Identification of Kaposin (Open Reading Frame K12) as a Human Herpesvirus 8 (Kaposi’s Sarcoma-Associated Herpesvirus) Transforming Gene

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The recently identified human herpesvirus 8 (HHV-8, or Kaposi’s sarcoma-associated herpesvirus) has been implicated in the etiology of both Kaposi’s sarcoma (KS) and primary effusion (body cavity-based) lymphoma (PEL) (Y. Chang et al., Science 266:1865–1869, 1994; P. S. Moore et al., J. Virol. 70:549–558, 1996). An important feature of the association of HHV-8 with these malignancies is the expression of an abundant, latency-associated 0.7-kb transcript, T0.7 (W. Zhong et al., Proc. Natl. Acad. Sci. USA 93:6641–6646, 1996). T0.7 is found in all stages in nearly all KS tumors of different epidemiologic origin, including AIDS-associated, African endemic, and classical KS (K. A. Staskus et al., J. Virol. 71:715–719, 1997), as well as in a body cavity-based lymphoma-derived cell line, BCBL-1, that is latently infected with HHV-8 (R. Renne et al., Nat. Med. 2:342–346, 1996). T0.7 encodes a unique HHV-8 open reading frame, K12, also known as kaposin. In this study, we report that the kaposin gene induced tumorigenic transformation. Constructs with kaposin expressed either from its endogenous promoter or from a heterologous promoter induced focal transformation upon transfection into Rat-3 cells. All transformed Rat-3 cell lines containing kaposin sequences produced high-grade, highly vascular, undifferentiated sarcomas upon subcutaneous injection of athymic nu/nu mice. Tumor-derived cell lines expressed kaposin mRNA, suggesting a role in the maintenance of the transformed phenotype. Furthermore, kaposin protein was detected in transformed and tumor-derived cells by immunofluorescence and localized to the cytoplasm. More importantly, expression of kaposin protein was also detected in the PEL cell lines BCBL-1 and KS-1. These findings demonstrate the oncogenic potential of kaposin and suggest its possible role in the development of KS and other HHV-8-associated malignancies.

Kaposi’s sarcoma (KS) is a vascular tumor most commonly occurring in patients with AIDS (5). KS lesions are histologically complex and contain proliferating spindle-shaped cells considered to be of endothelial origin, infiltrating mononuclear cells, plasma cells, and abundant neovascular spaces (74). A new member of the herpesvirus family, human herpesvirus 8 (HHV-8), also known as KS-associated herpesvirus, has been identified in KS tumors from both human immunodeficiency virus (HIV)-positive (2, 14, 64) and HIV-negative (6, 49, 60) patients. HHV-8 sequences have also been been identified in several rare lymphomas such as multicentric Castleman’s disease and primary effusion lymphoma (PEL), also known as body cavity-based large-cell lymphoma (10, 11, 13). The seroprevalence of HHV-8 in the general population exhibits variations with geographic distribution. Very low rates of prevalence have been reported for populations in Britain and North America, whereas high rates prevail in Africa and southern Europe (25, 36, 62). However, antibody kinetic studies have shown that a strong correlation exists between conversion to seropositivity and the risk for development of KS (25, 34). Thus, HHV-8 has been proposed as the etiologic agent for KS and other HHV-8-associated malignancies (25, 47, 50).

The nucleotide sequence of the HHV-8 long unique region (LUR) has been determined from viral sequences isolated from the PEL cell line BC-1 (56). Of 81 open reading frames (ORFs), 66 have homology to those in herpesvirus saimiri (HVS) and 15 (K1 to K15) are unique to HHV-8. Moreover, cellular homologs related to known oncogenes have also been identified in HHV-8; these include the genes encoding Bcl-2, cyclin D, interleukin-6 (IL-6), G-protein-coupled receptor (GPCR), and ribonucleotide reductase (1a, 12, 15, 17, 48, 52, 59). Some of these homologs have been shown to enhance cell proliferation. The HHV-8-encoded v-IL-6 supported the growth of an IL-6-dependent murine cell line, B9 (52), while the expression of v-cyclin D resulted in the induction of the S-phase in serum-starved quiescent NIH 3T3 cells (66). Expression of the v-GPCR in rat kidney fibroblasts (NRK-49F cells) enhanced cell proliferation (3). More recently, it has been reported that signaling by v-GPCR, which is associated with a switch to an angiogenic phenotype, leads to transformation and tumorigenesis in NIH 3T3 cells (4). Among the HHV-8 unique K ORFs, only K9 has been shown to induce tumorigenic transformation of NIH 3T3 cells (24). However, because K9 expression was detected in PEL cells but not KS tumors, Gao et al. concluded that K9 may play a role only in B-cell malignancies (24).

Analysis of HHV-8 gene expression in KS tumor spindle cells and the PEL cell line BCBL-1 showed a highly restricted pattern of latent HHV-8 RNA expression (74), i.e., two abundant polyadenylated transcripts of 1.1 and 0.7 kb (T1.1 and T0.7). T1.1 encoded only short ORFs and was primarily local-
ized in the nucleus (74). Recently, Sun et al. (65) demonstrated that the T1.1 polyadenylated nuclear RNA did not associate with polyribosomes and therefore was not translationally active. On the other hand, T0.7 contained three small ORFs, one of which, ORF K12, also known as kaposin (56), encoded a highly hydrophobic 60-amino-acid (aa) peptide. The hydrophobicity of kaposin is similar to that of the 45-aa E5 oncoprotein of bovine papillomavirus type 1 (BPV-1) (7, 61). T0.7 expression was observed in K5 tissue of all stages from earliest identifiable to advanced tumors (63). Moreover, in advanced KS tumors, approximately 85% of spindle cells expressed T0.7 RNA.

Due to the fact that T0.7 is an abundant, latency-associated transcript retained in K5 and PEL cell line BCBL-1, its transforming ability was tested and compared to that of HHV-8 fragments containing several other unique ORFs, including K4, K5, K6 (pBS/17), and K8 (pBS/199). Each of these three constructs encoding kaposin, including (i) pBS/23, a 4.4-kbp HHV-8 genomic fragment containing the T0.7 sequence cloned in the pBS vector, (ii) pBK/T0.7, the T0.7 sequence cloned in the mammalian expression vector pBK-CMV, and (iii) pBK/kap, the 225-bp kaposin gene cloned into pBK-CMV, induced morphologic transformation of Rat-3 cells. Constructs pBS/17 and pBS/199 containing the other unique HHV-8 ORFs, K4, K5, K6, and K8, were nontransforming. Focally transformed cell lines established from Rat-3 cells transfected with pBS/23, pBK/T0.7, and pBK/kap all produced high-grade, highly vascular, undifferentiated sarcomas by 1 to 2 weeks after injection into athymic nu/nu mice. Analysis of the tumor-derived cell lines demonstrated the expression of kaposin mRNA. Furthermore, kaposin protein was detected and localized to the cytoplasm of both the transformed and tumor-derived cells by an indirect immunofluorescence assay (IFA) using a polyclonal rabbit antikaposin antibody raised against a hydrophilic kaposin peptide, kap-2 (aa 42 to 55). More importantly, in this study kaposin protein was also detected in the HHV-8-containing PEL cell lines BCBL-1 (53) and KS-1 (57). The transforming ability of kaposin, its retention and expression in transformed and tumor-derived cells, and its detection in PEL cell lines demonstrate that kaposin is an HHV-8 transforming gene and suggest that kaposin could play a role in the etiology of KS and PEL as well as other HHV-8-associated malignancies.

MATERIALS AND METHODS

DNA and plasmids. HHV-8 DNA was isolated from the PEL cell line BC-1 (47). Three clones containing unique HHV-8 ORFs were constructed by cloning 4- to 6-kbp, partially digested Sau3A fragments into the BamHI site of pBluescript (pBS; Stratagene). One of these, clone pBS/23, contained nucleotides 116121 to 122049 of the HHV-8 LUR (56) which include the sequence of the T0.7 transcript and the kaposin ORF. The sequence of kaposin in the plasmids used in these studies (56) differed from the sequence of kaposin reported by which, ORF K12, also known as kaposin (56), encoded a highly hydrophobic 60-amino-acid (aa) peptide. The hydrophobicity of kaposin is similar to that of the 45-aa E5 oncoprotein of bovine papillomavirus type 1 (BPV-1) (7, 61). T0.7 expression was observed in K5 tissue of all stages from earliest identifiable to advanced tumors (63). Moreover, in advanced KS tumors, approximately 85% of spindle cells expressed T0.7 RNA.

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preimmune serum at 37°C for 2 h in a humidified chamber. Suspension cells (10^6 cells/ml) were spotted on polylysine-coated wells on a slide and placed at 4°C for 45 min. The medium was carefully removed, and the cells were air dried at room temperature for 10 min. Cells were then fixed in cold acetone at room temperature for 15 min and incubated with a 1:5 dilution of either the anti-kap-1 or anti-kap-2 antibody or with preimmune serum as described above. In both cases, following incubation with primary antibody, cells were washed with PBS as described above and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G at room temperature for 1 h in the dark. Following three washes with PBS, the cells were mounted with 50% glycerol in PBS containing 5% 4,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei. Immunofluorescence was detected in a Zeiss Axiophot fluorescence microscope.

RESULTS

Kaposin induced tumorigenic transformation of Rat-3 cells. Four- to 6-kbp fragments of HHV-8 genomic DNA, partially digested with Sau3A, were cloned into the BamHI site of pBS vector. Constructs which contained unique HHV-8 ORFs, i.e., pBS/17 (ORFs K4, K5, and K6), pBS/23 (ORF K12, i.e., kaposin), and pBS/199 (ORF K8) (Fig. 1), were transfected into Rat-3 cells to test for focus-forming ability. Background levels of foci were observed for clones pBS/17 and pBS/199 as well as for mock- and pBS-transfected cells (Table 1, experiment 1). In contrast, clone pBS/23 exhibited numerous morphologically altered, highly refractile foci not seen in mock- or pBS-transfected cells, indicating the transforming activity of this clone.

To determine if the transforming activity of pBS/23 was due to kaposin (ORF K12), both the T0.7 sequence and the kaposin gene were subcloned into the mammalian expression vector pBK-CMV and assessed for their transforming ability. Plasmid pBK/mtrII, containing the human cytomegalovirus (HCMV)
transforming gene mtrII (UL111a) (68), was used as a positive control. Like clone pBS/23, both pBK/T0.7 and pBK/kap induced similar levels of morphologically altered foci (Table 1, experiment 2). Background levels of foci were seen in mock- and vector-transfected cells. Similar levels of transforming activity observed with pBS/23, pBK/T0.7, and pBK/kap indicate that the transforming activity resides in kaposin.

Independent transformed foci were isolated and expanded into focal cell lines. Genomic DNA isolated from the cell lines was analyzed for the presence of kaposin sequences by PCR amplification. Kaposin-positive, transformed Rat-3 cell lines R3/23-1 (pBS/23 transfected), R3/T0.7-1, R3/T0.7-2, and R3/T0.7-3 (pBK/T0.7 transfected), and R3/kap-1 and R3/kap-2 (pBK/kap transfected) were tested for tumorigenicity by injection into athymic Ncr

nu/nu mice (Table 1). All cell lines containing kaposin sequences, whether derived from transfection by pBS/23, pBK/T0.7, or pBK/kap (Fig. 2B and C), induced highly vascular, high-grade, undifferentiated sarcomas within 1 to 2 weeks of injection, whereas Rat-3 cells did not.

In addition to the focal cell lines tested above, Rat-3 cells transfected with pBK-CMV or pBK/kap were selected for resistance to G418 and tested for tumorigenicity (Table 2, experiment 2). The R3/kap-G1 (pBK/kap-transfected) cell line exhibited a transformed morphology similar to focally derived R3/kap-1 and -2 cells and were tumorigenic. In contrast, the R3/BK-G1 (pBK-CMV vector-transfected) cell line maintained a morphology similar to parental Rat-3 cells and was not tumorigenic. Thus, both G418-selected as well as focally derived pBK/kap-transfected Rat-3 cells exhibited morphologic and tumorigenic transformation.

Kaposin mRNA is expressed in tumor-derived cell lines. Total RNA was extracted from tumor-derived cell lines R3/kap-TL1 and R3/kap-TL2, and kaposin mRNA was detected by a Northern blot analysis using 32P-labeled kaposin DNA probe (Fig. 3). Two kaposin-specific RNA species were detected at 0.8 and 1.1 kb in the RNA from the two tumor-derived lines, whereas no kaposin signal was observed in the RNA from control Rat-3 cells. The two RNA bands observed result from splicing of the message at the simian virus 40 3'-splice acceptor site downstream of the kaposin insertion site in the pBK/kap construct. Their sizes are consistent with expected sizes of polyadenylated spliced and unspliced messages, respectively, transcribed from pBK/kap. Kaposin-specific messages were also observed in poly(A) mRNA isolated from the above-specified two cell lines as well as from R3/T0.7-TL1, -TL2, and -TL3 tumor-derived cell lines (data not shown).

Demonstration of the retention and expression of the kaposin gene in the tumor-derived cell lines correlates with the observation of expression of the T0.7 kaposin message in all stages of KS tumors in vivo (63).

Antibodies to hydrophilic kaposin peptides recognize bacterially expressed kaposin. Polyclonal rabbit antisera were generated against two hydrophilic kaposin peptides, aa 15 to 29 (kap-1) and aa 42 to 55 (kap-2) (Fig. 4). The efficacy of anti-kap-2 antibody was determined by its reactivity to a 120-aa S-Tag/kaposin fusion protein produced in E. coli. A protein
band with an apparent molecular mass of 17 kDa was detected in the extract of IPTG-induced cells carrying plasmid pET30b/kap by Western blot analysis using the S-protein/alkaline phosphatase conjugate, which binds to the S-Tag (Fig. 5A). No corresponding band was detected in uninduced cells or in cells carrying the vector pET30b. The membrane was then stripped and analyzed by Western blotting using the anti-kap-2 antibody (Fig. 5B). The identical 17-kDa band was observed in the extract of induced cells with pET30b/kap but not in the other samples. Moreover, the 17-kDa band was also detected with the anti-kap-1 antibody in a Western blot but not with preimmune serum (data not shown). Taken together, these observations demonstrated that antikaposin antibody recognized both the linear and native conformations of kaposin protein.

Kaposin protein localized to the cytoplasm in transformed and tumor-derived cells. Kaposin protein was detected in transformed Rat-3 cells and tumor-derived cells by an IFA using the anti-kap-2 antibody. Positive anti-kap-2 antibody staining was observed in the tumor-derived cell line R3/kap-TL1 and predominantly localized to a restricted region of the cytoplasm (Fig. 6, panel 1A). Nuclei in the same field of cells were visualized by counterstaining with DAPI (panel 1B). No antibody staining of R3/kap-TL1 cells was observed with preimmune serum (panel 2A), while the presence of cells was demonstrated by DAPI counterstaining of nuclei (panel 2B). Moreover, no staining was observed with anti-kap-2 antibody in the stable Rat-3 cell line transfected with the vector pBK-CMV (panel 3A). Again, DAPI counterstaining revealed the presence of cells in the field (panel 3B). Positive staining was also observed with the kaposin-transformed cell line R3/kap-1, which produced tumors in athymic nu/nu mice (data not shown).
Kaposin protein was also detected by IFA in another HHV-8-containing PEL cell line, KS-1 (data not shown). With either anti-kap-2 or anti-kap-1 antibody, over 90% of BCBL-1 and KS-1 cells were stained. These findings demonstrate that kaposin protein is expressed in cell lines derived from HHV-8-associated lymphoid tumors like PEL and that this expression occurs in latently infected cells.

**DISCUSSION**

The data in this study demonstrated that the unique HHV-8 kaposin gene, ORF K12, morphologically transformed Rat-3 cells which were tumorigenic when inoculated into athymic nu/nu mice. The tumors developed with a very short latency period (1 to 2 weeks) and were highly vascular, high-grade, undifferentiated fibrosarcomas. Neoplastic transformation occurred whether kaposin was expressed from its endogenous promoter or from the heterologous CMV immediate-early promoter. Of significance, kaposin mRNA and protein were detected in both the focally transformed and tumor-derived cells (Fig. 6), suggesting that kaposin expression is required for both induction of transformation and maintenance of the tumorigenic phenotype. Retention and expression of viral oncoproteins is a hallmark of transformation by DNA tumor viruses including simian virus 40, polyomavirus, adenovirus, CMV, and HHV-6 (26, 33, 39, 51, 55, 68, 73). Although the abundant, latency-associated transcript T0.7 has been observed in all stages of KS tumors of different epidemiologic origin as well as in PEL (63, 74), expression of kaposin protein from this transcript has not been reported. This study has demonstrated, for the first time, the expression of kaposin protein in the PEL cell lines BCBL-1 and KS-1. As with the Rat-3-transformed and tumor-derived cells, kaposin protein localized predominantly to a restricted region of the cytoplasm of BCBL-1 and KS-1 cells. The ability of kaposin to induce tumorigenic transformation in rodents and its expression in PEL-derived cells suggests that kaposin may play a role in the development of KS and PEL.

The level of transforming activity of kaposin in the rodent cell focus-forming assay was 1 to 2 orders of magnitude lower than that reported for cellular or retroviral oncogenes in similar assay systems. However, the level is comparable to and representative of that reported for other herpesvirus transforming genes like HCMV mtrII (UL111a) (30, 31), HSV-2 mtrIII (UL39) (23, 32, 54), and HHV-6 ORF-1 (67). A similar low level of transforming activity has also been reported for the human papillomavirus type 16 oncogene E7 (69, 72).

Comparison of the genome of HHV-8 to those of HVS and Epstein-Barr virus (EBV) has revealed the presence of extensive conserved collinear regions as well as unique sequences (56). The transforming genes of HVS and EBV are unique to each of these herpesviruses. HVS is a T-cell-specific oncogenic virus capable of inducing lymphoproliferative disorders in natural or experimental hosts and transforming lymphoid cells in vitro (43, 44). Two HVS proteins have been associated with transforming activities (42). The saimiri transformation-associated protein is a membrane-associated phosphoprotein with a highly acidic amino terminus, central collagen-like repeats, and a hydrophobic carboxy terminus that has been shown to induce oncogenic transformation via interaction with cellular ras, resulting in activation of the Ras signaling pathway (35). Another protein, tyrosine kinase-interacting protein, binds to p56	extsuperscript{ck} tyrosine kinase and significantly increases its kinase activity. It has been postulated that this activated kinase feeds into the Ras and protein kinase C signaling pathways (40).
EBV, on the other hand, encodes a latent membrane protein, LMP2a, which binds stably to a Src family kinase in B cells and down-regulates Src kinase activity and thus maintains latency of the virus (9). However, none of the transforming genes of either HVS or EBV have been conserved in HHV-8. The kaposin transforming gene identified in this study is one of the unique HHV-8 ORFs.

The HHV-8 genome also contains ORFs that are functional homologs of cellular proto-oncogenes such as those encoding cyclin D (12, 15, 27, 38), GPCR (3), bcl-2 (17, 59), and IL-6 (48, 52). Of these, the v-IL-6, v-cyclin D, and v-GPCR genes have been shown to enhance cell proliferation (3, 52, 66). More recently, the v-GPCR gene has been reported to induce tumorigenic transformation in rodent cells and activate angiogenesis (4). However, v-GPCR mRNA is expressed as a lytic transcript upon tetradecanoyl phorbol acetate induction in the PEL cell line BC-1 (58). Furthermore, it has been reported that in HHV-8-containing cell lines such as BCBL-1 and BCP-1, only a minor population of cells are in the lytic state (4). In addition, using in situ hybridization with the T1.1 probe and colocalization with the major capsid protein RNA, Staksus et al. (63) have determined that only about 10% of HHV-8-infected cells in KS lesions are in the lytic state. The K9 ORF (vIRF), unique to HHV-8, has also been identified as an oncogene which inhibited the interferon signaling pathway (24). Because K9 expression was detected in an HHV-8-infected B-cell line but not in KS tissue, Gao et al. (24) proposed its putative role in B-cell malignancies. In contrast to both v-GPCR and K9, kaposin is expressed as an abundant, latency-associated transcript in both KS and PEL (63, 74).

In this study, fragments containing other ORFs unique to HHV-8 were tested for their transforming ability. Transformation was not observed with constructs of two HHV-8 fragments, pBS/17 containing unique HHV-8 ORFs K4, K5, and K6 and pBS/199 with K8. Although this observation indicated the specificity of transformation seen with pBS/23 containing kaposin, no definitive conclusions can be made about the oncogenic potential of the K4, K5, K6, and K8 ORFs because their expression in transfected cells was not analyzed. In conclusion, the kaposin transforming gene identified in this study is a unique HHV-8 ORF that is encoded by the abundant, latency-associated transcript expressed in both KS and PEL.

In vitro evidence suggests that autocrine and paracrine growth effects of cytokines, growth factors, and their receptors play an important role in the pathogenesis of KS, both AIDS-associated and non-AIDS-associated. AIDS-associated KS-derived cells have been shown to respond to and express high levels of an endothelial cell growth factor, IL-1β, basic fibroblast growth factor (bFGF), and IL-6 (21, 46). In addition, the HIV type 1 Tat protein has been implicated in the pathogenesis of AIDS-associated KS, either directly by activation of HHV-8 replication (29) or indirectly by activation of cytokines (22). The Tat gene has also been shown to act in synergy with bFGF in inducing KS-like lesions in mice (20). The importance of similar autocrine and paracrine growth effects in non-AIDS-associated KS has also been documented. Various growth factors and cytokines including bFGF, IL-6, platelet-derived growth factor B (PDGF-B), vascular endothelial growth factor, and oncostatin M can serve as modulators of KS cell proliferation in vitro (21, 37, 41, 45, 70). KS-derived cells have also been shown to express a functional FGF receptor, flg (37), and PDGF-A-type and PDGF-B-type receptors (71). Constitutive expression and activation of growth factors or their specific receptors could therefore serve as a mechanism by which viral oncogenes and oncoproteins create an autocrine growth loop that leads to self-sustained aberrant growth.

Similarities in size and hydrophobicity between kaposin and the BPV-1 E5 protein suggest that they might have common mechanisms for transformation (Fig. 4) (61). BPV-1 E5 is a small 44-aa protein with a strongly hydrophobic N terminus that has been localized to Golgi apparatus and endoplasmic reticulum (ER) membranes of transformed cells (8). In the present study, kaposin, a small 60-aa protein with N- and C-terminal hydrophobic domains, was also localized to the cytoplasm of transformed and tumor-derived cells by using an antibody raised against a hydrophilic kaposin peptide. Interestingly, kaposin protein was detected predominantly in a restricted area of the cytoplasm, suggestive of the Golgi apparatus and ER membranes. In support of these findings, AU1 epitope-tagged kaposin also localized to the Golgi apparatus and ER membranes in a transient transfection assay (28). Kaposin has been shown to bind to the 16-kDa component of the vacuolar H⁺-ATPase which is important in processing factor receptor (28). Moreover, the interaction of E5 with the type β PDGF receptor (PDGFR) transmembrane domain which results in constitutive PDGFR activation is required for transformation (19). Therefore, it is possible that kaposin also transforms cells by activation of growth factor receptors such as PDGFR which are processed in the Golgi complex. In summary, the data presented here demonstrate that the HHV-8 kaposin gene (ORF K12) induced tumorigenic transformation and that the kaposin protein is expressed in PEL-derived cell lines. Thus, it may play a role in the development of KS, PEL, and other HHV-8-associated malignancies.

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AUTHOR’S CORRECTION

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