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Kaposi's Sarcoma-Associated Herpesvirus Encodes a Functional Cyclin

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Kaposi's sarcoma-associated herpesvirus (KSHV) (also called human herpesvirus 8) is consistently found in Kaposi's sarcoma lesions and in body-cavity-based lymphomas. A 17-kb KSHV lambda clone was obtained directly from a Kaposi's sarcoma lesion. DNA sequence analysis of this clone identified an open reading frame which has 32% amino acid identity and 53% similarity to the virus-encoded cyclin (v-cyclin) of herpesvirus saimiri (HVS) and 31% identity and 53% similarity to human cellular cyclin D2. This KSHV open reading frame was shown to encode a 29- to 30-kDa protein with the properties of a v-cyclin. KSHV v-cyclin protein was found to associate predominantly with cdk6, a cellular cyclin-dependent kinase known to interact with cellular type D cyclins and HVS v-cyclin. The KSHV v-cyclin was also found to associate weakly with cdk4. KSHV v-cyclin–cdk6 complexes strongly phosphorylated glutathione *S*-transferase–Rb fusion protein and histone H1 as substrates in vitro. Thus, KSHV v-cyclin resembles the v-cyclin of the T-lymphocyte-transforming HVS in its specificity for association with cdk6 and in its ability to strongly activate cdk6 protein kinase activity.

The decision to enter the eukaryotic cell cycle is made in G_1, during which cells respond to both positive and negative growth signals (19). The ultimate recipients of these signals are cyclin-dependent protein kinases (cdks), which interact with regulatory subunits called cyclins (19, 39). cdk-cyclin complexes regulate passage through sequential cell cycle transitions (40). Eukaryotes from clams to humans express two classes of mitotic cyclins, A and B (19). These mitotic cyclins regulate the G_2/M transition. The G_1 cyclins C, D, and E regulate the G_1/S transition (11, 22–24, 26, 28–30, 34, 47). Type D cyclins, including D1, D2, and D3, were shown to associate with three different cdks, i.e., cdk2, cdk4, and cdk5, along with proliferating cell nuclear antigen and a kinase-inhibitory protein, p21 (28, 48, 49). Direct binding of cyclin D to the Rb protein and Rb phosphorylation by the cyclin D-dependent kinase cdk4 have been reported (10, 13, 22, 29). More recently, cdk6, the PLSTIRE gene product, was demonstrated to contain cell cycle-dependent kinase activity and to have 71% identity with cdk4 (2, 33). cdk6 protein was found to associate with cyclins D1, D2, and D3 in human cells, and cdk6 kinase activity with an Rb fusion protein was activated by coexpression of D-type cyclins (33).

Deregulated expression of cellular cyclins may be involved in at least some types of abnormal cell proliferation. In a human hepatocellular carcinoma, the cyclin A gene was identified as the site of clonal integration of hepatitis B virus (45). In adenovirus-transformed cells, cyclin A is associated with the viral transforming protein E1A (16, 36). Overexpression of cyclin D1 protein as a consequence of genetic rearrangement and deletion or mutation of the p16INK4 gene have been demonstrated in a large variety of human cancers, including parathyroid adenoma (34), centrocytic lymphoma (38), breast and a squamous cell carcinoma (25), and esophageal carcinoma (20). Additionally, cyclin D1 was recently shown to contribute to transforming activity by complementing a defective adenovirus E1A oncogene (18).

Kaposi's sarcoma (KS) has previously occurred rarely in elderly individuals of Mediterranean or eastern European origin and in central Africa. However, with the global pandemic of infection with human immunodeficiency virus (HIV), KS has surfaced as a major complication of AIDS (15). The lesion of KS is histologically complex, with many features that are atypical for a classical malignancy. It often occurs in a multifocal manner, and the spindle cells of individual nodules have been shown to be of clonal origin (15). Cultured KS cells secrete a host of cytokines, including basic fibroblast growth factor, interleukin-6, and platelet-derived growth factor (12).

Many lines of epidemiological evidence have suggested an infectious etiology for KS. Recently, DNA sequences of a novel member of the herpesvirus group, called KS-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8), have been widely identified in KS tumors from HIV-positive and HIV-negative patients (4, 5, 31, 50). This agent has been proposed as a possible etiologic factor for KS (4, 5, 31, 46, 50). Viral sequences of KSHV show greatest homology to herpesvirus saimiri (HVS), a member of the gamma-2 subfamily of herpesviruses.

HVS naturally infects squirrel monkeys (*Saimiri sciureus*), a common primate species of the South American rain forest, without any apparent disease association. Infection of marmosets, owl monkeys and other species of New World primates results in rapidly progressing, malignant lymphomas, leukemias, and lymphosarcomas (14). Furthermore, HVS can transform peripheral blood lymphocytes from monkey and human origins to continuous growth (3, 9). The complete 113-kb DNA sequence of the HVS genome has recently been published (1),

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and one open reading frame, called *eclf2*, displays 23% amino acid identity and 46% similarity to the human cyclin D1 (35). The *eclf2* gene product of HVS was shown to be a 29-kDa phosphoprotein that associates with cdk6, resulting in strong protein kinase activity toward Rb fusion proteins (21). This was the first virus-encoded cyclin (v-cyclin) to be identified.

While this report was in preparation, Chang et al. reported a partial sequence of a KSHV open reading frame which has similarity with the cyclin box of HVS v-cyclin and cellular type D2 cyclin (6). They also showed that expression of this open reading frame induced phosphorylation of cellular Rb, which is a hallmark of Rb inactivation (6). In this report, we describe the complete sequence of a KSHV cyclin-related gene, its similarity to other cyclin sequences, and the properties of the KSHV protein encoded by this gene.

MATERIALS AND METHODS

Cell culture and transfection. COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Sf9 cells were maintained at 27°C in Grace's medium containing 10% fetal calf serum, yeastolate, and lactalbumin hydrolysate. A DEAE-dextran transfection procedure was used for transient expression in COS-1 cells (7).

Cloning of KSHV from KS. A single KS biopsy specimen from a male AIDS patient was digested in melting buffer and extracted with phenol-chloroform twice. DNA was partially digested with restriction endonuclease *Sau*3A and ligated with *Bam*HI-digested arms of the bacteriophage-lambda vector DASH2 (Stratagene Inc., La Jolla, Calif.). The ligation reaction product was in vitro packaged and amplified once. A DNA corresponding to the ORF75 homolog of KSHV (5) was synthesized by PCR with primers H8-75-1 and H8-75-2. With this PCR product as a probe for plaque hybridization, the KSHV-specific phage lambda clone SY3-2 with an insert of about 17 kb was identified.

Shotgun cloning and DNA sequencing. The complete DNA sequence of the 17-kb insert of phage lambda clone SY3-2 was determined by a shotgun approach. Briefly, purified insert DNA of SY3-2 was sonicated (maximum output, 70% cycle, 60 s), and ends were filled in with Klenow and T4 DNA polymerases (New England Biolabs). DNA fragments ranging from 1 to 4 kb were prepared from agarose gels and ligated into the *Sma*I-digested vector BluescriptKSII-minus (Stratagene Inc.). DNA from the shotgun plasmids was sequenced on an ABI377 automated DNA sequencer by using the dye-terminator cycle sequencing chemistry according to the instructions of the manufacturer (Perkin-Elmer Inc., Foster City, Calif.). Sequence assembly and analysis of the contiguous sequence were performed with the suite of sequence analysis tools from the Genetics Computer Group, Madison, Wis.

Expression and purification of GST fusion proteins. Glutathione *S*-transferase (GST)–Rb (positions 379 to 928) fusion protein expression and purification were performed essentially as described by Smith and Johnson (41). For fusion protein recovery with glutathione-Sepharose, bacterial cell pellets were frozen once, resuspended with 1/10 volume lysis buffer (1% Triton X-100 and 0.1% sarcosinate in phosphate-buffered saline [PBS]) containing protease inhibitors, and disrupted by sonication. After centrifugation to remove cell debris, supernatant fluids were mixed with preequilibrated glutathione-Sepharose for 30 min at 4°C. The beads were then washed three times with PBS and once with buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris [pH 7.0]). A flag monoclonal antibody recognizing DYKDDDDK sequence was purchased from Eastman Kodak (New Haven, Conn.). Antisera against cdk2 and cdk5 were kindly supplied by Giulio Draetta, Michele Pagano, Tony Hunter, and David Beach. cdk6 and cdk3 antibodies were also kindly provided by Matthew Meyerson, Ed Harlow, and Michele Pagano. Polyclonal antibodies to cdc2, cdk2, cdk4, and cdk6 and monoclonal antibodies to PSTAIRE were purchased from Santa Cruz Biotech (Santa Cruz, Calif.).

Plasmid constructions. DNA containing the KSHV v-cyclin open reading frame was amplified from lambda DNA by PCR with primers containing *Bgl*II and *Eco*RI recognition sequences at the ends. Amplified DNA was ligated into the *Bam*HI and *Eco*RI cloning sites of the pcDNA3-flag vector for a flag tag at the amino terminus. For transient expression in COS-1 cells, flag-tagged KSHV v-cyclin DNA was amplified by PCR and subcloned into the pFJ vector (43). KSHV v-cyclin and flag-tagged KSHV v-cyclin DNAs were completely sequenced to verify 100% agreement with the original sequence. cdk2, cdk3, cdk4, and cdk5 genes were subcloned into the pFJ vector. The cdk6 expression vector was obtained from Matthew Meyerson and Ed Harlow, and the myc-tagged cdk7 expression vector was obtained from Robert Weinberg.

Metabolic labeling, immunoprecipitation, and immunoblotting. COS-1 cells at 80 to 90% confluence in a 25-cm^2 dish were rinsed three times with PBS, washed once with labeling medium (minimum essential medium without methionine and cysteine plus 10% dialyzed fetal calf serum), and then incubated with 2 ml of the same medium containing 200 μCi of methionine and cysteine (New England Nuclear, Boston, Mass.) for 5 to 7 h. In all cases, cells were incubated in labeling medium for 30 min prior to the addition of the radioisotopes. Cells were harvested and lysed with lysis buffer (0.3 M NaCl, 0.1% Nonidet P-40, and 50 mM HEPES buffer [pH 8.0]) or radioimmunoprecipitation assay buffer (0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5]) containing 0.1 mM Na_2VO_3 and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and bestatin). Immunoprecipitated proteins from cleared cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography of the dried gel slabs (21). For protein immunoblots, polypeptides in cell lysates corresponding to 10^5 cells were resolved by SDS-PAGE and transferred to nitrocellulose membrane filters. Immunoblot detection was performed with a 1:3,000 dilution of primary antibody as described previously (21).

Construction of recombinant baculoviruses. *Eco*RI-*Xho*I fragments containing flag-tagged KSHV v-cyclin genes were inserted into the *Eco*RI and *Xho*I sites of the baculovirus transfer vector pAcSG1 (Pharmingen, San Diego, Calif.). Vector plasmids were cotransfected into Sf9 cells with linearized baculovirus DNA. Four days later, virus-containing supernatants were harvested. The recombinant baculovirus was amplified to obtain a high-titer stock solution. The cdk4, cdk6, and v-cyclin baculoviruses have been described previously (21). Sf9 cells infected with baculovirus were assayed for expression of recombinant protein by labeling with [^{35}S]methionine or by immunoblotting. For routine production of recombinant proteins, 10^6 cells were infected with 0.2 ml of each baculovirus supernatant and lysed at 48 h postinfection with lysis buffer, and cleared cell lysates were used for immunoprecipitations.

In vitro kinase assays. For in vitro protein kinase assays, complexes prepared as described above were washed once more with kinase buffer and resuspended with 10 μl of the same buffer containing 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) for 15 min at room temperature. For some experiments, 5 μg of histone H1 protein and GST-Rb were added as substrates.

Nucleotide sequence accession number. The GenBank accession number for KSHV v-cyclin is U79416.

RESULTS

Sequence analysis of KSHV v-cyclin. The sequence presented in Fig. 1A is contained within the 17-kb lambda clone SY3-7. The open reading frame encodes a putative protein of 257 amino acids with a predicted relative molecular weight of 29,000. This open reading frame shows homology to v-cyclin of HVS and to several other cellular type D cyclins. KSHV v-cyclin has 32% identity and 53% similarity with the v-cyclin of HVS and 31 to 32% identity and 53 to 54% similarity with mammalian cyclin D2. The homology to v-cyclin and type D cyclins extends over the full length of the protein (257 amino acids) (Fig. 1B), whereas the homology to cyclins of type B, A, E, or F is less extensive and usually is limited to the typical cyclin box, which is located between amino acids 42 and 160 in the cyclin of KSHV (Table 1).

Identification of KSHV v-cyclin protein. To demonstrate expression of this viral open reading frame, the sequence was tagged with a flag epitope at the amino terminus. For transient expression in COS-1 cells, an expression vector containing the flag-tagged KSHV v-cyclin was constructed in plasmid pFJ containing the SRα-0 promoter (43). The flag antibody reacted specifically with a protein having an apparent molecular size of 29 to 30 kDa on immunoblots and by radioimmunoprecipitation assay and SDS-PAGE, both in COS-1 cells transfected with the flag-tagged KSHV v-cyclin expression vector (Fig. 2). No such protein was detected in control COS-1 cells lacking the KSHV v-cyclin gene.

Protein kinase activity associated with KSHV v-cyclin protein. To investigate possible protein kinase activity associated with KSHV v-cyclin protein, immune complexes prepared from COS-1 cells transiently expressing the KSHV v-cyclin gene were used for in vitro kinase reactions with histone H1 and GST-Rb proteins as substrates. Anti-KSHV v-cyclin immune complexes contained kinase activity with histone H1 and GST-Rb as substrates (Fig. 3, lanes 4). As a control, HVS v-cyclin immune complexes from COS-1 cells transiently expressing the HVS v-cyclin were used for in vitro kinase reactions. As shown previously, HVS v-cyclin complexes also contained kinase activity toward histone H1 and GST-Rb (Fig. 3,

A

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matannppsglldptlcedrifynileieprfltsdsvfgsfqqsltshm          50

rkllgtwmfsvcqeynlepnvvalalnlldrlllikqvskehfqktgsac          100

llvasklrsltpistsslcyaaadsfsrqelidqekelleklawrteavl          150

atdvtsflllkllggsqhldfwhhevdtlitkalvdpktgslpasiisaa          200

gcallvpanvipqdthsggvvpqlasilgcdvsvlqaaveqiltsvsdfd          250

lrildsy                                                     257
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B

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KSHV v-cyc      matannppsgl ------ LDPT LCE DR IFYNI LEI EP RFL TSD SVFGSF QQSL TSH MR KLL    54
HVS v-cyc       madspnrlnrak ----- IDST TMK DP RVLNN LKL RE LLL PKF TSLWEI QTEV TVD NR TIL    55
Cyclin D1       mehqllccevetirra  yPDAN LLN DR VLRAM LKA EE TCA PSV SYFKCV QKEV LPS MR KIV    60
Cyclin D2       mellchevdpvrravr  -DRNL LRD DR VLQNL LTI EE RYL PQC SYFKCV QKDI QPY MR RMV    59
Cyclin D3       mellccegtrhaprag  pDPRL LGD QR VLQSL LRL EE RYV PRA SYFQCV QREI KPH MR KML    60

KSHV v-cyc      GTWMFS VCQE YN LEP NVVALALNLL  DR LL LIK QVS KEH FQKT GSACLLVASKLR  SLT PIS  114
HVS v-cyc       LTWMHL LCES FE LDK SVFPLSVSIL  DR YL CKK QGT KKT LQKI GAACVLIGSKIR  TVK PMT  115
Cyclin D1       ATWMLE VCEE QK CEE EVFPLAMNYL  DR FL SLE PVK KSR LQLL GATCMFVASKMK  ETI PLT  120
Cyclin D2       ATWMLE VCEE QK CEE EVFPLAMNYL  DR FL AGV PTP KSH LQLL GAVCMFLASKLK  ETS PLT  119
Cyclin D3       AYWMLE VCEE QR CEE EVFPLAMNYL  DR YL SCV PTR KAQ LQLL GAVCMLLASKLR  ETT PLT  120

KSHV v-cyc      TSSL CYAAADSFSRQ EL IDQ EKE LL EKLA WRTE AV LA TDVTSF LL LK LLGGS QHLDFWHH    174
HVS v-cyc       VSKL TYLSCDCFTNL EL INQ EKD IL EALK WDTE AV LA TDFLIP LC NA LKIPE DLWPQLYE    175
Cyclin D1       AEKL CIYTDNSIRPE EL LQM ELL LV NKLK WNLA AM TP HDFIEH FL SK MPEAE ENKQIIRK    180
Cyclin D2       AEKL CIYTDNSIKPQ EL LEW ELV VL GKLK WNLA AV TP HDFIEH IL RK LPQQR EKLSLIRK    179
Cyclin D3       IEKL CIYTDHAVSPR QL RDW EVL VL GKLK WDLA AV IA HDFLAF IL HR LSLPR DRQALVKK    180

KSHV v-cyc      EV DTLI TK ALV DPK TGSL PASII SAA GCALL Vpanvipqdthsggvvp  qlasilgcdvsv    234
HVS v-cyc       AA STTI CK ALI QPN IALL SPGLI CAG GLLTT Ietdntncrpwtcyled  lssilnfstntv    235
Cyclin D1       HA QTFV AL CAT DVK FISN PPSMV AAG SVVAA Vqglnlrspnnflsyyr  ltrflsrvikcd    240
Cyclin D2       HA QTFI AL CAT DFK FAMY PPSMI ATG SVGAA Icglqqdeevssltcda  ltellakitntd    239
Cyclin D3       HA QTFL AL CAT DYT FAMY PPSMI ATG SIGAA Vqglgacsmsgdeltel  lagitgtevdcl    240

KSHV v-cyc      lqaaveqiltsvsdfdlrildsy  ------------------------------                  257
HVS v-cyc       rtvkdqvseafslydleil  ------------------------------------               254
Cyclin D1       pdclracqeqieallesslrqaq  qnmdpkaae eeeeeeeevdlactptd  vrdvdi          295
Cyclin D2       vdclkacqeqieavllnslqqyr  qdqrdgsks edeldqastptdvrdid  l-----          289
Cyclin D3       racqeqieaalreslreaaqtss  spapkaprg ssqgpsqtstptdvta  ihl---          292
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FIG. 1. Amino acid sequence of KSHV v-cyclin and alignment with sequences of cellular type D cyclins and HVS v-cyclin. (A) Amino acid sequence of KSHV v-cyclin. A typical cyclin box spans the region from nucleotide 132 (amino acid 44) to nucleotide 480 (amino acid 160). (B) Amino acid sequence alignment of KSHV v-cyclin with cellular type D cyclins and HVS v-cyclin. The five polypeptide sequences were aligned by using the MACAW program. Amino acids in the conserved region are in uppercase letters, and amino acids in the variable region are in lowercase letters. Identical (white letters) and homologous (shaded) amino acids are shown.

TABLE 1. Homology of KSHV v-cyclin with HVS v-cyclin and mammalian cellular cyclins[a]

Cyclin class	Species	Protein length (amino acids)	Alignment length (amino acids)	% Identity[b]	% Similarity[b]	Swissprot accession no.
V	HVS	254	253	32	53	Q01043
D2	*Homo sapiens*	263	262	31	53	P30279
	Mouse	289	282	32	54	P30280
	Rat	288	282	32	54	Q04827
A	*H. sapiens*	432	272	32	48	P20248
E	*H. sapiens*	395	269	18	47	P24864
C	*H. sapiens*	303	276	16	42	P24863
B	*H. sapiens*	433	269	18	42	P14635

[a] Sequence alignments were calculated with the program BESTFIT (Genetics Computer Group, Madison, Wis.).
[b] Percentage of the complete sequence of KSHV v-cyclin.

lanes 2). These results demonstrate that protein kinase activity is present in KSHV v-cyclin complexes from COS-1 cells.

Association of KSHV v-cyclin with cdk4 and cdk6. To identify possible serine/threonine kinases associated with KSHV v-cyclin protein, flag-tagged KSHV v-cyclin was cotransfected into COS-1 cells with an expression vector containing cdc2, cdk2, cdk4, cdk5, cdk6, or myc-tagged cdk7. Whole-cell lysates from COS-1 cells transiently expressing the KSHV v-cyclin and cdks were incubated with antibodies recognizing cyclin-dependent serine/threonine kinases. We used antibodies to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, and myc which have been used successfully by others and by us to demonstrate associations with cyclins (2, 19, 21, 29, 32, 33, 44, 49). Polypeptides present in anti-cdk immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with antiflag antibody. The 29- to 30-kDa KSHV v-cyclin protein was found to be associated with cdk6 and was strongly detected by the flag antibody, while much smaller amounts of 29- to 30-kDa KSHV v-cyclin were detected in cdk4 complexes (Fig. 4A). Additionally, half of ^{35}S-labeled cell lysates from COS-1 cells transiently expressing the KSHV v-cyclin and cdks were incubated with flag antibody, and the other half were incubated with antibodies against cdks. Results from these experiments showed that flag immune complexes contained the 40-kDa cdk6 protein in addition to the 29- to 30-kDa KSHV v-cyclin (Fig. 4B). Immunoprecipitations with anti-cdk antibodies showed equivalent expression of cdks in transient expression in COS-1 cells (data not shown). Finally, the presence of the cellular cdk4 and cdk6 in KSHV v-cyclin immune complexes was examined without overexpression of these cdks. COS-1 cells were transfected solely with pFJ-flag-KSHV v-cyclin DNA. Immune complexes of flag–KSHV v-cyclin were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with cdk4 and cdk6 anti-

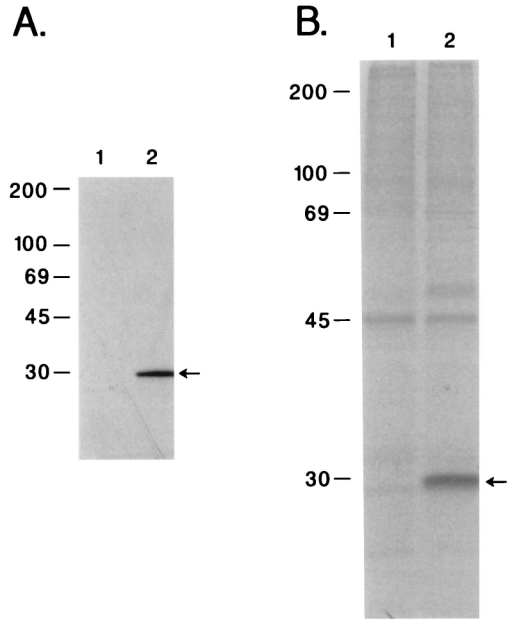

FIG. 2. Identification of KSHV v-cyclin protein. (A) Immunoblot detection of KSHV v-cyclin protein with flag antibody. Proteins in cell extracts were fractionated by SDS-PAGE, transferred to nitrocellulose, and reacted with flag antibody. (B) Immunoprecipitation of ^{35}S-labeled KSHV v-cyclin protein by flag antibody. Flag M2 antibody conjugated with beads was used for immunoprecipitation. After immunoprecipitation, proteins were separated by SDS-PAGE. Lanes 1, COS-1 cells transfected with pFJ; lanes 2, COS-1 cells transfected with pFJ-KSHV v-cyclin. The arrows indicate the KSHV v-cyclin protein. The molecular markers are lysozyme (14 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (29 to 30 kDa), ovalbumin (45 kDa), bovine serum albumin (69 kDa), phosphorylase *b* (97.4 kDa), and myosin (200 kDa).

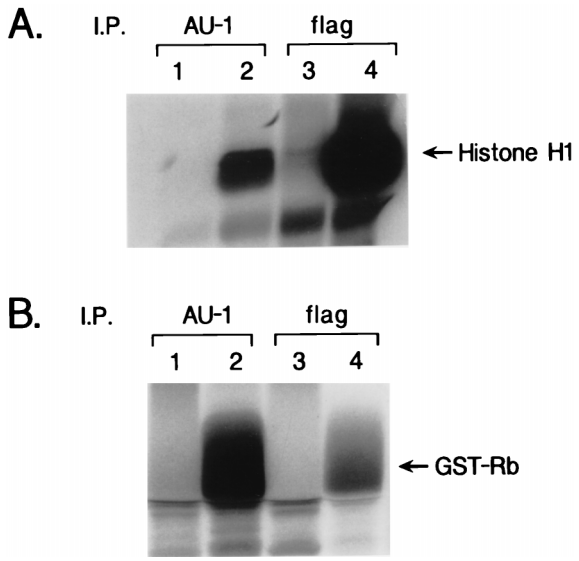

FIG. 3. Kinase activity associated with KSHV v-cyclin protein. Kinase activity with histone H1 (A) and GST-Rb (B) was measured in vitro in KSHV v-cyclin and HVS v-cyclin immune complexes. COS-1 cells were transfected with pFJ (lanes 1 and 3), pFJ-HVS-v-cyclin (lanes 2), or pFJ-KSHV-v-cyclin (lanes 4) by using DEAE-dextran. Proteins were immunoprecipitated (I.P.) with AU-1 antibody for HVS v-cyclin (lanes 1 and 2) or flag antibody for KSHV v-cyclin (lanes 3 and 4). For in vitro phosphorylation, immunoprecipitated HVS v-cyclin or KSHV v-cyclin complexes were incubated in a kinase reaction with [γ-^{32}P]ATP with histone H1 (A) or GST-Rb (B) as a substrate and then analyzed by SDS-PAGE.

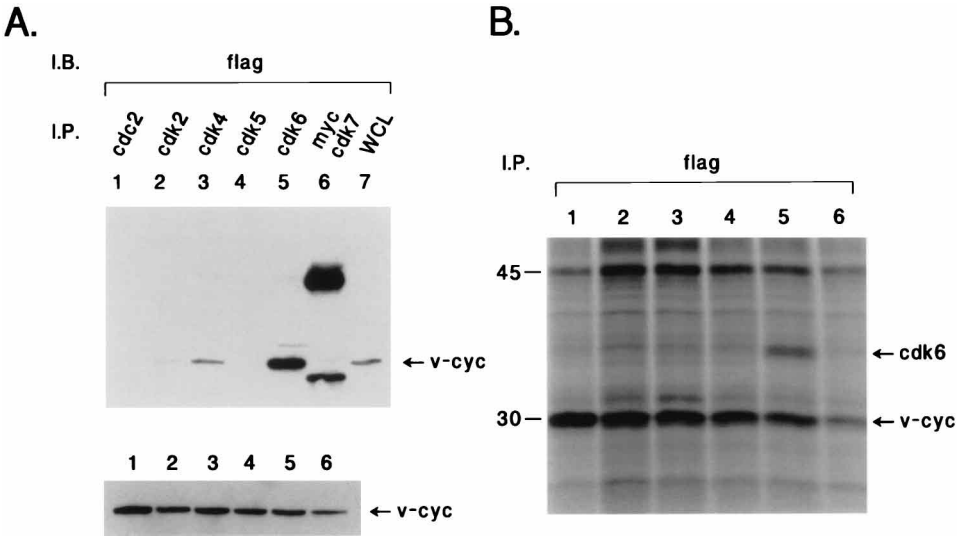

FIG. 4. Association of KSHV v-cyclin with cdks in COS-1 cells. (A) Formation of complexes between KSHV v-cyclin and cdks in COS-1 cells. COS-1 cells were transfected with pFJ-KSHV v-cyclin with pFJ-cdc2 (lane 1), pFJ-cdk2 (lane 2), pFJ-cdk4 (lane 3), pFJ-cdk5 (lane 4), pCMV-cdk6 (lane 5) or pCMV-myc-cdk7 (lane 6). Cell lysates were used for immunoprecipitation (I.P.) with antibodies for cdc2, cdk2, cdk4, cdk5, and cdk6 and with 9E10 myc antibody for myc-cdk7. Anti-cdk immune complexes were separated by SDS-PAGE, followed by Western blotting (I.B.) with flag antibody. Whole-cell lysates (WCL) containing flag–KSHV v-cyclin were used as a control. Detection in immunoblotting was with the enhanced chemiluminescence system. Whole-cell lysates from each transfection were used for immunoblotting with flag antibody to show the similar expression of KSHV v-cyclin (v-cyc) (bottom). The 28- and 45-kDa proteins in lane 7 are immunoglobulin light and heavy chains, respectively. (B) Association of cdk6 protein with KSHV v-cyclin. After transfection as described for panel A, COS-1 cells were labeled with [^{35}S]methionine-cysteine. Immunoprecipitations were performed with flag antibody. Radioactive polypeptides in flag immune complexes were separated by SDS-PAGE, followed by autoradiography.

bodies. As shown in Fig. 5A, cdk4 and cdk6 were detected in KSHV v-cyclin immune complexes. However, cdk4 in KSHV v-cyclin complexes was detected only after long exposure. Thus, while cellular type D cyclins are associated with cdk2, cdk4, cdk5, and cdk6, we detected restricted association of KSHV v-cyclin only with cdk6 and much weaker association with cdk4 under the same conditions.

Finally, the recombinant baculovirus system was also employed to study formation of complexes between cdk6 and KSHV v-cyclin. As shown in Fig. 6, coinfection of Sf9 insect cells with recombinant KSHV v-cyclin and cdk6 baculovirus resulted in the association of KSHV v-cyclin protein with cdk6. Thus, KSHV v-cyclin associates with cdk6 in COS-1 cells and in insect cells.

Activation of kinase activity of cdk6 by KSHV v-cyclin. COS-1 cells were transfected with KSHV v-cyclin together with cdk4 or cdk6. Cell lysates were used for immunoprecipitation with anti-cdk4 or anti-cdk6 antibody, and then these immune complexes were used for in vitro kinase assays with GST-Rb or

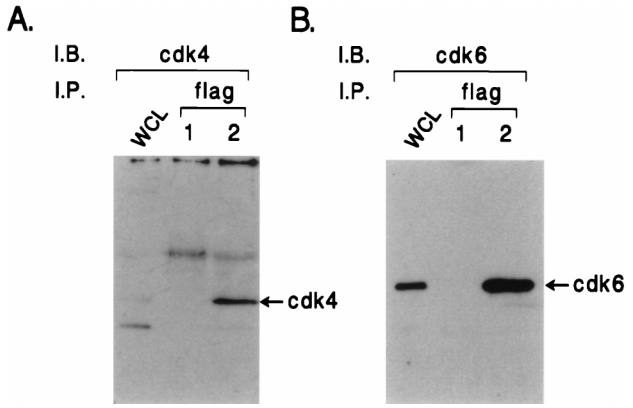

FIG. 5. Identification of cdk4 and cdk6 in KSHV v-cyclin complexes. COS-1 cells were transfected with pFJ vector (lanes 1) or pFJ-KSHV-v-cyclin (lanes 2). Flag immune complexes were separated by SDS-PAGE, followed by Western blotting (I.B.) with cdk6 antibody (B). After immunoblotting with cdk6, the membrane was stripped with 1% SDS and reblotted with cdk4 antibody (A). Immunoblot detection was by enhanced chemiluminescence with a 1-min exposure time for panel B and a 30-min exposure time for panel A. The 45-kDa protein in panel A is the immunoglobulin heavy chain in Fig. 4A. Whole-cell lysates (WCL) of COS-1 cells were used as a control.

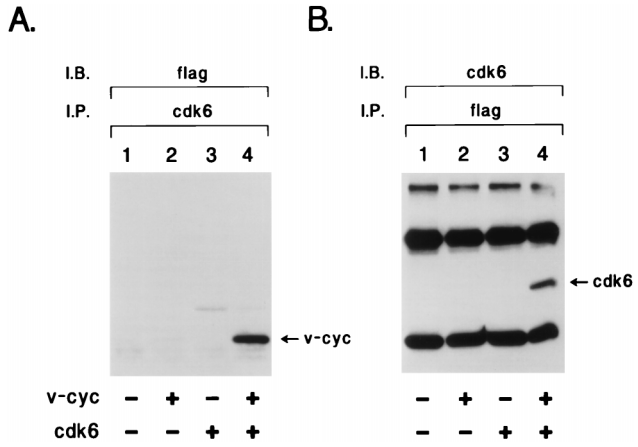

FIG. 6. Association of KSHV v-cyclin with cdk6 in insect cells. Sf9 insect cells were infected with recombinant baculovirus as indicated at the bottom. After 48 h of infection, cell lysates were used for precipitations (I.P.) with anti-cdk6 antibody (A) or flag antibody (B). Anti-cdk6 or anti-flag immune complexes were separated by SDS-PAGE, followed by Western blotting (I.B.) with flag or cdk6 antibody as indicated. Immunoblot detection was by enhanced chemiluminescence. The 28- and 45-kDa proteins in panel B are immunoglobulin light and heavy chains, respectively.

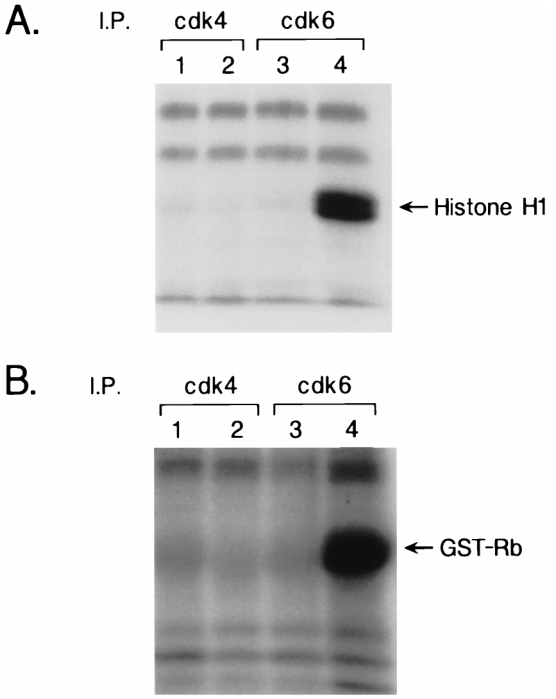

FIG. 7. Activation of kinase activity of cdk6 by KSHV v-cyclin. Kinase activity with histone H1 (A) and GST-Rb (B) was measured in vitro in cdk4 and cdk6 immune complexes. COS-1 cells were cotransfected with pFJ (lanes 1 and 3) or pFJ-KSHV-v-cyclin (lanes 2 and 4) together with pFJ-cdk4 (lanes 1 and 2) or pCMV-cdk6 (lanes 3 and 4). After 48 h of transfection, cell lysates were used for immunoprecipitation (I.P.) with anti-cdk4 (lanes 1 and 2) or anti-cdk6 (lanes 3 and 4). Immunoprecipitates were subjected to in vitro kinase reactions with histone H1 or GST-Rb as a substrate.

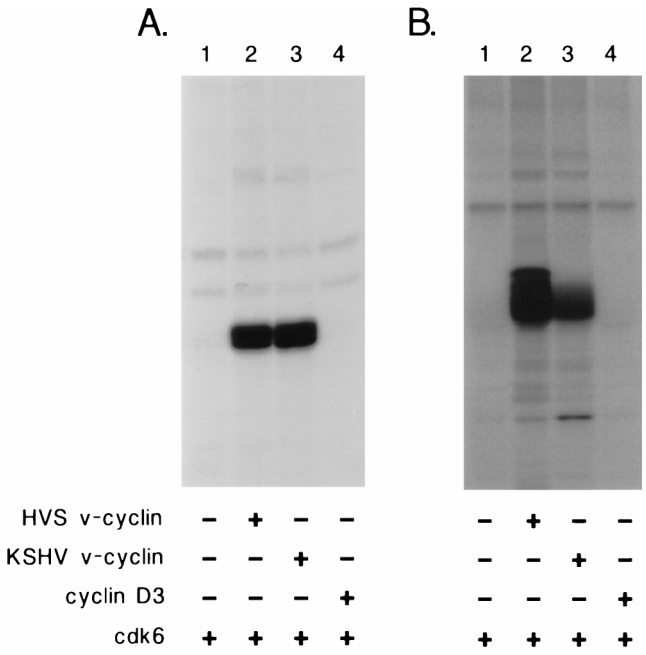

FIG. 8. Comparison of kinase activities of cdk6-cyclin D3, cdk6–HVS v-cyclin, and cdk6–KSHV v-cyclin complexes. Insect cells were infected with recombinant baculovirus as indicated at the bottom. After 48 h of infection, cell lysates were used for immunoprecipitation with anti-cdk6 antibody. cdk6 immune complexes from each cell lysate were assayed for kinase activity with 5 μg of GST-Rb fusion protein (B) or histone H1 (A) as a substrate. ^{32}P-labeled products were separated by SDS-PAGE, proteins were stained with Coomassie blue, and the gel was exposed to X-ray film.

histone H1 as a substrate. Complexes from cdk6 expression alone in COS-1 cells did not show detectable kinase activity with histone H1 or GST-Rb as the substrate (Fig. 7). In contrast, complexes from COS-1 cells transfected with cdk6 and KSHV v-cyclin showed a dramatic increase in kinase activity for histone H1 and GST-Rb (Fig. 7). cdk4 complexes with KSHV v-cyclin did not show any increase in kinase activity for histone H1 or GST-Rb. Thus, the association of KSHV v-cyclin with cdk4 was weak and not able to induce cdk4 kinase activity detectable under these conditions. In contrast, the association of KSHV v-cyclin with cdk6 was strong, and the induction of cdk6 kinase activity was dramatic. These results also provide strong evidence for the specificity of association of KSHV v-cyclin with cdk6.

Comparison of specificities of kinase activities of cdk6 complexes with cyclin D3, KSHV v-cyclin, and HVS v-cyclin in insect cells. cdk4 and cdk6 associated with cellular type D cyclins prefer Rb protein over histone H1 as a substrate (33). However, KSHV v-cyclin–cdk6 complexes exhibited very strong phosphorylation activity of histone H1 (Fig. 7). Thus, KSHV v-cyclin–cdk6 complexes were compared to complexes of cdk6 with cellular cyclin D3 or with HVS v-cyclin with regard to substrate specificity and strength of the kinase activity. Insect cells were infected with baculovirus expressing cdk6 along with baculovirus expressing cyclin D3, KSHV v-cyclin, or HVS v-cyclin. After 48 h of infection, half of the cells were used for in vitro kinase assays and the other half were used for labeling with [^{35}S]methionine. Anti-cdk6 antibody was used to precipitate the cdk6 and cdk6-cyclin D3, cdk6–KSHV v-cyclin, and cdk6–HVS v-cyclin complexes for in vitro kinase assay with GST-Rb and histone H1 proteins as substrates. Strong phosphorylation of GST-Rb and histone H1 proteins by cdk6–HVS v-cyclin and cdk6–KSHV v-cyclin complexes was detected (Fig. 8). With this short exposure, phosphorylation activity with cdk6-cyclin D3 was almost undetectable (Fig. 8). However, longer exposure showed specific phosphorylation activity of GST-Rb protein by cdk6-cyclin D3 (data not shown). Thus, cdk6 kinase activity for both histone H1 and GST-Rb appeared to be highly activated by association with KSHV v-cyclin. After labeling with [^{35}S]methionine, similar amounts of cyclin D3, KSHV v-cyclin, and HVS v-cyclin in cdk6 complexes were detected by anti-cdk6 antibody (data not shown).

DISCUSSION

In this report we have shown that the v-cyclin of KSHV/HHV-8 has the activities of a cell cycle regulator. It associates predominantly with cellular cdk6, and this association activates cdk6 kinase activity. These results suggest a role for KSHV v-cyclin in cell cycle regulation.

Cyclins are required for progression through the cell cycle, and this action is mediated through association with cdks (19, 37, 39). Sequence conservation is maintained among cyclins from different species, although it is less marked in the amino-terminal regions of the proteins. The KSHV v-cyclin gene product of KSHV/HHV-8 is comparable in length to v-cyclin of HVS and cellular type D cyclins. It displays 32% amino acid identity and 53% similarity with the v-cyclin of HVS, to which it is most closely related, and about 31% identity and 53% similarity with cellular type D cyclins. Homology of KSHV v-cyclin with cyclins A, B, C, and E is limited to the cyclin box. Highly conserved amino acid residues within the cyclin box of

cyclin A have been shown to represent a contact region for interaction with cdc2 and a functional domain for activation of cdc2 kinase activity (27). Our previous mutational analyses have shown that corresponding amino acids in HVS v-cyclin are also important for the association with cdk6 and activation of cdk6 kinase activity (21). KSHV v-cyclin also has a high degree of conservation in amino acids residues within the cyclin box, suggesting that this conserved region is likely to be a target site for binding to cdks (6). Notably, KSHV and HVS v-cyclins and cellular type D cyclins have substantial N-terminal truncations relative to other cellular cyclins.

Cellular cyclins are found in association with cdks, and their activity and function in regulating cell cycle progression are absolutely dependent on this interaction. Cyclins D1, D2, and D3 were previously found to be associated with four different cdks, i.e., cdk2, cdk4, cdk5, and cdk6, along with proliferating cell nuclear antigen and p21 or p16 kinase-inhibitory protein (17, 29, 48, 49). Also, recent reports showed that the mammalian cdc37 gene product associates with cyclin D-cdk4 complexes and that this association stabilizes cyclin D-cdk4 complexes (8, 42). Similarly to v-cyclin of HVS, which associates exclusively with cdk6, KSHV v-cyclin also associates predominantly with cdk6. Additionally, interaction with cdk4 was weakly detected in transient expression in COS-1 cells. The catalytic subunit for type D cyclins in macrophages and fibroblasts is mainly cdk4, while cdk6 as the catalytic subunit for type D cyclins appears to predominate in lymphoid cells (32, 33). Since T lymphocytes harbor HVS persistently in vivo and are the target of HVS transformation, a primary association between cdk6 and v-cyclin seems physiologically relevant. KSHV/HHV-8 has been localized to lymphocytes and endothelial cells in KS patients (5) and to the transformed B cells in body-cavity-based lymphomas (4) and primary effusion lymphomas (4). The specificity of KSHV v-cyclin for cdk6 is thus consistent with its presence in lymphocytes; the relevance of this association for endothelial cells and the spindle cells in KS remains to be explored.

Although the virus-encoded cyclins HVS v-cyclin and KSHV v-cyclin have sequence homology to type D cyclins and activate cellular cdk kinase activity, these cyclins differ from cellular type D cyclins in their specificity for cdk binding and their level of activation of cdk kinase activity. These virus-encoded cyclins were found to associate principally with cdk6, while cellular type D cyclins have been found to associate with four different cdks. Also, the kinase activity of cdk6 is much more greatly induced by association with v-cyclin than by association with the cellular D cyclin. Therefore, the virus-encoded cyclins exhibit a marked discrimination for the catalytic subunit with which they associate and a high level of kinase activation. A high level of activation of cdk kinase activity by these viral cyclins may result in much greater activation of cell cycle processes. The similarities in the activities of KSHV and HVS v-cyclins suggest that v-cyclin is not responsible for differences in the cell type specificities of these viruses or for differences in the diseases they induce. Whether v-cyclin in the context of either virus is important for growth transformation of one or more cell types remains to be determined.

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