 mutants. C, Rat-1 cells were infected with retroviruses encoding vFLIP K13-wt and the indicated mutants. 24 h postinfection, cells were plated in soft agar without prior drug selection. The values shown represent the number of colonies in soft agar per 1000 cells following 2 weeks of incubation. D, representative photomicrographs of colonies formed by the expression of wild-type and mutant K13 proteins following a 2-week incubation in soft agar.
HHV8-infected primary effusion lymphoma cell lines have constitutive NF-κB activation. In the present study, we demonstrate a key role of the NF-κB pathway in vFLIP K13-induced cellular transformation. The NF-κB pathway is also involved in the protective effect of K13 against growth factor withdrawal-induced apoptosis (29). Abnormal NF-κB activation has been previously implicated in the pathogenesis of several lymphoid malignancies. For example, Tax and latent membrane protein 1 are believed to play a key role in the transforming ability of the human T-cell leukemia virus-1 and Epstein-Barr virus, known to activate a number of genes involved in cell proliferation and apoptosis. Thus, it is conceivable that as in the case with Tax and latent membrane protein 1, vFLIP K13-induced NF-κB activation plays an important role in lymphoproliferative disorders seen in association with HHV8 infection.

The exact mechanism of cellular transformation induced by vFLIP K13 is not known at present. However, the NF-κB pathway is known to activate a number of genes involved in cell cycle progression and protection against apoptosis, such as cyclin D1, bcl2, bclXL, cell inhibitors of apoptosis protein, and X-linked inhibitor of apoptosis protein (30), and it is conceivable that some or all of these genes are involved in vFLIP K13-induced transformation. Furthermore, it is possible that vFLIP K13-induced NF-κB activation cooperates with additional signaling pathways activated by this protein as well as other HHV8-encoded proteins (e.g. K1, K9, K12, vIRF1, LANA, and vGPCR) to induce cellular transformation.

At the time of their discovery, it was speculated that the main biological function of vFLIPs is to function as inhibitors of caspase-8 activation and thereby protect the virally infected cells from apoptosis induced by the death receptors (10–12). It can be argued that the tumorigenic potential of vFLIP K13 is not related to its transforming ability but is due to increased apoptotic resistance against the residual immune system of nude mice. However, several lines of evidence argue against this hypothesis. First, the tumorigenic potential of vFLIP K13 expressing cells in nude mice is associated with several in vitro properties commonly seen in transformed cells, such as increased cellular proliferation, lack of contact inhibition, and anchorage-independent growth in soft agar. It is highly unlikely that the above attributes of K13-expressing cells are due to its antiapoptotic property. Second, the ability of vFLIP K13 to transform Rat-1 cells is not shared by other vFLIPs. For example, E8 and MC159L, two vFLIPs known to block death receptor-induced apoptosis, failed to induce cellular transformation and/or form colonies in nude mice. Third, we have demonstrated that K13-induced cellular transformation is associated with NF-κB activation, a signaling pathway that, as discussed above, has been implicated in cellular transformation and tumorigenesis by a number of other viral proteins (17).

Fourth, we have previously demonstrated that, unlike cFLIP, vFLIP K13 has no significant protective effect against death receptor-induced apoptosis (29, 31). Furthermore, a recent study found that vFLIP K13 primarily interacts with proteins involved in NF-κB activation and did not detect any interaction with proteins involved in death receptor-induced caspase activation (15). Thus, taken together, our data suggest that vFLIP K13 can induce cellular transformation and tumorigenesis via activation of the NF-κB pathway and independent of its effect on death receptor-induced apoptosis.

Recent studies, based on the genetic and molecular analysis of the processes of cell division and apoptosis, suggest that cell proliferation and apoptosis are coupled; the tendency of cells to undergo apoptosis is a normal consequence of engaging the cell’s proliferative machinery (32, 33). Thus, cellular transformation by a number of oncogenes is frequently coupled to increased sensitivity to apoptosis. For example, whereas both MYC and adenoviral E1A protein promote cellular proliferation, they are also powerful inducers of apoptosis, especially under conditions of stress, depleted survival factors, and genotoxic stress (32, 34–36). It is believed that this innate apoptotic potential of oncogenes serves as an in-built foil to their tumorigenic capacity and limits the potential size of tumors induced by them (32, 33). In this context, we point out that we recently demonstrated that HHV8 vFLIP possesses the unique ability to protect TF-1 leukemia cells against growth factor withdrawal-induced apoptosis by up-regulating the expression of several antiapoptotic proteins (29). This protective effect was dependent on NF-κB activation and independent of its protective effect against death receptor-induced apoptosis (29). Therefore, it is conceivable that in addition to inducing cellular transformation, the K13-induced NF-κB pathway might also promote tumor growth by protecting the transformed cells against apoptosis by up-regulating the expression of antiapoptotic genes.

Consistent with the above hypothesis, expression of HHV8 vFLIP in murine B lymphoma cells was shown to promote tumor development and progression when injected into immunocompetent mice, thereby establishing it as a new class of tumor progression factor (37). Thus, HHV8 vFLIP might con-
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