Cyclin-dependent kinases (Cdks) are activated by cyclin binding and phosphorylation by the Cdk-activating kinase (CAK). Activation of Cdk6 by the D-type cyclins requires phosphorylation of Cdk6 by CAK on threonine 177. In contrast, Cdk6 is activated by the Kaposi’s sarcoma-associated herpesvirus (KSHV)-cyclin in the absence and presence of CAK phosphorylation. The activity of Cdk6/KSHV-cyclin complexes was investigated here by analyzing mutants of the KSHV-cyclin and Cdk6 in vitro as well as in U2OS cells. Deletion of the N terminus of the KSHV-cyclin affects the substrate specificity indicating that the N terminus is required for phosphorylation of histone H1 but not for other substrates. Mutation of residues in the region 180–200 of the KSHV-cyclin decreases the binding affinity to Cdk6 in U2OS cells but increases the activity of Cdk6/KSHV-cyclin complexes in vitro indicating that low affinity binding of cyclins to the Cdk subunit might favor increase on- or off-rates of Cdk substrates. Expression of high levels of p16INK4a in cells leads to the formation of a heterotrimeric complex composed of Cdk6, KSHV-cyclin, and p16INK4a. Some of the Cdk6/KSHV-cyclin-p16 complexes were found to be active indicating that there might be different modes of p16 binding to Cdk6-cyclin complexes.

The activities of cyclin-dependent kinases (Cdks) are thought to promote the transitions between the different phases of the cell cycle. For this reason, Cdk activities are tightly controlled by several mechanisms: cyclin binding, activating phosphorylation by the Cdk-activating kinase (CAK), inhibitory phosphorylation by Wee1/Mylt1 kinases, dephosphorylation by the dual specificity phosphatase Cdc25, transcriptional regulation, ubiquitin-mediated degradation, subcellular localization, binding of inhibitors.

Eleven different Cdks have been identified in mammals. Of these, Cdk4 and Cdk6 bind to the three D-type cyclins and are active in the G1 phase. Cdk2 binds to E- and A-type cyclins and has been shown to promote entry into S-phase and DNA replication. Cdc2 binds to the A- and B-type cyclins and is responsible for entry into mitosis. In general, the activities of Cdk4 and Cdk6 are hard to differentiate although there are indications that they do not have identical functions. For example, Cdk4 knock-out mice display specific defects (1, 2) that are not compensated by the presence of Cdk6.

Cyclin binding to the Cdk catalytic subunit is an important step in the activation of Cdks since it leads to a realignment of the activation segment/T-loop (substrate binding region) and the P-loop (ATP binding site) (3). For example, cyclin A binds to the PSTAIR helix, the T-loop, and the C-terminal lobe of Cdk2. For full activation of Cdks, threonine 177 in Cdk6 (Thr-160 in Cdk2) needs to be phosphorylated by CAK (for review see Ref. 4). Phosphorylation of the activating threonine causes further movement of the T-loop (5). Mutation of the activating threonine in Cdk6 leads to inactivation in vitro (6–10) and in vivo (11, 12).

Cdk activity can be inhibited by binding of inhibitory proteins (for review see Refs. 13 and 14). The Cip/Kip family of Cdk inhibitors (CKIs) consists of p21Cip1/Waf1 (15–17), p27Kip1 (18–20), and p57Kip2 (21, 22) and bind to all Cdks. The Ink4 family of CKIs specifically inhibits Cdk4 and Cdk6 and consist of p15INK4B (23), p16INK4A (24), p18INK4C (25, 26), and p19INK4D (26, 27). The Cip/Kip inhibitors bind to both the Cdk and cyclin subunit (28) whereas the Ink4 inhibitors bind exclusively to the N-terminal lobe of Cdk4 or Cdk6 (29, 30). Interestingly, immuno precipitation of p16 from cell extracts did not yield a heterotrimeric complex composed of Cdk6, KSHV-cyclin, and p16INK4a. Some of the Cdk6/KSHV-cyclin-p16 complexes were found to be active indicating that there might be different modes of p16 binding to Cdk6-cyclin complexes.

The substrate specificity of Cdk4/cyclin complexes is an important determinant of their function and is an area we know very little about. It has been shown that the cyclin subunits can influence substrate specificity since binding of different cyclins to the same Cdk can change its substrate specificity (32–34). Furthermore, there is a substrate binding site in the cyclin subunits, termed Cy or RXL motif (35, 36), where p27 binds. Mutation of the Cy/RXL motif leads to decreased phosphorylation of Rb without affecting other substrates (e.g. histone H1) (37).

Kaposi’s sarcoma and B-cell lymphoproliferative disorders can be caused by the Kaposi’s sarcoma-associated herpesvirus (KSHV or HHV8; for review see Refs. 38–40). This large DNA tumor virus encodes many open reading frames (ORFs) and one of them, ORF72, shares homology with the D-type cyclins (41). KSHV-cyclin and the related v-cyclin (42) from herpesvirus saimiri when bound to Cdk6, phosphorylate the retinoblastoma (Rb) protein (42–45). In contrast to Cdk6/cyclin D complexes, Cdk6/KSHV-cyclin complexes also phosphorylate histone H1 (42, 44–47), p57Kip1 (46, 47), Orc1 (48), Cdc6 (48), Cdc25A (47), and the C-terminal domain of the large subunit of RNA polymerase II (CTD) (49). Cdk6/KSHV-cyclin complexes are less sensitive to inhibition by p16 and p27 (46, 50). The KSHV-cyclin

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‡ The abbreviations used are: Cdk, cyclin-dependent kinases; CAK, Cdk-activating kinase; RB, retinoblastoma protein; RIPA, radioimmune precipitation assay buffer; KSHV, Kaposi’s sarcoma-associated herpesvirus; HA, hemagglutinin.
has been shown to activate Cdk6 in the absence of CAK phosphorylation (49, 51). Thr-177 phosphorylation of Cdk6 prevents inhibition by p16 in vitro while forming a heterotrimERIC complex of KSHV-cyclin, Cdk6, and p16 (49). Taken together, KSHV-cyclin-Cdk6 complexes evade growth regulatory mechanisms and can induce S phase (48). Crystal structures of Cdk6-KSHV-cyclin complexes in the presence (52) and absence of p18 (53) indicate that the interaction between the KSHV-cyclin and Cdk6 is limited to the PSTAIR helix, whereas cyclin A binds to the PSTAIR helix, the T-loop, and the C-terminal lobe of Cdk2 (3, 5). Transfection of cells with KSHV-cyclin and Cdk6 plasmids leads to cell death (54) that is dependent on phosphorylation of histone H1 whereas other substrates are not affected. To study the complex formation of p16, Cdk6, and KSHV-cyclin, p16 was expressed at high levels in U2OS cells, and a heterotrimERIC complex of p16-Cdk6-KSHV-cyclin was detected. Nevertheless, p16 was not bound to KSHV-cyclin mutants because of the weak interaction with Cdk6 in cells.

### EXPERIMENTAL PROCEDURES

**Plasmids and Mutagenesis**—Wild-type GST-Cdk6 (pGEX-KG; PKB512, Ref. 49), GST-KSHV-cyclin (pCooL; PKB522, Ref. 49), Cdk6-HA (pCI-neo; PKB396, Ref. 49), Cdk6D163N-HA (pCI-neo; PKB252, Ref. 58), Cdk6D163N-HA (pCMV-Bam; PKB396, Ref. 49), Cdk6 (pCMV-Bam; PKB707, Ref. 58), GFP-cyclin D1T286A (PKB750, provided by Y. Guo), p16 (pcDNA3; PKB613, provided by K. Vousden), GST-p27 (PKB397, Ref. 58), and Myc-KSHV-cyclin (pcDNA3; PKB618, obtained from S. Mittnacht, Ref. 46) have been transfected into the BamHI site of EGFP-C1 (Clontech). GFP-KSHV-cyclin (pcDNA3; PKB618, obtained from S. Mittnacht, Ref. 46) have been transfected into the BamHI site of EGFP-C1 (Clontech). GFP-cyclin D1T286A (PKB750, provided by Y. Guo), p16 (pcDNA3; PKB613, provided by K. Vousden), GST-p27 (PKB397, Ref. 58), and Myc-KSHV-cyclin (pcDNA3; PKB618, obtained from S. Mittnacht, Ref. 46) have been transfected into the BamHI site of EGFP-C1 (Clontech). All mutants have been generated using the QuikChange system (Stratagene) according to the manufacturer's instructions using the indicated oligonucleotides for KSHV-cyclin mutants.

### Table I

| KSHV-cyclin | Sequence 5' → 3' | Oligo |
|-------------|-----------------|-------|
| AN1-19      | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0333 |
| AN1-45      | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0334 |
| T3A         | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0335 |
| S9A/15A     | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0336 |
| T34A/S35A/S37A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0337 |
| S41A/S45A/S47A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0338 |
| S50A        | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0339 |
| S59A        | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0340 |
| K106E       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0341 |
| S100A/T111A/S114A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0342 |
| T115A/S116A/S117A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0343 |
| T125A/S127A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0344 |
| T146A       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0345 |
| T152A       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0346 |
| T155A/S156A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0347 |
| T166A       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0348 |
| T178A       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0349 |
| T181A       | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0350 |
| T185A/S189A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0351 |
| T195A/S198A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0352 |
| T187A/S181A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0353 |
| S215A/S217A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0354 |
| S226A/S233A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0355 |
| T244A/S245A/S247A | GTAATCCGATGTTCTTTGCATACATTTTACAGAGCGCGAAGCGGCATACAGCAAGAGCTTGATGGCCGAGGCTGGATTCGTACAG | PKO0356 |
cycin (Table I) or Cdk6 (Table II). N-terminal deletion mutants of KSHV-cyclin were generated by PCR using oligonucleotides that are listed in Table I.

Protein Expression and Purification—Wild-type human GST-Cdk6, Cdk7/cyclin H (29, 61), and GST-Cak1p (62) were expressed in baculovirus-infected insect cells and purified. The following proteins were expressed in bacteria and purified as described; Cdk-cyclin A-(173–432) human GST-Cdk6, wild type, and mutant GST-KSHV-cyclin proteins, GST-Rb-(605–928), GST-p27, GST-p53, and GST-Cdc25C (49, 63).

Antibodies and Reagents—Mouse monoclonal antibodies recognizing the Myc epitope (9E10, Clontech/BD, 3800-1), anti-Myc antibodies coupled to agarose (Clontech/BD, 3843-1), peptide antibodies against the HA epitope (raised against the peptide NH2-CYPYDVPDYASLG-432), human GST-Cdk2, wild type, and mutant GST-KSHV-cyclin proteins, GST-Rb-(605–928), GST-p27, GST-p53, and GST-Cdc25C (49, 63).

Results

Activation of Cdk6 by KSHV-cyclin—Cyclin-dependent kinases are activated both by cyclin binding and by phosphorylation of an activating threonine residue (Thr-177 in Cdk6 or Thr-160 in Cdk2) by CAK (for review see Ref. 4). The KSHV-cyclin has been shown previously to activate Cdk6 in the absence of CAK (49). I aimed to investigate the mechanism of Cdk6 activation by the KSHV-cyclin under identical conditions in the presence or absence of CAK (Fig. 1A). The assay consists of a 4.5-h preincubation period followed by 15 min labeling of substrate in the presence of radiolabeled ATP (for details see “Experimental Procedures”). CAK phosphorylated baculovirus produced GST-Cdk6 was readily activated by bacteria produced GST-KSHV-cyclin (Fig. 1B, lane 3). Interestingly, incubation of Cdk6 with the KSHV-cyclin and ATP resulted almost in the same degree of activation as CAK phosphorylation (Fig. 1B, compare lanes 2 and 3). When Cdk6 was incubated with the KSHV-cyclin in the absence of ATP, very little activation was achieved (Fig. 1B, lane 1). As a control, identical experiments were performed with Cdk2 and cyclin A-(174–432). Cdk2 was only activated by CAK, ATP, and cyclin A-(174–432) (Fig. 1B, lane 6) but not by incubation with ATP and cyclin A-(174–432)
Cdk6-KSHV-cyclin Activity

**Fig. 1. Activation of Cdk6 by KSHV-cyclin.** A, activation of baculovirus produced GST-Cdk6 was studied by setting up three reaction conditions: buffer treatment, GST-Cdk6 was incubated with GST-KSHV-cyclin for 4.5 h at room temperature, followed by the addition of ATP, histone H1, or GST-Rb and [γ-32P]ATP. ATP treatment, same as buffer treatment but ATP was present during incubation of GST-Cdk6 and GST-KSHV-cyclin; CAK+ATP treatment, GST-Cdk6 was phosphorylated by CAK in the presence of ATP for 4.5 h at room temperature, followed by the addition of GST-KSHV-cyclin, histone H1, or GST-Rb, and [γ-32P]ATP. B, reactions were set up as described in A using GST-Cdk6 and GST-KSHV-cyclin (KSHV-cyclin (lanes 1–3), GST-Cdk2 and GST-cyclin A (lanes 4–6), GST-Cdk2 and GST-KSHV-cyclin (lanes 7–9), or GST-Cdk6 and GST-cyclin A (lanes 10–12). C, Cdk6-GST-KSHV-cyclin were incubated with 0, 10 μM, or 1 mM ATP. The KSHV-cyclin displays a mobility shift reminiscent of phosphorylation. D, Myc-KSHV-cyclin (lanes 1) or Cdk6-HA/Myc-KSHV-cyclin complexes (lane 2) were immunoprecipitated and incubated with [γ-32P]ATP. Both Cdk6 and the KSHV-cyclin were labeled in this phosphorylation reaction. Reactions were run on 12.5% SDS-polyacrylamide gels. The gels were dried and analyzed by phosphorimaging.

(FIG 1B, lane 5). Next, I investigated if these results were related to the intrinsic properties of the purified Cdk6 or KSHV-cyclin preparations. The KSHV-cyclin could only activate CAK-phosphorylated Cdk2 (FIG 1B, lane 9), as has been reported before (49). Furthermore, Cdk6 was not activated by cyclin A (173–432) (FIG 1B, lanes 10–12). Thus, activation of Cdk6 in the absence of CAK is restricted to the combination of Cdk6 and the KSHV-cyclin. The fact that ATP-activated Cdk6-KSHV-cyclin complexes indicate at least two possibilities: (1) Cdk6-KSHV-cyclin complexes autophosphorylate at one or several residues and this contributes to the activation; and/or (2) ATP causes conformational changes in Cdk6-KSHV-cyclin complexes, which activate these complexes. When Cdk6-KSHV-cyclin complexes were incubated in vitro with increasing amounts of ATP, a substantial mobility shift of the KSHV-cyclin was observed (FIG 1C, compare lane 1 to lanes 2 and 3). In addition, when Cdk6-KSHV-cyclin complexes were immunoprecipitated from transfected cell extracts and incubated in vitro with radiolabeled ATP (FIG 1D, lane 2), both Cdk6 and the KSHV-cyclin incorporated radiolabeled phosphate. The KSHV-cyclin alone (in the absence of transfected Cdk6) was not labeled (FIG 1D, lane 1). These results were consistent with the possibility that Cdk6-KSHV-cyclin phosphorylation might be related to their activation. To investigate this possibility further, I generated mutations in a number of potential phosphorylation sites.

**Mutation of the KSHV-cyclin Affects Its Ability to Activate Cdk6**—Bacteria produced GST-KSHV-cyclin proteins bearing one or more mutations were assayed for activation of baculovirus produced GST-Cdk6 using the conditions described in Fig. 1A (buffer, ATP, and CAK+ATP treatment). All mutant KSHV-cyclin proteins were able to activate Cdk6 toward the substrates Rb (FIG 2A) and histone H1 (FIG 2B). Wild-type or mutant GST-KSHV-cyclin complexes autophosphorylate at one or several residues and this contributes to the amount of time it takes to activate Cdk6 (essentially during the 15 min of labeling) (see Table III and also discussion). When the KSHV-cyclin mutants were assayed for activation of Cdk6 toward the substrate histone H1, very similar results were obtained (FIG 2B). There was one notable

**Fig. 2. Mutagenesis of the KSHV-cyclin and its effect on Cdk6 activation.** Wild-type or mutant GST-KSHV-cyclin together with GST-Cdk6 was assayed for their ability to phosphorylate GST-Rb (605–928) (A) or histone H1 (B). Conditions for the reactions were identical to the ones described in Fig. 1. GST-KSHV-cyclin was produced in bacteria and GST-Cdk6 was produced in insect cells. Each measurement corresponds to the average of three to eight independent experiments. Black bars correspond to buffer, speckled bars to ATP, and striped bars to CAK+ATP treatment.
The differences observed for the KSHV-cyclin mutants (see Fig. 2) could be due to the time it takes to activate Cdk6 or due to changed binding affinity. Therefore, I performed experiments where I varied the length of incubation or the amount of KSHV-cyclin added in the reaction (Table III). To achieve 50% of the maximal Cdk6 activation, it required incubation of ATP with Cdk6 and wild-type KSHV-cyclin for 160 min using the substrate Rb or 311 min using the substrate histone H1 (Table III). Most of the KSHV-cyclin mutants required only 10–30 min for activation of Cdk6 for both Rb and histone H1. The exception was KSHV-cyclinT56A, which required approximately half the time than wild-type KSHV-cyclin needed. KSHV-cyclinT56A needed ∼9 times longer to activate Cdk6 toward histone H1 than Rb, consistent with a specific defect when the substrate histone H1 was used (see also Fig. 3). An inverse effect was observed with KSHV-cyclinS178A, which took four times longer to activate Cdk6 toward Rb compared with histone H1. The difference between these two mutants was KSHV-cyclinT56A. Cdk6 complexes reached similar maximal activity for Rb and histone H1 as all other mutants. In contrast, the theoretical maximal activity for ∆N1–19 using the substrate histone H1 was ∼6-fold lower than all other mutants (and wild type) KSHV-cyclin. 0.5 μg of wild-type KSHV-cyclin was required to achieve 50% Cdk6 activity toward histone H1. Two KSHV-cyclin mutants (T34–37A and T181A) required 10–15 times less protein (0.03–0.04 μg) compared with wild-type KSHV-cyclin. KSHV-cyclinT56A required two times more (1 μg), whereas most of the other mutants required 2–4 times less protein compared with wild-type KSHV-cyclin (Table III).

The N Terminus of the KSHV-cyclin and Its Involvement in Substrate Specificity—In Fig. 2, it was shown that deletion of the N terminus of KSHV-cyclin resulted in low levels of histone H1 activity whereas the activity toward Rb was comparable to wild-type KSHV-cyclin. To investigate this phenomenon in more detail, the activity toward five different substrates, Rb, histone H1, p27, p53, and Cdc25 was compared. Wild-type KSHV-cyclin-Cdk6 complexes phosphorylated all substrates (Fig. 3, lanes 1–3). Similar results were obtained with two other mutants, T181A and S178–198A (Fig. 3, lanes 7–12). The mutant KSHV-cyclinT34–37A phosphorylated Rb to similar levels as wild-type KSHV-cyclin in complex with Cdk6 (Fig. 3A, lanes 4–6). In contrast, histone H1 was poorly phosphorylated by ∆N1–45, leading to a 10–15-fold decreased phosphorylation efficiency (Fig. 3B, lanes 4–6). Nevertheless, phosphorylation of p27, p53, or Cdc25 by Cdk6/KSHV-cyclinT34–37A complexes was similar as wild type. These results suggested that the N terminus of the KSHV-cyclin contributes to the recognition of the substrate histone H1 (see also discussion).

To further investigate the function of the N terminus of the KSHV-cyclin, a synthetic peptide corresponding to the first 20 amino acids was synthesized (NH₂-MATANNPPSGLLDPTL-CEDR-COOH). This peptide was used in competition experiments at concentrations up to 1 μM and had no effect on the ability of Cdk6/KSHV-cyclin complexes to phosphorylate either Rb or histone H1 (data not shown). Furthermore, the N terminus of cyclin D1 was replaced by the N terminus of the KSHV-cyclin. Such a cyclin D1 mutant was able to phosphorylate Rb but not histone H1 (data not shown). These results indicated that the N terminus of the KSHV-cyclin is not sufficient to confer the ability to phosphorylate histone H1 in the context of cyclin D1. Most likely other domains of the KSHV-cyclin in addition to the N terminus contribute to the substrate specificity.

Expression of KSHV-cyclin Mutants in U2OS Cells—Although the KSHV-cyclin mutants tested in vitro (see Fig. 2) did not prevent activation of Cdk6/KSHV-cyclin complexes in the absence of CAK, I aimed to test them in a cellular system. The
Cdk6-KSHV-cyclin Activity

To investigate if mutations in the KSHV-cyclin had any effect on the binding to Cdk6, I assayed immunoprecipitates using stringent conditions. Myc-KSHV-cyclin was co-transfected with Cdk6 and cell lysates were prepared. Myc-KSHV-cyclin-Cdk6 complexes were then immunoprecipitated in RIPA buffer (which contains Triton X-100, sodium deoxycholate, and SDS; see “Experimental Procedures”) and immunoprecipitates were analyzed for their kinase activity and Cdk6 binding (Fig. 4B). Cdk6 did not bind to Myc-KSHV-cyclinT181A (Fig. 4B, third panel from top) and bound to a lesser extent to Myc-KSHV-cyclin T189–198A, T244–247A (lanes 12, 15, 16, and 17). All other mutant Myc-KSHV-cyclin proteins bound similar amounts of Cdk6 as wild-type Myc-KSHV-cyclin. The amount of Cdk6 bound to Myc-KSHV-cyclin directly correlated with the observed kinase activity toward Rb (Fig. 4B, top panel) and histone H1 (Fig. 4B, second panel from top).

Expression of Cdk6 mutants in U2OS Cells—To learn more about the interaction of the KSHV-cyclin and Cdk6, I investigated the binding and resulting kinase activity of Cdk6/KSHV-cyclin complexes. Cdk6-HA was co-transfected with wild-type GFP-KSHV-cyclin into U2OS cells and after 48 h of incubation, cell extracts were prepared. Such lysates were analyzed for their ability to phosphorylate GST-Rb-(605–928) or histone H1 in vitro. When Cdk6-HA or KSHV-cyclin alone was transfected, only background activity was detected (data not shown and see Fig. 4A, lane 1). Extracts prepared from cells co-transfected with wild-type Cdk6-HA and Myc-KSHV-cyclin resulted in robust Rb kinase activity (Fig. 5A, third panel from top, lane 1) and expressed Cdk6 (Fig. 5A, fourth panel from top) and Myc-KSHV-cyclin (Fig. 5A, third panel from bottom) at similar level. The Cdk6 mutants, D163N, T177A, and T177A/S178A displayed only low activity. All other mutants, displayed similar activity when compared with wild-type Cdk6. To exclude contribution of other kinases that are present in cell lysates and of the endogenous wild-type Cdk6, Myc-KSHV-cyclin-Cdk6 complexes were immunoprecipitated and analyzed for kinase activity (Fig. 5A, top panel). After immunoprecipitation, all Cdk6 mutants displayed similar activity as wild-type Cdk6 with the exception of D163N, T177A, and T177A/S178A. Cdk6D163N was completely inactive, as expected and previously described (49, 58, 63). Low levels of Rb kinase activity were detected for Cdk6T177A and Cdk6T177A/S178A (Fig. 5A, top panel, lanes 11–12), consistent with previous findings (49, 51). I also verified that similar amounts of Cdk6-HA were co-immunoprecipitated with KSHV-cyclin (Fig. 5A, second panel from top). As comparison, activation of Cdk6 by cyclin D1 was investigated. When

drawback of the cellular system is that autophosphorylation cannot be investigated since there is plenty of CAK activity and high levels of ATP present. In the following experiments, it can be assumed that Cdk6 is phosphorylated at Thr-177 at all times. To test KSHV-cyclin mutants in cells, Myc-tagged KSHV-cyclin was co-transfected with GFP-Cdk6 or Cdk6-HA into U2OS cells. Cell extracts were assayed for their total kinase activity toward Rb (Fig. 4A, third panel from the bottom). Transferase of the vector without coding region resulted in little background activity (Fig. 4A, lane 1). Cell extracts expressing wild-type Myc-KSHV-cyclin together with GFP-Cdk6 yielded robust activity toward Rb (Fig. 4A, lane 2). Most of the KSHV-cyclin mutants displayed activity similar to wild-type KSHV-cyclin. However, four mutants displayed decreased activity; T34A/S35A/S37A, K106E, T189–198A, and T244–247A (Fig. 4A, lanes 5, 12, 21, and 24). Myc-KSHV-cyclin-GFP-Cdk6 complexes were immunoprecipitated and assayed for their activity toward Rb (Fig. 4A, top panel) and histone H1 (Fig. 4A, second panel from top). The kinase activities from the immunoprecipitates were similar as the ones from the lysates. To analyze the cyclin-Cdk6 complexes, I analyzed the amount of Cdk6 protein bound to Myc-KSHV-cyclin (Fig. 4A, third panel from top). Similar amounts of Cdk6 were bound to most mutant KSHV-cyclin proteins compared with wild-type KSHV-cyclin. There were only four mutant KSHV-cyclins (T189–198A, S215A/S217A, S226A/S233A, and T244–247A) that displayed decreased Cdk6 binding, although the expression of Myc-KSHV-cyclin was also decreased in the extracts (Fig. 4A, bottom panel, lanes 21–24).

FIG. 4. Activity of wild-type and mutant Myc-KSHV-cyclin in U2OS cells. Wild-type or mutant Myc-KSHV-cyclin was co-transfected with GFP-Cdk6 into U2OS cells. Myc-KSHV-cyclin/GFP-Cdk6 complexes were immunoprecipitated and assayed for phosphorylation of GST-Rb-(605–928) (A, top panel), histone H1 (A, second panel from top) in the presence of [γ-32P]ATP, binding of Cdk6 (A, third panel from top) and Myc-KSHV-cyclin (A, fourth panel from top). Cell extracts (inp) were assayed for their ability to phosphorylate GST-Rb-(605–928) (A, third panel from the bottom) and the expression of Cdk6 (A, second panel from the bottom) and Myc-KSHV-cyclin (A, bottom panel). B, same experiment as in A but immunoprecipitation was done under stringent conditions (RIPA buffer, see “Experimental Procedures”). Immunoprecipitates were assayed for their ability to phosphorylate Rb (B, top panel), histone H1 (B, second panel from top), binding of Cdk6 (B, third panel from top). Cell extracts (inp) were blotted for the expression of Cdk6 (B, second panel from the bottom) and Myc-KSHV-cyclin (B, bottom panel).
Cdk6-HA was co-transfected with GFP-cyclin D1T286A, a low level of activity was detected. Interestingly, Cdk6T58A, Cdk6S57A, and Cdk6S57A/T58A displayed elevated levels of cyclin D1 kinase activity toward Rb (Fig. 5A, second panel from bottom).

Since mutations in Cdk6 had little effect on the activity of KSHV-cyclin-Cdk6 complexes, the immunoprecipitation experiments were repeated under stringent conditions (Fig. 5B). Using these stringent conditions, KSHV-cyclin bound poorly to mutant Cdk6S290A, Cdk6T58A, Cdk6T93A, Cdk6S57A, and Cdk6S290A (Fig. 5, third panel from top, lanes 3, 4, 9, 11, and 14). These results were different from Fig. 5A where gentle conditions (0.5% Nonidet P-40) were used. Nevertheless, this indicated that these mutations affected the ability of Cdk6 to bind to KSHV-cyclin. The resulting kinase activity toward Rb (Fig. 5B, top panel) and histone H1 (Fig. 5B, second panel from top) correlated with the amount of bound KSHV-cyclin to Cdk6 (Fig. 5B, third panel from top).

**FIG.5. Activity of wild-type and mutant Cdk6 in cells.** Wild-type or mutant Cdk6-HA was co-transfected with Myc-KSHV-cyclin into U2OS cells. Myc-KSHV-cyclin-Cdk6-HA complexes were immunoprecipitated and phosphorylation of GST-Rb-(605–928) was determined (A, top panel) in the presence of [γ-32P]ATP. Western blot analysis of immunoprecipitated samples using antibodies against Cdk6-HA (A, second panel from top). Cell extracts (inp) were assayed for their ability to phosphorylate GST-Rb-(605–928) (A, third panel from top), and the expression of Myc-KSHV-cyclin (third panel from bottom). Wild-type and mutant Cdk6-HA was co-transfected with GFP-cyclin D1T286A into U2OS cells. The expression of GFP-cyclin D1 was analyzed by Western blotting (A, bottom panel, inp). Cdk6-HA-GFP-cyclin D1 complexes were immunoprecipitated and phosphorylation of GST-Rb-(605–928) was assayed (A, second panel from bottom). B, same experiment as A but immunoprecipitation was done under stringent conditions (RIPA buffer, see "Experimental Procedures"). Immunoprecipitates were assayed for their ability to phosphorylate Rb (B, top panel), histone H1 (B, second panel from top), and binding of Cdk6 (B, third panel from top). Cell extracts (inp) were blotted for the expression of Cdk6 (B, second panel from the bottom) and Myc-KSHV-cyclin (B, bottom panel).

Heterotrimeric Complex of Cdk6-KSHV-cyclinp16—From crystallographic studies, it has been shown that Cdk6, KSHV-cyclin, and p16 form a heterotrimeric complex in vitro (52). Here, I tried to confirm these results when these proteins were expressed in mammalian cells. First of all, I checked if high levels of p16 were able to inhibit Myc-KSHV-cyclin-Cdk6 complexes in U2OS cells. High levels of p16 inhibited wild type Myc-KSHV-cyclin-Cdk6 but even more so Myc-KSHV-cyclin-S41–48A and Myc-KSHV-cyclinS178–198A (Fig. 6A). Transfection of more wild-type Myc-KSHV-cyclin plasmid resulted in larger amounts of protein that was immunoprecipitated (Fig. 6A, third and fourth panel from top, compare lanes 1, 2, 3, and 4). Similar results were observed for Myc-KSHV-cyclinS41–48A and Myc-KSHV-cyclinS178–198A (lanes 9–12 and 5–8). Myc-KSHV-cyclinS178–198A resulted in low levels of expression compared with wild type (lanes 13–16) and these results were consistent in several repetitions of the experiment (data not shown), which might be due to low stability of this mutant. Cdk6 binding was similar to wild-type Myc-KSHV-cyclin as to Myc-KSHV-cyclinS41–48A or Myc-KSHV-cyclinS178–198A (Fig. 6A, second panel from top). Nevertheless, Cdk6 binding to Myc-KSHV-cyclinS178–198A was clearly reduced (lanes 13–16). The kinase activity toward Rb (Fig. 6A, top panel) and histone H1 (Fig. 6A, second panel from top) was dose-dependent for wild-type Myc-KSHV-cyclin and Myc-KSHV-cyclinS41–48A (lanes 1–4 and 9–12). Myc-KSHV-cyclinS178–198A displayed only low activity toward histone H1, similar as has been shown for bacteria produced KSHV-cyclinS178–198A (see Figs. 2 and 3). Although Myc-KSHV-cyclinS178–198A bound similar amounts of Cdk6 as Myc-KSHV-cyclinS41–48A, the activity toward Rb was reduced (compare lane 2 and 10 to lane 6). These results indicated the deletion of the N terminus of KSHV-cyclin affected activity and substrate specificity in cells.
Cdk6-KSHV-cyclin Activity

**FIG. 6.** N terminus of KSHV-cyclin affects activity and p16 binding. A, increasing amounts of wild-type (lanes 1–4) or mutant Myc-KSHV-cyclin (∆N1–19 (lanes 5–8), S41–48A (lanes 9–12), or S178–198A (lanes 13–16)) were co-transfected with GFP-Cdk6. Myc-KSHV-cyclin was immunoprecipitated and assayed for phosphorylation of GST-Rb-(605–928) (A, top panel), histone H1 (A, second panel from top), binding to Cdk6 (A, third panel from top), and Myc-KSHV-cyclin (A, fourth panel from top). Cell extracts (inp) were analyzed by Western blotting for the expression of Cdk6 (A, second panel from bottom) and Myc-KSHV-cyclin (A, bottom panel). B, increasing amounts of p16 were co-transfected with wild-type (lanes 1–4) or mutant Myc-KSHV-cyclin (∆N1–19 (lanes 5–8) or S178–198A (lanes 9–12)), and GFP-Cdk6. Complexes were immunoprecipitated with antibodies against Myc and assayed for phosphorylation of GST-Rb-(605–928) (B, top panel) and histone H1 (B, second panel from top) in the presence of [γ-32P]ATP. To determine the complex composition, immunoprecipitates of Myc-KSHV-cyclin were blotted for Cdk6 (B, third panel from top), KSHV-cyclin (B, fourth panel from top), and p16 (B, fifth panel from top). Cell extracts (inp) were analyzed by Western blotting for the expression of Cdk6 (B, third panel from bottom), Myc-KSHV-cyclin (B, second panel from bottom), and p16 (B, bottom panel).

Myc-KSHV-cyclin-Cdk6 complex. The two mutant Myc-KSHV-cyclins were much more sensitive to p16 and in that case the binding of Cdk6 to the Myc-KSHV-cyclin was decreased. Therefore, Cdk6 binding to mutant Myc-KSHV-cyclin^{∆N1–19} or S178–198A is weakened, especially in the presence of p16.

**DISCUSSION**

In this study, I have investigated the activation of Cdk6 by the KSHV-cyclin. Several mutants of the KSHV-cyclin were able to activate Cdk6 more effectively in the absence than in the presence of CAK. None of the mutations (or combination of mutations) rendered the KSHV-cyclin inactive or nonfunctional. Deletion of the N terminus of the KSHV-cyclin led to a change in substrate specificity. Expression of high levels of p16 in U2OS cells resulted in inactivation of KSHV-cyclin-Cdk6 complexes while forming a heterotrimERIC complex.

Mutants were generated of the KSHV-cyclin that could only activate CAK-phosphorylated Cdk6 but not Cdk6 activated by ATP treatment. Some of the mutant KSHV-cyclins were activated preferentially by CAK phosphorylated Cdk6, others displayed higher activity after ATP treatment but none of them were activated only by one treatment. There are many potential reasons for this outcome. First of all, I have speculated that the effect of the ATP treatment among other possibilities could be caused by autophosphorylation or an ATP induced conformational change. To peruse phosphorylation events, I made a number of mutants that could not be phosphorylated at specific residues but these mutations had little effect on the activation of Cdk6-KSHV-cyclin complexes. It is possible that the KSHV-cyclin is phosphorylated at several residues and that I have not mutated the right combination of residues. Another possibility is that both the KSHV-cyclin and Cdk6 are phosphorylated. Here I have not tested a large number of Cdk6 mutants since Cdk6 consist of only a minimal kinase domain and are notorious for being inactivated by even single residue mutations, which is not related to phosphorylation but rather to folding. Therefore, this question remains to be answered in the future. Nevertheless, some of the KSHV-cyclin mutants turned out to be quite interesting since the mutated residues affect the binding affinity to Cdk6. Furthermore, it is interesting to note that all KSHV-cyclin mutants remained active even when homologous cyclin D mutants were not active (e.g. T156A, Ref. 66; K112E, Ref. 67).

ATP treatment activated Cdk6 to similar levels than CAK phosphorylation *in vitro* (see Figs. 1 and 2). In contrast, when Cdk6^{∆N/H18528} was expressed in U2OS cells only a low level of activity was detected (Fig. 5, A and B, lane 11). Similar results have been described previously (49, 51). There are two distinct possibilities that could lead to this difference: (1) the mutation at residue 177 could lead to structural distortions in the T-loop, which are unrelated to the phosphorylation state of threonine 177, and (2) in vitro, ATP treatment of Cdk6-KSHV-cyclin complexes results in (auto)-phosphorylation of Thr-177 in Cdk6. By phosphopeptide mapping, we have shown that Cdk6 was phosphorylated on Thr-177 in these conditions (data not shown). It is possible that both possibilities apply under certain conditions.

The determination of the substrate specificity of Cdk6-cyclin complexes is of great importance since only few substrates have been identified. Cyclins have been implied to be determinants of the substrate specificity (32–34). For example, Cdk2/cyclin D complexes phosphorylate Rb only, whereas Cdk6/KSHV-cyclin complexes phosphorylate a wide range of substrates (see Introduction). It was not known which regions of the KSHV-cyclin contribute to the change in substrate specificity. I have shown here that two different deletions of the N terminus of the KSHV-cyclin display reduced phosphorylation of histone H1 whereas phosphorylation of other substrates was unchanged (see Fig. 3). This indicates that the N terminus of the KSHV-cyclin is required to phosphorylate histone H1. It is interesting to note that the N terminus of the KSHV-cyclin directly interacts with the T-loop of Cdk6 (53). Therefore, it is possible that...
deletion of the N terminus of the KSHV-cyclin leads to a conformational change of the T-loop, which then affects histone H1 phosphorylation. Nevertheless, this possibility is unlikely since phosphorylation of other substrates (Rb, p27, p53, Cdc25) was not affected (see Fig. 3). The N terminus of cellular cyclins has not been shown to affect substrate specificity. Deletion of the N terminus of cyclin A has no obvious effect on phosphorylation of histone H1 (3, 69) or Rb2 but deletion of the N terminus of cyclin D (70) or cyclin H (71) abolishes activity. Nevertheless, it is difficult to compare cyclin A to the KSHV-cyclin (or to the D-type cyclins) since cyclin A contains an N-terminal extension (D-type cyclins) since cyclin A contains an N-terminal extension which then might be two different modes of p16 binding to the active or not (Fig. 6, compare lane 3 and lane 4). In addition, this heterotrimeric complex can be formed in cells expressing the KSHV-cyclin complex in cells that do not express histone H5 but was lower than in UV-treated cells (data not shown). This indicates that the cell death induced by the KSHV-cyclin-KSHV-cyclin is independent on caspase activation, which is consistent with Bel-2 not being able to rescue this phenotype (55).

The Ink4 inhibitor p16 binds to the N-terminal lobe of Cdk6 (or Cdk4) (29, 30). Immunoprecipitation of p16 from cell extracts indicated that only Cdk4 bound but cyclin D was not co-immunoprecipitated (24). This led to the conclusion that p16 would displace cyclin D when it binds to Cdk6 (or Cdk4). Nevertheless, it is also possible that cyclin D is such an unstable protein (68) that its levels are too low to detect in such experiments. In vitro, p16 was shown to form a heterotrimeric complex with purified Cdk6 and the KSHV-cyclin (49, 52) but p16 does not interact directly with the cyclin subunit (52). In this study, p16 binds to the Cdk6-KSHV-cyclin complex in cells (see Fig. 6B). In addition, this heterotrimeric complex can be active or not (Fig. 6B, compare lane 3 to lane 4) indicating that there might be two different modes of p16 binding to the Cdk6-KSHV-cyclin complex. Interestingly, deletion of the N terminus or mutation of the region around residue 180–200 of the KSHV-cyclin led to destabilization of the heterotrimeric p16 complex formation.

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