Constitutively Active K-cyclin/cdk6 Kinase in Kaposi Sarcoma–Associated Herpesvirus–Infected Cells

Rukiyah Van Dross, Shan Yao, Shaheena Asad, Grant Westlake, Deborah J. Mays, Laura Barquero, Stephanie Duell, Jennifer A. Pietenpol, Philip J. Browning

Background: Kaposi sarcoma–associated human herpesvirus (KSHV) encodes K-cyclin, a homologue of D-type cellular cyclins, which binds cyclin-dependent kinases to phosphorylate various substrates. K-cyclin/cdk phosphorylates a subset of substrates normally targeted by cyclins D, E, and A. We used cells naturally infected with KSHV to further characterize the biochemical features of K-cyclin. Methods: We used immunoprecipitation with K-cyclin antibodies to examine the association of K-cyclin with cdk2, cdk6, p21Cip1, and p27Kip1 proteins in BC3 cells. We separated populations of BC3 cells enriched in cells in G1, S, or G2/M phases by elutriation and measured K-cyclin protein and the kinase activity of K-cyclin/cdk6 complexes. The half-life of K-cyclin and cyclin D2 proteins was determined by blocking protein synthesis with cycloheximide and measuring proteins in cell lysates by western blot analysis. We fused the entire K-cyclin sequence to the carboxyl-terminal sequence of cellular cyclin D that contains the PEST degradation sequence to produce K-cyclin/D2 and transfected K-cyclin/D2 into K-cyclin–negative cells to investigate the effect of the PEST sequence on K-cyclin’s stability. Results: Viral K-cyclin interacted with cyclin-dependent kinases cdk2, cdk4, and cdk6 and with the cyclin/cdk inhibitory proteins p21Cip1 and p27Kip1 in BC3 cell lysates. Unlike D-type cyclins, whose expression is cell cycle dependent, the level of K-cyclin was stable throughout the cell cycle, and the kinase associated with the K-cyclin/cdk6 complex was constitutively active. The half-life of K-cyclin (6.9 hours) was much longer than that of cellular cyclin D2 (0.6 hour) and that of K-cyclin/D2 (0.5 hour), probably because K-cyclin lacks the PEST degradation sequence present in D-type cyclins. Conclusion: The constitutive activation of K-cyclin/cdk complexes in KSHV-infected cells appears to result from the extended half-life of K-cyclin and may explain its role in Kaposi sarcoma. [J Natl Cancer Inst 2005;97:656–666]

Kaposi sarcoma herpesvirus (KSHV) has been implicated as the causative agent of Kaposi sarcoma (KS), primary effusion lymphoma, and multicentric Castleman’s disease (1,2). KSHV is a member of the γ-2 herpesvirus family, and development of malignant tumors is a common pathologic event after γ-2 herpesvirus infection (3,4). KSHV and other γ-2 herpesviruses encode homologues of cellular genes that may deregulate cell signaling pathways, promote cellular proliferation, and prevent apoptosis (5,6). One of the viral-encoded genes, K-cyclin, is homologous to cellular cyclin D and shares structural and functional similarities with cyclin D, as well as cyclins encoded by other γ-2 herpesviruses, including V-cyclin encoded by herpesvirus saimiri, M-cyclin encoded by murine herpesvirus 68, and A-cyclin encoded by herpesvirus Atelles (2,7). K-cyclin is one of the few viral-encoded genes expressed in latent, KSHV-containing malignant cells, suggesting that it may be critical for tumor formation and for maintenance of the transformed state. Enforced expression of K-cyclin induces chromosome instability and tumorigenesis in p53−/− mice but not in wild-type mice expressing functional p53 tumor suppressor protein (8,9).

Transition from the G0/G1 phase to S phase of the cell cycle requires sequential activation and/or deactivation of specific complexes of cyclin and cyclin-dependent kinase (cdk). Cyclin D is the first cyclin expressed during G1 phase, and its associated kinase activity is required for passage through the G1/S-phase transition in cells containing functional retinoblastoma protein (10,11). Cellular D-type cyclins form complexes with cdk4 and with cdk6, and these complexes phosphorylate the retinoblastoma protein and initiate S-phase entry (12). Because the kinase activity associated with cyclin D initiates the cascade of events that leads to cell cycle progression, cyclin D activation is tightly regulated. Mitogen stimulation of cells increases the stability of cyclin D, so that it accumulates in the cytoplasm during G1 phase (13,14). Assembly of cyclin D/cdk4 and cyclin D/cdk6 complexes is facilitated by interactions with the cyclin-dependent kinase inhibitors (CDKIs) p21Cip1 and p27Kip1, and the assembly of cdk4 with cyclin D is blocked by the interaction of p16INK4A family proteins with cdk4 or cdk6 (15,16). Assembled cyclin D/cdk complexes translocate to the nucleus by use of nuclear localization signals present in the CDKIs (16,17). Nuclear cyclin D/cdk complexes are then fully activated by posttranslational modification of cdk by the cdk-activating kinase and cdc25A phosphatase (18,19).

Regulation of cyclin D/cdk kinase activity is also influenced by the stability of cyclin D. In early S phase, glycogen synthase kinase 3β (GSK-3β) is activated, and the active kinase phosphorylates cyclin D at amino acid threonine 286 (20,21). Phosphorylation of cyclin D at threonine 286, in turn, facilitates its association with the nuclear export protein CRM1, resulting in diffusion of cyclin D from the nucleus to the cytoplasm through nuclear pores (22). Cytoplasmic cyclin D is then targeted for ubiquitin-mediated proteosomal degradation by the PEST motif located at the carboxyl-terminal end of the protein. [PEST motifs are sequences enriched in proline (P), glutamic acid (E), serine (S),...
or threonine (T) residues, and these motifs are present in many rapidly degraded proteins (23,24). Disruption of the PEST motif in cellular cyclin D by a mutation of threonine-286 to alanine prevents its cytoplasmic translocation and ubiquitin-mediated degradation (21,22).

Viral K-cyclin associates with the cdk2, cdk4, and cdk6 cyclin-dependent kinases, albeit primarily with cdk6 (12,25,26), and K-cyclin complexes with cdk6 and cdk2 form an active kinase that phosphorylates the retinoblastoma protein and induces entry into S phase (27–29). The kinase activity of K-cyclin, however, is not tightly regulated, and this lack of regulation may create conditions that are favorable for unscheduled cell cycle entry and, consequently, malignant transformation. For example, members of the p16\(^{ink4A}\) inhibitor family do not inhibit the assembly of K-cyclin/cdk complexes and do not reduce its kinase activity (27). The phosphorylation of cdk6, which is induced by the cdk-activating kinase, is not required for K-cyclin/cdk-mediated phosphorylation of retinoblastoma protein, but it is necessary for S-phase entry of K-cyclin–expressing NIH 3T3 cells (30,31). K-cyclin/cdk6 complexes phosphorylate not only the cellular cyclin D substrate retinoblastoma protein but also substrates normally targeted by cyclins E and A, including histone H1, p27\(^{kip1}\), cdc25A, Orc1, and Cdc6 [for review, see (5,32)].

In this study, we investigated the expression of K-cyclin, the formation of complexes between K-cyclin and cdk5, the kinase activity of this complex, and K-cyclin turnover, and we compared these characteristics with those of cellular cyclin D. Our goal was to identify molecular properties of K-cyclin that could explain the lack of regulation of this kinase and provide further support that K-cyclin plays an important role in Kaposi sarcoma tumorigenesis.

**Materials and Methods**

**Cell Culture**

Primary effusion lymphoma (PEL) cell lines BC1, BC2, and BC3 are KSHV-containing human cells. They were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA). Epstein-Barr virus–positive, KSHV-negative human Daudi cells were obtained from ATCC and cultured in RPMI 1640 medium. U-2OS cells (KSHV-negative human osteosarcoma cell line) stably transfected with the pTet-On vector (which is active in the presence of doxycycline, a derivative of tetracycline, but not in its absence) was obtained from BD Biosciences (Palo Alto, CA), transfected with the pTRE-K-cyclin or pTRE-K-cyclin/D2 vector, and cultured in McCoy’s 5A medium. RPMI 1640 and McCoy’s 5A media were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin G at 100 U/mL, and streptomycin at 100 μg/mL (Invitrogen). RPMI 1640 cell culture medium for BC2 cells contained 15% fetal bovine serum, penicillin at 100 U/mL, and streptomycin at 100 μg/mL. SAOS cells were obtained from ATCC and cultured in McCoy’s 5A medium containing 10% fetal bovine serum, penicillin at 100 U/mL, and streptomycin at 100 μg/mL.

**Reagents**

Sheep K-cyclin and control sheep immunoglobulin G (IgG) antibody were developed by Exalpha Biologicals, Inc. (Watertown, MA). Antibodies against cyclin D2 (product C-17), cyclin B1 (product GNS1), p21\(^{Cip1}\) (product 187), p27\(^{kip1}\) (product C-19), cdk2 (product H-298), cdk4 (product C-22), and cdk6 (product C-21) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lamin B1 antibody was obtained from Abcam Ltd. (Hartford, CT). Doxycycline, cycloheximide, aprotinin, RNase A, ATP, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and Pefabloc were from Sigma Chemical Co. (St. Louis, MO). \(^{35}\)S-labeled methionine/cysteine (Tran\(^{35}\)S-label) was obtained from MP Biomedicals (Irvine, CA). Adenosine 5’-[\(^{32}\)P]triphosphate, triethylammonium salt, was obtained from Amersham Biosciences (Piscataway, NJ). All other reagents used in this study were reagent grade.

**Generation and Characterization of K-Cyclin Antibody**

K-cyclin cDNA was subcloned into the pET histidine (His) fusion protein expression vector and expressed in *Escherichia coli* BL21(DE3) by induction with 1.0 mM isopropyl β-D-thiogalactoside, by the manufacturer’s protocol (EMD Biosciences, Madison, WI). Cells were collected by centrifugation at 6000g for 10 minutes at 4 °C, resuspended in phosphate-buffered saline (PBS), and broken by repeated freeze–thaw cycles. Crude extract was then obtained by removing the insoluble material with centrifugation at 10000g for 10 minutes at 4 °C. The fusion protein His/K-cyclin was then purified by the addition of 1.0 mL of Ni\(^{2+}\)-immobilized agarose beads, and the bound proteins were washed and eluted with the pET vector kit components, as directed by the manufacturer (EMD Biosciences). The beads were removed by centrifugation at 500g for 5 minutes at room temperature. The resulting supernatant containing His/K-cyclin fusion protein was dialyzed three times against dialysis buffer (20 mM HEPES [pH 7.4], 0.2 mM EDTA, 0.1 M KCl, and 20% glycerol). Sheep and rabbit IgG polyclonal antibodies directed against the recombinant K-cyclin protein were produced by Exalpha Biologicals (Boston, MA). We characterized the K-cyclin antibodies by western blot analysis of the expressed, recombinant protein or of cell lysates from KSHV-containing cell lines (BC1, BC2, and BC3 cells; data not shown) and KSHV-negative cell lines (Daudi, U-2OS, and SAOS cells; data not shown). A single band was observed in all KSHV-positive but no KSHV-negative cell lines; the band corresponded to a molecular mass of slightly less than 30 kDa, as predicted for K-cyclin.

**Immunoprecipitation and Immunoprecipitation-Coupled Western Blot Analyses**

BC3 cells \((2 \times 10^6 \text{ cells})\) cultured in RPMI 1640 medium were washed twice in ice-cold PBS, and the cell membrane was disrupted by resuspension in 1 mL of kinase lysis buffer (KLB = 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 4 mM EDTA, 4 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 0.1% bovine serum albumin) containing protease inhibitors (phenylmethylsulfonyl fluoride at 50 ng/mL, aprotinin at 5 μg/mL, leupeptin at 5 μg/mL, pepstatin at 5 μg/mL, and Pefabloc at 150 μg/mL). Immunoprecipitation was carried out with antibodies, as indicated, that had been covalently attached to protein A– or G-Sepharose beads through the chemical cross-linker disuccinimidyl suberate (Pierce Chemical Company, Rockford, IL). Cellular proteins that interact nonspecifically with Sepharose beads or sheep IgG were removed by incubating the cell lysate with 40 μL of sheep IgG–Sepharose beads (1 μg of
antibody per 10 μL of beads) for 90 minutes at 4 °C. The cell lysates were centrifuged at 1000g for 1 minute, and the supernatant was transferred to a clean microcentrifuge tube. After this pre-clearing step, the cell lysate was quantified, and 200 μg of protein was incubated with 20 μL of the K-cyclin antibody–Sepharose bead conjugate at a concentration of 1 μg of antibody per 10 μL of beads for 2 hours at 4 °C. Immune complexes were washed three times with 1 mL of KLB containing phenylmethylsulfonyl fluoride at 50 ng/mL and centrifuged for 1 minute at 1000g, and the buffer was discarded. Immunoprecipitated proteins were dissociated from the beads by boiling for 3 minutes in one bed volume of 2 × sodium dodecyl sulfate (SDS)–loading dye (126 mM Tris–HCl [pH 6], 20% glycerol, 4% SDS, 0.02% bromophenol blue, and 1% 2-mercaptoethanol), and the entire sample was loaded onto an SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel by use of pipetman gel loading tips (Continental Lab Products, San Diego, CA) that have a small bore size to exclude the beads.

For immunoprecipitation-coupled western blot analysis, the cell lysate was precleared with sheep IgG– and rabbit IgG–Sepharose beads as described above. Immunoprecipitation was then conducted with Sepharose beads coupled to sheep K-cyclin antibodies or to rabbit antibodies against cdk2, cdk4, cdk6, cyclin D2, and p27Kip1. Immune complexes were washed and resolved by SDS–PAGE, as described above. Western blot analysis of the immunoprecipitated samples was performed with rabbit K-cyclin antibodies or with goat antibodies against cdk2, cdk4, cdk6, cyclin D2, p27Kip1, or p21Cip1, as indicated below.

**Western Blot Analysis**

Proteins resolved by SDS–PAGE were transferred to Immobilon P membranes as instructed by the manufacturer (Millipore Corp., Bedford, MA). The membrane was then incubated in a solution of Tris-buffered saline and Tween-20 (TTBS = 0.02 M Tris–HCl [pH 7.6], 0.14 M NaCl, and 0.05% Tween-20) containing 5% nonfat dry milk (BioRad, Hercules, CA) at room temperature for 1 hour. The membrane was incubated with antibodies against K-cyclin, cyclin D2, cyclin B1, cdk2, cdk4, cdk6, p27Kip1, p21Cip1, lamin B1, or actin at a 1 : 1000 dilution in TTBS containing 5% nonfat dry milk. Membranes were then washed twice in TTBS and incubated with the appropriate secondary antibody at a 1 : 10000 dilution for 1 hour in TTBS containing 5% nonfat dry milk. After three 5-minute washes in TTBS and one 5-minute wash in TBS (0.02 M Tris–HCl [pH 7.6] and 0.14 M NaCl), the membrane was incubated with Enhanced Chemiluminescence Plus reagents (ECL-Plus; Amersham-Pharmacia, Piscataway, NJ) for 1 minute, as described in the instruction manual. To visualize western blot protein bands, the membranes were exposed to x-ray film at room temperature and developed in a film processor.

**Metabolic Labeling and Pulse-Chase Analyses**

For metabolic labeling, 2 × 10^6 BC3 cells were cultured in methionine-free/cysteine-free RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone), penicillin G at 100 U/mL, and streptomycin at 100 μg/mL (Invitrogen). Tran35S-Label (MP Biomedicals) was added to the cell culture medium to a final concentration of 500 μCi/mL, and the cell culture was incubated for 6 hours at 37 °C. Cells were harvested. Cells were washed in PBS, and cell membranes were broken by resuspension in KLB. The insoluble cellular material was removed by centrifugation at 12000g for 20 minutes at 4 °C. Radiolabeled proteins were isolated by immunoprecipitation with K-cyclin antibody or sheep IgG antibody as described above. Samples were loaded onto an SDS–polyacrylamide gel and separated by electrophoresis. Autoradiographic signals were enhanced with Amplify reagent (Amersham-Pharmacia), and the gel was exposed to film for visualization of protein bands.

For determination of protein half-life by pulse-chase analysis, BC3 cells were metabolically labeled for 30 minutes, as described above, and centrifuged at 1000g for 3 minutes at room temperature. The cell pellet was resuspended in complete medium supplemented with 2 mM methionine and 2 mM cysteine; cells were harvested at 0, 4, 6, 8, 12, and 24 hours; and cell lysates were prepared as described above. The total protein content of the cell lysates was determined with the Bio-Rad DC Protein Assay Reagent as directed by the manufacturer (Hercules, CA). A total of 200 μg of lysate protein was immunoprecipitated with 20 μL of K-cyclin antibody–bead conjugate as described above. Immunopurified proteins were resolved by SDS–PAGE and visualized with the InstantImager Electronic Autoradiography System (Packard BioScience Company, Meriden CT).

**Nuclear and Cytoplasmic Cell Fractionation**

Cytoplasmic extracts were prepared by washing 1 × 10^6 BC3 cells in PBS, centrifuging cells as described above, and resuspending the cell pellet in cytoplasmic buffer A (10 mM Tris–HCl [pH 7.5], 25 μM sodium fluoride, 5 mM magnesium chloride, 1 mM EGTA, 1 mM diithiothreitol, 0.1 mM sodium vanadate, aprotinin at 5 μg/mL, leupeptin at 5 μg/mL, pepstatin at 5 μg/mL, and Pefabloc at 150 μg/mL). Cells were allowed to swell on ice for 15 minutes and then homogenized in a Dounce homogenizer with 20 strokes of pestle B. Nuclear material was removed from the cytoplasmic extract by centrifugation at 500g for 10 minutes at 4 °C, and the supernatant was further purified by centrifugation at 315000g for 30 minutes. The supernatant from this centrifugation was the cytoplasmic lysate.

Nuclear lysates were prepared from nuclei purified by sucrose gradient centrifugation. In brief, 1 × 10^6 BC3 cells were washed in PBS and resuspended in 4 mL of ice-cold sucrose buffer A (0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween-20, 10 mM Tris [pH 8.0], 0.1 mM sodium vanadate, aprotinin at 5 μg/mL, leupeptin at 5 μg/mL, pepstatin at 5 μg/mL, and Pefabloc at 150 μg/mL). Cells were transferred to a Dounce homogenizer and broken with 50 strokes of pestle B. Ruptured cells were mixed with 4 mL of ice-cold sucrose buffer B (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris [pH 8.0], 0.1 mM sodium vanadate, aprotinin at 5 μg/mL, leupeptin at 5 μg/mL, pepstatin at 5 μg/mL, and Pefabloc at 150 μg/mL). The cell suspension was layered onto a 4.4-mL cushion of sucrose buffer B, the gradient was centrifuged at 300000g for 45 minutes, and the supernatant was removed by vacuum aspiration. The remaining nuclear pellet was lysed with KLB, as described above for whole-cell lysates. Protein in cytoplasmic and nuclear lysates was quantified, and aliquots were stored at −80 °C. To determine whether cytoplasmic fractions were free from nuclear contamination, 50 μg of nuclear and cytoplasmic cell lysate proteins was separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane as described above. To determine whether the nuclear
and cytoplasmic lysates were properly isolated, western blot membranes were also probed with antibody directed against lamin B1, a protein that is expressed in the nuclear compartment but not the cytoplasm.

**Elutriation**

BC3 cells were fractionated into G1–phase–, S-phase–, and G2/M-phase–enriched populations by use of a J6-MC centrifuge with a JE-5.0 preparative scale rotor (Beckman Coulter, Inc., Fullerton, CA). The medium flow rate was controlled with a Masterflex pump and model 7518–12 pump head (Cole-Palmer, Vernon Hills, IL), as previously described (33). Briefly, elutriation medium consisted of equal parts of PBS and RPMI 1640 medium supplemented with 1% fetal bovine serum. Approximately 1.9 × 10⁹ cells were introduced into the elutriation chamber at a flow rate of 17 mL/minute for 60 minutes with a rotor speed of 2000 rpm. The cell gradient was allowed to form for 20 minutes at a pump rate of 54 mL/minute. The first liter fraction was collected at a flow rate of 73 mL/minute, and then the flow rate was increased by 10 mL/minute for each successive fraction. Approximately 1 × 10⁶ intact cells were removed from an elutriated fraction to analyze DNA content by flow cytometry. Aliquots of the remaining cells in the fraction were concentrated by centrifugation at 500g, and cell pellets were stored for future use at −80 °C.

**DNA Content Analyses**

The DNA content of a population of cells was determined by flow cytometry. In brief, approximately 1 × 10⁶ BC3 cells were suspended in a buffer of propidium iodide at 50 μg/mL, sodium citrate at 1 μg/mL, Triton X-100 at 1 μg/mL, and RNase A at 5 μg/mL; incubated at room temperature for 30 minutes; filtered through a 40-μm (pore size) mesh (Small Parts, Inc., Miami Lakes, FL), and subjected to flow cytometry on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Fifteen thousand events were analyzed for each sample, and DNA histogram analyses were performed with ModFit LT (Verity Software House, Topsham, ME).

**In Vitro Kinase Assays**

For kinase assay, 2 × 10⁶ BC3 cells were washed in PBS, lysed in KLB, and immunoprecipitated with K-cyclin, cdk6, or cyclin B1 antibodies, as described above. Immune complexes were washed twice with KLB (without protease inhibitors) and twice with 2× kinase buffer (100 mM Tris–HCl [pH 7.4], 20 mM MgCl₂, and 2 mM phenylmethylsulfonyl fluoride) and then incubated for 10 minutes at 30 °C in kinase buffer containing 12.5 μM ATP, 2 μCi of [γ-³²P]ATP (Amersham-Pharmacia), and 1 μg of a fusion protein containing glutathione S-transferrase and retinoblastoma protein (GST-Rb), histone H1, or no substrate in a total volume of 20 μL. After this incubation, kinase reactions were stopped by the addition of an equal volume of 2× SDS–loading dye. Reaction products were separated by SDS–PAGE, and phosphorylated products were visualized by autoradiography.

**Identification of PEST Domains**

We used the program PESTfind (http://www.at.embnet.org/embnet/tools/bio/PESTfind) to identify potential PEST sequences in human cellular cyclins D1, D2, and D3 (GenBank accession numbers = NM_053056, X68452, and NM_001760, respectively) and in cyclins encoded by human herpesvirus 8, herpesvirus saimiri, and murine herpesvirus 68 (GenBank accession numbers = U40667, NP_040274, and U91858, respectively). PEST sequences are hydrophilic peptide regions that contain at least one proline (P), one glutamic acid (E) or one aspartic acid (D), and one serine (S) or threonine (T) flanked by lysine (K), arginine (R), or histidine (H). The results of PESTfind analysis are a score ranging from −50 to +50. Any positive score indicates a possible PEST region, but values greater than +5 are of more interest.

**Generation of K-Cyclin/cyclin D2 Chimeric Protein and Stable Expression in U-2OS Cells**

The K-cyclin/cyclin D2 chimeric sequence was designed to express the entire 257-amino acid sequence of K-cyclin fused to amino acids 262–289 of cellular cyclin D2, which contains the PEST sequence of cyclin D2. Each DNA fragment was amplified by the polymerase chain reaction (PCR) with Pfu polymerase (Stratagene, CA) and the following oligonucleotide primer pairs: 5′ cyclin D2 (GTCGACCTGCAGTACCGTCAGGAC) and 3′ cyclin D2 (TCACAGGTGATCCGCA); and 5′ K-cyclin Nhel (GCTAGCCACTCTATGGCAACTGCAAACTAC) and 3′ K-cyclin Xhol (CTCGAGAAGTCTGTCGTAAGGCTCG). PCR fragments were ligated at the unique Xhol and Nhel restriction enzyme sites added to K-cyclin and the carboxyl-terminal end of the cyclin D2 gene sequence. The nucleotide sequence of the K-cyclin/cellular cyclin D2 chimera (K-cyclin/D2) was verified by dideoxynucleotide sequencing in the DNA sequencing core facility at Vanderbilt University. The DNA sequence corresponding to wild-type K-cyclin or the K-cyclin/D2 chimera was subcloned into the tacrycycline-regulated plasmid pTRE2-Hyg, and the plasmids were stably transfected in U-2OS cells by use of the Tet-On Expression System (BD Biosciences, Palo Alto, CA). The Tet-On expression vector expresses a mutated form of the Tet repressor protein fused to the herpes simplex virus VP16 activation domain (rtTas). The rtTas protein binds to the responsive element in a second plasmid, the tet response element plasmid (pTRE). The U-2OS-pTet-On stable cell line was transfected with the pTRE-Hyg-K-cyclin or pTRE-Hyg-K-cyclin/D2 vectors, and colonies arising from single cells were selected with hygromycin (Hyg) at a final concentration of 75 μg/mL. The expression of K-cyclin or the K-cyclin/D2 is induced when doxycycline (a tetracycline derivative) interacts with the rtTa protein and binds to the responsive element in the pTRE vector.

**Protein Half-Life Analysis With Cycloheximide**

Protein half-life was measured by blocking protein synthesis with cycloheximide and harvesting the cells at various times. To determine the half-life of K-cyclin, cellular cyclin D2, cdk6, and p27Kip1 in BC1, BC2, and BC3 cells, we added cycloheximide to the culture medium to a final concentration of 50 μg/mL. The half-life of K-cyclin, the K-cyclin/D2 chimera, and cellular cyclin D2 was determined in U-2OS cells by adding doxycycline (to a final concentration of 1 μg/mL) to the cell culture medium 16 hours before the addition of cycloheximide. For all half-life determinations with cycloheximide, cells were collected as indicated by centrifugation, and western blot analysis of cyclins, cdk6, and CDK1 proteins was carried out as described above. Cells collected at time zero were cultured in the absence of cycloheximide.
Statistical Analysis

Student’s \( t \) test was used to determine the statistical significance of the increased half-life of K-cyclin in comparison with that of cellular cyclin D2 in BC3 cells. Half-life values were determined by digitizing x-ray images of western blot bands and quantifying the density of each protein band with Gel-Pro Analyzer version 3.1 software (Media Cybernetics, Silver Spring, MD). The fold increase in band density in comparison with that at zero time was determined for each point. Band densities were then plotted, and the time corresponding to half of the protein density was determined by extrapolation. All calculations were performed with the Microsoft Excel 2002 software package. The half-lives of K-cyclin and cellular cyclin D2 were expressed as mean values. Values were determined to be statistically significantly different at \( P < .05 \). All statistical tests were two-sided.

Results

K-Cyclin and K-Cyclin–Associated Proteins in KSHV-Infected Cells

We generated antibodies against KSHV-encoded K-cyclin and used them to characterize K-cyclin in KSHV-infected cells. We found that immune complexes isolated from metabolically labeled BC3 cells with this K-cyclin antibody contained a protein of approximately 30 kDa, which is consistent with the predicted size of K-cyclin (Fig. 1). Immune complexes containing K-cyclin also contained proteins with molecular masses of 40 and 33 kDa, corresponding to known K-cyclin–interacting proteins (i.e., cdk6 and cdk2, respectively), in addition to unknown, high molecular mass proteins of approximately 97 and 110 kDa.

Subcellular Distribution of K-Cyclin and K-Cyclin–Associated Proteins

To determine the subcellular distribution of K-cyclin, we isolated cytoplasmic and nuclear cell fractions from BC3 cells. We detected K-cyclin protein by western blot analysis in both cytoplasmic and nuclear cell fractions (Fig. 2, A, upper), with the level of K-cyclin being greater in the cytoplasmic fraction than in the nuclear fraction. We determined that the cytoplasmic fraction was not contaminated with nuclear material by probing the western blots with antibody against the nuclear matrix protein lamin B1 (Fig. 2, A, lower). As a negative control, we also probed fractions of KSHV-negative Daudi cells, which do not express K-cyclin, and found no labeled bands. Thus, the K-cyclin antibodies appeared to specifically recognize the KSHV-encoded K-cyclin and did not cross-react with other 30-kDa proteins (Fig. 2, A).

Because the kinase activity of cellular cyclin D is regulated by its association with cdks in the cytoplasm and by CDKI-mediated nuclear translocation (12,15,20), we investigated whether the K-cyclin antibody could immunoprecipitate K-cyclin complexes containing cdks and CDKIs from the nuclear and cytoplasmic lysate fractions of BC3 cells by use of western blot analysis. We found that proteins immunoprecipitated with the K-cyclin antibody included K-cyclin, cdk2, and cdk6 in both subcellular compartments, indicating that K-cyclin interacts with these two cdks (Fig. 2, B and C). In these complexes, the level of cdk6 was much higher than that of cdk2.

In contrast, when we used antibodies against cellular cyclin D2 in immunoprecipitation experiments, cellular cyclin D2 apparently did not interact with cdk6 but did form complexes cdk4 and cdk2, as expected. Immune complexes made with cdk4 or cdk2 antibody contained cdk4 or cdk2 proteins but did not contain cyclin D2 probably because the cdk4 and cdk2 antibody bound to cdk in a region that was important for the interaction between cyclin and cdk. These results show that K-cyclin and cyclin D2 interact with cdks, consistent with previous results from whole-cell lysates (25,26,29).

We used western blot analysis of proteins precipitated with the K-cyclin antibody to show that K-cyclin also interacted with p21Cip1 and p27Kip1 in cytoplasmic and nuclear fractions of BC3 cells (Fig. 2, B and C). In addition, we found that immune complexes precipitated with antibodies against cellular cyclin D contained p21Cip1 and p27Kip1, as well as cellular cyclin D. Because K-cyclin/cdk complexes were detected in both the cytoplasmic and nuclear subcellular compartments and because the immune complexes precipitated with antibodies against K-cyclin contained p21Cip1 and p27Kip1, these CDKIs may facilitate the assembly of K-cyclin/cdk complexes and their nuclear translocation in the same way as they facilitate the assembly and nuclear translocation of cellular cyclin D/cdk complexes.

Cell Cycle–Specific Expression and Kinase Activity of K-Cyclin

To characterize the cell cycle-specific expression and kinase activity of K-cyclin, we isolated populations of BC3 cells enriched in cells in G1, S, or G2/M phase by elutriation and assessed...
their DNA content by flow cytometry to verify that each population was enriched as expected. We found the expected enrichment of cells: G1-phase–enriched cells were in fractions 1–4, S-phase–enriched cells were in fractions 5–8, and G2/M-phase–enriched cells were in fractions 9 and 10 (Fig. 3, A). We also used a portion of each cell fraction to determine its protein content. As shown in Fig. 3, B, the same level of K-cyclin protein was found in cells in each phase of the cell cycle; however, the level of cellular cyclin D2 protein was higher in cells in G1 and S phases and lower in cells in G2/M phase. We also examined the expression of cellular cyclin B1 in the elutriated BC3 cell fractions and observed that cyclin B1 was undetectable in cells in early G1 phase but was clearly detected in cells in S and G2/M phase, as expected. The expression patterns of cellular cyclin B1 and cyclin D proteins are consistent with those in other reports (11,34) indicating that K-cyclin appears to be constitutively expressed and that its synthesis and/or degradation may be improperly regulated in BC3 cells.

To determine whether the K-cyclin/cdk6 kinase was active, we carried out immunoprecipitation-coupled kinase assays with retinoblastoma protein as substrate (Fig. 3, C). BC3 cells in all phases of the cell cycle contained the same level of K-cyclin–associated kinase activity, indicating that the kinase is active throughout the cell cycle. In addition, the kinase activity of cdk6 paralleled that of K-cyclin. However, when we examined cyclin B1 kinase activity with histone H1 as substrate, we observed the expected lack of kinase activity in cells in early G1 phase, followed by increased cyclin B1 activity in cells in S and G2/M phases (Fig. 3, C). Thus, the protein expression and the kinase activity of K-cyclin/cdk6 complexes appear to be constitutive in KSHV-infected cells, but the protein expression and kinase activity of cyclins D2 and B1 appear to be cell cycle dependent.

### K-Cyclin Protein Stability in KSHV-Containing Cell Lines

To determine whether the constitutive kinase activity of K-cyclin/cdk complexes is due to increased K-cyclin stability, we analyzed K-cyclin stability by pulse-chase analysis and by blocking protein synthesis with cycloheximide (Fig. 4, A and B). We found that the average half-life of K-cyclin in BC3 cells was 6.9 hours. In contrast, cellular cyclin D2 was labile, with a half-life of approximately 0.6 hours, consistent with previous reports (21,35). These results show that the half-life of K-cyclin was statistically significantly longer than cellular cyclin D (difference = 6.3 hours, 95% confidence interval = 5.3 to 7.4 hours; P = .004). The half-lives for cdk6 (4 hours) and p27kip1 (3 hours) proteins in BC3 cells were consistent with those previously reported (36,37).

We also examined the half-life of K-cyclin in BC1 and BC2 cells, two other KSHV-infected cell lines. The BC1 and BC2 cell lines were isolated by Cesaran et al. (38) from a primary effusion lymphoma and were described as being dually infected with KSHV and Epstein-Barr virus. We found that the half-life of K-cyclin in BC2 cells (Fig. 4, C) and BC1 cells (data not shown) was between 36 and 48 hours. We also observed that the half-life of cdk6 in BC2 cells (>8 hours) was also dramatically increased compared with its half-life in BC3 cells (4 hours) (Fig. 4, D). In contrast, the half-lives of cellular cyclin D2 (58 minutes) (Fig. 4, C) and p27kip1 (2.3 hours) (Fig. 4, D) in BC2 cells were similar to those in BC3 cells, suggesting that the increased half-life of K-cyclin and cdk6 is specific to the K-cyclin/cdk6 complex.

### Cyclin D2 PEST Sequence and K-Cyclin Protein Stability

To determine whether structural features may account for the extended half-life of the viral-encoded K-cyclin, we compared the PEST sequence of K-cyclin with that of cellular cyclin D2.
the amino acid sequences of cyclins encoded by murine herpesvirus 68, herpesvirus saimiri, and KSHV with those of the cellular D-type cyclins. The viral proteins were smaller, primarily because of truncation at the carboxyl terminus (Fig. 5, A). The carboxyl terminus of cellular cyclin D contains a PEST sequence that targets this protein for ubiquitin-mediated degradation. We used the algorithm PESTfind to search for PEST motifs in the viral and cellular cyclin protein sequence (23,39,40), and we found that none of the viral cyclin sequences contained a PEST motif but that the cellular cyclin D1, D2, and D3 sequences did contain a PEST motif. The region with the PEST motif contains a threonine (threonine-280 in cyclin D2 or threonine-286 in cyclin D1) that is phosphorylated by glycosyl synthase kinase-3β to initiate its translocation to the cytoplasm and degradation by the proteasome (20,21).

From the amino acid sequences of D-type cyclins, we hypothesized that the increased half-life of K-cyclin was related to the absence of a PEST motif. To test this hypothesis, we constructed a chimeric protein, K-cyclin/D2, that included the complete 257-amino acid sequence of K-cyclin fused to the carboxyl-terminal 27 amino acids of cellular cyclin D2, which contain a PEST motif (Fig. 5, B). We then stably transfected K-cyclin/D2 and wild-type K-cyclin into U-2OS cells by use of expression vectors driven by the tetracycline promoter, which was activated with doxycycline (a derivative of tetracycline), and confirmed that the expression of the corresponding proteins was positively regulated by the tetracycline promoter (Fig. 5, C). Using this system, we were able to examine the half-life of K-cyclin away from any influence of KSHV- or Epstein-Barr virus–encoded proteins. Western blot analysis of the doxycycline-induced K-cyclin/D2 chimeric protein showed that it was efficiently expressed and was recognized by our K-cyclin antibody (Fig. 5, C). Next, we performed immunoprecipitation-coupled kinase assays with immune-purified K-cyclin/D2 chimeric protein or cyclin D2 protein. Mitogen stimulation is required for activation of cyclin D/cdk kinase activity but not for activation of K-cyclin/cdk kinase activity (27,31). K-cyclin/D2 kinase was able to phosphorylate the retinoblastoma protein and histone H1 in the absence of mitogen stimulation. Our results indicated that the chimeric protein retained kinase activity toward retinoblastoma protein and also its promiscuous kinase activity toward histone H1 (Fig. 5, D). As expected, cellular cyclin D was unable to phosphorylate the retinoblastoma protein or histone H1 above background levels in the absence of mitogen stimulation.

Finally, we examined the half-life of K-cyclin protein, K-cyclin/D2 chimeric protein, and cellular cyclin D2 protein by use of the doxycycline-inducible system. The half-life of K-cyclin protein was between 36 and 48 hours (Fig. 5, E). When the PEST motif from cyclin D2 was fused to K-cyclin, however, the half-life of the chimeric protein was reduced to 30 minutes, which was similar to the half-life of cellular cyclin D2 (23 minutes) in U-2OS cells (Fig. 5, E and F). Thus, the longer half-life of the protein appears to be related to the absence of PEST-containing sequences. The extended half-life of K-cyclin may contribute to the constitutive activity of the K-cyclin/cdk complex.

**Discussion**

In this study, we showed that K-cyclin, a cyclin D homologue that is produced in KSHV-infected cells, forms complexes with proteins that play a role in cell cycle control, including the CDKIs p21Cip1 and p27Kip1 and the cdk proteins cdk6 and cdk2. The protein levels and kinase activity of K-cyclin protein were stable in all phases of the cell cycle. The half-life of K-cyclin protein (6.9 hours) was much longer than that of cellular cyclin D2 (0.6 hours). In addition, we obtained evidence that the increased half-life of K-cyclin was related to the absence of the PEST degradation motif that is present in cellular D-type cyclins. Thus, the constitutive K-cyclin/cdk kinase activity may result from the increased half-life of K-cyclin.

Several groups have demonstrated that the CDKIs p21Cip1 and p27Kip1 enhance formation of the cyclin D/cdk complex and facilitate the nuclear translocation of cyclin D. Parry et al. (15) showed that p21Cip1 or p27Kip1 associated with the cyclin D/cdk complex and that immunodepletion of either CDK1 from EL4 cell lysates effectively eliminated cyclin D/cdk complex formation. LaBaer et al. (16) demonstrated that immunodepletion of p21Cip1 from U-2OS cells eliminated cdk4-associated kinase activity and that p21Cip1, p27Kip1, and p57Kip2 could all direct the nuclear localization of cyclinD1/cdk4 complexes. Cheng et al.
K-cyclin formed complexes with both p21 Cip1 and p27 Kip1 in and did not localize properly to the nucleus. We found that have kinase activity and to induce cells to enter S phase expressed K-cyclin in unstimulated NIH 3T3 cells was shown to be similar to those formed with cellular cyclin D, and p21 Cip1 may have a role in the assembly of K-cyclin/cdk complexes. This result is also supported by the following studies. Ectopically D, which is regulated in a cell cycle phase-dependent fashion. Stitutive, unlike the kinase activity associated with cellular cyclin D, in the carboxyl-terminal PEST degradation motif, resulted in a mutant cyclin D protein with a half-life that was compared with that of wild-type cyclin D due to its enhanced stability. We found that, in BC3 cells, K-cyclin protein had a longer half-life than cellular cyclin D2 (Fig. 4, A). To investigate the half-life of K-cyclin and cellular cyclin D2, we constructed a chimeric protein that expressed the coding region of K-cyclin fused to the carboxyl-terminal PEST degradation domain of cyclin D2 and found that, in cells transfected with this chimeric protein, the half-life of wild-type K-cyclin was longer than that of the K-cyclin/D2 chimeric protein, which was comparable to the half-life of cellular cyclin D2. Thus, the inability to degrade K-cyclin may result in the constitutive kinase activity of K-cyclin/cdk complexes.

We also examined the stability of K-cyclin protein in cells that were dually infected with KSHV and Epstein-Barr virus and observed that the half-life of K-cyclin was increased by between 36 and 48 hours, with a concomitant increase in the half-life of cyclin D2. Sinclair et al. reported that, in mouse embryo fibroblasts lacking p21 Cip1 and/or p27 Kip1, cyclin D/cdk complexes assembled inefficiently and did not localize properly to the nucleus. We found that K-cyclin formed complexes with both p21 Cip1 and p27 Kip1 in KHSV-infected cells and detected these complexes in both the nucleus and cytoplasm (Fig. 2, B and C). We found that K-cyclin immune complexes also contained cdk6 and/or cdk2 (Fig. 2, B and C) and efficiently phosphorylated retinoblastoma protein (Fig. 3, C). Thus, K-cyclin/cdk6/CDKI ternary complexes appear to be similar to those formed with cellular cyclin D, and p21 Cip1 and p27 Kip1 may have a role in the assembly of K-cyclin/cdk complexes and their localization to the nucleus.

We isolated populations of KHSV-infected cells enriched in cells in G1, S, or G2/M phases of the cell cycle and found K-cyclin/cdk kinase activity in all populations (Fig. 2, C). These results indicate that K-cyclin, associated kinase activity is constitutive, unlike the kinase activity associated with cellular cyclin D, which is regulated in a cell cycle phase-dependent fashion. This result is also supported by the following studies. Ectopically K-cyclin in unstimulated NIH 3T3 cells was shown to have kinase activity and to induce cells to enter S phase (27,31). Posttranslational modification of cdk6 by cdk-activating kinase is not required for K-cyclin/cdk-mediated phosphorylation of retinoblastoma protein but is necessary for cells to progress through S phase (30,31). K-cyclin/cdk kinase activity is not inhibited by CDKI p16 Ink4A, p21 Cip1, or p27 Kip1, and K-cyclin/cdk-mediated phosphorylation induces degradation of p27 (27,41,42).

Thus, the apparently unregulated kinase activity associated with K-cyclin is consistent with our hypothesis that the kinase associated with the K-cyclin/cdk6 complex is constitutively active. Inappropriate expression of cellular D-type cyclins is a common feature in many types of cancer and may be a critical step in tumorigenesis. For example, cyclin D1 mRNA and protein levels are amplified in breast tumors and in benign parathyroid tumors, compared with normal breast tissue (10,43). Overexpression of cyclin D2 is observed in B-cell chronic lymphocytic leukemia and in T-cell leukemias compared with normal B and T cells (44,45).

We also examined the stability of K-cyclin protein in cells that were dually infected with KSHV and Epstein-Barr virus and observed that the half-life of K-cyclin was increased by between 36 and 48 hours, with a concomitant increase in the half-life of cyclin D2 (Fig. 4, D). An earlier study by Palmero et al. (45) indicated that cyclin D2 is overexpressed in many Epstein-Barr virus–immortalized cell lines. Sinclair et al. (47) reported that, in B lymphocytes, the Epstein-Barr virus–encoded proteins EBNA-2 and EBNA-LP were directly involved in inducing cellular cyclin D2, indicating that Epstein-Barr virus–encoded proteins may...
influence the half-life of K-cyclin. However, the half-life of K-cyclin protein in the U-2OS cell line was also between 36 and 48 hours (Figs. 4, C and 5, E), indicating that cellular proteins may influence the stability of K-cyclin. Thus, the half-life of K-cyclin may be influenced by cellular or viral proteins.

The finding that KSHV, a γ-2 herpesvirus, has an altered viral homologue (i.e., K-cyclin) of a cellular gene is reminiscent of the finding that transforming retroviruses have viral oncogenes that are homologues of corresponding cellular genes. An example is the feline sarcoma virus, which encodes v-fms, the viral homolog of the monocyte-macrophage colony-stimulating factor receptor. v-fms lacks the negative regulatory tyrosine residue found in its cellular counterpart and so is constitutively active in the absence of ligand and has increased transformation potential (48,49).

KSHV appears to have a similar strategy because the primary amino acid sequence of K-cyclin lacks the PEST degradation motif found in cellular cyclins. We expected that a stabilized K-cyclin would be associated with persistent kinase activity, as we observed, and would persistently phosphorylate its cellular substrates, including retinoblastoma protein, p27<sup>Kip1</sup>, cdc25A, Orc1, and Cdc6. For example, continuous K-cyclin–mediated phosphorylation of p27<sup>Kip1</sup> and retinoblastoma protein could remove the G<sub>1</sub>-phase block imposed by these proteins and activate expression of E2F-responsive genes (27,41,42). K-cyclin–mediated phosphorylation of cdc25A would then stimulate the phosphatase activity of cdc25A, resulting in unregulated activation of cdk2 and premature entry of cells into S phase (19,50).

Unabated phosphorylation of the replication initiation proteins Orc1 and Cdc6 could also provide a continuous stimulus for entry into S phase (30,51). However, the overall effect of continuous K-cyclin/cdk activation is likely to be dependent upon the functional status of regulatory host cell proteins. For example, Ojala et al. (52,53) reported that expression of K-cyclin in U-2OS cells produces an increase in apoptosis by K-cyclin/cdk6-mediated phosphorylation and inactivation the cellular antiapoptotic protein Bcl-2. Verschuren and colleagues (8,9) showed that expression of K-cyclin in mice promotes lymphomagenesis only in the absence of the tumor suppressor activity of p53. Identification of additional proteins that modulate K-cyclin activity may provide more clues about the cellular requirements for K-cyclin-mediated transformation.

**Fig. 5.** PEST motif and K-cyclin turnover. (A) Carboxyl-terminal amino acid sequences of cyclin D from Kaposi sarcoma–associated human herpesvirus (KSHV) cyclin, murine herpesvirus 68 (MHV-68), herpesvirus saimiri (HVS), and human cellular cyclins D1, D2, and D3. (B) Schematic diagram of the K-cyclin/D2 chimeric protein. Carboxyl-terminal nucleotide sequence of cyclin D2 containing the PEST motif was fused to the 3′-terminal end of the nucleotide sequence of K-cyclin. (C) Western blot analyses of U-2OS cells stably expressing the doxycycline-regulatable K-cyclin/D2 fusion protein in the presence (+Dox) or absence (−Dox) of doxycycline. Lane labeled with BC3 indicates the position of migration of wild-type K-cyclin protein. Lanes labeled with K-cyclin/D2 show the position of migration and level of expression of the K-cyclin/D2 chimeric protein in U-2OS cells in the absence (−) and presence (+) of doxycycline. (D) In vitro kinase analyses of U-2OS cells expressing K-cyclin/D2 chimeric protein in the absence (−) and presence (+) of doxycycline. Cellular cyclin D protein (lanes cycD) and K-cyclin/D2 chimeric protein (lanes K-cyclin/D2) were immunoprecipitated and subjected to analysis in kinase assays with glutathione S-transferase–retinoblastoma protein or histone H1 as substrate. (E) Half-life of K-cyclin. Expression of K-cyclin and the K-cyclin/D2 chimeric proteins was induced with doxycycline. Protein half-life was determined by adding cycloheximide to the cell medium. Western blot analysis was conducted with antibody directed toward K-cyclin (that reacts with K-cyclin and K-cyclin/D2 chimeric protein) or antibody directed against cyclin D2. The fold increase in protein band density is indicated (below). (F) Schematic representation of the half-lives of K-cyclin, K-cyclin/D2 fusion protein, and cyclin D2 from results in panel E. Results are representative of three independent experiments.
REFERENCES


NOTES

Rukiyah Van Dross, Shan Yao, Shaheena Asad, Grant Westlake, Deborah J. Mays, Laura Barquero, Stephanie Duell, and Jennifer A. Pietenpol dedicate this manuscript to the memory of our colleague Philip J. Browning, M.D. (1953–2004), his wife Renee, and his sons Philip II and Andrew. Dr. Browning was a clinical oncologist and a cancer researcher specializing in AIDS-related cancers.

Supported by Public Health Service Grants CA75535 (PJB), CA83216 (PJB), CA070856 (JAP), CA098695 (RVD), and P30 CA68485 (PJB) from the National Cancer Institute.

Manuscript received July 18, 2003; revised February 17, 2005; accepted March 8, 2005.