The Cyclin Encoded by Kaposi’s Sarcoma-Associated Herpesvirus Stimulates cdk6 To Phosphorylate the Retinoblastoma Protein and Histone H1

DIANA GODDEN-KENT,1 SIMON J. TALBOT,2 CHRIS BOSHOFF,2 YUAN CHANG,3 PATRICK MOORE,3 ROBIN A. WEISS,2 AND SIBYLLE MITTNACHT1*

Centre for Molecular and Cell Biology and Centre for Virology,2 The Institute of Cancer Research, Chester Beatty Laboratories, London, United Kingdom, and Division of Epidemiology, School of Public Health and Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, New York3

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Kaposi’s sarcoma-associated herpesvirus (KSHV or human herpesvirus 8) is a novel gammaherpesvirus implicated in the cause of Kaposi’s sarcoma and certain malignancies of lymphatic origin. One of the candidate genes possibly involved in promoting tumor development is an open reading frame (ORF) with sequence similarity to human type D cyclin genes. This cyclin-like gene, when expressed in tissue culture cells, promotes phosphorylation and inactivation of the retinoblastoma tumor suppressor protein and thereby may result in deregulation of cell division control. We report here the biochemical characterization of this cyclin (KSHV-cyc) and the kinase activity that it elicits upon expression in tissue culture cells. We demonstrate that the kinase activity associated with KSHV-cyc is sensitive to the cdk inhibitor p27 (KIP) and due to activation of cdk6. However, in contrast to cdk6 activated by cellular type D cyclins, the cdk6 activated by KSHV-cyc is capable of phosphorylating not only the retinoblastoma protein but also histone H1. This finding implies that activation by KSHV-cyc alters the substrate preference of this cdk. This may have important physiological consequences in that the kinase activity triggered by this viral cyclin may abrogate cell cycle checkpoints in addition to those targeted by cellular cyclin D-ckd6 kinase.

Kaposi’s sarcoma (KS) is a vascular tumor characterized by multicentric lesions that most likely originate from hyperproliferation of endothelial cells (reviewed in reference 53). Classic KS, which presents in elderly men of Mediterranean origin, and endemic KS, found in human immunodeficiency virus (HIV)-negative individuals in parts of central Africa, are both usually indolent tumors which mainly affect the skin. KS associated with immunosuppression caused by HIV and iatrogenic KS after an organ transplant are more aggressive forms of the disease and can affect systemic organs (6). DNA belonging to a novel gammaherpesvirus was isolated from a primary AIDS-associated KS biopsy (12). Since then, evidence that implicates this KS-associated herpesvirus (KSHV or human herpesvirus 8) as the cause of KS has accumulated. Viral DNA is found in KS lesions of all epidemiological forms of the disease (i.e., classic, endemic, AIDS-associated, and posttransplant KS) (1, 8, 9, 17, 37, 48), and detection of KSHV DNA in peripheral blood of HIV-infected individuals by PCR predicts the subsequent development of KS. Furthermore, first-generation serological assays suggest that KSHV is not ubiquitous but is confined mainly to those at risk for developing KS (24, 25, 34, 51). Sequencing of the viral genome has demonstrated colinearity with the oncogenic gammaherpesviruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), a simian herpesvirus with the oncogenic gammaherpesviruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), a simian herpesvirus that causes fulminant lymphoproliferation in New World primates (36, 37). Like EBV, KSHV infects CD19+ lymphocytes (1, 31). KSHV is also found in cells from primary effusion lymphomas, a rare form of B-cell lymphoma seen mainly in patients with advanced AIDS (10). The lymphatic system appears to be the primary site of KSHV replication, while in KS tumors the virus is found in a latent, nonlytic state with only a subset of its genes expressed (14, 44, 58).

KSHV lacks several genes implicated in EBV-induced cell immortalization and oncogenesis (such as those encoding EBNA-1 and -2, LMP-1 and -2, and Gp 350/220) and two genes implicated in HVS transformation (those encoding StpC and Tip) (46). However, KSHV encodes other genes whose products could play a role in tumor induction, including a bcl-2 homolog, genes encoding chemokines interleukin-6, macrophage inflammatory protein, an interferon regulatory factor, an interleukin-8 receptor, and an NCAM-like protein (35, 46, 47), and a gene with cyclin homology (11), similar to that encoded by HVS (39). Transcripts encoding this cyclin are found in KS tumor cells and cells from primary effusion lymphomas, suggesting that the product of this gene could be involved in KS and lymphoma tumorigenesis (7a, 11).

Like the HVS cyclin, the cyclin encoded by KSHV (KSHV-cyc) is closest related in sequence to the cellular type D cyclins (11, 39) suggesting that these viral cyclins may be evolutionarily derived from this subgroup of cellular cyclins.

Cellular cyclins are components of kinases, many of which regulate cell proliferation and cell cycle progression (reviewed in references 21, 41, and 49). When ectopically expressed in tissue culture cells, the cell cycle-regulating G1 cyclins D and E accelerate transit through the G1 phase of the cell cycle, and the mitotic type B cyclin has been associated with cellular hyperproliferation in plants (15, 45, 54; reviewed in reference 16). Moreover, the aberrant expression of cellular type D cyclins is strongly implicated in various human cancers (7, 20, 27; reviewed in references 5, 20, 21, 41, and 50). A hallmark of cyclins is their ability to associate with and activate cellular kinase subunits (cyclin-dependent kinases [cdks]) (reviewed in...
reference 38). When active, certain of these kinases phosphor- ylate cell cycle checkpoint molecules and thereby facilitate progression of cells through the cell cycle. The KSHV-encoded cyclin-like gene, when ectopically expressed in cells, promotes phosphorylation of the retinoblastoma protein (pRB) and can overcome a cell cycle checkpoint imposed by this tumor suppressor (13), suggesting that the encoded cyclin protein may act to stimulate cellular cdks and, in ways analogous to cell cycle-regulating cellular cyclins, promote cell cycle progression.

We provide evidence here that the KSHV-cyc functions as a genuine cyclin protein. Upon expression in Cos cells or insect cells it stimulates cdks, a kinase subunit normally activated by the cellular type D cyclins. KSHV-cyc-activated cdks, however, has features divergent from those inherent to cyclin D-activated cdks and therefore may fulfill functions beyond those elicited by cellular cyclin D-cdk complexes.

MATERIALS AND METHODS

Materials. A 9E10 (c-myc) epitope-tagged version of the KSHV-cyc was generated by PCR, using a cloned genomic fragment of KSHV covering open reading frame (ORF) 72 (11) as a template. Sac/XbaI and NorI sites were attached to the 5’ and 3’ ends, respectively, using the following primers: 5’ gatacgctgtagatatgaacgtgtacctas3’ and 5’ gatacgctgtagatatgaacgtgtacctagc 3’. The PCR product was designed such that the natural stop codon of ORF 72 was deleted. A cloned version of the 9E10 (c-myc) epitope (coding for amino acids EQLISEEDL-stop) was subsequently inserted into the 3’ NorI site. Similarly, a NorI site was introduced via PCR prior to the stop codon of human cyclin D1, D2, and E to facilitate in-frame insertion of the above 9E10 epitope sequence. Cloned versions of human cyclin D1 and D2 were provided by G. Peters (Imperial Cancer Research Fund, London, England). Epitope-tagged cyclins were cloned into RcCMV (Invitrogen, San Diego, Calif.) for expression in mammalian cells or pVL13923 (Pharmingen, San Diego, Calif.) to generate cyclin-expressing baculovirus. Baculovirus encoding cdks was obtained from C. Sherr (Howard Hughes Medical Institute, St. Jude’s Hospital, Memphis, Tenn.). Cloned versions of human cdk2, cdk5, and cdk6 were obtained from E. Harlow (Massachusetts General Hospital, Charlestown, Mass.) and engineered into pVL13923 to generate baculovirus. Baculoviruses were produced by using a Baculovirus Gold kit (Pharmingen). A construct allowing expression of human p27 (KIP) as a glutathione S-transferase (GST) fusion in Escherichia coli was obtained from T. Hunter (Salk Institute, La Jolla, Calif.). Specific anti-cdk rabbit sera were purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.) (cdk2) and Santa Cruz Biotechnology (Santa Cruz, Calif.) (cdk4, cdk6, and cdk7).

Characterization of KSHV-cyc by Cos cell transfection. Cos cells were transfected with plasmid via DEAE dextran-mediated DNA transfer essentially as described previously (26). To determine the intracellular distribution of the KSHV-cyc, transfected Cos cell monolayers or MDCK cells microinjected with plasmid encoding 9E10 epitope-tagged KSHV-cyc were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton, and reacted with 9E10 antibody followed by fluorescein isothiocyanate (FITC)-coupled anti-mouse antibody. Cell morphology was assessed by confocal microscopy.

Lysates were prepared from transfected Cos cells 42 h posttransfection in HB (50 mM HEPES-NaOH [pH 7.4])-150 mM NaCl-20 mM EDTA-0.5% Triton X-100–2 mM dithiothreitol with 1% aprotinin, 2.5 μg of leupeptin per ml, and 1 mM phosphomonoester sulfonyl fluoride (PMSF) to inhibit proteinases and 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM sodium vanadate to inhibit phosphatas, using 150 μl per 106 cells. Lysis was performed on ice, and lysates were cleared by centrifugation (10 min at 10,000 × g, 4°C).

Expression of the 9E10 epitope-tagged cyclins was monitored by probing Western blots with 9E10 antibody followed by horse-radish peroxidase-coupled mouse-specific secondary antibody (Amersham, Little Chalfont, England) and enhanced chemiluminescence (Amersham).

For immunoprecipitation of epitope-tagged cyclins, 30 μl of cleared lysate was diluted with 500 μl of HB and reacted for 2 h with 20 μg of 9E10 antibody prebound to 15 μl of protein G-agarose (Sigma, Poole, England). The immunocomplexes were washed three times in HB and analyzed by Western blotting for copurifying cdk subunits.

For measurement of kinase activity, beads were washed once in kinase buffer (50 mM HEPES-KOH [pH 7.4])-10 mM MgCl2–10 mM MnCl2–1 mM dithiothreitol containing 0.1 mM protein kinase A inhibitor [Sigma], 10 mM β-glycerophosphate, 1 mM phosphomonoester sulfonyl fluoride (PMSF), and 2.5 μg of leupeptin per ml). Kinase assays were performed by adding 15 μl of kinase buffer, containing 500 ng of substrate, 10 μM ATP, and 0.1 μCi of γ[32P]-ATP, directly to the bead pellet. For a substrate, we used either purified histone H1 (a gift of G. Goodwin, Institute of Cancer Research, London, England), full-length pRB, or a fragment (amino acids 763 to 928) containing the cluster of cdk consensus phosphorylation

RESULTS

Expression of KSHV-cyc in Cos cells. To study the biochemical properties of the KSHV-encoded cyclin, we engineered a cyclin version tagged at the carboxy terminus with a 9E10 (c-myc) epitope. When probing lysates from Cos cells transfected with plasmid to express this cyclin with the tag-specific antibody 9E10, we detected a reactive protein with a molecular mass (28 kDa) close to that predicted for the 257-amino-acid-long KSHV-cyc ORF (Fig. 1a).

Immunofluorescence on Cos cells transfected, or MDCK cells microinjected, with this cyclin-expressing plasmid demonstrated that the encoded cyclin protein is found predominantly in the nuclear compartment (Fig. 1b), a subcellular localization common to cellular G1/S cyclins (3, 28, 40).

When immunopurified via the epitope tag from extracts of transfected Cos cells, KSHV-cyc copurified with kinase activity (Fig. 2) capable of phosphorylating classical cyclin D substrates such as full-length pRB and a C-terminal fragment of pRB. However, in contrast to reports on cyclin D-associated kinases (29, 30), the KSHV-cyc-associated activity is also able to phosphorylate histone H1.

Inhibition of KSHV-cyc-associated kinases by p27 (KIP). To gain evidence for whether the activity associated with KSHV-cyc was due to stimulation of cellular cdks, we tested whether this kinase activity was affected by p27 (KIP), a cellular cdk inhibitor that regulates a wide variety of cyclin-cdk complexes, including those containing cellular D, E, and A cyclins (19, 42, 43, 53). We found that recombinant p27, when added to cellular extracts containing KSHV-cyc, acted as a potent repressor of both the histone H1 and pRB kinase activities associated with this cyclin (Fig. 3). This result suggests that the majority of activity associated with KSHV-cyc is mediated by p27-sensitive kinase subunits. We note that a residual amount of both histone H1 and pRB kinase activity remained detectable in precipitates of KSHV-cyc even at a high concentration of p27. Using phosphorymager-based analysis, we determined that between 5 and 7% of the initial activity was retained in immunoprecipitates treated with p27. This activity is inhibited by 100 μM of aprotinin, 1 mM PMSF, and 2.5 μg of leupeptin per ml. The p27 concentration was increased 25-fold. In contrast, the activity associated with cellular cyclin E (epitope-tagged and expressed in Cos cells like KSHV-cyc) was completely blocked

sites in the carboxy terminus of pRB, both expressed as GST fusions in E. coli. GST-pRB fusion proteins were constructed and purified as described previously (57). Kinase reactions were allowed to proceed for 10 min at 27°C, after which the reaction was terminated by adding sodium dodecyl sulfate (SDS)-containing gel loading buffer. Reactions were separated on SDS-polyacrylamide gels. Gels were fixed in 40% methanol–10% acetic acid, dried, and exposed to X-ray film. Signals were quantified with a phosphorimager.

Lysates were depleted for individual cdks by treatment with 20 μg of anti-cdk antisera bound to protein A-agarose (Bethesda Research Laboratories, Gaithersburg, Md.). Depleted lysates were subsequently subjected to 9E10 precipitation, and the resulting precipitates were assayed for kinase activity. For p27 (KIP) inhibition, lysates was mixed with purified GST-p27 followed by incubation at room temperature for 15 min. Cyclins were immunoprecipitated from these lysates, and kinase activity was determined as described above.

Expression of KSHV-cyc and stimulation of cdk activity in insect cells. Spodoptera frugiperda SF9 insect cells were cultured and infected with baculovirus by using standard procedures, with modifications as described previously (57). Extracts from virus-infected cells were prepared 40 h postinfection. Cells were collected by centrifugation (800 × g for 10 min, 4°C) and lysed in hypotonic buffer (10 mM HEPES-KOH [pH 7.0])–10 mM KCl-1 mM EDTA containing protease inhibitors PMSF, aprotinin, and leupeptin and phosphatase inhibitors b-glycerophosphate, NaF, and sodium vanadate at concentrations indicated above), using 100 μl per 5 × 106 cells. Prior to the kinase assay, the hypotonic lysis was diluted fivefold in kinase buffer, and insoluble material was removed by centrifugation (10,000 × g for 10 min, 4°C). Kinase reactions were performed as described above, using 5 μl of diluted insect cell extract per reaction.

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by 5 nM p27. These data raise the possibility that a small portion of the activity associated with the viral cyclin is mediated via a p27-resistant kinase.

**Association of KSHV-cyc with cdk subunits.** To identify which cdk subunits bind to and may be activated by KSHV-cyc, we probed KSHV-cyc immunoprecipitates from transfected Cos cells with antisera that specifically recognize individual cdks. To determine the specificity of our assay, we examined, in parallel, cellular cyclins known to complex with distinct subsets of these cdks. As with KSHV-cyc, all of these cellular cyclins were engineered to express the 9E10 epitope tag at their carboxy termini.

For the experiment shown in Fig. 4, precipitates for these individual cyclins were divided into four equal aliquots and, following separation on SDS-gels, probed with different anti-cdk antisera. We found that both cdk5 and cdk6 efficiently copurified with KSHV-cyc. Both of these cdks, in agreement with earlier reports (4, 29, 33, 55), also copurified with the cellular cyclins D1 and D2 but not with cyclin E (Fig. 4). We further detected a very small amount of cdk2 in complexes with KSHV-cyc. Interestingly, we did not detect association of this cyclin with cdk4, a kinase subunit that is very closely related in sequence to cdk6 (32, 33) and which, in accord with previous reports, efficiently copurified with the cellular type D cyclins (Fig. 4). These results suggest that KSHV-cyc can bind to a panel of cdks similar but not identical to those associating with cellular type D cyclins.
Specific activation of cdk6 by KSHV-cyc. To determine which, if any, of the cdk subunits associated with KSHV-cyc was responsible for the observed kinase activities, we used cdk-specific antibodies to remove individual cdks from KSHV-cyc-containing Cos cell lysates. We then precipitated for the viral cyclin by using the tag-specific antibody and assayed the resulting immunoprecipitates for kinase activity by using either histone H1 or pRB as a substrate. Figure 5 shows that the kinase activities directed against pRB and histone H1 were greatly reduced (i.e., by 96 and 91%, respectively, as quantified by phosphorimager analysis) when lysates were precleared with the anti-cdk6 antibody. Preclearing with either the anti-cdk5 serum or a serum against cdk4 or cdk2 had no effect. This result suggests that the vast majority of kinase activity associated with the viral cyclin in transfected Cos cells is mediated via complexes that contain cdk6. Furthermore, our results suggest that these cdk6 complexes not only are responsible for pRB phosphorylation but also mediate the phosphorylation of histone H1.

cdk activation in insect cells. To ascertain that our results reflect genuine biochemical features of KSHV-cyc, we sought to confirm our findings by an independent approach, namely, baculovirus-directed expression in insect cells. We generated recombinant baculoviruses expressing 9E10 epitope-tagged KSHV-cyc and studied the ability of this virus to facilitate kinase activation upon coinfection with baculoviruses encoding individual cdks (Fig. 6). To compare the features of the viral cyclin with those of cellular type D cyclins, we also engineered a virus encoding 9E10-tagged cellular cyclin D1. Infection of insect cells with virus encoding either of these cyclins alone or various cdk catalytic subunits alone resulted in only a low level of background activity. Kinase activity significantly above this background level was seen when virus encoding KSHV-cyc was used in combination with cdk6 and, to a lesser extent, in conjunction with cdk4, but not with cdk2 or cdk5. Similarly, combined infection of cdk4 or cdk6 with cellular cyclin D1 resulted in increased kinase activity. Probing of the lysates by using cdk-specific antibodies demonstrated that similar levels of cdk4 and cdk6 catalytic subunits were expressed in singly and doubly infected cells, indicating that the increase in kinase activity upon cyclin coinfection was not due to differences in expression of these cdks but reflect a genuine increase in their specific activities.

Significantly, we found that the kinase activity evoked upon coexpression of KSHV-cyc with cdk6 (and also cdk4) was capable of phosphorylating efficiently both pRB and histone H1. This is in stark contrast to the situation seen when cellular cyclin D1 is coexpressed with these cdk subunits. The kinase activity generated under these conditions was specific to pRB and did not yield histone H1 phosphorylation above the background level.

Thus, in agreement with our results for Cos cells, expression of the KSHV-cyc in insect cells results in efficient stimulation of cdk6. However, in insect cells, where both kinase subunit and cyclin are overexpressed, this cyclin appears also capable of stimulating cdk4. More importantly, activation via KSHV-cyc appears to modify the substrate preference of these cdk complexes that contain cdk6. Furthermore, our results suggested that these cdk6 complexes not only are responsible for pRB phosphorylation but also mediate the phosphorylation of histone H1.

DISCUSSION

Our results demonstrate that the cyclin-like reading frame encoded by KSHV can operate as a genuine cyclin in that it can associate with and activate cdk catalytic subunits. When ectopically expressed in tissue culture cells, it complexes with a...
set of kinases similar but not identical to those bound by cellular type D cyclins. The kinase activity associated with this viral cyclin appears to be mediated largely through cdk6. Although we found that also cdk5 associates with this cyclin, the significance of this is not clear, as complexes containing cdk5 appear not to generate activity (at least when assayed under the conditions used). This finding parallels observations with cellular type D cyclins. While association of these cyclins with cdk5 can be demonstrated (reference 56 and this report), activity of such cyclin D–cdk5 complexes has not been reported to date.

Interestingly, the kinase activity associated with the KSHV-cyc–cdk6 complex catalyzes efficiently phosphate transfer onto both histone H1 and pRB. This contrasts with results obtained for the cyclin D–cdk6 complex, the substrate specificity of which appears confined to pRB itself and a few pRB-related proteins, and which is virtually incapable of phosphorylating histone H1 (reference 33 and this report). Such confined substrate preference may reflect the fact that type D cyclins are specifically designed to catalyze progression through the G1 phase of the cell cycle. The ability of the KSHV-cyc–cdk complex to target additional substrates, however, opens the possibility that this kinase is able to fulfill functions other than promoting G1 progression, e.g., to mediate progression through further checkpoints imposed later in the cell cycle.

Various features of KSHV-cyc appear to closely resemble those previously described for the cyclin encoded by HVS (23). Like KSHV-cyc, the cyclin encoded by HVS associates with and activates cdk6 in insect cells, and like the kinase activated by KSHV-cyc, this kinase can phosphorylate histone H1. Such conservation of biochemical features appears remarkable, in particular when one considers that the amino acid conservation between these two viral cyclins is moderate—only 33% of their amino acids are identical (11). Despite error-prone viral replication, these biochemical features appear to have been preserved, perhaps indicating their importance for the functioning of these cyclins in the context of virus propagation. An interesting question may be how these biochemical features were derived in the first place. Likely, the viral cyclins are descendants of an ancient type D cyclin scavenged by these viruses from their hosts. The features of the viral cyclins reflect characteristics of this ancient cyclin D precursor or, alternatively, comprise adaptations to a special need of these viruses. The function of the virally encoded cyclins in virus replication is not clear, though we found that also cdk5 associates with this cyclin, the viral cyclin appears to be mediated largely through cdk6. Alternatively, comprise adaptations to a special need of these viruses.

Methods of triggering cell proliferation have independently evolved in a wide variety of other DNA viruses. For example, the papillomavirus E6 and E7 proteins, adenovirus E1A and E1B, and polyomavirus-encoded T antigens all bind and inactivate pRB and the tumor suppressor p53 (for reviews, see references 18 and 22), while two of EBV’s immortalizing genes (encoding EBNA-2 and LMP-1) promote cell cycle progression via the induction of cellular G1 cyclins (2, 52). Viruses may require genes that modulate cellular growth to execute their replication cycle. However, many of these growth-promoting genes elicit tumorigenic effects when expressed in isolation or in the absence of lytic virus replication.

An important cofactor for the establishment of KSHV-associated tumors is immunodeficiency, and this condition appears to determine the severity and clinical presentation of the disease (6). In KSHV-infected individuals, occasional infection of cells that cannot support virus replication may occur. While such cells would be eliminated in immunocompetent hosts, in immunocompromised individuals they may prevail and as a result of residual viral gene expression adopt a quasi-tumorous, hyperproliferative nature. The cyclin-like reading frame is one of the few genes transcribed under such conditions. By aberrantly stimulating cellular cdk5, it may trigger cell cycle progression and thereby contribute to the pathogenesis of KS.

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