Molecular Biology of the Cell
Vol. 12, 3987–3999, December 2001

CAK-independent Activation of CDK6 by a Viral Cyclin

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Submitted June 15, 2001; Revised October 5, 2001; Accepted October 12, 2001
Monitoring Editor: Tim Stearns

In normal cells, activation of cyclin-dependent kinases (cdks) requires binding to a cyclin and phosphorylation by the cdk-activating kinase (CAK). The Kaposi's sarcoma-associated herpesvirus encodes a protein with similarity to D-type cyclins. This KSHV-cyclin activates CDK6, alters its substrate specificity, and renders CDK6 insensitive to inhibition by the cdk inhibitor $p16^{INK4a}$. Here we investigate the regulation of the CDK6/KSHV-cyclin kinase with the use of purified proteins and a cell-based assay. We find that KSHV-cyclin can activate CDK6 independent of phosphorylation by CAK in vitro. In addition, CAK phosphorylation decreased the $p16^{INK4a}$ sensitivity of CDK6/KSHV-cyclin complexes. In cells, expression of CDK6 or to a lesser degree of a nonphosphorylatable $CDK6^{T177A}$ together with KSHV-cyclin induced apoptosis, indicating that CDK6 activation by KSHV-cyclin can proceed in the absence of phosphorylation by CAK in vivo. Coexpression of p16 partially protected cells from cell death. p16 and KSHV-cyclin can form a ternary complex with CDK6 that can be detected by binding assays as well as by conformational changes in CDK6. The Kaposi's sarcoma-associated herpesvirus has adopted a clever strategy to render cell cycle progression independent of mitogenic signals, cdk inhibition, or phosphorylation by CAK.

INTRODUCTION

The sequential activation of cyclin-dependent kinases (cdks) promotes cell cycle transitions. CDK4 and CDK6 bind to D-type cyclins and are active in G1, CDK2/cyclin E complexes function in late G1, CDK2/cyclin A complexes function in S phase, and CDC2/cyclin B and A complexes function in G2/M. The activities of cdks are regulated by protein–protein interactions (with cyclins, inhibitors, and assembly factors), protein degradation, transcriptional control, subcellular localization, and multiple phosphorylations (Pines, 1995; Sherr and Roberts, 1995; King *et al.*, 1996; Solomon and Kaldis, 1998). Viral infection frequently targets downstream targets of cdks, resulting in inappropriate cell cycle progression.

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Cyclin binding activates cdks by inducing conformational changes in the structure of cdks (Jeffrey *et al.*, 1995; Pavletich, 1999). Cyclins are unstable proteins that are synthesized and degraded periodically during the cell cycle. Transcriptional control (Koch and Nasmyth, 1994) and ubiquitin-mediated degradation (King *et al.*, 1996) ensure the proper and irreversible timing of cell cycle regulatory events. Cyclins have also been shown to affect the substrate specificity of cdks (Peeper *et al.*, 1993; Kelly *et al.*, 1998; Schulman *et al.*, 1998; Cross *et al.*, 1999).

Maximal activation of cdks requires phosphorylation of certain residues as well as dephosphorylation of others. The dual-specificity phosphatase CDC25 removes phosphates from inhibitory phosphorylation sites (Thr-14 and Tyr-15 in human CDK2) that have been phosphorylated by the WEE1/MYT1 protein kinases (Solomon and Kaldis, 1998). Phosphorylation of an activating threonine (Thr-160 in CDK2 and Thr-177 in CDK6) by the cdk-activating kinase (CAK; reviewed by Kaldis, 1999) is accompanied by structural changes in the T-loop (also called the activation segment), allowing the phosphate group to interact with several other residues and thereby acting as an organizing center in the catalytic cleft (Russo *et al.*, 1996b). Mutation of the acti-

vating threonine to an unphosphorylatable amino acid prevents activation of vertebrate cdks (Desai *et al.*, 1992; Solomon *et al.*, 1992; Connell-Crowley *et al.*, 1993; Kato *et al.*, 1994; Matsuoka *et al.*, 1994) and equivalent mutants are unable to support growth in yeast (Gould *et al.*, 1991; Cismowski *et al.*, 1995).

Two very different CAKs have been identified. In species other than budding yeast, CAK is composed of a catalytic subunit, CDK7 (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993; Tassan *et al.*, 1994; also called MO15); a regulatory subunit, cyclin H (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994); and an assembly factor, MAT1 (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). All three of these proteins have also been found to be subunits of the general transcription factor TFIIH (Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995; Adamczewski *et al.*, 1996), which phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II. CDK7 prefers to phosphorylate cdk/cyclin complexes; monomeric cdks are poor substrates (Fisher and Morgan, 1994; Kaldis *et al.*, 1998). In contrast, the budding yeast CAK consists of a single, distantly related protein kinase, Cak1p (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996; Thuret *et al.*, 1996). Cak1p is an essential kinase that is responsible for phosphorylation and activation of the yeast cdk Cdc28p in vivo (Kaldis *et al.*, 1996; Thuret *et al.*, 1996). Cak1p preferentially phosphorylates monomeric cdks; cyclin binding to cdks decreases phosphorylation by Cak1p (Kaldis *et al.*, 1998; Brown *et al.*, 1999). Furthermore, Cak1p phosphorylates and activates several human cdks (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996, 1998; Thuret *et al.*, 1996).

Cdks can be inhibited by the binding of inhibitory proteins termed CKIs. Two families of CKIs have been identified (reviewed by Sherr and Roberts, 1995, 1999). Members of the Cip/Kip family inhibit all cdks and include $p21^{Cip1}$ (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993), $p27^{Kip1}$ (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994), and $p57^{Kip2}$ (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). Members of the INK4 family are specific for CDK4 and CDK6 and include $p15^{INK4b}$ (Hannon and Beach, 1994), $p16^{INK4a}$ (Serrano *et al.*, 1993), $p18^{INK4c}$ (Guan *et al.*, 1994; Hirai *et al.*, 1995), and $p19^{INK4d}$ (Chan *et al.*, 1995; Hirai *et al.*, 1995). All CKIs prevent the activating phosphorylation of cdks by CDK7, either by inducing a conformational change or by steric hindrance (Aprelikova *et al.*, 1995; Kaldis *et al.*, 1998); phosphorylation of cdks by Cak1p is not affected by CKIs (Kaldis *et al.*, 1998).

The Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8; reviewed by Brooks *et al.*, 1997; Ganem, 1997; Boshoff and Weiss, 1998; Moore and Chang, 1998) encodes a functional cyclin D homolog (Russo *et al.*, 1996a) termed v-cyclin or KSHV-cyclin. Like the related v-cyclin from herpesvirus saimiri (Jung *et al.*, 1994), the KSHV-cyclin can activate CDK6 (Jung *et al.*, 1994; Chang *et al.*, 1996; Godden-Kent *et al.*, 1997; Li *et al.*, 1997). CDK6/KSHV-cyclin complexes can phosphorylate the Retinoblastoma protein (Rb) and, unlike CDK6/cyclin D complexes, can also phosphorylate histone H1 (Jung *et al.*, 1994; Godden-Kent *et al.*, 1997; Li *et al.*, 1997; Ellis *et al.*, 1999; Mann *et al.*, 1999), p27 (Ellis *et al.*, 1999; Mann *et al.*, 1999), Id-2 (Mann *et al.*, 1999), Orc1 (Laman *et al.*, 2001), and CDC25A (Mann *et al.*, 1999). CDK6/KSHV-cyclin complexes have been reported to be insensitive to inhibition by p16 (Swanton *et al.*, 1997) and to be insensitive (Swanton *et al.*, 1997) or less sensitive (Ellis *et al.*, 1999) to inhibition by p27 in vitro. Ectopic expression of KSHV-cyclin in mammalian cell lines overcomes p16-induced cell cycle arrest (Swanton *et al.*, 1997) and induces phosphorylation and degradation of p27 (Ellis *et al.*, 1999; Mann *et al.*, 1999). Therefore, KSHV-cyclin expression can bypass normal growth regulatory mechanisms and induce S-phase (Laman *et al.*, 2001) in infected cells (reviewed by Swanton *et al.*, 1999). Transfection of cells with CDK6 and KSHV-cyclin leads to a high level of cell death (Ojala *et al.*, 1999). Recent results suggest that phosphorylation and subsequent inactivation of Bcl-2 may be required for this apoptosis (Ojala *et al.*, 2000). Recently the crystal structure of the CDK6/KSHV-cyclin/$p18^{INK4c}$ complex has been solved and demonstrates that the KSHV-cyclin binds almost exclusively to the "PSTAIRE"-helix (Jeffrey *et al.*, 2000). In contrast, cyclin A binds to CDK2 via the "PSTAIRE"-helix, the T-loop, and the C-terminal lobe (Jeffrey *et al.*, 1995; Russo *et al.*, 1996b).

Here we examine the regulation of the CDK6/KSHV-cyclin kinase in vitro using purified proteins as well as in a cell-based assay. We find that KSHV-cyclin can activate CDK6 in the absence of CAK phosphorylation and that the insensitivity of CDK6/KSHV-cyclin toward p16 is dependent on the phosphorylation status of CDK6. Both CDK6/KSHV-cyclin and to a lesser extent $CDK6^{T177A}$/KSHV-cyclin complexes can induce apoptosis in vivo. These results indicate that KSHV-cyclin activates CDK6 in a different way than endogenous cyclin D.

MATERIALS AND METHODS

Protein Expression and Purification

Wild-type human CDK2, GST-CDK6, CDK6/p16 complexes, and CDK7/cyclin H (Russo, 1997; Russo *et al.*, 1998), and GST-Cak1p (Kaldis *et al.*, 2000) were expressed in baculovirus-infected insect cells and purified. The following proteins were expressed in bacteria and purified as described: cyclin $A_{173–432}$ (Russo, 1997) human GST-CDK2, $GST\text{-}CDK2^{T160A}$, GST-CDK6, $GST\text{-}CDK6^{T177A}$, p16, $GST\text{-}Rb_{605–928}$, and His_6-p27 (Kaldis *et al.*, 1998).

KSHV-cyclin was cloned into the *Nco*I (5′) and *Sal*I (3′) sites of pCool (a modified version of pGEX-2T [Amersham Pharmacia Biotech, Piscataway, NJ] (N. Pavletich, unpublished data) via polymerase chain reaction (PCR). The GST-KSHV-cyclin fusion protein was expressed in *Escherichia coli* and affinity purified by glutathione-agarose chromatography in 25 mM Tris pH 7.5, 200 mM NaCl, and 5 mM dithiothreitol (DTT). Human CDK6 was expressed in insect cells as described (Russo *et al.*, 1998). CDK6/GST-KSHV-cyclin complexes were expressed and purified as described (Jeffrey *et al.*, 2000). For in vitro transcription and translation, an *Nco*I-*Bam*HI fragment containing KSHV-cyclin was removed from pCool and cloned into the Δ13Tb vector (Gautier *et al.*, 1991) to create PKB375. KSHV-cyclin was transcribed and translated in vitro with the use of the TNT-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions with 1 μCi of [^{35}S]methionine (PerkinElmer Life Sciences, Boston, MA) per microliter of reaction volume.

A C-terminal PKA site was introduced into p16 by PCR using GST-p16 (Russo *et al.*, 1998) as a template and the following oligonucleotides: 5′-GCC GTG ACG TCA GAA TTC ATG GAG CCT TCG GCT GAC-3′ and 5′-GCT AGG CAT GTC GGA TCC CTA AAC ACT GGC CCG CCG ACT ACT GCC ATC GGG GAT GTC TGA-3′. The PCR product was digested with *Eco*RI (5′) and *Bam*HI (3′) (underlined sequences in oligonucleotides) and ligated into

pCool cut with the same enzymes. The expression and purification of $p16^{PKA}$ was identical to that of GST-KSHV-cyclin (see above).

Antibodies and Reagents

Mouse monoclonal antibodies recognizing the Myc epitope (9E10) or HA epitope (12CA5) were from Babco (Berkeley, CA), whereas rabbit polyclonal antibodies against CDK6 (C-21), cyclin D1 (HD11), and p16 (H-156) were from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst 33342 was obtained from Sigma (St. Louis, MO).

Prephosphorylation of cdks

Prephosphorylation of cdks was performed essentially as described (Kaldis *et al.*, 1998). Five microliters of CAK (19 ng of GST-Cak1p or 30 ng of CDK7/cyclin H complexes) was incubated for 30 min at room temperature with 5 μl of substrate mix containing 0.1 μg of GST-CDK6, CDK6/p16 complexes, or CDK2, 0.5 mM ATP, 5 mM $MgCl_2$, 50 μg/ml creatine kinase, and 35 mM phosphocreatine in EB (80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 10 mM DTT, 1 mg/ml ovalbumin, and 1× protease inhibitors [10 μg/ml each of leupeptin, chymostatin, and pepstatin; Chemicon, Temecula, CA]).

Complex Formation

GST-CDK6 (0.1 μg) (with or without prephosphorylation on Thr-177 by Cak1p), $GST\text{-}CDK6^{T177A}$, CDK2 (with or without prephosphorylation on Thr-160 by Cak1p), or $GST\text{-}CDK2^{T160A}$ was incubated with 0.1 μg of KSHV-cyclin (Figures 2 and 4) or with the indicated amounts of KSHV-cyclin (Figures 1 and 3) in buffer A (100 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mg/ml ovalbumin, 10 mM DTT, 1× protease inhibitors [see above]; total volume 10 μl). After incubation for 15 min at room temperature, complexes were assayed for their activities as described below.

Incubation with Inhibitors

Prephosphorylated cdk or unphosphorylated cdk (0.1 μg) bound to 0.1 μg of cyclin was incubated for 30 min at room temperature with the indicated amounts of p16 or His_6-p27 (Figures 2 and 4) in buffer A (total volume 10 μl). Complexes were assayed for their activities as described below.

Kinase Assays

Histone H1 Phosphorylation. Ten microliters of each sample was incubated with 1.5 μCi of [γ-^{32}P]ATP, 0.375 mM ATP, and 1.5 μg of histone H1 (Roche Molecular Biochemicals, Indianapolis, IN) in EB (final volume 16 μl). After incubation for 15 min at room temperature, reactions were terminated by the addition of 7 μl of 5× SDS-PAGE sample buffer. After electrophoresis in 10% polyacrylamide gels, phosphorylation was analyzed by autoradiography and quantified by phosphorimaging (Molecular Imager GS-250; Bio-Rad, Hercules, CA).

Rb Kinase Assay. Activity was determined as described above except that buffer A was used instead of EB. Ten microliters of each sample was incubated with 1.5 μCi of [γ-^{32}P]ATP, 0.375 mM ATP, and 5 μl of $GST\text{-}Rb_{605–928}$ (Kaldis *et al.*, 1998) bound to glutathione-agarose beads in buffer A (final volume 16 μl). After incubation for 15 min at room temperature, reactions were terminated by the addition of 10 μl of 5× SDS-PAGE sample buffer. Samples were processed as described above.

CTD Phosphorylation. CTD kinase assays were performed essentially as described (Cismowski *et al.*, 1995; Kaldis *et al.*, 1998). Briefly, 10-μl samples were incubated in the presence of 3 μCi of [γ-^{32}P]ATP, 0.375 μM ATP, and 4 μg of CTD peptide [$(YSPTSPS)_4$] in buffer A (see above). Reactions were terminated after 15 min at room temperature by the addition of 7 μl of 5× SDS-PAGE sample buffer. Samples were processed as described above.

Phosphorylation of CDK6 by Cak1p and CDK7

CDK6 or CDK6/p16 complexes (0.1 μg) were incubated with the indicated amounts of KSHV-cyclin or with 0.1 μg of KSHV-cyclin and with the indicated amounts of p16 in buffer A (total volume 8 μl). Five microliters of CAK (19 ng of GST-Cak1p or 30 ng CDK7/cyclin H complexes) was incubated with the substrates in the presence of 5 μCi of [γ-^{32}P]ATP, 10 μM ATP, and 20 mM $MgCl_2$ in buffer A (final volume 16 μl). The reactions were terminated after 30 min at room temperature by the addition of 7 μl of 5× SDS-PAGE sample buffer and analyzed as described above for the CAK assay.

Binding of p16, KSHV-Cyclin, and CDK6

Binding of Radiolabeled p16 to CDK6/KSHV-Cyclin. CDK6/GST-KSHV-cyclin complexes (0.1 μg) were prephosphorylated by CDK7/cyclin H as described above for 150 min at room temperature. Five micrograms of $p16^{PKA}$ was phosphorylated using 60 U of PKA (P-2645; Sigma) in the presence of 20 μCi of [γ-^{32}P]ATP, 20 μM ATP, 200 mM $MgCl_2$, 25 mM Tris pH 7.5, 200 mM NaCl, and 1 mM DTT (total volume 20 μl). After incubation for 150 min at room temperature, 200 ng of PKI (P-0300; Sigma) was added. Radiolabeled $p16^{PKA}$ (0.24 μg) was incubated with the cyclin/cdk complexes for 60 min at room temperature, followed by the addition of 150 μl of EB containing 0.5% NP-40 and 20 μl of glutathione-agarose beads (Sigma). After rotating the slurry for 120 min at room temperature, beads were pelleted and washed three times with 300 μl of EB containing 0.5% NP-40 followed by four washings in EB. Beads were resuspended in 13 μl of 5× SDS-PAGE sample buffer and run on 10% SDS-PAGE, followed by autoradiography and phosphorimaging.

Binding of Radiolabeled KSHV-Cyclin to CDK6. Unphosphorylated GST-CDK6, phosphorylated GST-CDK6, $GST\text{-}CDK6^{T177A}$, unphosphorylated GST-CDK6/p16 complexes, or phosphorylated GST-CDK6/p16 complexes (0.2 μg) (see above) were incubated with 5 μl of ^{35}S-labeled KSHV-cyclin, precipitated, run on SDS-PAGE gels, and analyzed by phosphorimaging as has been described (Kaldis *et al.*, 2000).

Cell Culture and Transfections

U2OS human osteosarcoma cells were routinely cultured in a humidified 5% CO_2 atmosphere at 37°C in DMEM, supplemented with 10% (wt/vol) fetal calf serum. Transient transfection into U2OS cells was performed as previously described (Ojala *et al.*, 1999). DNA precipitates were washed at 20 h and the cells were placed in fresh medium. Cells were analyzed 28 h later.

Kinase Activities from Transfected Cells

Transfected U2OS cells were lysed into 1% NP-40 lysis buffer (20 mM $NaPO_4$ pH 7.4, 1% NP-40, 250 mM NaCl, 5 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1.5 μg/ml aprotinin) supplemented with 25 mM β-glycerophosphate. For measurement of CDK6-associated activity in vitro, the complexes were immunoprecipitated for 2 h at 4°C using anti-hemagglutinin (HA) antibody. Immunocomplexes were bound to protein A-Sepharose beads for an additional hour at 4°C and washed four times with the lysis buffer followed by one wash with the kinase buffer (20 mM Tris pH 7.5, 50 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1.5 μg/ml aprotinin). Kinase reactions were performed in the presence of 2 μCi of [γ-^{32}P]ATP for 15 min at 30°C using 5 μg of GST-Rb (prepared according to Matsus-

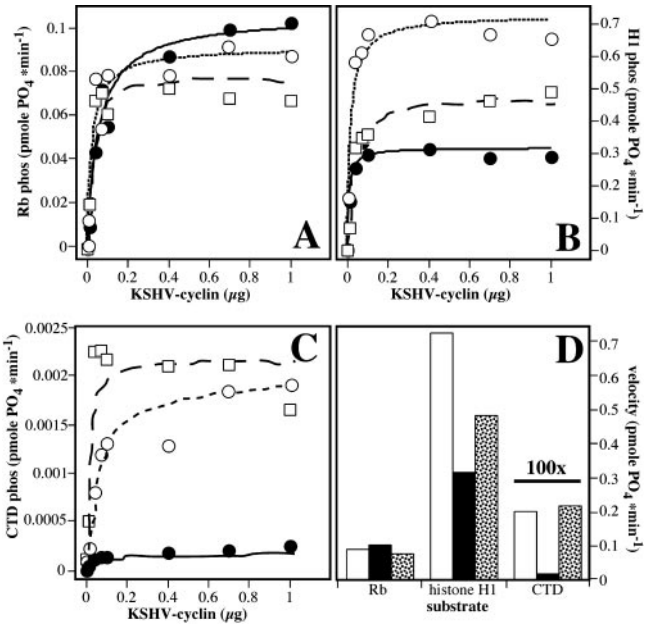


Figure 1. CAK-independent activation of CDK6 by KSHV-cyclin. GST-CDK6 was incubated with increasing amounts of purified KSHV-cyclin and then assayed for Rb kinase (A), histone H1 kinase (B), and CTD kinase (C) activities. The following forms of CDK6 were used: GST-CDK6 (○), GST-CDK6 phosphorylated on Thr-177 by Cak1p (●), and GST-CDK6^{T177A} (□). Mass ratios of KSHV-cyclin: CDK6 were 0, 1:10, 1:2.5, 1:1.4, 1:1, 4:1, 7:1, and 10:1. Data were fit to the Michealis-Menten equation and the corresponding velocities are shown in D. Note that wild-type GST-CDK6 was purified after baculoviral infection from insect cells, whereas GST-CDK6^{T177A} was purified from bacteria.

hime *et al.*, 1994) and 4 μg of histone H1 as substrates in kinase buffer. Phosphorylated proteins were analyzed on 10% SDS-polyacrylamide gels followed by autoradiography.

Indirect Immunofluorescence and Apoptosis Assay

Transfected U2OS cells on coverslips were fixed with 3.5% (wt/vol) paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, and labeled as described previously (Ojala *et al.*, 1999). DNA was stained with Hoechst 33342 (0.5 μg/ml) for 5 min, and the coverslips were mounted in 50% glycerol in phosphate-buffered saline on glass slides and viewed under a fluorescence microscope. Transfected cells were scored by expression of Myc-tagged KSHV-cyclin (detected with 9E10). Apoptotic and normal morphologies in transfected KSHV-cyclin–positive cells were scored and quantified from the Hoechst morphology of nuclei, and the results were displayed as the percentage of apoptotic or protected cells, respectively. At least 100 transfected cells were scored for each sample and the results are based on three independent experiments.

RESULTS

CAK-independent Activation of CDK6 by KSHV-Cyclin

We studied the activation of recombinant purified CDK6 by KSHV-cyclin toward Rb (Figure 1A), histone H1 (Figure 1B), and the CTD of RNA polymerase II peptide (Figure 1C). CDK6 prephosphorylated on Thr-177 (filled circles) by yeast Cak1p displayed good activity. (We used Cak1p for these experiments because it can efficiently phosphorylate monomeric cdks, unlike CDK7 [Kaldis *et al.*, 1998]; see below.) Surprisingly, even unphosphorylated CDK6 (open circles) was activated toward all three substrates by KSHV-cyclin (Figure 1). Activation of CDK6 by cellular D-type cyclins depends absolutely on Thr-177 phosphorylation (Aprelikova *et al.*, 1995; Iavarone and Massagué, 1997; Kaldis *et al.*, 1998; negative data not shown). Confirming these results, bacterially expressed CDK6^{T177A} (open squares) was also activated by KSHV-cyclin toward Rb, histone H1, and the CTD peptide. We have shown previously that CDK6^{T177A} cannot be phosphorylated by CAK and cannot be activated by D-type cyclins (Kaldis *et al.*, 1998; negative data not shown). Quantification of the results revealed that CDK6/KSHV-cyclin complexes used histone H1 more efficiently than the physiological CDK6 substrate Rb (Figure 1D). The CTD peptide was a rather poor substrate. Interestingly, Rb was equally well phosphorylated by all forms of CDK6 (phosphorylated CDK6, unphosphorylated CDK6, and CDK6^{T177A}) with KSHV-cyclin (Figure 1A), whereas there were clear differences in the phosphorylation of the other substrates by the different forms of CDK6. For instance, the phosphorylated CDK6/KSHV-cyclin complex was a poor kinase for both CTD and histone H1 (Figure 1, B and C). Unphosphorylated CDK6/KSHV-cyclin, and to some extent the CDK6^{T177A} mutant, phosphorylated the CTD peptide and histone H1 much better than did phosphorylated CDK6. These results indicate that phosphorylation of the activating threonine (Thr-177) influences substrate specificity, similar to what was reported for CDK2/cyclin A (Kaldis *et al.*, 2000).

CDK6/KSHV-Cyclin Inhibition by p16

In preliminary experiments, we found that KSHV-cyclin was unable to activate unphosphorylated CDK6/p16 complexes (our unpublished data), despite a previous report indicating that CDK6/KSHV-cyclin complexes are insensitive to p16 (Swanton *et al.*, 1997). We traced this discrepancy to whether CDK6 was phosphorylated on Thr-177. This finding led us to investigate the inhibition of CDK6/KSHV-cyclin by CKIs in more detail.

First, we verified that our purified CKIs were active. Both p16 and p27 inhibited the ability of CDK6/cyclin D1 complexes to phosphorylate Rb (Figure 2A). The CDK6/cyclin D1 complexes used here were phosphorylated on the activating threonine, which is essential for the activity of these complexes (see DISCUSSION). We next incubated CDK6/KSHV-cyclin complexes with increasing amounts of p16 or p27 and then assayed kinase activity toward Rb, histone H1, and the CTD-peptide. Both p16 and p27 inhibited the activity of unphosphorylated CDK6/KSHV-cyclin complexes toward all three substrates (Figure 2B). Because these results were inconsistent with previous reports showing that CDK6/KSHV-cyclin complexes could evade inhibition by p16 (Swanton *et al.*, 1997), we repeated the experiment using CDK6/KSHV-cyclin complexes that were prephosphorylated on Thr-177 of CDK6. Interestingly, p16 was unable to inhibit these prephosphorylated CDK6/KSHV-cyclin complexes (Figure 2C), whereas Thr-177 phosphorylation had no effect on inhibition by p27 (Figure 2C). Nevertheless, high concentrations of p16 were still able to partially inhibit Thr-

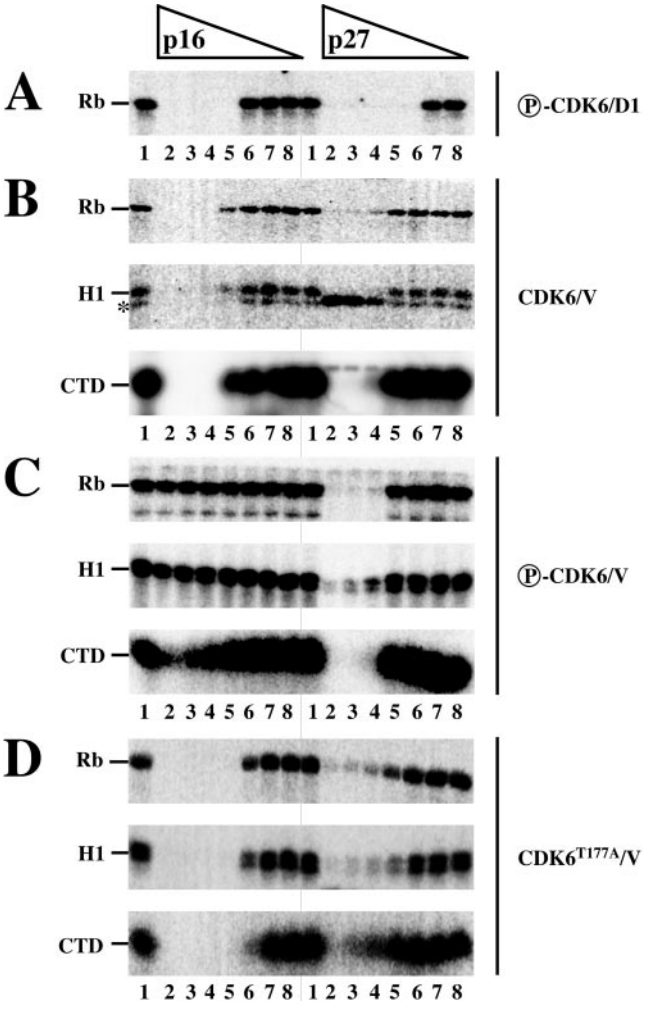


Figure 2. Phosphorylation-dependent sensitivity of CDK6 to inhibition by p16. (A) GST-CDK6/cyclin D1 complexes were treated with decreasing amounts of p16 (left) or p27 (right) and then assayed for their Rb kinase activity. (B) CDK6/KSHV-cyclin complexes were treated with decreasing amounts of p16 or p27 and assayed for their Rb kinase (top), histone H1 kinase (middle), and CTD kinase (bottom) activities. (C) Same experiment as B except that CDK6 was first phosphorylated on Thr-177 by Cak1p. (D) Same experiment as B using mutant GST-CDK6^{T177A}/KSHV–cyclin complexes (that cannot be phosphorylated by CAK). In A–D, lanes 1 contained no inhibitors. Mass ratios of inhibitor: CDK6 were 0 (lanes 1), 10:1 (lanes 2), 5:1 (lanes 3), 2.5:1 (lanes 4), 1.2:1 (lanes 5), 0.3:1 (lanes 6), 0.09:1 (lanes 7), and 0.03:1 (lanes 8). Note that GST-CDK6/cyclin D1 complexes were purified from insect cells after coinfection with baculoviruses. Monomeric GST-CDK6 was obtained from singly infected insect cells and GST-CDK6^{T177A} was purified from bacteria. Asterisk represents phosphorylation of the KSHV-cyclin.

177 phosphorylated CDK6/KSHV-cyclin complexes toward the CTD-peptide (Figure 2C, bottom, lanes 2 and 3), which seems to be more sensitive than Rb and histone H1. As expected, mutant CDK6^{T177A}/KSHV-cyclin complexes (in the absence or presence of Cak1p, which is unable to phosphorylate CDK6^{T177A}; Kaldis *et al.*, 1998) remained sensitive to p16 and p27 (Figure 2D). Previous reports have disagreed

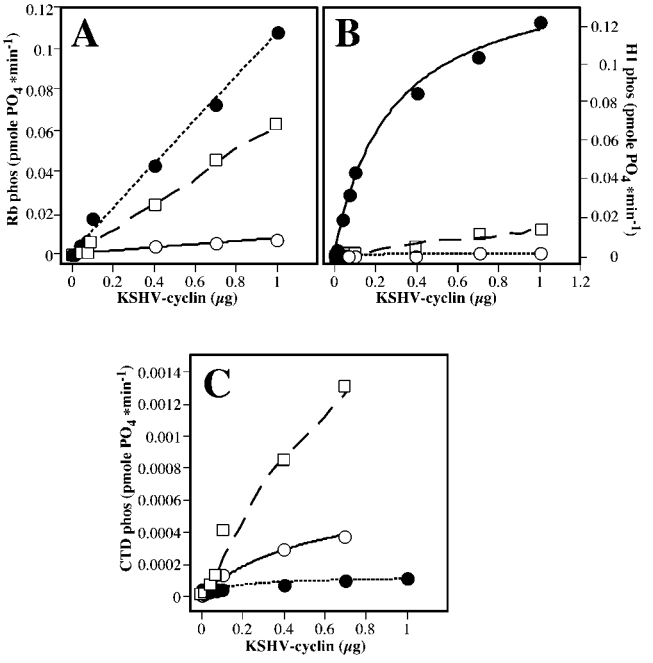


Figure 3. Activation of CDK2 by KSHV-cyclin is CAK dependent. CDK2 was incubated with increasing amounts of purified KSHV-cyclin and assayed for Rb kinase (A), histone H1 kinase (B), and CTD kinase (C) activities. The following forms of CDK2 were used: CDK2 phosphorylated on Thr-160 by Cak1p (●), CDK2 (○), and GST-CDK2^{T160A} (□). First data point contained no KSHV-cyclin. Mass ratios of KSHV-cyclin: CDK2 were 0, 1:10, 1:2.5, 1:1.4, 1:1, 4:1, 7:1, and 10:1. CDK2 was purified from insect cells after infection with a baculovirus; GST-CDK2^{T160A} and KSHV-cyclin were purified from bacteria.

over whether p27 can inhibit CDK6/KSHV-cyclin complexes (Godden-Kent *et al.*, 1997; Swanton *et al.*, 1997; Ellis *et al.*, 1999; Mann *et al.*, 1999). Thus, although some forms of CDK6/KSHV-cyclin can resist inhibition by p16, all forms of CDK6/KSHV-cyclin appear to be sensitive to p27 under the conditions used in our experiments.

Activation of CDK2 by KSHV-Cyclin

We next investigated the activation of CDK2 by KSHV-cyclin. Although the KSHV-cyclin shares similarity with D-type cyclins and activates CDK6 (a physiological partner of D-type cyclins), KSHV-cyclin was also shown to bind and activate CDK2 (Mann *et al.*, 1999; Laman *et al.*, 2001). The ability of KSHV-cyclin to activate a G1-S cdk (CDK2) as well as a G1 cdk (CDK6) could have profound implications for the mechanism of viral control of the cell cycle. We found that KSHV-cyclin weakly activated Thr-160 phosphorylated CDK2 toward Rb and histone H1 (Figure 3A). The activity toward the CTD peptide was low compared with that of CDK2/cyclin A, which is a good CTD kinase (Figure 4A). In contrast, KSHV-cyclin failed to activate the histone H1 kinase activity of unphosphorylated CDK2, weakly activated its Rb kinase activity, and only activated its CTD kinase activity at high KSHV-cyclin concentrations (Figure 3B). KSHV-cyclin did not activate a CDK2^{T160A} mutant toward

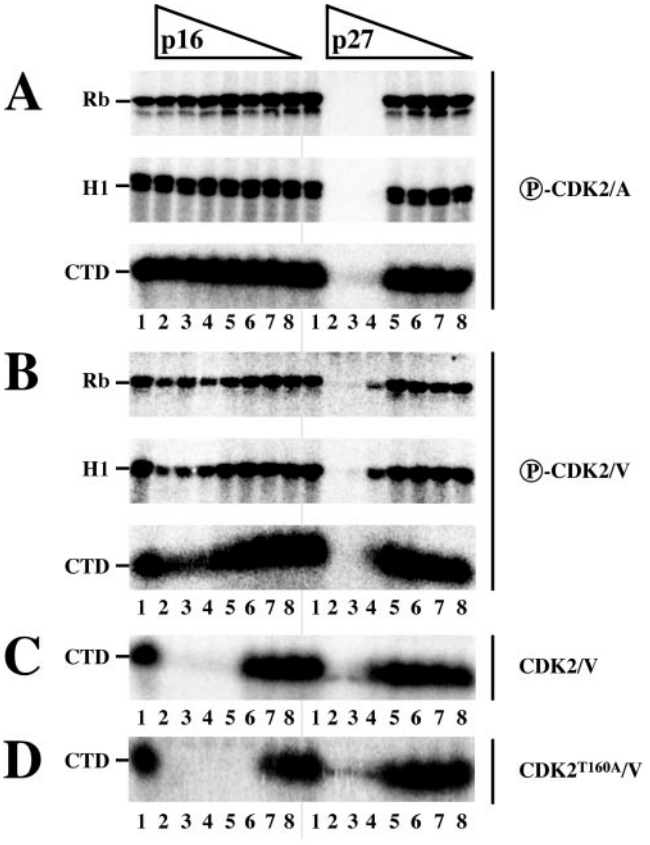


Figure 4. CDK2/KSHV-cyclin complexes can be inhibited by p16. CDK2 complexes were incubated with decreasing amounts of p16 (left) or p27 (right) and then assayed for kinase activities toward the indicated substrates. The following CDK2 complexes were used: CDK2/cyclin $A_{173–432}$ complexes in which CDK2 was phosphorylated on Thr-160 by Cak1p (A), CDK2/KSHV-cyclin complexes in which CDK2 was phosphorylated on Thr-160 (B), unphosphorylated CDK2/KSHV-cyclin complexes (C), and $CDK2^{T160A}$/KSHV–cyclin complexes (D, which cannot be phosphorylated by CAK). The amounts of inhibitors are identical to those used in Figure 2. Note that unphosphorylated CDK2/KSHV-cyclin complexes are not active toward Rb and histone H1 (Figure 3B). CDK2 was purified from insect cells after infection with a baculovirus; cyclin $A_{173–432}$, $CDK2^{T160A}$, and KSHV-cyclin were purified from bacteria.

histone H1 and partially activated it toward Rb (Figure 3, A and B), although $CDK2^{T160A}$/KSHV-cyclin proved to be the best CTD kinase (Figure 3C). The activation of CDK2 by KSHV-cyclin increased much more gradually with increasing KSHV-cyclin concentration and required higher concentrations of KSHV-cyclin than were needed to activate CDK6.

Inhibition of CDK2/KSHV-Cyclin by p16

We examined the sensitivity of CDK2/KSHV-cyclin complexes to inhibition by p27 and p16. p16 is a specific inhibitor of CDK4 and CDK6 and normally cannot inhibit CDK2/cyclin complexes (Serrano *et al*., 1993). We confirmed this observation using CDK2/cyclin A complexes that were phosphorylated on Thr-160 by Cak1p. As expected, p27 inhibited CDK2/cyclin A toward all substrates, whereas p16 had no effect (Figure 4A). p27 also inhibited all CDK2/KSHV-cyclin complexes, whether or not CDK2 was phosphorylated on Thr-160 (Figure 4, B and C). However, although phosphorylated CDK2/KSHV-cyclin complexes were largely resistant to inhibition by p16 (Figure 4B), the weak CTD kinase activity of the unphosphorylated CDK2/KSHV-cyclin complexes was completely inhibited (Figure 4C). We only used the CTD peptide as a substrate in this experiment because neither histone H1 nor Rb was significantly phosphorylated by this complex (Figure 3C). Furthermore, mutant $CDK2^{T160A}$/KSHV-cyclin complexes (that cannot be phosphorylated by Cak1p) remained sensitive to p16 and p27, as expected (Figure 4D). Thus, KSHV-cyclin can confer p16 sensitivity on CDK2, which is normally insensitive to the INK4 family of CKIs.

Direct Binding of CDK6, KSHV-Cyclin, and p16

The above-mentioned experiments suggested that a trimeric CDK6/KSHV-cyclin/p16 complex is formed. To test this possibility directly, we incubated p16 with CDK6 and GST-KSHV-cyclin, and recovered GST-KSHV-cyclin and associated proteins via binding to glutathione-agarose beads. Before binding, the p16 was radiolabeled by phosphorylating an engineered C-terminal site with PKA. Only background binding of p16 to GST-KSHV-cyclin was observed in the absence of CDK6 (Figure 5A, column 1). p16 associated with GST-KSHV-cyclin in the presence of unphosphorylated CDK6 (Figure 5A, column 2), demonstrating ternary complex formation. Interestingly, p16 displayed a significant but reduced binding for Thr-177 phosphorylated CDK6/KSHV-cyclin complexes in this experiment (Figure 5A, column 3).

In a second approach to assess the impact of p16 on the ability of KSHV-cyclin to bind to CDK6 (Figure 5B), radiolabeled KSHV-cyclin was incubated with various forms of CDK6 and the amount of KSHV-cyclin associated with CDK6 at different times was quantitated. We found that KSHV-cyclin bound most efficiently to phosphorylated CDK6; binding was reduced by ~50% to unphosphorylated CDK6 or to $CDK6^{T177A}$. Prebinding of p16 either to phosphorylated or unphosphorylated CDK6 reduced binding of KSHV-cyclin by 25–50%. Half-maximal binding occurred in 7–11 s and binding did not increase appreciably at much longer incubation times. These kinetics are similar to that obtained using cyclin A and CDK2 (Kaldis *et al*., 2000). The results in Figure 5, A and B, demonstrate that heterotrimeric CDK6/KSHV-cyclin/p16 complexes are formed, but that the relative binding is affected by the phosphorylation state of CDK6 and appears to be dependent on the order of complex formation.

Probing the Conformation of the T-loop by Phosphorylation by CAK

As an alternative way to detect the heterotrimeric complex, we used yeast Cak1p and human CDK7/cyclin H as probes for T-loop conformations after binding of CDK6 to p16 and/or KSHV-cyclin. We previously reported that Cak1p prefers to phosphorylate the activating site on monomeric cdks and that binding of CKIs had little effect on the phosphorylation of either monomeric or cyclin-bound cdks (Kaldis *et al*., 1998). In contrast, CDK7/cyclin H preferentially

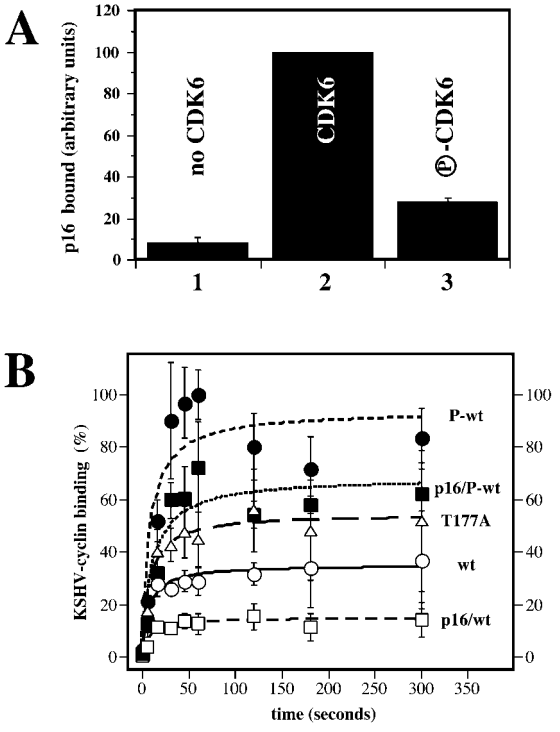


Figure 5. CDK6, KSHV-cyclin, and p16 form a ternary complex. (A) Radiolabeled p16 was incubated with GST-KSHV-cyclin alone (column 1), with GST-KSHV-cyclin and unphosphorylated CDK6 (column 2), or with GST-KSHV-cyclin and Thr-177 phosphorylated CDK6 (column 3). GST-KSHV-cyclin and associated proteins were precipitated by binding to glutathione-agarose, washed extensively, and the amount of bound p16 was quantified by phosphorimaging analysis following SDS-PAGE. p16 binding to unphosphorylated CDK6/GST-KSHV-cyclin (column 2) was assigned a value of 100. (B) [^{35}S]methionine labeled KSHV-cyclin was incubated with unphosphorylated CDK6 (○), phosphorylated CDK6 (●), CDK6^{T177A} (△), unphosphorylated CDK6/p16 complexes (□), and phosphorylated CDK6/p16 complexes (■). GST-CDK6 was precipitated and the amount of bound KSHV-cyclin was analyzed on SDS-PAGE gels followed by phosphorimaging. Six independent sets of data were averaged and fit to the Michaelis-Menten equation.

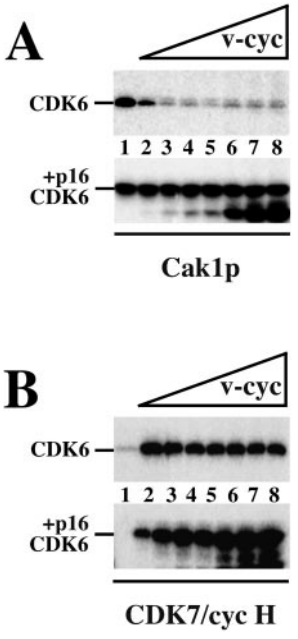


Figure 6. Conformational changes in CDK6 due to binding of KSHV-cyclin and CKIs are detectable by Cak1p and CDK7. (A) Phosphorylation of GST-CDK6 and CDK6/p16 complexes by Cak1p in the presence of increasing amounts of KSHV-cyclin (v-cyc). The amounts of KSHV-cyclin are identical to those used in Figure 1. (B) Same experiment as A but Cak1p was replaced with CDK7/cyclin H.

phosphorylates cdk/cyclin complexes and this phosphorylation is inhibited by binding of CKIs. We examined the phosphorylation of CDK6 by Cak1p and CDK7/cyclin H in the presence of increasing concentrations of KSHV-cyclin (Figure 6, A and B). As reported for CDK2 and cyclin A (Kaldis *et al.*, 1998), Cak1p phosphorylated CDK6 very well in the absence of cyclin but progressively less well as more KSHV-cyclin was present (Figure 6A, top). When we used CDK7/cyclin H, phosphorylation of CDK6 was stimulated by increasing amounts of KSHV-cyclin (Figure 6B, top).

Interestingly, KSHV-cyclin had no effect on the Cak1p phosphorylation of CDK6 in CDK6/p16 complexes (Figure 6A, bottom), indicating that p16 could maintain CDK6 in a conformation available for phosphorylation by Cak1p. The level of phosphorylation indicated that essentially all CDK6 molecules were bound to a p16 molecule. In contrast, KSHV-cyclin stimulated the phosphorylation of CDK6 in CDK6/p16 complexes by CDK7/cyclin H (Figure 6B, bottom). Thus, KSHV-cyclin maintains CDK6 in a conformation available for phosphorylation by CDK7/cyclin H even in the presence of p16, which usually inhibits phosphorylation by CDK7/cyclin H. The level of phosphorylation indicated that most of the CDK6 molecules were bound to KSHV-cyclin. Taken together, these results imply that most CDK6 molecules are in a CDK6/KSHV-cyclin/p16 complex.

Activity of CDK6/KSHV–Cyclin Complexes In Vivo

To test whether our findings applied in vivo, we transfected cells with CDK6 or CDK6^{T177A}, KSHV-cyclin or cyclin D1, and p16 or vector. The complexes were immunoprecipitated via an HA-tag on the CDK6 subunit and assayed for Rb kinase activity in vitro (Figure 7). Both CDK6/cyclin D1 and CDK6/KSHV-cyclin complexes displayed strong activity toward Rb (lanes 2 and 6), whereas in the absence of cyclin no activity was observed (lane 1). Immunoblotting with an antibody against CDK6 showed that similar amounts of CDK6 were present in each of the KSHV-cyclin samples (Figure 7, bottom). Importantly, this blot also demonstrates the modest level of CDK6-HA overexpression compared with endogenous CDK6 in these experiments. In contrast, although CDK6^{T177A}–cyclin D1 complexes had no detectable Rb kinase activity (lane 7), CDK6^{T177A}/KSHV-cyclin complexes retained significant Rb kinase activity (compare lanes 2 and 3), indicating that KSHV-cyclin could at least partially overcome the requirement for activating phosphorylation in vivo. The activity of CDK6^{T177A}/KSHV-cyclin was higher than that of CDK6/cyclin D. In addition, whereas the

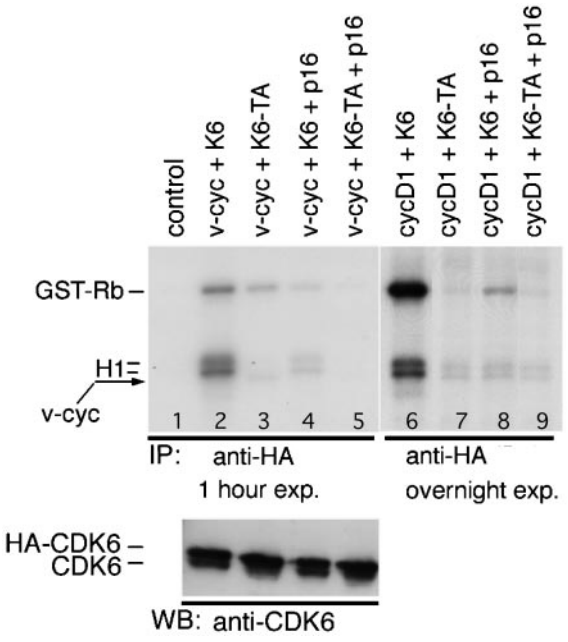


Figure 7. Activity of CDK6/KSHV-cyclin complexes in cells. U2OS cells transfected with Myc-KSHV-cyclin, cyclin D1, HA-CDK6, HA-CDK6^{T177A}, and p16 expression vectors as indicated were lysed at 48 h post-transfection, and CDK6 was immunoprecipitated via its HA-tag. Kinase activities toward GST-Rb and histone H1 were determined as detailed in MATERIALS AND METHODS. Phosphorylated substrates were detected by autoradiography following 10% SDS-PAGE. Bottom, immunoblot of samples with antibody against CDK6. The upper band represents the transfected CDK6-HA and the lower band represents the endogenous CDK6.

activity of CDK6/cyclin D1 complexes was completely inhibited by p16 (compare lanes 6 and 8), the activity of CDK6/KSHV-cyclin complexes partially resisted the action of p16 (compare lanes 2 and 4). This finding is consistent with the existence of both phosphorylated and unphosphorylated subpopulations of CDK6. In the presence of KSHV-cyclin, p16 could inhibit the unphosphorylated subpopulation of CDK6, but not the phosphorylated subpopulation of CDK6.

p16 Reduces Apoptosis Induced by CDK6/KSHV-Cyclin

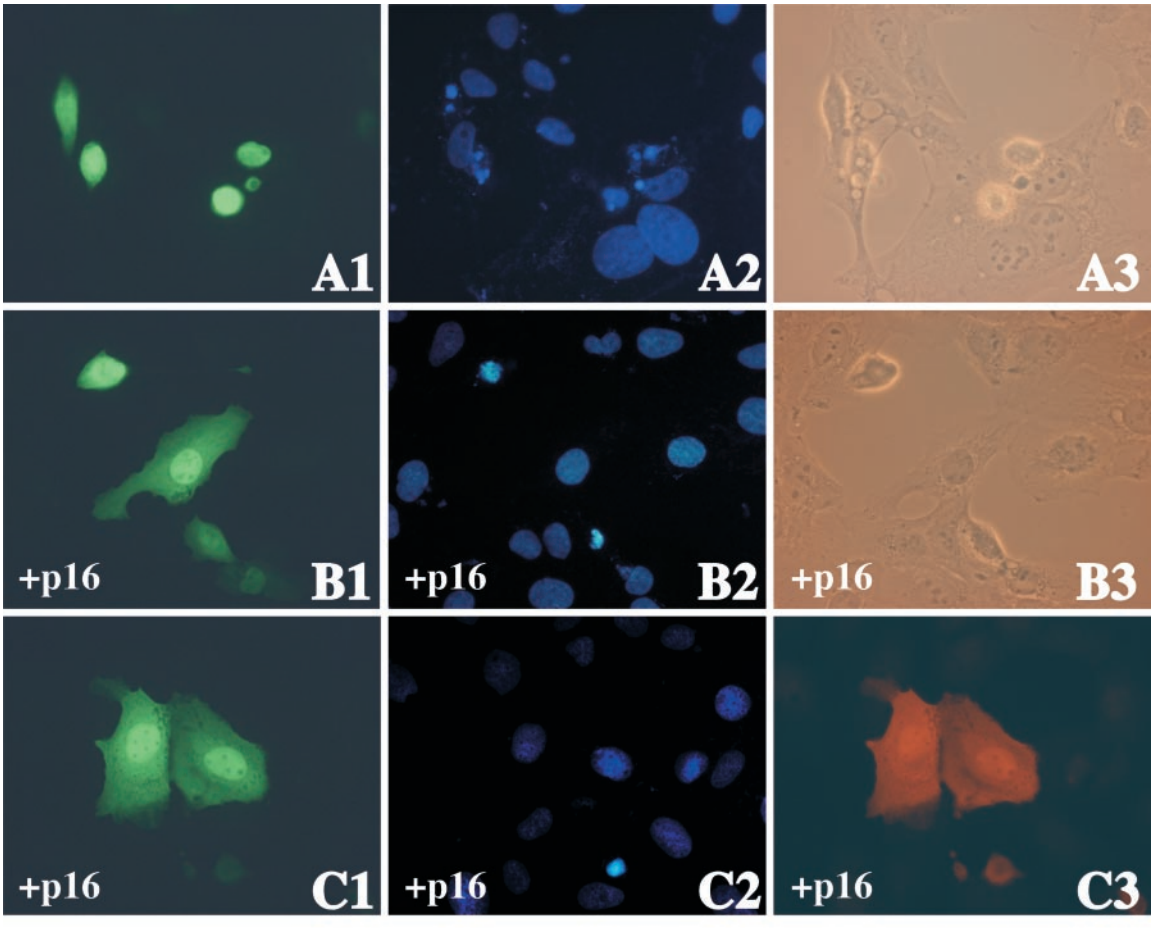
To determine whether CAK-independent activation of CDK6 by KSHV-cyclin plays a physiological role in vivo, we took advantage of a previous observation indicating that expression of CDK6 together with KSHV-cyclin results in high levels of apoptosis (Ojala *et al.*, 1999). This is a sensitive assay because 80% of the cells undergo cell death within 24 h of transfection. We tested the CDK6^{T177A} mutant and the effect of p16 in this system (Figure 8). Expression of CDK6^{T177A} with KSHV-cyclin led to cell death in 40% of the cells, whereas catalytically inactive CDK6^{D163N} had no effect (our unpublished data; Ojala *et al.*, 1999). This indicates that CDK6^{T177A} displays biological activity when expressed in cells. p16 expression in combination with CDK6 and KSHV-cyclin reduced apoptosis roughly 50% (from 80 to 40%, indicating that there are CDK6 molecules in these cells that can be inhibited by p16; Figure 8, B–D), possibly because they are not phosphorylated on Thr-177. This result correlates nicely with our findings in Figures 2 and 7.

DISCUSSION

In this study, we have investigated the activation of CDK6 by a cyclin encoded by the Kaposi's sarcoma-associated herpesvirus. In vitro, KSHV-cyclin could activate CDK6 in the absence of phosphorylation by CAK. However, unphosphorylated CDK6/KSHV-cyclin complexes were sensitive to p16^{INK4a}, whereas CAK-phosphorylated CDK6/KSHV-cyclin complexes were not sensitive. Unlike D-type cyclins, KSHV-cyclin could also weakly activate CDK2, although this activation was CAK dependent. Furthermore, the residual activity of unphosphorylated CDK2/KSHV-cyclin complexes could be inhibited by p16. When expressed in cells, nonphosphorylatable CDK6^{T177A} displayed kinase activity preferentially toward Rb (our unpublished data) and was able to induce apoptosis when coexpressed with KSHV-cyclin, although to a lesser extent than wild-type CDK6. p16 could suppress the ability of CDK6/KSHV-cyclin to induce cell death.

Activation of CDK6 by KSHV-cyclin is unconventional and very strong. So far all cell cycle cdk/cyclin complexes examined have required phosphorylation of the activating threonine within the T-loop by CAK for full activity (Gould *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992; Connell-Crowley *et al.*, 1993; Fisher and Morgan, 1994; Kato *et al.*, 1994; Matsuoka *et al.*, 1994; Aprelikova *et al.*, 1995; Kaldis *et al.*, 1996, 1998). The only exception is CDK7 and its orthologs, where binding of the assembly factor MAT1 to the CDK7/cyclin H complex can substitute for activating phosphorylation (Fisher *et al.*, 1995; Kimmelman *et al.*, 1999). Studies in yeast demonstrated that mutation of the activating threonine of Cdc28p (in *Saccharomyces cerevisiae)* or of Cdc2 (in *Schizosaccharomyces pombe*) rendered yeast cells inviable (Gould *et al.*, 1991; Cismowski *et al.*, 1995), demonstrating that the activating phosphorylation was essential. How does activation of CDK6 by KSHV-cyclin, but not by cellular D-type cyclins, bypass this requirement? One intriguing possibility is that KSHV-cyclin binding induces a conformation in the unphosphorylated T-loop of CDK6 that resembles that of the phosphorylated T-loop of CDK2 (see below). In contrast to CDK6, activation of CDK2 by KSHV-cyclin was mostly CAK dependent, weak, and linearly correlated to the amount of KSHV-cyclin used.

Our conclusions would be weakened if some of the cdks that have been purified from insect cells were partially phosphorylated on the activating threonine by an endogenous insect cell kinase. We are confident, however, that our insect cell-expressed monomeric cdks are completely unphosphorylated because 1) mass spectroscopy analysis of CDK2 (Jeffrey *et al.*, 1995) and CDK6 (Russo *et al.*, 1998) used in this study showed only masses for the unphosphorylated forms; 2) unphosphorylated CDK2/cyclin $A_{173-432}$ complexes displayed undetectable activity toward histone H1 (Kaldis *et al.*, 1998); and 3) mutant GST-CDK6^{T177A}, which cannot be phosphorylated by CAK (Kaldis *et al.*, 1998), was still activated by the KSHV-cyclin (Figure 1).



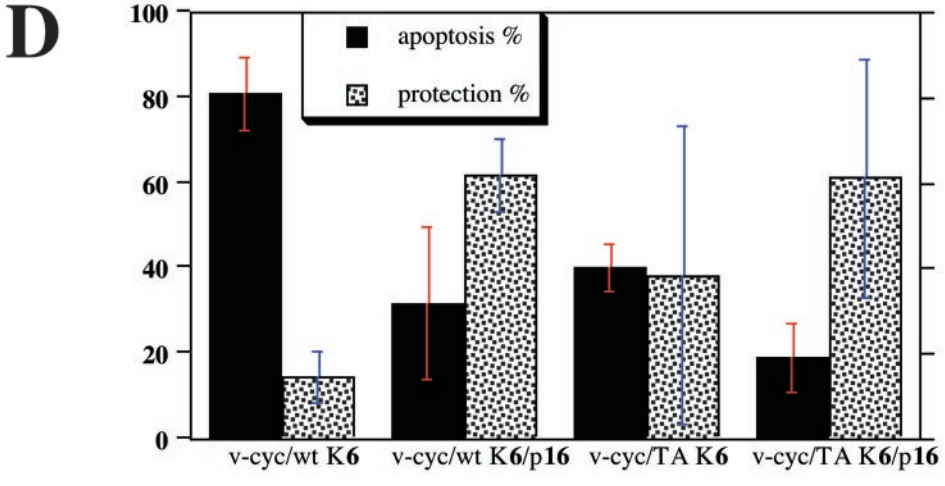


Figure 8. CDK6/KSHV-cyclin induced apoptosis. U2OS cells cotransfected with Myc-KSHV-cyclin and CDK6 expression vectors (A1-A3) or Myc-KSHV-cyclin and CDK6 together with p16 (B1–B3 and C1–C3) were analyzed by immunofluorescence with α-Myc (green; A1, B1, and C1) or α-CDK6 (red; C3) antibodies or by phase contrast (A3 and B3) at 28 h post-transfection. DNA was visualized by Hoechst staining (blue; A2, B2, and C2) to reveal apoptotic nuclei. Protection of apoptosis by $CDK6^{T177A}$ and p16 (D). U2OS cells were transfected with the indicated expression vectors and treated as indicated in top panels. Cells were analyzed by immunofluorescence with α-Myc antibodies (KSHV-cyclin), Hoechst staining, and phase contrast at 28 h post-transfection. Percentage of apoptosis or protection from apoptosis induced by CDK6/KSHV-cyclin are shown from three experiments with SDs. Transfected apoptotic cells were scored as detailed in MATERIALS AND METHODS.

Inhibition by CKIs

p16 belongs to the INK4 family of inhibitors that are specific for CDK4 and CDK6 and are normally unable to inhibit CDK2 (Serrano *et al.*, 1993). Ectopic expression of KSHV-cyclin overcomes p16 induced cell cycle arrest and CDK6/KSHV-cyclin complexes have been reported to be resistant to p16 (Swanton *et al.*, 1997). Our experiments extend this understanding by showing that unphosphorylated CDK6/KSHV-cyclin complexes are inhibited by p16 (Figure 2C) but that phosphorylation by CAK makes these complexes less sensitive to p16 (Figure 2B). Similar results have recently been reported for p18 (Jeffrey *et al.*, 2000). These findings suggest that CDK6 in the immunoprecipitated CDK6/KSHV-cyclin complexes used by others (Swanton *et al.*, 1997) were already phosphorylated on Thr-177. Nevertheless, when p16 is expressed in cells it is partially able to inhibit CDK6/KHSV-cyclin activity (Figure 7) and to reduce CDK6/KSHV-cyclin–induced apoptosis (Figure 8). Furthermore, the affinity of p16 for CDK6 in such complexes was reduced upon activating phosphorylation of CDK6, a finding that has not previously been observed for CKI–cdk interactions. Such an effect was unexpected because p16 binds to CDK6 far from the T-loop (Brotherton *et al.*, 1998; Russo *et al.*, 1998; Jeffrey *et al.*, 2000).

In contrast to p16, p27 inhibited every cdk/cyclin complex we tested. Godden-Kent *et al.* (1997) also found that p27 inhibited CDK6/KSHV-cyclin complexes but others found that CDK6/KSHV-cyclin complexes were partially or completely resistant to p27 (Swanton *et al.*, 1997; Ellis *et al.*, 1999). We cannot explain these contradictory findings, although it should be noted that we used purified proteins throughout, whereas other studies used cell extracts or immunoprecipitated proteins. Nevertheless, p27 inhibition does not seem to play an important role in KSHV-infected cells because KSHV-cyclin induces the phosphorylation and degradation of p27 (Ellis *et al.*, 1999; Mann *et al.*, 1999), resulting in a low level and short half-life of p27.

Structural Implications

Recently, the crystal structure of the squirrel monkey herpes virus cyclin (Schulze-Gahmen *et al.*, 1999) and of the unphosphorylated CDK6/KSHV-cyclin/p18^{INK4c} complex (Jeffrey *et al.*, 2000) have been solved. Interestingly, the structure of the viral cyclin, despite considerable sequence diversity, folds very similarly to cyclin A (Jeffrey *et al.*, 1995, 2000). The structure of CDK6/KSHV-cyclin is remarkable because p18 binds to the identical domain as in monomeric CDK6 and induces the same conformational changes (Brotherton *et al.*, 1998; Russo *et al.*, 1998). Nevertheless, KSHV-cyclin binds exclusively to the PSTAIRE helix of CDK6 without contacting the T-loop or the C-terminal lobe (Jeffrey *et al.*, 2000). This is very different from how cyclin A binds CDK2 via the PSTAIRE helix, the T-loop, and the C-terminal lobe (Jeffrey *et al.*, 1995). The question is whether this is an effect of p18 binding to CDK6 or a general characteristic of the KSHV-cyclin. Despite binding only to the PSTAIRE helix, KSHV-cyclin binds strongly to CDK6 and fully activates it. The other remarkable aspect of the CDK6/KSHV-cyclin/p18 structure concerns the T-loop. The position of the T-loop is >30 Å away from the active site in the CDK6/KSHV-cyclin/p18 and the CDK6/p16 structure (Jeffrey *et al.*, 2000). Clearly, both the structures of CDK6/p16 and CDK6/KSHV-cyclin/p18 are snapshots from inactive kinases and further work will be needed to determine whether the phosphorylated T-loop is closer to the active site (as in CDK2) or whether the structure of CDK6 differs fundamentally from that of CDK2.

Our own data indicate that there is a delicate balance between activating phosphorylation, p16 binding, and KSHV-cyclin binding. Depending on the combination of theses effects, CDK6 is active or not, suggesting that the activating phosphorylation in this case is as important as p16 or KSHV-cyclin. We think that p16 induces a conformational change to the T-loop of CDK6 in the CDK6/KSHV-cyclin complex because it makes the T-loop available for phosphorylation by Cak1p (Figure 6A, bottom). On the other hand, p16 does not prevent CDK7/cyclin H from phosphorylating CDK6 in the CDK6/KSHV-cyclin complex (Figure 6B, bottom), whereas in the CDK6/cyclin D/p16 complex it does (Kaldis *et al.*, 1998). Taken together, these results suggest that the T-loop in the CDK6/KSHV-cyclin/p16 complex might be much closer to the active site in the active conformation than in the unphosphorylated, inactive conformation (Jeffrey *et al.*, 2000).

Surprisingly, binding to KSHV-cyclin makes CDK2 susceptible to p16 inhibition (Figure 4). Because p16 appears to contact the cdk only, and not the cyclin subunit, this finding suggests that the reason CDK2 is normally insensitive to p16 lies in its tertiary structure, not its primary structure. KSHV-cyclin appears to alter the structure of CDK2 so that it resembles CDK6 and can interact with p16.

Viral Strategies to Run the Cell Cycle

DNA tumor viruses have a common goal: to hijack cells and run their cell cycles independent of normal mitogenic signals. Only a few viruses (including KSHV and herpesvirus saimiri) encode D-type cyclins like the KSHV-cyclin. The otherwise closely related Epstein-Barr virus does not encode a cyclin but induces the expression of the cellular cyclin D2 (Sinclair *et al.*, 1994). Many DNA tumor viruses instead inactivate Rb (Jansen-Dürr, 1996), a downstream target of cyclin D-associated kinases. The KSHV-cyclin is such a strong activator of CDK6 (and to a lesser extent of CDK2) that there might be no need for other cellular cyclin/cdk complexes to promote cell cycle progression. The Kaposi's sarcoma-associated herpesvirus has adopted a clever strategy to make cell cycle progression independent of mitogenic signals, inhibition by CKIs, or phosphorylation by CAK.

There are still many unanswered questions regarding KSHV-cyclin function. What makes KSHV-cyclin such a potent activator of CDK6 compared with the cellular cyclins? Is the activation of CDK6 by KSHV-cyclin sufficient to promote full cell cycle progression? If we could inhibit KSHV-cyclin–mediated activation of CDK6, would that prevent HHV8 infection? Future studies will be needed to answer these important questions.

ACKNOWLEDGMENTS

We thank Nikola Pavletich and Alicia Russo for generous supply of reagents and exchange of results before publication. We also thank Adrienne Natrillo for technical support. For discussion, support, and comments on the manuscript, we thank Janet Burton, Aiyang

Cheng, Daniel DiMaio, and Karen Ross. This work was supported by a long-term fellowship from the Swiss National Science Foundation (to P.K.) and by National Institutes of Health grant GM-47830 (to M.J.S.). M.J.S. was a Leukemia Society of America Scholar.

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