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Degradation of p27^{Kip} cdk inhibitor triggered by Kaposi's sarcoma virus cyclin–cdk6 complex

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The Kaposi's sarcoma-associated human herpesvirus 8 (KSHV/HHV8) encodes a protein similar to cellular cyclins. This cyclin is most closely related to cellular D-type cyclins, but biochemically it behaves atypically in various respects. Complexes formed between the viral cyclin and the cyclin-dependent kinase subunit, cdk6, can phosphorylate a wider range of substrates and are resistant to cdk inhibitory proteins. We show here that the KSHV-cyclin–cdk6 complex phosphorylates p27^{Kip} on a C-terminal threonine that is implicated in destabilization of this cdk inhibitor. Expression of the viral cyclin in tissue culture cells overcomes a cell cycle block by p27^{Kip}. However, full cell-cycle transit of these cells appears to depend on C-terminal phosphorylation of p27^{Kip} and seems to involve transactivation of other cellular cyclin-dependent kinases. A p27^{Kip}-phosphorylating cdk6 complex exists in cell lines derived from primary effusion lymphoma and in Kaposi's sarcoma, this indicating that virally induced p27^{Kip} degradation may occur in KSHV-associated tumours.

Keywords: cyclin/KSHV/HHV8/p27^{Kip}/phosphorylation/protein degradation

Introduction

Progression of cells through the division cycle is accompanied by an ordered activation of cyclin–cdk complexes. Transit through the different cell cycle phases requires activation of different members of this serine/threonine kinase family (reviewed in Hunter and Pines, 1991; Sherr, 1996; Morgan, 1997).

Entry of cells into S phase depends on activation of kinase complexes containing D-type cyclins and the catalytic subunits cdk4 or cdk6, and complexes containing cyclin E and cdk2 (reviewed in Weinberg, 1995; Sherr, 1996). Inhibitory proteins, collectively referred to as cyclin-dependent kinase inhibitors (CDKIs), control the activation of these kinase complexes (reviewed in Morgan, 1995, 1997). The KIP/CIP family of CDKIs, exemplified by p21^{Cip}, p27^{Kip} and p57^{Kip2}, inhibits both cyclin D- and cyclin E-activated kinases, whereas those of the INK family, exemplified by p16^{Ink4a}, p15^{Ink4b} and p18^{Ink4c}, specifically inhibit activation of cdk4 and cdk6 by D-type cyclins.

CDKI activity is affected in many tumours, indicating that these proteins are critical for the control of cellular proliferation (reviewed in Miller and Koeffler, 1997). Loss of INK inhibitors through mutation or transcriptional silencing is a frequent event during tumourigenesis (reviewed in Palmero and Peters, 1996; Carnero and Hannon, 1998). Although described, mutations in the *CIP/KIP*-type inhibitors are rarely seen (Morosetti *et al*., 1995; Shi *et al*., 1996; Spirin *et al*., 1996). However, *p21^{Cip}* induction is affected by mutations in the tumour suppressor *p53*. Furthermore, evidence now indicates that p27^{Kip} is destabilized in many types of cancer, an event which also correlates with poor prognosis and progression of the disease (Esposito *et al*., 1997; Loda *et al*., 1997; Porter *et al*., 1997; Tan *et al*., 1997; Yang *et al*., 1998).

The molecular events that result in p27^{Kip} destabilization are currently not understood. However, destabilization of p27^{Kip} is seen to occur in cells in response to growth factor signalling and can be mediated via pathways involving the proto-oncogene *Ras* (Aktas *et al*., 1997; Kawada *et al*., 1997; Takuwa and Takuwa, 1997; reviewed in Herwig and Strauss, 1997; Lloyd, 1998; Lundberg and Weinberg, 1998; Mittnacht, 1998).

Destabilization of p27^{Kip} in cells is initiated by phosphorylation of this inhibitor. Members of the mitogen-activated family of ERK kinases but also cyclin E-activated cdk2 have been implicated in p27^{Kip} phosphorylation and its subsequent degradation (Kawada *et al*., 1997; Muller *et al*., 1997; Sheaff *et al*., 1997; Vlach *et al*., 1997). From current evidence it would appear that different kinases trigger p27^{Kip} degradation in response to different upstream signalling pathways. Activation of ERK kinases, for example, can occur through Ras while rapid activation of cyclin E–cdk2 is seen upon Myc induction (Steiner *et al*., 1995; Perez *et al*., 1997).

We report here that, apart from the cellular kinases mentioned, the kinase complex between cdk6 and the D-like cyclin encoded by the Kaposi's sarcoma-associated herpesvirus also triggers p27^{Kip} mentioned destabilization. Kaposi's sarcoma-associated herpesvirus/HHV8 has been implicated as the causative agent of Kaposi's sarcoma and primary effusion lymphoma (Chang *et al*., 1994; Cesarman *et al*., 1995; reviewed in Boshoff *et al*., 1998; Moore and Chang, 1998). We present evidence here that the ability of the viral cyclin to promote cell cycle progression in p27^{Kip}-expressing cells depends on p27^{Kip} phosphorylation and consequential transactivation of cellular cyclin–cdks. The presence of p27^{Kip}-phosphorylating cdk6 complex in KSHV-containing tumours suggests that this mechanism is operational in KSHV-associated cancers.

Results

Phosphorylation of p27^{Kip} by KSHV-cyc–cdk6

When examining the effects of the cdk inhibitor p27^{Kip} on crude preparations of KSHV-cyc–cdk6 enzyme from baculovirus-programmed insect cells, we found, in accordance with recently published observations (Swanton *et al*., 1997), that the activity of this kinase was virtually unaffected by p27^{Kip} concentrations that completely inhibited preparations of cyclin E–cdk2. We noted, however, that in reactions containing KSHV-cyc–cdk6 enzyme another protein species became phosphorylated, apart from the added substrate (Figure 1A). This species closely resembled in size the recombinant glutathione *S*-transferase (GST)–p27^{Kip} that had been added to the reaction as a source of inhibitor. Moreover, this phosphorylated species was most obvious in reactions that contained high concentrations of the recombinant p27^{Kip} suggesting that the inhibitor itself might have become phosphorylated.

Phosphorylation of p27^{Kip} is a known regulatory event that can be executed by cyclin E–cdk2, resulting in destabilization of this cdk inhibitor. However, p27^{Kip} phosphorylation is only detectable when the kinase is exposed to p27^{Kip} under conditions that allow catalysis and not when this kinase is pre-incubated with inhibitor in the absence of Mg^{2+}/ATP (Sheaff *et al*., 1997). The relative resistance of the KSHV-cyc–cdk6 kinase complex to p27^{Kip} might allow phosphorylation of the inhibitor to occur even when administered before initiating catalysis.

To confirm that KSHV-cyc–cdk6 can phosphorylate GST–p27^{Kip} and to investigate whether this phosphorylation resides in the p27^{Kip} moiety of the GST–p27^{Kip} fusion, we immunopurified this enzyme from insect cell lysate and performed kinase assays using Histone H1, GST–p27^{Kip} and GST alone as substrates in separate reactions (Figure 1B). The GST–p27^{Kip} protein was significantly phosphorylated in reactions containing the purified enzyme but not when lysate containing cdk6 alone was used. Importantly, recombinant GST–protein was not phosphorylated, indicating that the GST moiety does not serve as a phosphate acceptor in its own right. Significantly, neither cyclin D1–cdk4 nor cyclin D2–cdk6 were capable of phosphorylating p27^{Kip} in similar assays, although both of these kinases phosphorylated a GST–pRB fragment to a similar and even greater extent than did the KSHV-cyc–cdk6 complex (Figure 1C). This indicates that p27^{Kip} is a substrate for the KSHV-cyc–cdk6 complex whereas it is not for kinase complexes involving the cellular cyclin D cousins of KSHV-cyc.

Locating the site of p27^{Kip} phosphorylation by KSHV-cyc–cdk6

While our experiments so far reveal that p27^{Kip} becomes phosphorylated by KSHV-cyc–cdk6, they do not indicate whether such phosphorylation is of functional significance. There are three phosphate acceptor sites in p27^{Kip} that conform to the presumed minimal consensus for cyclin-dependent kinases, i.e. a serine or threonine followed by a proline. These are a serine in the N-terminus at position 10 (S10), a further serine at position 178 (S178) and a threonine at position 187 (T187) (see Figure 2A). Phosphorylation of T187 has been implicated in p27^{Kip} regulation by cyclin E–cdk2 (Sheaff *et al*., 1997). To

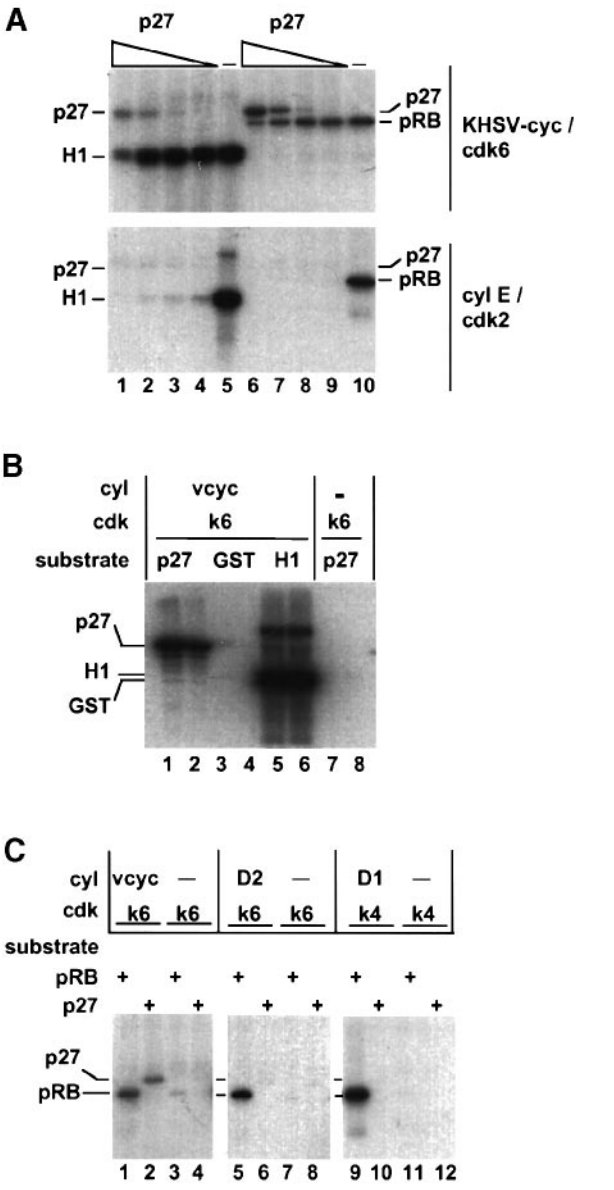

Fig. 1. Phosphorylation of p27^{Kip} by KSHV-cyc–cdk6. (**A**) Substrate phosphorylation following pre-incubation with p27^{Kip}. Insect cell lysate programmed by recombinant baculovirus encoding KSHV-cyc and cdk6 (KSHV-cyc/cdk6), or human cyclin E and cdk2 (cyl E/cdk2) was pre-incubated with recombinant GST–p27^{Kip}. The amounts of p27^{Kip} used were 1 μg (lanes 1 and 6), 200 ng (lanes 2 and 7), 40 ng (lanes 3 and 8) and 8 ng (lanes 4 and 9), and no inhibitor (lanes 5 and 10). Kinase activity remaining in the reactions was determined subsequently by adding a reaction mix containing radioactive ATP and substrate [either Histone H1 (H1) or GST fused to the C-terminus of pRB (pRB)]. (**B**) Phosphorylation of GST–p27^{Kip} by immunopurified KSHV-cyc–cdk6 complex. KSHV-cyc–cdk6 from baculovirus-programmed insect cell lysate was immunopurified via a C-terminal 9E10 tag present on KSHV-cyc using 9E10 monoclonal antibody. Kinase reactions were performed using 0.5 μg of either GST–p27^{Kip} (lanes 1, 2 and 7, 8), GST (lanes 3 and 4) or Histone H1 (lanes 5 and 6) for a substrate. Immunoprecipitates from insect cells programmed with cdk6 alone were used as a baseline control. (**C**) Phosphorylation of p27^{Kip} by cellular D cyclin–cdk complexes. Cyclin D1–cdk4, cyclin D2–cdk6 and KSHV-cyc–cdk6 were immunopurified from baculovirus-programmed insect cell lysate using 9E10 antibody. Kinase reactions were performed as in (B) using for a substrate either GST–pRB or GST–p27^{Kip}. Immunoprecipitates using lysates programmed with cdk4 or cdk6 alone were used as a baseline control. The position of substrate and the GST–p27^{Kip} inhibitor, revealed by Coomassie Brilliant Blue staining, are indicated.

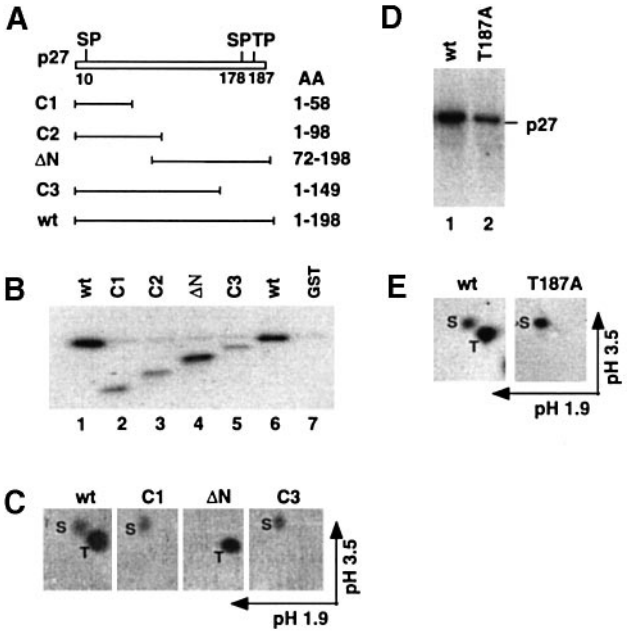

Fig. 2. Mapping of $p27^{Kip}$ residues phosphorylated by KSHV-cyc–cdk6. (**A**) Schematic showing the position of cdk consensus phosphorylation sites in $p27^{Kip}$ and the $p27^{Kip}$ deletion mutants used in (B). (**B**) Localization of phosphorylated residues using $p27^{Kip}$ deletion mutants. Full-length $p27^{Kip}$ and the $p27^{Kip}$ deletion mutants shown in (A) were used as substrates for immunopurified KSHV-cyc–cdk6. All substrates were expressed as GST fusions in *E.coli* and 0.5 μg of each was used per reaction. (**C**) Two-dimensional phosphoamino acid analysis. Phosphorylated substrates were excised from gels, subjected to acid hydrolysis and the nature of phosphorylated residues was determined by two-dimensional electrophoresis on cellulose acetate sheets. The position of phospho-serine (S) and phospho-threonine (T) is indicated. Direction of the first (pH 1.9) and second (pH 3.5) electrophoretic dimension is indicated. (**D**) Phosphorylation of wt $p27^{Kip}$ and the mutant $p27^{Kip}$(T187A). Purified GST–wt $p27^{Kip}$ and GST–$p27^{Kip}$(T187A), a mutant in which T187 was changed to an alanine residue, were used as substrates for immunopurified KSHV–cdk6 complex. (**E**) Two-dimensional phosphoamino acid analysis of wt $p27^{Kip}$ and $p27^{Kip}$(T187A) following phosphorylation by KSHV-cyc–cdk6.

address whether this threonine is phosphorylated by KSHV-cyc–cdk6, we performed kinase assays using a series of $p27^{Kip}$ deletion constructs and determined the nature of the phosphorylated residues by two-dimensional phosphopeptide mapping (Figure 2B and C). These experiments reveal that phosphorylation occurs on a serine residue located within the N-terminal 58 residues, most likely representing S10, and a threonine located within the most C-terminal 49 amino acids, thus most likely representing T187. To confirm independently that the C-terminal threonine phosphorylation was due to modification of T187, we generated a mutated version of $p27^{Kip}$ in which this threonine was exchanged with alanine. This mutated form of $p27^{Kip}$ was significantly less phosphorylated by immunopurified KSHV-cyc–cdk6 complex (Figure 2C) and phosphoamino acid analysis revealed absence of threonine phosphorylation (Figure 2E). This confirms that KSHV-cyc–cdk6 *in vitro* can trigger phosphorylation on T187, which in turn suggests that expression of this viral cyclin in cells could mediate $p27^{Kip}$ destabilization.

Destabilization of $p27^{Kip}$ in KSHV-cyc-expressing cells

To address whether KSHV-cyc expression can affect the level of $p27^{Kip}$ in living cells we transfected a plasmid encoding a 9E10-tagged version of this cyclin into H/p27 cells, which express human $p27^{Kip}$ from an Ecdysone-responsive promoter and into Cal51, a mammary cancer-derived cell line in which expression of endogenous $p27^{Kip}$ is readily detectable. In either case, the level of $p27^{Kip}$ fluorescence present in the cell nuclei was reduced to near undetectable levels (Figure 3A and B). Statistical analysis showed that the number of $p27^{Kip}$-negative cells was significantly increased in KSHV-cyc expressing cells of either type, both when compared with KSHV-cyc-negative cells from the same culture dish or to cells transfected with plasmid encoding a DNA binding-defective (and thus functionally disabled) version of the E2F transcription factor (Figure 3C and D).

To obtain evidence as to whether KSHV-cyc can operate to decrease $p27^{Kip}$ stability, we examined the turnover rate of $p27^{Kip}$ in U2OS osteosarcoma cells transiently transfected with expression constructs encoding 9E10-tagged $p27^{Kip}$ using a pulse–chase approach. We chose U2OS cells for these experiments since these cells are readily transfectable and can also be used for DNA-fluorescence-based cell cycle analysis because of their homogeneous DNA content. While $p27^{Kip}$ was stable over several hours when expressed by itself in these cells, its life was greatly decreased when co-expressed with KSHV-cyc (Figure 4A). Moreover, co-expression of KSHV-cyc reduced the steady-state expression level of transfected $p27^{Kip}$ (Figure 4B). In contrast, the expression levels of $p27^{Kip}$ with mutations in T187 or T187 in combination with S10 were not obviously affected. Mutation of S10 alone seemed not to result in an appreciable stabilization of the inhibitor protein (Figure 4C).

First, these experiments indicate that KSHV-cyc expression can operate to reduce $p27^{Kip}$ levels in cells. Furthermore, this appears to be affected by conversion of the T187 of $p27^{Kip}$ into a non-phosphorylatable alanine residue, thus complying with the notion that phosphorylation of this threonine may trigger the reduction in $p27^{Kip}$.

Dependence of KSHV-cyc-mediated cell cycle rescue on $p27^{Kip}$ phosphorylation

KSHV-cyc can overcome $p27^{Kip}$-mediated cell cycle arrest, an ability that recently has been accredited to the inherent resistance of the KSHV-cyc–cdk6 complex to this inhibitor (Swanton *et al.*, 1997). The results presented above, however, imply an alternative explanation, namely that this viral cyclin might operate in cells by eliminating $p27^{Kip}$. To distinguish between these two alternatives we examined whether expression of the KSHV cyclin can drive U2OS cells into the G_2/M compartment of the cell cycle in the presence of phosphorylation-defective $p27^{Kip}$ (Figure 5A).

As can be seen in Figure 5, transfection of U2OS cells with vector encoding wild-type (wt) $p27^{Kip}$ resulted in an accumulation of the transfectants in G_1 while significantly reducing the number of cells that succeeded to progress into G_2/M when compared with vector-transfected cells. Similarly, the expression of the different $p27^{Kip}$ mutants resulted in an increased amount of G_1 cells while reducing

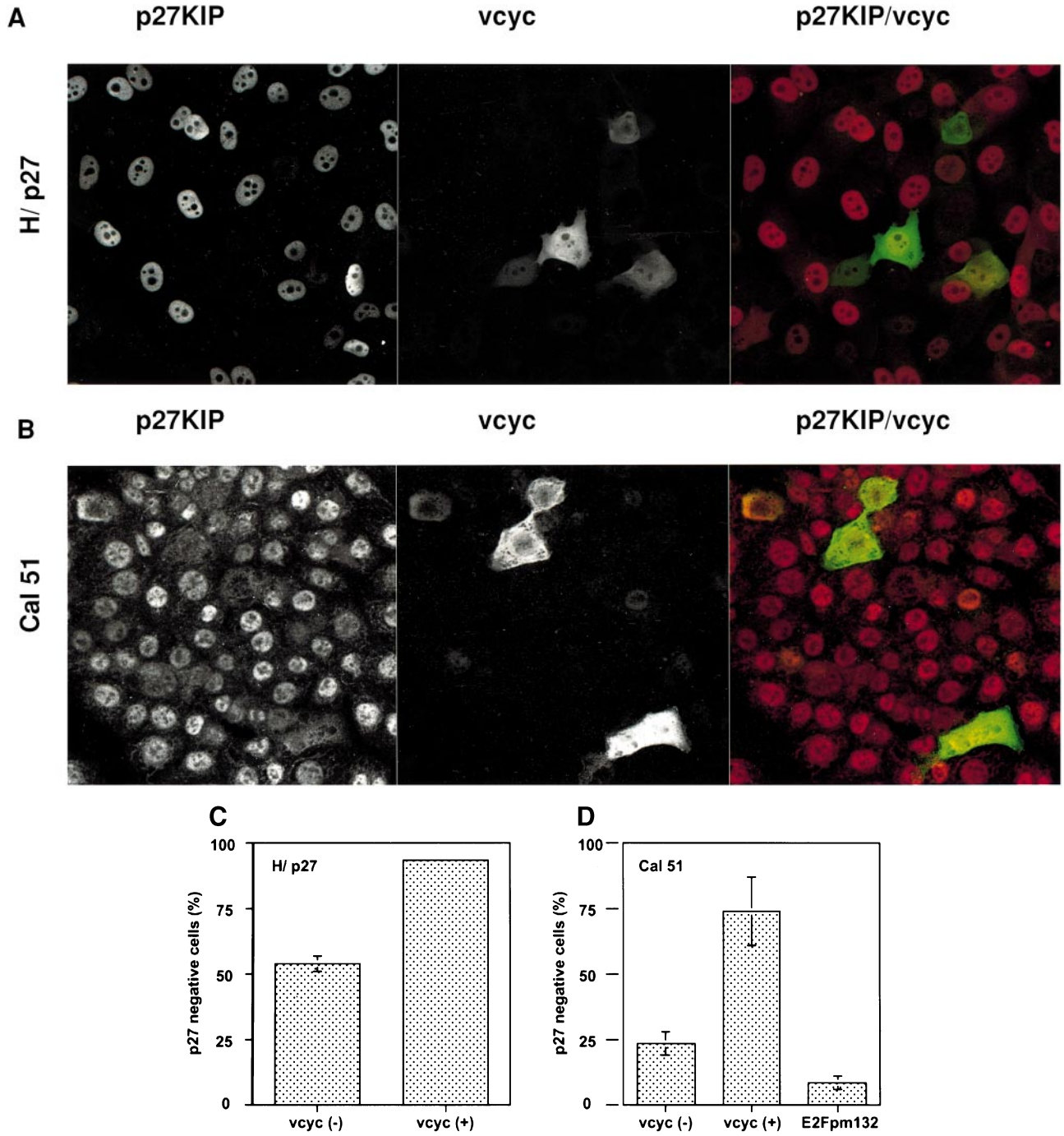

Fig. 3. Effect of KSHV-cyc on $p27^{Kip}$ expression. (**A**) $p27^{Kip}$ accumulation in KSHV-cyc-expressing H/p27 cells. KSHV-cyc was transfected into H/p27, a H1299-derived line containing Ecdysone-inducible $p27^{Kip}$. $p27^{Kip}$ was induced immediately after removing the transfection medium. Cell monolayers were fixed after 24 h and immunostained using 9E10 antibody to identify KSHV-cyc expressing cells, and anti-human $p27^{Kip}$ rabbit serum to determine the level of $p27^{Kip}$ in these cells. (**B**) Loss of endogenous $p27^{Kip}$ upon KSHV-cyc expression. Cal51 cells were transfected with plasmid encoding 9E10 epitope-tagged KSHV-cyc. Cells were fixed and stained 30 h post-transfection as described in (A). Note the absence of $p27^{Kip}$ nuclear staining in KSHV-cyc expressing cells. (**C** and **D**) Statistics on p27 expression in KSHV-cyc transfected H/p27 or Cal51 cells. Results are derived from two independent experiments, respectively. Data were compiled by examining $p27^{Kip}$ expression in a minimum of 80 KSHV-cyc positive [vcyc(+)] or negative [vcyc(–)] cells per experiment. E2Fpm132 refers to a control in which a plasmid encoding a 9E10-tagged, DNA binding-defective mutant of the E2F transcription factor was used in place of KSHV-cyc. Note that expression of this construct does not result in an increase of $p27^{Kip}$-negative cells.

the number of cells that reached G_2/M. However, when the KSHV cyclin was co-expressed with wt $p27^{Kip}$, the ability of the transfectants to exit G_1 and to proceed with their transit into G_2/M was partially restored. A similar result is seen in cells that express $p27^{Kip}$ mutated in S10 (S/A). In contrast, transfectants expressing KSHV-cyc and $p27^{Kip}$ mutated in either T187 (T/A) or T187 and S10 (T/A S/A) do not apparently succeed to proceed into the G_2/M phase to any greater degree than transfectants expressing the $p27^{Kip}$ mutants alone. However, we note that they seem to have acquired the ability to exit G_1 and enter S phase.

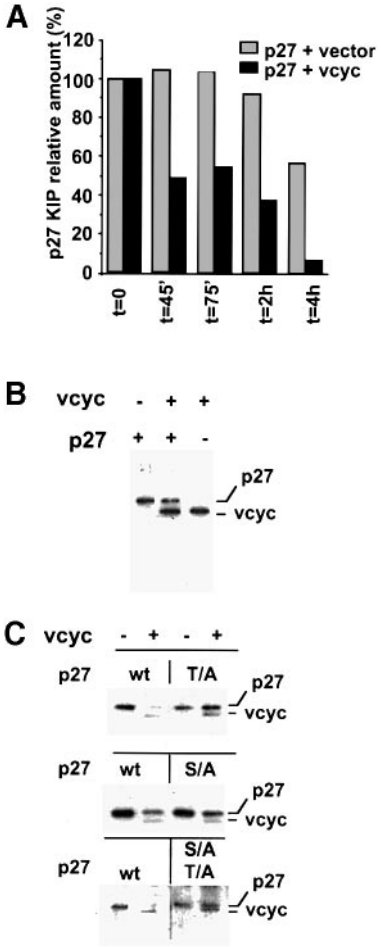

Fig. 4. $p27^{Kip}$ stability and accumulation in KSHV-cyc expressing cells. (**A**) Impact of KSHV-cyc on $p27^{Kip}$ stability. U2OS cells were transfected with plasmid encoding 9E10 epitope-tagged $p27^{Kip}$ alone or in combination with plasmid encoding KSHV-cyc. Turnover of $p27^{Kip}$ in the transfected cells was determined in a pulse–chase experiment. Cells were pulse-labelled with [^{35}S]methionine for 1 h and subsequently cultured in methionine-rich growth medium for the periods indicated. $p27^{Kip}$ was immunopurified using the epitope-specific antibody 9E10. Immunoprecipitates were separated by gel electrophoresis and the amount of radioactivity associated with $p27^{Kip}$ was determined by PhosphorImager analysis. (**B**) Accumulation of transfected $p27^{Kip}$ in KSHV-cyc-transfected cells. U2OS cells were transfected with vector encoding epitope-tagged $p27^{Kip}$ (0.2 μg), a β-galactosidase construct in the same vector (2.5 μg) and KSHV-cyc or empty vector (4 μg). Cell lysates were prepared 30 h post transfection. Lysates were normalized for the expression of β-galactosidase activity and analysed for $p27^{Kip}$ expression by Western blot using 9E10 antibody. (**C**) Accumulation of phosphorylation-defective mutant $p27^{Kip}$. U2OS cells were transfected as in (B), except that the amount of cyclin encoding vector was reduced to 2 μg. Cell lysates were prepared and analysed essentially as described in (B). T/A refers to mutation of Thr187, S/A to mutation of Ser10 and S/A-T/A to mutation of Ser10 and Thr187 into alanine, respectively.

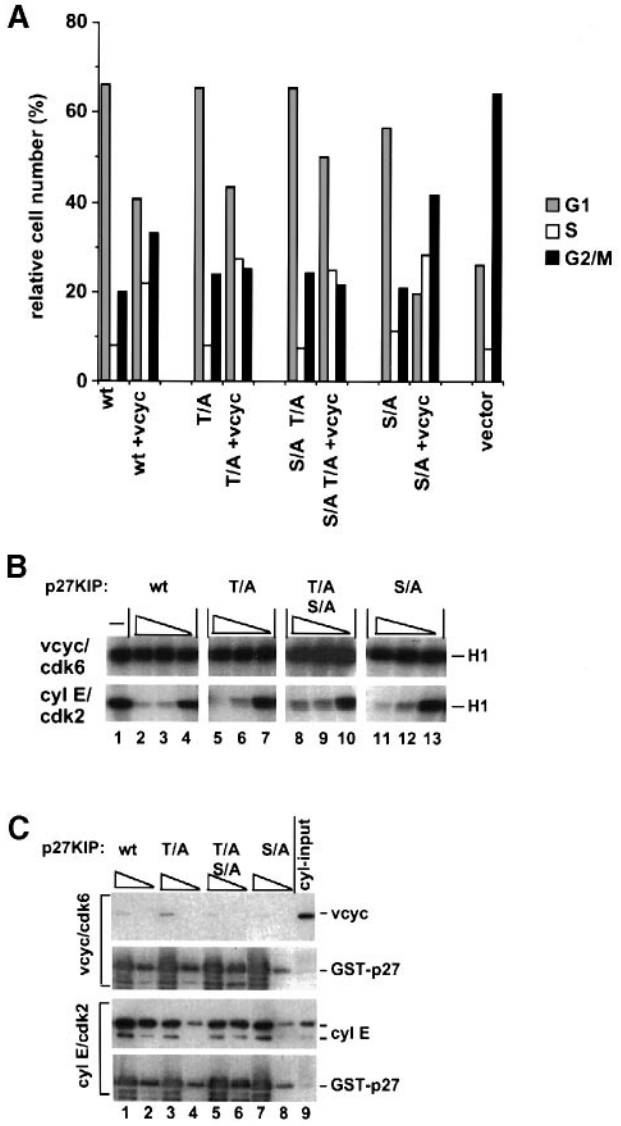

Fig. 5. Effect of KSHV-cyc on cell cycle progression of $p27^{Kip}$-expressing cells. (**A**) U2OS cells were co-transfected with a plasmid encoding CD20 surface marker and the combination of plasmids indicated. Thirty hours post-transfection, cells were treated with Nocodazole for 10 h. The cell cycle position of CD20-expressing cells was determined by fluorescence-activated cell scan (FACScan). (vcyc) denotes use of a plasmid encoding KSHV-cyc, (wt) refers to plasmid encoding wt $p27^{Kip}$, (T/A), (S/A) and (T/A-S/A) to plasmids encoding the $p27^{Kip}$ mutants described in Figure 4. Results for $p27^{Kip}$ T/A were confirmed by four independent experiments, those for S/A and the S/A-T/A double mutant by two independent experiments. (**B**) Effect of wt $p27^{Kip}$ and the various $p27^{Kip}$ phosphorylation site mutants on kinase activity of KSHV-cyc–cdk6. Insect cell lysate programmed with KSHV-cyc and cdk6 (vcyc/ k6) or cyclin E and cdk2 (cyl E/ k2) were incubated with increasing amounts of GST–wt $p27^{Kip}$ and $p27^{Kip}$ mutants as described for Figure 1. The amount of kinase activity in the various samples was subsequently determined using Histone H1 (H1) as a substrate. Lane 1 shows the activity in mock-treated samples. (**C**) Binding of $p27^{Kip}$ to KSHV-cyc–cdk6 complex. Cyclin–cdk programmed insect lysates as indicated were incubated with GST–wt $p27^{Kip}$ or $p27^{Kip}$ mutants using either 1 μg (lanes 1, 3, 5 and 7) or 0.2 μg (lanes 2, 4, 6, 8) protein, respectively. Following incubation, the $p27^{Kip}$ fusions were recovered from the reaction using glutathione–Sepharose and analysed for co-purification of the respective cyclins by Western blot. The lower panel in each display shows the GST fusion protein recovered for each of the samples as determined by probing the membranes with anti-$p27^{Kip}$ antibody. Cyclins (upper panel) were detected using the 9E10 (anti-tag) antibody. Lane 9 was loaded with half the amount of insect cell lysate used in the respective reactions to serve as an input control.

The inability of KSHV-cyc to promote progression into G_2/M in cells expressing phosphorylation-defective $p27^{Kip}$ would not appear to be explained by gain of the mutated $p27^{Kip}$ to inhibit KSHV-cyc–cdk6 directly. Treatment of recombinant KSHV-cyc–cdk6 with either the $p27^{Kip}$ phosphorylation site mutants or wt $p27^{Kip}$ did not result in loss of phosphorylating activity, while these inhibitors efficiently and to a similar extent inhibited cyclin E–cdk2 in parallel experiments (Figure 5B). Furthermore, the inability of KSHV-cyc to promote G_2/M progression of

cells expressing T187-mutated p27^{Kip} cannot be explained by a differential ability to sequester the mutated inhibitor. In agreement with a previous observation (Swanton *et al*., 1997), we find that the recombinant KSHV-cyc–cdk6 complex binds only poorly to p27^{Kip}, an association being detectable only at very high doses of the cdk inhibitor (Figure 5C). More importantly, the viral cyclin–cdk complexes bind with similar efficiency to wt and mutated p27^{Kip}, making it unlikely that the inability of the KSHV-cyclin to overcome the cell cycle inhibition by T187-mutated p27^{Kip} is due to a reduced affinity for binding to the p27^{Kip} mutant.

Together, the above experiments indicate that the ability of KSHV-cyc to promote G_2/M progression of cells expressing p27^{Kip} is compromised by mutations that abolish phosphorylation of p27^{Kip} on T187, suggesting that KSHV-cyc may achieve cell cycle progression in p27^{Kip}-expressing cells via a route involving phosphorylation and concomitant destabilization of p27^{Kip}. Our experiments do not indicate the significance for S10 phosphorylation although phosphorylation of this site could be important under conditions that cannot be reproduced by the transient transfection assays employed here.

Evidence for a requirement of cdk2 activation in KSHV-cyc-expressing cells

Our observation that KSHV-cyc expression cannot support progression of cells into G_2/M under conditions that do not allow elimination of p27^{Kip} suggests that the kinase activity elicited by the KSHV cyclin may not in itself be sufficient to promote cell cycle completion. Indeed, progression through S-phase and entry into G_2/M may rely on the activation of other cellular cyclin–cdk complexes whose activity is inhibited by p27^{Kip}, but restored if the inhibitor can be eliminated. For example, S-phase completion in cells normally requires the activation of cdk2 by cyclin E and by cyclin A (reviewed in Hunter and Pines, 1991; Sherr, 1996).

To examine whether S-phase completion in p27^{Kip}/KSHV-cyc-expressing cells might depend on activation of these cdk2-containing kinases, we tested whether roscovitine, a cdk2-selective chemical inhibitor (Heijer *et al*., 1997), would hinder progression into G_2/M of U2OS cells transfected with KSHV-cyc and p27^{Kip}.

We find that addition of roscovitine effectively inhibited progression into G_2/M of cells co-transfected with KSHV-cyc and wt p27^{Kip} (Figure 6A). This was observed, although in cells treated with this kinase inhibitor the kinase activity associated with KSHV-cyc was not reduced (Figure 6B), nor did this drug inhibit KSHV-cyc-associated kinase from transfected cells (Figure 6C) or recombinant KSHV-cyc–cdk6 (Figure 6D) *in vitro*, even when used at concentrations $>$60 times that sufficient to affect cyclin E–cdk2 activity. Thus, roscovitine would seem to achieve the observed inhibition of cell cycle transit, not via inhibiting KSHV-cyc-associated kinase but possibly by inhibiting cyclin–cdk complexes required for cell cycle transit in addition to those involving KSHV-cyc.

To gain additional support for such a concept we conducted further experiments employing dominant-negative cdks (dn-cdks) as a means to inhibit specific types of cdks in the transfected cells. We found that co-expression of both dn-cdk6 and dn-cdk2 but not dn-cdk4 impaired the ability of the KSHV cyclin to drive cells into G_2/M.

Previous experiments indicate that the KSHV-cyc-associated activity in transfected cells is predominately mediated via activation of cdk6 (Godden Kent *et al*., 1997). Thus, the effects of dn-cdk6 may be explained by its ability to interfere with kinase activation by the KSHV cyclin itself. In accord with this, we find that the KSHV-cyc-associated kinase activity is significantly lowered in lysate from the dn-cdk6-transfected cells (Figure 6F). In contrast, the KSHV-cyc-associated activity was only marginally affected in cells expressing dn-cdk2 or dn-cdk4. Thus, the effects of dn-cdk2 may not rely on directly inhibiting complexes involving the KSHV cyclin, but more likely is due to the inhibition of cellular cyclin–cdk complexes that use cdk2 as their catalytic partner.

Together, our experiments thus support a concept whereby KSHV-cyc–cdk6 per se may not be sufficient to achieve full cell cycle progression in p27^{Kip}-expressing cells. Its ability to do so would seem to rely on phosphorylation and destabilization of p27^{Kip}, an event which may result in activation of one or several p27^{Kip}-sensitive kinases that are affected by roscovitine and/or dn-cdk2.

p27^{Kip}-phosphorylating cdk6 in tumours associated with KSHV infection

Our experiments demonstrate that KSHV-cyc when expressed in tissue culture cells causes p27^{Kip} destabilization and through this can trigger cell cycle transit in p27^{Kip}-expressing cells. It is, however, unclear whether a similar activity exists in the tumours arising in KSHV-infected patients. To address this, we examined the activity of cdk6 in lysates from such tumours.

In transfected tissue culture cells, KSHV-cyc forms complexes with cdk6 and the majority, if not all of the detectable kinase activity is associated with such KSHV-cyc–cdk6 complexes (Godden Kent *et al*., 1997). We therefore precipitated cdk6 from lysates of two cell lines, BCP-1 and HBL-6, derived from KSHV positive primary effusion lymphomas (Gaidano *et al*., 1996; Boshoff *et al*., 1998) and a primary Kaposi's sarcoma biopsy. We find that cdk6 precipitates from these lysates contain an extremely high level of pRB phosphorylating activity when compared with precipitates from U2OS cells or various KSHV-negative lymphoblastoid cell lines. Importantly, this cdk6 activity is capable not only of phosphorylating pRB but also p27^{Kip}, and does so with a relative efficiency similar to cdk6 from KSHV-cyc-transfected U2OS cells. Conversely, p27^{Kip} phosphorylation was not elicited by cdk6 from any of the KSHV-negative lymphoma lines or non-transfected U2OS cells. While these results do not prove the involvement of the KSHV cyclin in this activity, they indicate that a cdk6 complex with features resembling those of recombinant KSHV-cyc–cdk6 complex exists in KSHV-associated tumours. Furthermore, the unusual ability of this cdk6 complex to phosphorylate p27^{Kip} suggests that p27^{Kip} destabilization may occur in these tumours.

Discussion

We provide evidence here that expression of the KSHV-encoded D-type cyclin triggers degradation of p27^{Kip} in

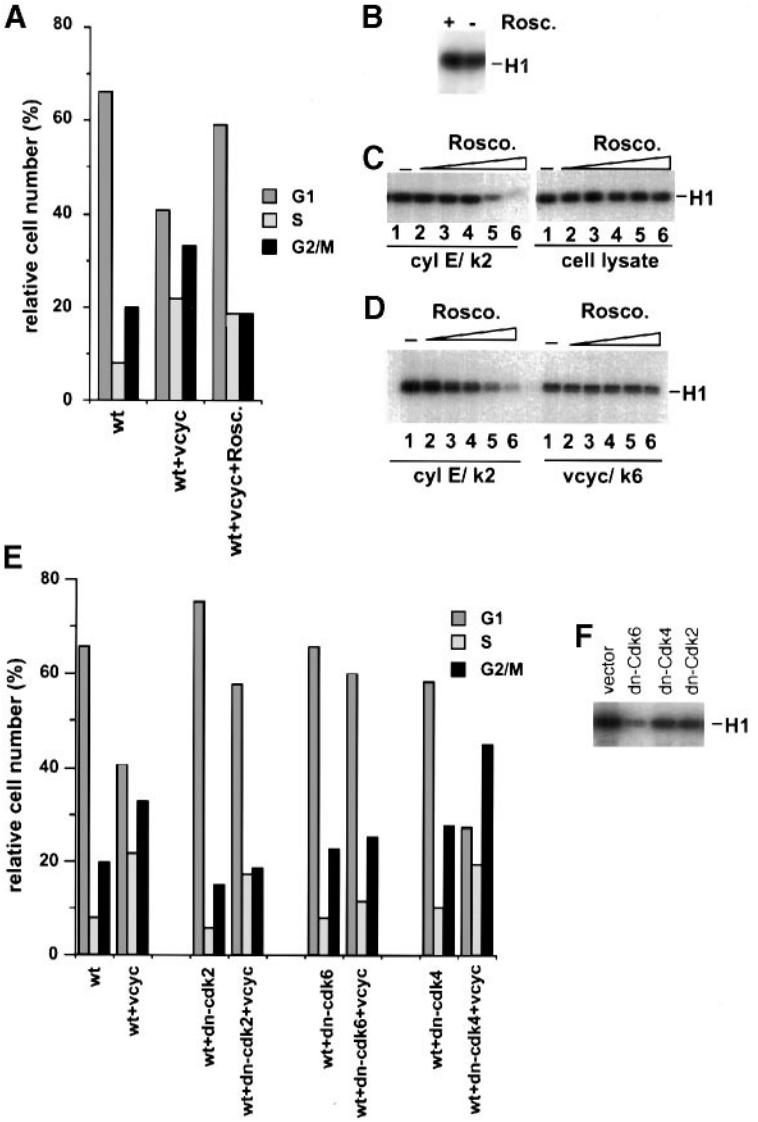

Fig. 6. Impairment of the KSHV-cyc-mediated cell cycle rescue by roscovitine and dn-cdks. (**A**) Effect of roscovitine treatment on KSHV-cyc mediated cell cycle progression. U2OS cells were transfected with plasmids encoding wt $p27^{Kip}$ and KSHV-cyc as indicated. Twenty hours post-transfection, cultures were treated with 25 μM roscovitine where indicated (Rosc.). Cells were harvested following Nocodazole treatment, stained for CD20 expression and analysed by FACScan as in Figure 5. Data are representative of three independent experiments. (**B**) KSHV-cyc associated kinase activity in roscovitine treated cells. Cells were transfected with plasmid encoding KSHV-cyc. Twenty-four hours post-transfection they were split into two and treated with 25 μM roscovitine (+) or left untreated (–). Cell lysate was prepared 30 h later and the amount of kinase activity associated with KSHV-cyc was determined by 9E10 immunoprecipitation/kinase assays. (**C**) Roscovitine resistance of vcyc-associated kinase from transfected cells. Lysates from U2OS cells transfected with KSHV-cyc-encoding plasmid were subjected to immunoprecipitation using 9E10 antibody. The precipitate was split into six equal aliquots and kinase reactions performed in the presence of increasing concentrations of roscovitine. Roscovitine concentrations used were 0.26 μM (lanes 2), 1 μM (lanes 3), 4 μM (lanes 4), 14 μM (lanes 5), 56 μM (lanes 6), and 0 μM (lanes 1). Insect cell lysate programmed with cyclin E and cdk2 was used as a control for roscovitine function. Histone H1 (H1) was used as a kinase substrate. (**D**) Roscovitine resistance of recombinant vcyc–cdk6. Kinase assays were performed in the presence of increasing concentrations of roscovitine as described for (C) but using insect cell lysate programmed with KSHV-cyc and cdk6 or cyclin E and cdk2. (**E**) Impairment of cell cycle progression by dominant-negative cdk proteins. U2-OS cells were transfected with combinations of $p27^{Kip}$ and KSHV-cyc and dominant-negative versions of either cdk2 (dn-cdk2), cdk4 (dn-cdk4) or cdk6 (dn-cdk6). Cell cycle analysis was conducted as described for Figure 5. The data are representative of two independent experiments. (**F**) Effect of dn-cdk expression on KSHV-cyc-associated kinase activity. Cells lysates was prepared from cells transfected with KSHV-cyc, $p27^{Kip}$ and the dn-cdk constructs and subjected to immunoprecipitation via 9E10 antibody followed by kinase assays. Histone H1 was used as a substrate.

cells and, more importantly, that rescue of cell cycle transit in $p27^{Kip}$-expressing cells depends critically on the degradation of this inhibitor. These findings provide novel insight into both the functional principles by which this cyclin undermines cell cycle control and how it might contribute to cell transformation.

$p27^{Kip}$ phosphorylation is critical for KSHV-cyc to mediate cell cycle progression

We show that *in vitro* KSHV-cyc–cdk6 phosphorylates $p27^{Kip}$ on a C-terminal threonine, implicating this cyclin–cdk complex in promoting proteolysis of the cdk inhibitor. Notably, KSHV-cyc cannot promote cell cycle progression

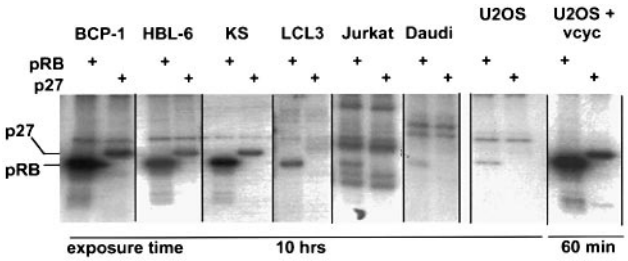

Fig. 7. p27^{Kip} phosphorylating activity in tumours with KSHV involvement. Anti-cdk6 immunoprecipitates were prepared from lysate of cell lines as indicated. The cell lines used were as follows: BCP-1, body cavity-based lymphoma; HBL-6, body cavity-based lymphoma; LCL-3, EBV-immortalized B cell line; Jurkat, T cell lymphoma; and Daudi, Burkitt lymphoma. U2OS osteosarcoma cells, either mock-transfected or transfected with expression plasmid encoding KSHV-cyc, were taken along as controls. KS denotes the use of lysate from a freshly frozen Kaposi's sarcoma biopsy. Immunoprecipitates were assayed for kinase activity using either GST–pRB or GST–p27^{Kip} as a substrate. Note that the autoradiographic exposure was 60 min for KSHV-cyc-transfected U2OS cells but 10 h for all other cell lines. The equivalent of 2×10^6 cells was used per assay and an amount matched for protein content was used of the KS lysate.

in cells expressing p27^{Kip}(T187A), a mutant in which this threonine is exchanged for the non-phosphorylatable alanine. These observations have various implications: first, that p27^{Kip} phosphorylation and its subsequent elimination via proteolysis are critical for the viral cyclin to overcome growth inhibition by this inhibitor and secondly, that the inherent activity of the KSHV-cyc kinase complex is not sufficient for completion of the cell cycle.

To complete a full division cycle, cells require several CDKs that promote cell cycle progression at different points of the division cycle, supposedly by phosphorylating different substrates. *In vitro*, the viral cyclin–cdk6 complex can phosphorylate a far greater range of substrates than can cdk6 in association with cellular D-type cyclins, including Histone H1, E2F1 and myb proteins (Godden Kent *et al*., 1997; Li *et al*., 1997; S.Mittnacht, unpublished; R.Watson, personal communication). The evidence presented above, however, would seem to indicate that certain substrates that require phosphorylation for S-phase completion may not be phosphorylated by KSHV-cyc-activated kinases. Our results are consistent with the notion that full cell cycle progression requires the activity of several different cyclin-dependent kinases and indicate that this is also true for KSHV-cyc-expressing tumour cells. Therefore, growth of such tumours should be amenable to newly emerging anti-cancer approaches that target activation of cellular cyclin–cdks (reviewed in Meijer and Kim, 1997).

KSHV-cyc expression represents a novel means for driving p27 degradation

Several lines of evidence suggest that p27^{Kip} inactivation is an important step in cancer progression. p27^{Kip} accumulates in an active form in growth factor-deprived cells and is induced by growth-restricting morphogens such as tumour growth factor β and through cell contact (Polyak *et al*., 1994; Reynisdottir *et al*., 1995). Yet classically, proliferation of tumour cells is seen under such conditions. Destabilization of p27^{Kip} has been described for various tumour types, and appears to correlate with poor prognosis and tumour progression. How p27^{Kip} destabilization is achieved in these tumours has not been studied in detail. It is conceivable that this destabilization is a consequence of hyper-activation of signal transduction pathways that normally trigger p27^{Kip} inactivation. Expression of KSHV-cyc in cells would appear to be an alternative means to achieve this end. This in turn suggests that KSHV-cyc expression could act in place of defects normally resulting in p27^{Kip} elimination, for example, activating Ras mutations and deregulated c-myc expression resulting in activation of cyclin E–cdk2. In support of this idea, although deregulated c-myc expression is frequently seen in human lymphomas, this is never observed in KSHV-associated primary effusion lymphoma (Nador *et al*., 1996).

p27^{Kip} inactivation is a common feature of oncogenic viruses

Inactivation of p27^{Kip} function is not unique to KSHV and possibly other cyclin-expressing herpesviruses. Oncoproteins encoded by classical DNA tumour viruses such as the adenoviral E1A and the papillomavirus E7 protein also impair the molecular functioning of p27^{Kip} (Mal *et al*., 1996; Zerfass *et al*., 1996). Apart from their ability to inactivate the tumour suppressor pRB, KSHV-cyc and these other viral oncoproteins appear to share another feature with potential to cause oncogenesis in infected hosts, a remarkable degree of convergence considering that these viruses are otherwise unrelated. The induction of tumours by these viruses undoubtedly must be considered a biological accident, which raises the question of why the different viruses would have developed and retained these functions. The need to restore proliferation competence in infected host cells in order to aid viral genome replication would be one explanation.

However, the expression pattern of KSHV-cyc strongly argues for its requirement in KSHV latency. Transcripts of this viral gene are commonly found in latently infected lymphoma cells and their abundance is not significantly increased upon induction of lytic replication in these cells (Zhong *et al*., 1996; Sarid *et al*., 1998). Various lines of evidence suggest that pRB and the cdk inhibitors are involved in establishing replication incompetence preceding cell differentiation and cell senescence (reviewed in Chellappan *et al*., 1998). The expression of KSHV-cyc during latency could be a means to prevent replication incompetence, an event that would make re-activation of viral genome replication and progeny production impossible.

It should be noted at the end of this discussion that currently no direct evidence exists for the involvement of the cyclin in the tumourigenic activity ascribed to HHV8. While the biochemical features of this cyclin suggest that it could trigger events known to be associated with tumourigenesis, it is clear that HHV8 encodes other gene products with documented oncogenic features (reviewed in Moore and Chang, 1998) that could either contribute or be solely responsible for the oncogenic activities of this virus.

Materials and methods

Plasmids and recombinant baculoviruses

Recombinant baculoviruses expressing KSHV-cyc, the human cellular cyclins D1, D2 and E, and the cyclin-dependent kinase subunits cdk4, cdk6 and cdk2 were constructed as described (Godden Kent *et al*., 1997; Zarkowska and Mittnacht, 1997). All cyclins contained a 9E10 (c-myc)

epitope tag fused to their C-terminal end. Vectors for expression of KSHV-cyc and $p27^{Kip}$ in tissue culture cells were constructed by cloning PCR-derived fragments covering the coding sequence for these proteins into the plasmid pCDNA3(9E10). To construct pCDNA3(9E10), a 60 bp *Hin*dIII–*Nco*I fragment comprising the β-globin 5′ untranslated region followed by a 9E10 (c-myc) epitope tag was inserted into the polylinker of pCDNA3 (Invitrogen). To express $p27^{Kip}$ in *Escherichia coli*, the $p27^{Kip}$ coding sequence was isolated from pCDNA3(9E10) and inserted into pGEX-KG (Pharmacia). Deletion mutants of $p27^{Kip}$ were derived by PCR using full-length pCDNA3(9E10)$p27^{Kip}$ as a template and likewise cloned into pGEX-KG. The $p27^{Kip}$ phosphorylation site mutants were generated by PCR-based mutagenesis using pCDNA(9E10)$p27^{Kip}$ as a template. Mutagenesis was performed using the quickchange™ mutagenesis Kit (Stratagene) and primers 5′-GTGGAGCAGGCGCCCAAGAAGC-3′ and 5′-GCTTCTTGGGCGCCTGCTCCAC-3′ for mutation of Thr187 or 5′-GTCTAACGGGGCCCCTAGCCT-3′ and 5′-CCAGGCTAGGGGCCCC-CGTTAG-3′ for mutation of Ser10 (mutated codons underlined). The mutated reading frame was excised from pCDNA3(9E10) and inserted into pGEX-KG. All PCR-generated constructs were fully sequenced. Construct expressing human CD20 surface marker and 9E10-tagged E2Fpm132 have been described previously (Hsieh *et al*., 1997). Plasmids encoding dominant-negative versions of cdk2, cdk4 and cdk6 were a gift from E.Harlow and are described in van den Heuvel and Harlow (1993).

Antibodies and immune procedures

The tag-specific 9E10 antibody was purified from tissue culture supernatant of 9E10 hybridoma cells (a gift of G.Evan, ICRF) using DEAE chromatography. The rabbit anti-cdk6 antibody (for immunoprecipitation) and the rabbit anti-$p27^{Kip}$ antibody (for immunostaining) were purchased from Santa Cruz. Fluorescein isothiocyanate (FITC)-coupled anti-human CD20 antibody was purchased from Becton Dickinson. Detection of proteins by Western blot followed standard procedure (Harlow and Lane, 1988). Blots were reacted with Horseradish peroxidase-coupled secondary antibodies (Amersham) and developed using enhanced chemiluminescence (Amersham). Immunofluorescence procedures were essentially as described previously (Godden Kent *et al*., 1997). Cell monolayers were fixed in 4% w/v paraformaldehyde followed by permeabilization with 0.5% Triton. Specimens were reacted with 9E10 mouse monoclonal antibody followed by FITC-coupled donkey anti-mouse immunoglobulin G (IgG) secondary antibody to detect KSHV-cyc expressing cells and counter-stained for $p27^{Kip}$ using rabbit anti-$p27^{Kip}$ antiserum followed by Rhodamine-coupled donkey anti-rabbit IgG antiserum. Specimens were examined by fluorescence microscopy. Immunoprecipitation was performed essentially as described (Godden Kent *et al*., 1997).

In vitro kinase assays

Production of insect cell lysate containing recombinant cyclin–cdk complex was as described (Godden Kent *et al*., 1997). To measure kinase activity, 2 μl of crude lysate was mixed with 20 μl substrate mix containing 10 μM ATP, 0.1 μCi [γ-^{32}P]ATP and 0.5 μg of substrate in reaction buffer [25 mM HEPES–KOH pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 0.1 μM protein kinase A inhibitor peptide (Sigma), 10 mM β-glycerophosphate, 2.5 μg/ ml Leupetin (Sigma), 1 mM phenylmethylsulfonide (PMSF) and 1% Aprotenin (Sigma)]. Samples were incubated at 27°C for 15 min. To assess inhibition by $p27^{Kip}$, lysates was pre-incubated with recombinant GST–$p27^{Kip}$ in the absence of ATP. Typically, 2 μl of insect cell lysate was mixed with 10 μl recombinant GST–$p27^{Kip}$ (diluted in reaction buffer) followed by incubation at 27°C for 15 min. Subsequently, 10 μl of double-concentrated substrate mix was added and incubation was continued for a further 15 min at 27°C. Reactions were stopped by adding sodium dodecyl sulfate (SDS)-containing sample buffer followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were fixed and stained with Coomassie Brilliant Blue to visualize the substrates, then dried and exposed to X-ray film to assess the degree of substrate phosphorylation. Recombinant GST–pRB substrate and GST–$p27^{Kip}$ were produced in *E.coli* and purified by affinity chromatography using glutathione–Sepharose as described (Zarkowska and Mittnacht, 1997). For immunoprecipitation kinase assays, lysates were prepared in HEPES-buffer (HB) [25 mM HEPES–KOH pH 7.4, 20 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, containing protease inhibitors Leupetin (2.5 μg/ml), Aprotenin (1%) and PMSF (1 mM), and phosphatase inhibitors β-glycerophosphate (10 mM), NaF (10 mM) and sodium vanadate (1 mM)]. Lysates were spun at 10 000 *g* to remove insolubles and incubated for 60 min with 5 μg 9E10 monoclonal antibody bound to protein G–agarose (Sigma) or 5 μg anti-cdk6 rabbit serum (Santa Cruz) bound to protein A–Sepharose (Bio-Rad). Immunoprecipitates were washed twice in HB and once in kinase buffer. Substrate mix was added to the washed beads and kinase activity was determined as described above. Phosphoamino acid maps were performed essentially as described previously (Zarkowska and Mittnacht, 1997), except that separation in two dimensions was performed as described in Boyle *et al*. (1991).

$p27^{Kip}$ binding assay

Insect cell lysate programmed with cyclin–cdks was mixed with GST–$p27^{Kip}$ inhibitor and incubated at 27°C as described for $p27^{Kip}$ inhibition of kinase activity. Subsequently, samples were diluted with 10 vol. HB and incubated with 20 μl of glutathione–Sepharose (Pharmacia) for 30 min at 4°C. Beads were collected in mini-columns attached to a vacuum manifold (Promega) washed two times with 1 ml HB. The proteins retained were eluted with SDS-containing loading buffer and analysed by Western blot.

Cell culture, transfection and cell cycle analysis

U2OS cells were cultured in Dulbecco's modified essential medium (DMEM), 10% fetal calf serum (FCS). The transfection procedure via $CaCl_2$ and the analysis of the cell cycle distribution of transfected cells followed published procedures (Hsieh *et al*., 1997). Typically cells were transfected with a total of 15 μg DNA containing 0.2 μg pCDNA3(9E10) $p27^{Kip}$, 4 μg pCDNA3(9E10)vcyc and 2.5 μg CD20 expression plasmid. Where indicated, 5 μg of plasmids encoding dn-cdks were included into the transfection mixture. Cells were harvested and analysed 48 h post transfection. Prior to this, transfected cell cultures were treated with 400 ng/ml microtubule inhibitor Nocodazole for 8–10 h. Where indicated, the cdk2 inhibitor roscovitine (a gift of L.Meijer, CNRS Roscoff) was added at a concentration of 25 μM. H/p27 are a derivative of H1299 mammary carcinoma cells and engineered to express human $p27^{Kip}$ under the control of an Ecdysone-responsive promoter (Invitrogen). Construction and maintenance of the cell line followed instructions by Invitrogen. Expression of $p27^{Kip}$ was induced following transfection of the cells by addition of Muristerone A at 1 μM to the culture medium. Cal51 mammary carcinoma cells were cultured in DMEM and 10% heat-inactivated FCS. Transfection of both these cell lines by $CaCl_2$ followed published procedures (Hsieh *et al*., 1997). Primary effusion lymphoma-derived cell lines BCP-1 and HBL-6 were cultured in Rouswell Park Memorial Institute (RPMI) medium 20% inactivated FCS, and Daudi Burkitt's lymphoma cells, the T-cell lymphoma-derived Jurkat cells and LCL-3, an EBV-immortalized human B-cell line (a gift of P.Farrell, Ludwig Institute, UK), were grown in RPMI medium containing 10% inactivated FCS.

Pulse–chase analysis

U2OS were transfected with plasmid DNA in 10 cm dishes. Cells were trypsinized 24 h post-transfection, re-seeded into six individual 5 cm dishes and cultured for an additional 12 h. Subsequently, cell monolayers were exposed for 1 h to [^{35}S]methionine (200 μCi/ml) in methionine-free labelling medium followed by a variable incubation period in methionine-rich growth medium. Cell lysates were prepared in HB and $p27^{Kip}$ from the transfected cells were isolated by immunoprecipitation using the monoclonal antibody 9E10. The amount of ^{35}S-labelled $p27^{Kip}$ in the precipitates was quantified by PhosphorImager analysis (Molecular Dynamics) following SDS–PAGE.

$p27^{Kip}$ stability assay

U2OS cells were transfected as for cell cycle analysis except that pCDNA3-gal, a plasmid in its backbone identical to those used for expression of $p27^{Kip}$ and KSHV-cyc but encoding β-galactosidase, was included instead of CD20-encoding plasmid. β-galactosidase activity was determined following cell lysis using a kit by Promega according to the manufacturer's instructions. Amounts of cell lysate equalling identical β-galactosidase activities were subjected to Western blot analysis using 9E10 antibody to detect $p27^{Kip}$ and KSHV-cyc.

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