Human Herpesvirus 8 K1-Associated Nuclear Factor-kappa B-Dependent Promoter Activity: Role in Kaposi’s Sarcoma Inflammation?

Felipe Samaniego, Shibani Pati, Judith E. Karp, Om Prakash, Debashish Bose

**Background:** The growing number of human immunodeficiency virus type 1 (HIV-1) infections worldwide and the increasing use of immunosuppressive modalities for organ transplantation have contributed to an epidemic of Kaposi’s sarcoma (KS), which has been etiologically linked to human herpesvirus 8 (HHV8) or KS-associated virus. Since the onset of the acquired immunodeficiency syndrome epidemic, inflammation has been recognized as an essential component of KS pathology. HHV8 bears a gene (K1) encoding a transmembrane protein with an immunoreceptor tyrosine-based activation motif. This motif is present in receptors that mediate inflammation. **Purpose:** To dissect the cellular effects of K1 function and the eventual role of K1 in KS, we developed a cell model for studying K1 expression. **Methods:** K1 was cloned from BC-3 lymphoma cells. To monitor transcriptional activation, K1 was coexpressed with plasmids containing luciferase under control of various promoters. K1 expression was monitored by indirect immunofluorescence and by combined immunoprecipitation/immunoblot analysis. Inflammatory cytokines were measured by enzyme-linked immunosorbent assay. **Results:** Cellular transfection of the K1 gene induced reporter expression under control of nuclear factor-kappa B (NF-κB), which controls the transcription of numerous proteins involved in inflammation. Treatment of cells with aspirin, an agent that targets this intracellular pathway and blocks cell inflammatory responses, blocked K1-induced NF-κB-dependent promoter activity. When a second KS cofactor, i.e., the HIV-1-transactivating gene tat, was coexpressed with K1, we observed an additive effect on NF-κB-dependent transcription. K1 transfection stimulated the secretion of cytokines interleukin (IL) 6, granulocyte-macrophage colony-stimulating factor, and IL-12. Cells treated with the conditioned media of K1 transfectants exhibited similar characteristics of K1 transfectants, indicating that a paracrine loop was being activated. **Conclusion:** Thus, K1 may activate cells in which it is expressed, as well as other cells in a paracrine manner. K1 cooperates in signaling with HIV-1 Tat, suggesting that both of the proteins from these viruses converge to reach an enhanced level of inflammation that may underlie progressive KS. [J Natl Cancer Inst Monogr 2000;28:15–23]

Kaposi’s sarcoma (KS) can be a lethal disorder that preferentially occurs in clinical settings of altered immunity, as is found in human immunodeficiency virus type 1 (HIV-1)-infected individuals and in recipients of solid-organ transplants. The growing number of cases of HIV-1 infection worldwide and the increasing use of immunosuppressive drugs for organ transplantation are accompanied by a concomitant increase in cases of KS. Human herpesvirus 8 (HHV8) has been etiologically implicated in KS, and the presence of HIV-1 further increases the rates of KS by more than 20 000-fold (1–5). HIV-1 infection appears to contribute directly to KS because other retroviruses (i.e., human T-cell leukemia/lymphoma virus) that also induce immunosuppression do not predispose individuals to develop KS. In addition, other contributing factors have yet to be identified because the vast majority of the world’s population of HHV8-infected persons, including those from geographic regions with high (>50%) seroprevalence, do not develop KS (6–8).

HHV8 is a recently isolated gamma herpesvirus that is related to the tumorigenic viruses herpesvirus saimiri and Epstein-Barr virus. These viruses are associated with tumor induction, particularly during immunosuppression. Their genomes contain numerous human oncogene homologues, such as cyclin D, immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling proteins (see below), bcl-2, and cytokine homologues of monocyte inflammatory protein I (MIP-I) and MIP-II, thereby making them resourceful models for comparative studies of viral oncogenesis (9,10).

The unique hyperplastic features of KS lesions have led investigators to conclude that these lesions are distinctly different from tumors of neoplastic cells. The early stages of KS are characterized by the presence of activated endothelial cells (ECs), inflammatory cell infiltration, spindle-shaped cells of vascular origin, and angiogenesis (11,12). The spindle cell population is dominated by activated ECs and macrophages (13,14) that over time proliferate to become the predominant cell phenotype.

We have shown that inflammation is a feature of KS and that inflammatory cytokines are necessary for maintaining the spindle cell phenotype found in these lesions. ECs and monocytes are considered to be the progenitors of KS spindle cells because they acquire the spindle cell morphology, marker expression, and functional features of KS spindle cells when exposed to inflammatory cytokines (tumor necrosis factor-α, interleukin [IL] 1β, and interferon gamma) (15–17). Inflammatory cytokines induce ECs to produce (30-fold) more basic fibroblast growth factor and vascular endothelial growth factor that are essential for generating the angiogenic features of KS lesions (15,16,18,19). Inflammatory cytokines also render primary ECs with a capacity to induce angiogenic KS-like lesions when im-

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planted in nude mice. Moreover, inflammatory cytokines also increase the level of HHV8 DNA in blood-derived cells (11,20). Taken together, inflammation plays a key role in the development of many of the features of KS, and it is possible that the local source for inflammation may stem from the expression of the HHV8 K1 gene (see below).

HIV-1 contributes directly to KS development through production of HIV-1 Tat (21–23). Tat is essential for viral gene expression, yet it also exhibits the capacity to exit live cells, disseminates systemically, and enters other cells where it can activate latent HIV-1 and promoters of other genes (i.e., transforming growth factor-β and tumor necrosis factor-α) (24). Mice made transgenic for tat express the protein in blood and show preferential KS tumor growth when inoculated with KS cells (21,25,26). Thus, Tat is a systemically distributed viral cytokine that can participate in inflammation and tumor promotion.

Recipients of solid-organ transplants, especially renal transplant patients, are at increased risk of developing KS. It is believed that immune stimulation from engrafted allogeneic tissue and challenges by microbes in the setting of drug-induced immunosuppression provide the necessary conditions that would promote tissue inflammation.

Multiple studies, including our current data, suggest that HHV8 infection may play a role in host cell activation and proliferation to produce KS lesions. Even though a minority (approximately 5%) of KS spindle cells express HHV8 lytic-phase genes, expression of selected HHV8 genes may influence host cells sufficiently to induce diffusible inflammatory cytokines in a paracrine manner. It is interesting that EC proliferation with HHV8 infection has been demonstrated by Flore et al. (27). Despite the fact that only 5% of the ECs were infected with HHV8, all cells, including uninfected cells, exhibited an extended replicative life span beyond senescence with acquired telomerase activity, thereby indicating a potent paracrine effect of HHV8 (27). Also, HHV8 and ECs inoculated under the human skin of a human skin severe combined immunodeficiency (SCID) mouse chimera produced angiogenic lesions similar to human KS (28).

Among HHV8 genes, K1 is a promising candidate for mediating activation signal pathways based on its characteristic cytoplasmic motif (29,30). K1 contains a cytoplasmic ITAM (31). ITAMs are contained in subunits of multiprotein complexes, B-cell receptor, and T-cell receptor that are critically involved in inflammatory responses (32). Thus, we anticipate K1 to signal in and activate inflammation-related pathways by mimicking the functions of host ITAM proteins (33).

The ITAM of K1 has been shown to transmit signals when this motif is tested as a chimeric protein fused to the extracellular domain of CD8 (31). This construct, however, may not reliably reflect native K1 function because the vast majority of this fused protein is CD8. ITAMs can also exhibit paradoxical effects that clearly depend on the position on the polypeptide. One isolate of K1 (clade 3A) has been shown to stimulate nuclear factor of activated T-cell (NFAT)-dependent promoter activity (30). Because ITAM-dependent signaling typically stimulates several types of promoters and because multiple isolates of K1 exist, we sought to examine 1) whether other isolates of K1 are active in promoter activity, 2) whether K1 expression would stimulate promoters of other types, 3) whether K1 is expressed in KS tumor and cell lines, and 4) whether K1 cooperates with other factors in promoting inflammation of KS.

METHODS
Cloning

The open reading frame K1 of HHV8 was cloned by polymerase chain reaction (PCR) from DNA of BC-3 cells (provided by E. Cesaram, Cornell Medical College, New York, NY) (34,35). Thirty-five cycles (95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 90 seconds) were performed by PCR (1) with the use of the AmpliTaq Gold PCR Kit (The Perkin-Elmer Corp., Foster City, CA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 0.001% (wt/vol) gelatin and oligomers (5’-GACGGATCCACACGTGTGAGGACATCCTG-3’ and 5’-TTTATATGAAAATATCAGCCCTAGGGTG-3’). Gel electrophoresis was performed, and a single prominent band of separated PCR products of approximately 1 kilobase (kb) was extracted from the gel and cloned into pcR2.1 with the use of TA cloning (Invitrogen Corp., Carlsbad, CA). The insert was sequenced. The BamHI fragment containing K1 was cloned into pSG5 and pCR3.1. To epitope tag K1, we generated a fusion construct in pcR3.1 by PCR with the use of pcR2.1K1 and with oligomers 5’-CACAAAGCTTGCGAATTCATGTTCCTG-TATGTGTTTGCAGTCCTGG-3’ and 5’-GATATCCGAGATCCCTACAGATCTTCTTCAAGAAAAATTTTTGT-TCGTACAACTCCTAGGGTGTA-3’ that directed the addition of the DNA coding for c-myc epitope that is recognized by monoclonal antibody 9E10: EQKLISEEDL. Constructs were sequenced to confirm DNA sequence.

Cells and Transfection

KS-1 and BC-3 cells are HHV8-infected lymphoma cells derived from primary effusion lymphoma (provided by H. P. Koefler, University of california at Los Angeles, and E. Cesaram) (36,37). They were propagated in RPMI-1640 medium with 15% fetal bovine serum and supplemented with 1 mM glutamine, penicillin G (100 U/ml), and streptomycin (100 mg/ml). Cos-1 (American Type Culture Collection, Manassas, VA) cells were selected for studying K1 expression because of their ease of transfection and their support of high-level plasmid-driven expression. KS Y-1 cells are transformed KS-derived cells isolated from an HIV-1-infected individual with KS (38). KS Y-1 cells exhibit characteristics of KS spindle cells and, like other KS-derived cell lines, lack HHV8 DNA. Cos-1 cells and KS Y-1 cells were transfected (4 × 106) with the use of the Fugene Transfection Reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN), and, after 48 hours, 20 µg of cell extract was mixed with the luciferase assay reagent (Promega Corp., Madison, WI) and light emission was measured over a 15-second period on a luminometer (Turner Designs, Sunnyvale, CA). Because cell stress and various stimuli (e.g., oxidation stress and high serum levels) may contribute to a nuclear factor-kappa B (NF-κB) transcriptional response, we optimized the assay conditions by minimizing nonspecific procedure-related cell activation (in RPMI-1640 medium with 0.5% fetal bovine serum) and the minimal amount of plasmid DNA (1–2 µg) to elicit a nuclear transcription response. In other studies, transgene expression was accomplished by mixing 107 cells with a total of 30 µg of total plasmid and electroporation of cells (0.28 V, 25 µF) on the Bio-Rad Electroporator (Bio-Rad Laboratories, Hercules, CA). Assays were done in triplicate, and the means were reported. A second plasmid, using simian virus 40 promoter to direct expression of green fluorescent protein (pGreenLantern; Stratagene, San Diego, CA).
gene, La Jolla, CA), was used to compare transfection efficiencies. Reporter plasmids used in these studies were pAP-1-Luc and pNFκB Luc (Stratagene). Test plasmids pCR3.1K1, pCR3.1K1myc, and pSG5K1myc contained K1 from BC-3 cells. Control plasmid pFC-MEKK, which directs expression of extracellular-regulated kinase kinase (MEKK), was used as a potent intracellular stimulus for NF-κB activation (Stratagene). To begin to define the pathway K1 uses in activation, we determined whether NF-κB transscriptional activity was sensitive to the blocking effects of aspirin (1 mM) or cyclosporin (1 μM) that are known to abrogate NF-κB signaling (39). After transfection, cells were refed media with or without each inhibitor and incubated for another 24 hours. Whole-cell extracts were made, and 20 μg was added to the luciferase reagent and emissions were assayed on luminometer. Cells transfected with vector alone and cells transfected with pFC-MEKK served as negative and positive controls, respectively.

Immunofluorescence Staining and Cytokine Levels

Immediately after transfection with pSG5K1myc or pSG5, cells were seeded on 12-well slides (Erie Scientific, Portsmouth, NH) and incubated in RPMI-1640 medium with 10% fetal bovine serum. The cells were washed, air-dried, fixed with acetone at −20°C, and stained by indirect immunofluorescence. The slides were dried and permeabilized with phosphate-buffered saline, 1% bovine serum albumin, 0.1% Tween, and 5% wt/vol sucrose. The cells were treated with primary antibody 9E10 (1:100) and secondary antibody anti-mouse fluorescein isothiocyanate-conjugated antibody (1:50) (mixed with Evans blue) each for 0.5 hour at 37°C. The cells were rehydrated, and anti-fade solution (Molecular Probes, Inc., Eugene, OR) was applied before sealing with coverslips. Representative cell fields were captured on 1600 film. Cells transfected with plasmid pSVK3-βcatenin-myc were used as a positive control (D. Sussman, University of Maryland). After transfection, cells were refed, and the medium was collected after 24 hours. The supernatants were handled with the use of plastic ware precoated with 0.1% bovine serum albumin in phosphate-buffered saline to avoid adherence of cytokines to surfaces. Supernatants were frozen at −80°C before measurement by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Reverse Transcription (RT)-PCR and Northern Blot Analysis

Tissue from human KS was obtained after diagnostic evaluation was completed and after consent was signed, according to the policy of our Institutional Review Board. Tissue samples were snap-frozen in a dry ice/methanol bath, and total RNA was isolated with the use of Trizol according to the instructions of the manufacturer (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Cultured cell RNA was isolated with the use of the Trizol reagent. RT was performed with the Titan RT–PCR Kit (Boehringer Mannheim Biochemicals) with the use of avian myeloblastosis virus, RT, a Taq/Pwo DNA polymerase blend, and 5′-TTTGTTGCCCCTAGAGTGGTT-3′ and 5′-TGACTGTTGTTGATGTTGGT-3′. For northern blots, 15 μg of total-cell RNA was separated in 0.8% formaldehyde gel and transferred to a nylon membrane. The K1 DNA sequence was labeled by the random primer method and was used for northern blot hybridization at 42°C, and washing was done at 45°C in 1 x standard saline citrate/0.1% sodium dodecyl sulfate (SDS).

Immunoprecipitation/Immunoblot Analysis

Cos-1 cells were electroporated with pSG5K1myc or with pSG5. After 48 hours, cell extracts were made in 1% Nonidet buffer (40). Immunoprecipitation was conducted with anti-myc antibody (1 μg) and rabbit anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA, and ICN, Costa Mesa, CA), respectively, and protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) and tumbled for 12 hours at 4°C. Immunoprecipitants were size-separated on an 8% SDS polyacrylamide gel and transferred to nitrocellulose. Blotting was carried out with anti-myc antibody (1 μg/mL), and a duplicate filter was blotted with antiphosphotyrosine (4G10) (0.5 μg/mL) (Upstate Biotechnology, Lake Placid, NY), and the signal was read with the use of chemiluminescence (Amersham Pharmacia Biotech).

Statistical Analysis

Where appropriate, the means are shown with standard deviation. Student’s t test was applied to estimate the statistical significance of the mean differences (41). All P values are two-sided.

RESULTS

To determine whether K1 might play a role in the clinical manifestations of KS, we analyzed human KS for the presence of K1 RNA. RT–PCR was performed on total RNA isolated from a KS lesion from an HIV-1-infected individual. RT–PCR analysis showed a product of approximately 280 base pairs (bp), and the band was not generated by PCR after treatment of the template with ribonuclease (RNase) A (Fig. 1, A). The band remained after treatment of the templated RNA with deoxyribonuclease (DNase) I (not shown). RNA isolated from 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated BC-3 cells (reported to contain approximately 40 viral genomes per cell during latency) used as a positive control showed a more intense signal. Thus, K1 is expressed in human KS, and TPA enhances its RNA levels in cell lines. These results, combined with recent evidence that HHV8-infected humans develop cytotoxic T-cell lymphocyte responses targeting K1 (42), indicate that K1 is expressed at the protein level in the course of HHV8 infection.

Because viral gene expression is anticipated to be tightly regulated, particularly in chronic viral infection, and viral transcripts are produced, we attempted to stimulate and characterize K1 gene expression of latent HHV8-infected cells. Cellular RNA of TPA-treated KS-1 and BC-3 cells was subjected to northern blot analysis with the use of K1 DNA as the labeled probe. Transcripts of 1.3 and 3.6 kb were observed to be either induced or enhanced with TPA treatment (Fig. 1, B). The smaller band corresponds to the length of a K1 transcript, whereas the higher molecular weight transcript likely represents a complex transcript encompassing K1 (43,44). Ethidium bromide staining of 28S ribosomal RNA served to indicate the relative levels of RNA loading. Thus, K1 is expressed in these cells, consistent with lytic-phase gene expression in vitro and by extrapolation during lytic-phase HHV8 expression in human KS.

Of the HHV8 genes considered as candidates for mediating inflammation in KS, K1 is a promising candidate. The K1 protein sequence exhibits a highly conserved cytoplasmic ITAM that is involved in inflammation (45,46). We cloned K1 (GenBank accession No. AF170531) from the primary effusion lymphoma-derived cell line BC-3, which is chronically infected with
HHV8 (see below). The predicted protein sequence from the K1 DNA from BC-3 cells reveals a transmembrane domain protein with an extracellular immunoglobulin light chain-like domain, two hypervariable regions (29), and a short cytoplasmic tail that contains an ITAM (YxxLxxxxxxYxxL) (Fig. 1, C). Amino acid sequence shared by clades A and C is shown, and the ITAM is identified. The rightmost leucine (L) is substituted by proline (P) in K1. K1’s highly variable protein sequence can be classified into several clades A-D (BC-3 is C); however, thus far, a clade-specific clinical entity or pathology has not emerged (29).

K1 DNA from BC-3 cells was tagged at its cytoplasmic car-

boxy terminus with a myc epitope that is targeted by 9E10 antibody. Also, to speed up the evaluation of K1 function, we performed initial studies on Cos-1 cells because of their relative ease of transfection and their reproducibly high level of expression of plasmids. These studies serve as the basis for further studies with KS-derived spindle cells (38). Expression of the tagged protein was confirmed by detection of K1myc protein in transfected cells (Fig. 2, A). Transfectants were stained with anti-myc and fluorescein isothiocyanate-conjugated second antibody, which localized activity to cell membrane in K1myc transfectants but showed no staining in vector transfectant controls (not shown). Transfection with a plasmid-directing expression of a cytosolic protein β catenin-myc showed activity in a different distribution and predominantly in dividing cells (Fig. 2, B).

For the investigation of a possible role for the ITAM-containing HHV8 K1 protein in inducing inflammation, K1 was expressed in Cos-1 cells, and cell extracts were analyzed for NF-κB-dependent promoter activity (Fig. 3, A). Cells expressing K1myc showed NF-κB-driven luciferase activity of 19 200 U and its vector control transfectant level of 4400 U (Fig. 3, A). Of interest, no cross-linking reagents were necessary for K1 to generate NF-κB-driven expression. Cells expressing MEKK displayed enhanced luciferase activity, whereas mock transfectants displayed luciferase activity comparable to vector transflectants (not shown). Transfection of plasmid-directing expression of green fluorescent protein showed equivalent cell transfection rates in K1 versus vector transflectants, indicating that the levels of transfection were similar regardless of the plasmid used. Transfection of cells with K1 that lacked a myc-tag produced similar NF-κB-dependent activation, indicating lack of interference from the myc epitope (not shown).

To determine if another NF-κB-related promoter could be activated by K1, we coexpressed K1 with plasmid containing luciferase driven by a promoter containing AP-1 sites. K1 expression showed a greater than twofold increase in AP-1-dependent luciferase activity (Fig. 3, B). To begin analyzing the effects of K1 expression in KS cells, we transfected pCR3.1K1myc into KS Y-1 cells along with the luciferase re-
porter construct. NF-κB-dependent luciferase activity in K1 transfectants was three times the activity observed in vector (control) transfectants (Fig. 3, C). In all cases, K1-stimulated activity was consistently higher than vector transfectant controls. Thus, K1 stimulates transcriptional activity at two promoter/enhancer sites, AP-1 and NF-κB, and suggests that KS cells are competent to handle ITAM-related signaling.

NF-κB plays a central role in the induction of numerous immunoregulatory responses, including expression of IL-1, IL-2, IL-3, IL-6, IL-8, tumor necrosis factor-α, and interferon gamma. KS Y-1 cells transected with K1 compared with vector transfectants showed 11-fold higher levels of IL-12 and significantly higher levels of IL-6 and granulocyte–macrophage colony-stimulating factor (P<.05) (Table 1). K1-transfected Cos-1 cells also showed enhanced IL-8 secretion. These cytokines bear NF-κB-response elements in their promoters (47). This cytokine production was not a generalized effect because K1 expression did not induce elevation of other inflammatory cytokines, such as RANTES, monocyte inflammatory protein 1 (MIP-I), and MIP-II. The cytokines induced by K1 are inducible by cell stimuli that operate through NF-κB (47), and the cytokines are also implicated in KS lesion formation (15–18, 20, 23).

We have hypothesized that the inflammation in KS lesions is mediated by a few lytically active cells that possess pervasive inflammatory effects on other cells. To evaluate the possibility of these paracrine effects, we examined the cells’ conditioned media for their ability to promote NF-κB-dependent activation. Cos-1 cells incubated with conditioned media of K1 transfectants exhibited enhanced NF-κB-promoter activity, indicating that K1 mediates a paracrine effect (not shown). Taken together, the secretion of numerous cytokines and a second wave of NF-κB activation would support the development of diffuse tissue inflammation present in KS lesions.

Table 1. Cytokines secreted by K1 transfectants

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vector transfectants, pg/mL</th>
<th>K1 transfectants, pg/mL</th>
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<tbody>
<tr>
<td>GM-CSF</td>
<td>66 (±8.5)</td>
<td>171 (±11.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>1587 (±18)</td>
<td>1870 (±42)</td>
</tr>
<tr>
<td>IL-8</td>
<td>32 (±28)</td>
<td>123 (±59)</td>
</tr>
<tr>
<td>IL-12</td>
<td>12 (±1)</td>
<td>132 (±5)</td>
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*Cells were transfected with pCR3.1K1myc or pCR3.1, and cell supernatants were collected at 24 hours. The cells’ conditioned media were analyzed for human cytokines by enzyme-linked immunosorbent assay (ELISA). Results are reported from Kaposi’s sarcoma (KS) Y-1 transfectants with the exception of the analysis of interleukin (IL)-8, which was done with Cos-1 cells with the use of ELISA for human IL-8. No statistically significant differences were noted in the levels of RANTES, monocyte inflammatory protein 1 (MIP-I), and MIP-II. The means of cytokine levels of granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8, and IL-12 of K1 transfectant supernatants were statistically significant (each P<.05).

Activation pathways can be blocked by specific therapeutic anti-inflammatory or immunoregulatory agents, such as aspirin and cyclosporin (48, 49). To determine whether aspirin or cyclosporin would block K1-triggered activation, we treated pCR3.1K1myc and pCR3.1 transfectants with either aspirin or cyclosporin for 24 hours after transfection. K1 transfectants demonstrated enhanced NF-κB-dependent activity over untreated vector transfectants (Fig. 4). Treatment of cells with aspirin blocked the K1-dependent promoter activity down to the activity of vector-alone transfectants, indicating selective blockage of inducible luciferase activity. Treatment of cells with cyclosporin also abrogated the K1-induced luciferase activity (Fig. 4). However, in contrast to aspirin treatment, the vector transfectants treated with cyclosporin also showed significantly lower luciferase activity, indicating that baseline activity was also affected. Thus, drugs that block inflammation and cell activation demonstrate the ability to block inflammation-related K1 signaling and further substantiate that K1 signals via host NF-κB-dependent pathways.

Because expression of the cell membrane-associated K1 activates NF-κB in the absence of an added ligand and viral receptors generally mimic activated host receptors, we surmised that K1 from BC-3 might be constitutively activated. ITAM-
receptor binding and/or aggregation is typically required for phosphorylation of ITAMs that constitutes an activated state. Therefore, we examined K1 for phosphorylation by combined immunoprecipitation/immunoblot analysis by transfecting Cos-1 cells with pSG5K1myc and detection with anti-myc antibody. Our studies show that K1 appeared as a protein of approximately 47 kilodaltons with apparent lower molecular weight diffuse bands (Fig. 5). K1 can be glycosylated, and the lower molecular weight bands likely represent incompletely glycosylated forms of K1 (50). No apparent multimers of K1 were observed in contrast to other studies (30,50). The bases for this difference may rest on the different clades of K1 or on the differences in methods of detection. Similar immunoprecipitation/immunoblot of β catenin-myc transfecteds showed an expected band of β catenin-myc, and vector-transfected Cos-1 cells lacked either of these bands (not shown). To determine whether the immunoprecipitated K1myc was also phosphorylated, we blotted a duplicate filter with anti-phosphotyrosine antibody and noted the appearance of a phosphoprotein approximating the size of the largest K1 band (Fig. 5). There was no phosphorylation signal in the region immediately below the K1 band, even after extended exposure of the film. Of interest, only the high-molecular-weight K1 band was phosphorylated (at the present level of detection), suggesting that only the completely processed K1 (glycosylated) is preferentially phosphorylated. This finding indicates that K1 that is myc-tagged can serve as a substrate for phosphorylation and is active in NF-κB-dependent transcription. Thus, even in the absence of a ligand, K1 is expressed as a phosphorylated protein, consistent with its being a constitutively activated protein capable of signaling through ITAM-dependent pathways.

Although HHV8 infection alone, without intercurrent illnesses, is sometimes associated with milder forms of KS, the presence of a second virus, i.e., HIV-1, usually dictates an aggressive clinical course of KS that parallels the activity of HIV-1. HIV-1 appears to stimulate KS cell proliferation indirectly by inducing inflammatory cytokine production, and this production depends, in part, on NF-κB-dependent promoters (51). To assess for a possible contribution of HIV-1 Tat to the ability of K1 to stimulate NF-κB-dependent transcription, we coexpressed K1 and Tat (52). Transfection of pCR3.1K1 or pCMVtat stimulated NF-κB-dependent luciferase activity of 490 and 380 arbitrary units, respectively, over vector-alone transfecants (Fig. 6). However, transfection with the combination of plasmids showed enhanced promoter activity in an additive fashion. Thus, in this model, two genes from disparate origins converge on NF-κB-dependent transcriptional activity in a cooperative fashion. In this regard, HIV-1 and HHV8 share signaling targets to bring about a cellular inflammatory response at a level not observed with each viral product acting alone.

**DISCUSSION**

The variable clinical course of KS and the histologic features of KS lesions suggest that the inflammatory process is central to the promotion of KS lesions. HHV8 may be the stimulus for
inflammation and, to that end, we have presented data implicating HHV8 K1 in activating pathways that operate in cell activation and inflammation. K1, like other ITAM-containing proteins, may provide cells with a critical signal that ultimately determines cell activation, at least, in part, by inducing NF-κB and AP-1-dependent promoter activity.

The ITAM from K1 can transmit signals when tested as a chimeric protein joined to the extracellular domain of a known receptor (CD8) (31). Even though signaling through ITAM can transmit inhibitory signals that are dependent on context, K1 ITAM fused to CD8 positively stimulated signaling (31,45, 53,54). More recently, full-length K1 from body cavity-based lymphoma-1 cells (clade A3) was shown to induce NFAT-dependent promoter activity (30,50). NFAT is another ITAM-dependent factor described in lymphocytes that regulates promoter-driven expression of proteins associated with inflammation and proliferation (55). Thus, K1 leads to activation of NFAT- and NF-κB-dependent promoter activities that, in turn, orchestrate the transcription of an array of proteins involved in cell activation and inflammation.

K1 is likely to activate pathways used by host cell ITAM-containing proteins. However, unlike the K1 protein product, which is constitutionally active, host cell ITAM-containing proteins generally reside in the resting state, and, on ligand binding or receptor aggregation, they undergo phosphorylation and become competent for signaling. ITAM-containing receptors include subunits of the B-cell receptor, T-cell receptor, and Fc receptor, which contain one or more copies of ITAMs within their cytoplasmic tails (56). Specifically, these ITAM-bearing proteins include human proteins TCR-ζ, CD3ζ, and FcεRI and viral proteins, such as HHV8 LMP2A-like protein, Hantavirus glycoprotein G1, bovine leukemia virus gp30, Epstein-Barr virus LMP2A, and rhesus monkey rhadinovirus R1 (32,45,53,57–61).

NF-κB-dependent signaling is modulated by viral proteins as well as by ligands and drugs that affect inflammation and cell activation. HIV-1 Tat is shown to contribute to NF-κB-dependent promoter activity that is additive to that of K1. Because HIV-1 infection substantially accelerates the aggressive course of KS, additive effects at NF-κB-dependent promoter activity may be one key mechanism in which HIV-1 and HHV8 converge to stimulate cell activation and inflammation and account for an aggressive course of KS in HIV-1 infection (62).

Although lymphocytes were first discovered and extensively studied for expression of ITAM receptors, other cells are known to harbor functional ITAM-bearing receptors as well. Indeed, monocytes and macrophages (MO/MC) contain ITAM-containing receptors, such as the immunoglobulin Fc receptors. In MO/MC, ligand binding activates Fc receptors to induce cell activation by expression of the IL-2 receptor and secretion of inflammatory cytokines (tumor necrosis factor-α and IL-12), which together can synergize in mounting a greatly amplified inflammatory response (63,64). The copious cytokines released in K1-expressing cells would predict that K1 expression in MO/MC would mount a wave of cytokine secretion and would contribute toward an overall activation of MO/MC. In KS tissue, despite the fact that most cells contain HHV8 in the latent phase, some lytic viral replication does occur in cells that share markers of activated MO/MC and ECs (CD68) (65). These cells are expected to express K1 as part of their lytic-phase expression program that would be implicated in KS inflammation (Fig. 7).

MO/MC, in particular the subendothelial MO/MC, have been noted to express substantial levels of activation markers, and their location in the subendothelium suggests that they play pivotal roles in transluminal trafficking.

The small percentage of cells in KS lesions that undergo lytic-phase replication are anticipated to express K1 and to dictate the inflammatory status of KS tissue. Therapy targeting herpesvirus in humans leads to regression of KS and lowers the frequency of KS development (66). Antiviral therapy in experimental models has reduced the levels of HHV8 in human SCID mouse models infected with HHV8 (14,33). In our cell model, we show that K1 stimulates NF-κB and that agents known to block inflammation or cell activation also block K1-mediated NF-κB-dependent promoter activity. Thus, the link between inflammation and HHV8 gene regulation and replication appears as a central event to the development of KS. In summary, K1, which functions by mimicking the activity of host ITAM proteins, is likely to be a major trigger for cell activation and inflammation in KS.

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NOTES

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