Characterization of murine gammaherpesvirus 68 v-cyclin interactions with cellular cdks

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Abstract

All known γ2-herpesviruses encode a cyclin homolog with significant homology to mammalian D-type cyclins. The murine gammaherpesvirus 68 (γHV68) viral cyclin (v-cyclin) has been shown to be oncogenic when expression is targeted to thymocytes in transgenic mice and to be critical for virus reactivation from latency. Here, we investigate the interaction of the γHV68 v-cyclin with cellular cyclin-dependent kinases (cdks). We show that, in contrast to the Kaposi's sarcoma-associated herpesvirus (KSHV) v-cyclin, the γHV68 v-cyclin preferentially interacts with cdk2 and cdc2 but does not interact with either cdk4 or cdk6. Mutation of conserved residues, predicted to be involved in cdk binding based on the γHV68 v-cyclin:cdk2 crystal structure, resulted in the loss of both cdk binding and the ability to mediate phosphorylation of substrates. Like the KSHV v-cyclin, the γHV68 v-cyclin appears to confer expanded substrate specificity to the cellular cdk binding partners. As expected, the γHV68 v-cyclin:cdk complexes are able to target phosphorylation of histone H1, the retinoblastoma protein (pRb), and p27^{Kip1} as assessed using in vitro kinase assays. Notably, hyperphosphorylation of pRb was observed during wt γHV68 replication in serum-starved murine fibroblasts, but not in cells that were either mock-infected or infected with a v-cyclin null γHV68. In addition, infection of serum-starved murine fibroblasts also results in a v-cyclin-dependent increase in cdk2-associated kinase activity and a concomitant decrease in the levels of p27^{Kip1}. Taken together, the latter studies served to validate the results of the in vitro kinase assays. Finally, in vitro kinase assays revealed that the γHV68 v-cyclin:cdk complexes can also phosphorylate p21^{Cip1}, Bcl-2, and p53. The latter suggests that, at least in vitro, the γHV68 v-cyclin exhibits functional characteristics of both cyclin E and cyclin A.
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Keywords: Gammaherpesvirus; v-cyclin; cdk

Introduction

The mammalian cell cycle is a series of tightly controlled and elegantly coordinated events leading to, and including, the replication of cellular DNA and the segregation of this material to daughter cells. Sequential activation of cyclin-dependent kinases (cdks) by their cognate cyclin, during each phase of the cell cycle, is critical to maintaining an ordered and controlled cycle (Arellano and Moreno, 1997; Lees, 1995). D-type cyclins are upregulated early in G1 in response to mitogens, bind cdk4 and/or cdk6, and this complex phosphorylates the retinoblastoma protein (pRb), thereby mediating G1 progression (Harbour et al., 1999; Sherr, 1993). E-type cyclins, expressed later in G1, bind cdk2 and mediate transition through the restriction point (the point at which mitogenic signals are no longer required for continued cell cycle progression) and the G1/S transition. Cyclin E:cdk2 hyperphosphorylates pRb to allow E2F-driven transcription, which in turn upregulates a number of cell cycle genes (Harbour and Dean, 2000; Zhang et al., 2000). Cyclin A:cdk2 complexes form during S-phase and drive S-phase to G2/M, where cyclinB:cdc2 (cdk1) controls the transition into mitosis. In addition to

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cyclin binding, cdk activity is regulated by post-translational modifications and through direct protein–protein interactions with members of the INK4 and Cip1/Kip1 families of cdk inhibitors (CKIs) (Sherr and Roberts, 1999). These basic components of the cell cycle machinery interact as part of a vast signaling pathway to carefully control the rate, timing, and execution of each replicative cycle, and failure to do so can result in the unrestrained cell division seen as a hallmark of cancers and other proliferative diseases.

Perturbation of host cell cycle is a common strategy employed by DNA viruses to achieve a cellular environment conducive to viral growth. Adenovirus, polyoma virus, papilloma virus, and many herpesviruses encode genes that directly alter the host cell cycle or interact with host gene products to the same end (Swanton and Jones, 2001). γ2-herpesviruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV), herpesvirus saimiri (HVS), and murine gammaherpesvirus 68 (γHV68), each encode a homologue of mammalian D-type cyclins (Chang et al., 1996; Jung et al., 1994; Li et al., 1997; Nicholas et al., 1992; Virgin et al., 1997). These viral cyclins (v-cyclins) likely represent a direct mechanism by which these viruses interrupt the normal host cell cycle in favor of the viral life cycle.

The biochemical properties of the KSHV and HVS v-cyclins have been extensively characterized (Laman et al., 2000; Mittnacht and Boshoff, 2000; Verschuren et al., 2004a, 2004b). The KSHV v-cyclin preferentially binds to and activates cdk6, although it also binds cdk2 and cdk4 with lower affinity (Godden-Kent et al., 1997; Li et al., 1997; Mann et al., 1999). In contrast, the HVS v-cyclin only binds cdk6 (Jung et al., 1994). In addition to the traditional cdk6 substrate pRb, KSHV v-cyclin confers on cdk6 a wider substrate repertoire, mediating the phosphorylation of a variety of substrates involved in cell cycle regulation and survival, including histones, p27^{Kip1}, cdc25A, cdc6, Orc1, and Bcl2 (Ellis et al., 1999; Godden-Kent et al., 1997; Laman et al., 2001; Li et al., 1997; Mann et al., 1999; Ojala et al., 2000; Swanton et al., 1997). Similarly, the HVS v-cyclin:cdk6 complex has been shown to also phosphorylate histone H1 and pRb (Jung et al., 1994). However, unlike cellular D-type cyclins, the KSHV and HVS v-cyclin:cdk complexes are resistant to inhibition by cellular cdk inhibitors (CKIs) of the Cip1/Kip1 and INK4 families (Swanton et al., 1997), and the KSHV v-cyclin can form active complexes without activating phosphorylation of the cdk by CAK (Child and Mann, 2001; Kaldis et al., 2001). Potential explanations for many of these unique features were revealed by the analysis of the crystal structures of the HVS v-cyclin in complex with cdk6 (Schulze-Gahmen et al., 1999) and the KSHV v-cyclin in complex with cdk6 and the CKI p18^{INK4c} (Jeffrey et al., 2000). Recent studies have demonstrated the oncogenic potential of transgenic KSHV v-cyclin expression in conjunction with inactivation of the p53 tumor suppressor (Verschuren et al., 2002, 2004a, 2004b). The individual effects of these biochemical properties are likely to act in a synergistic manner to force cell cycle progression to a point conducive to viral replication. However, the role of the KSHV v-cyclin in the context of viral infection remains unclear due to the narrow host range of KSHV.

In comparison to the KSHV v-cyclin and HVS v-cyclin, much less is known about the γHV68 viral cyclin. When expressed as a transgene in mice, it promotes cell cycle progression of primary lymphocytes and functions as an oncogene, causing tumors in more than 60% of transgenic mice before 1 year of age (van Dyk et al., 1999). In the context of the viral life cycle, the γHV68 v-cyclin is a critical regulator of reactivation from latency (Hoge et al., 2000; van Dyk et al., 2000). The crystal structure of the γHV68 v-cyclin has been determined in complex with cdk2, showing that, although it makes several novel interactions with cdk2 when compared to cyclin A, its mechanism for activation of cdk2 appears similar (Card et al., 2000). The structural model also offers insights into sequence and conformational features of the γHV68 v-cyclin that allow it to evade inhibition by cellular CKIs. However, the biochemical properties of the γHV68 v-cyclin remain poorly understood. While it is known that the γHV68 v-cyclin binds cdk2, phosphorylates pRb, and evades inhibition by p27^{Kip1} in vitro, the γHV68 v-cyclin has not been afforded the attention of the KSHV v-cyclin, despite the advantage of a tractable animal model in which to extend in vitro structure/function studies.

As discussed above, we have previously shown that the γHV68 v-cyclin is both an oncogene and a critical regulator of γHV68 reactivation (van Dyk et al., 1999, 2000). Here, we present an initial characterization of γHV68 v-cyclin interaction with cellular cdks and identify potential target substrates. These studies provide potential mechanisms by which the γHV68 v-cyclin mediates both cell cycle progression and requirements for virus reactivation.

Results

γHV68 v-cyclin interacts with cdk2 and cdc2, but not cdk4 or cdk6

To begin the characterization of the γHV68 v-cyclin functional properties, we focused on interactions of the v-cyclin with cellular cdks. Following transient transfection of Cos-1 cells with FLAG-v-cyclin and cdk expression vectors, cells were harvested, immunoprecipitated, and immunoblotted to determine which cdks were found in the FLAG-v-cyclin immunocomplex. In agreement with previous studies (Card et al., 2000), cdk2 was shown to co-immunoprecipitate with γHV68 v-cyclin (Fig. 1A). However, neither cdk4 nor cdk6 efficiently co-immunoprecipitated with the v-cyclin, despite overexpression of each cdk and comparable immunoprecipitation of FLAG-v-cyclin (Fig. 1B).

Based on inhibition studies showing that the cdc2/cdk2 inhibitor roscovitine can inhibit v-cyclin function (JU and
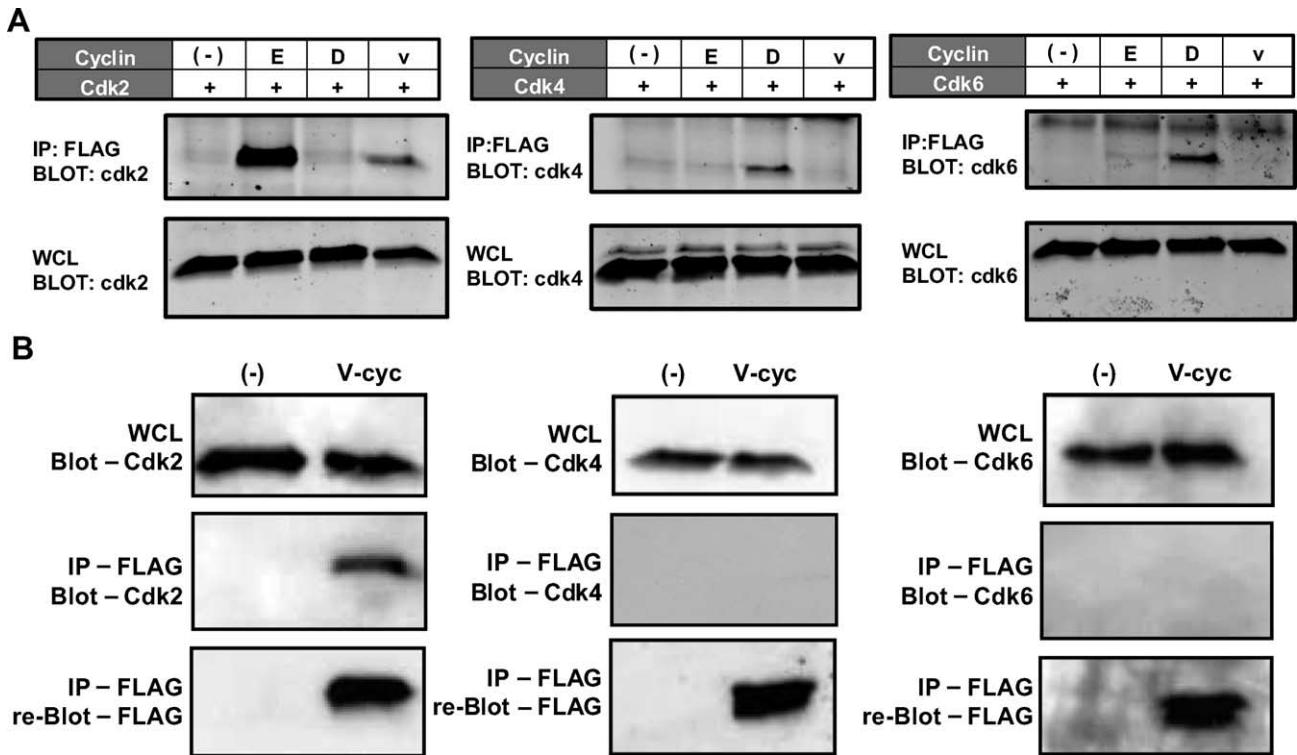
Fig. 1. γHV68 v-cyclin binds to cdk2, but not cdk4 or cdk6. (A) Cos-1 cells were transfected with pCMV-TAG2B vector control or FLAG epitope-tagged cyclins E, D or v-cyclin and pCMV-cdk2 (left panel), pCMV-cdk6 (middle panel), or pCMV-cdk4 (right panel). In the analyses of cyclin association with cdk2 and cdk4, a cyclin D1 expression vector was used, while for the analysis of association with cdk6, a cyclin D3 expression vector was used (see Materials and methods). (B) Cos-1 cells were transfected with pCMV-TAG2B vector control or FLAG epitope-tagged v-cyclin and pCMV-cdk2 (left panel), pCMV-cdk6 (middle panel), or pCMV-cdk4 (right panel). Cells were harvested 48 h post-transfection, lysates prepared, and immunoprecipitations carried out with agarose-conjugated anti-FLAG antibodies (Sigma). Immunoprecipitates were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the indicated anti-cdk antibody. Membranes were stripped and reprobed with anti-FLAG antibody to verify FLAG-v-cyclin immunoprecipitation (panel B only). WCL, whole cell lysates (5% of that used for immunoprecipitation).
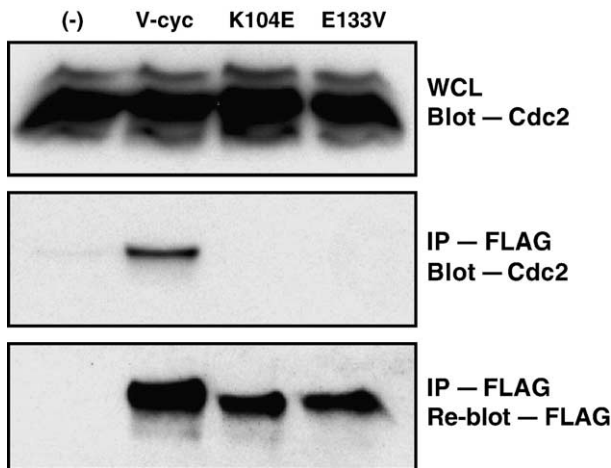

Fig. 2. γHV68 v-cyclin binds cdc2 in vitro. Cos-1 cells were transfected with pCMV-TAG2B vector control (lane 1), FLAG-v-cyclin (lane 2), FLAG-v-cyclin.K104E (lane 3), or FLAG-v-cyclin.E133V (lane 4) and pCMV-cdc2. 48 h post-transfection, cells were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibody (Sigma). Immunoprecipitated complexes were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibody against cdc2. Membranes were stripped and reprobed with anti-FLAG antibody. WCL, whole cell lysates (5% of that used for immunoprecipitation).

SHS, unpublished data; see also Fig. 7), we also assessed binding of the γHV68 v-cyclin to cdc2. As shown in Fig. 2, co-immunoprecipitations from transfected Cos-1 cells demonstrated that the γHV68 v-cyclin can indeed interact with cdc2, although less efficiently than with cdk2 (Fig. 2), and that mutations introduced into the v-cyclin that eliminate binding to cdk2 (see Fig. 3 and discussion below) also disrupt interaction with cdc2. Thus, the γHV68 v-cyclin is distinct from the KSHV v-cyclin in terms of which cellular cdks it interacts with.

Mutation of conserved residues within the cyclin box of γHV68 v-cyclin eliminates binding to cellular cdks

Sequence and structural analysis indicated that γHV68 v-cyclin possessed a number of features conserved among all cyclins (Card et al., 2000), suggesting that these common sequences and structures are important in the engagement and activation of cdks (Lees and Harlow, 1993). We generated two independent amino acid substitution mutations at conserved sites within the cyclin box, one which mutated the lysine at residue 104 to a glutamic acid (K104E) and the other which mutated a glutamic acid at position 133
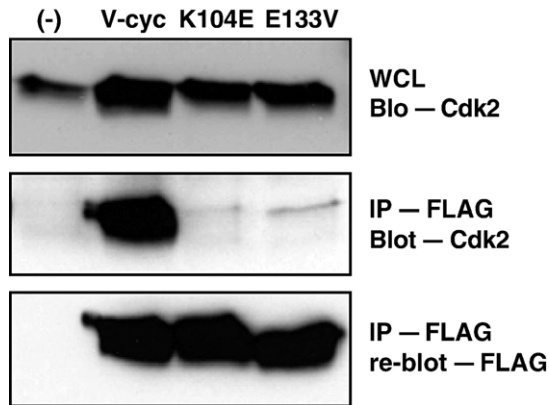
Fig. 3. Mutations of conserved residues within the cyclin box inhibit binding of cdk2 to the γHV68 v-cyclin. Cos-1 cells were transfected with pCMV-TAG2B vector control (lane 1), FLAG-v-cyclin (lane 2), FLAG-v-cyclin.K104E (lane 3), or FLAG-v-cyclin.E133V (lane 4) and pCMV-cdk2. 48 h post-transfection, cells were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibodies (Sigma). Immunoprecipitates were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibody against cdk2. Membranes were stripped and reprobed with anti-FLAG antibody. WCL, whole cell lysates (5% of that used for immunoprecipitation).

to a valine (E133V). From the crystal structure, both of these residues were shown to form critical interactions with cdk2 (Card et al., 2000). In Cos-1 cells, these mutations nearly or completely eliminated binding of cdk2 and cdc2 to the γHV68 v-cyclin (Figs. 2, 3), indicating that the γHV68 v-cyclin binds and activates cdks, in a manner similar to cellular cyclins. Importantly, neither mutation appeared to affect the stability of the v-cyclin since both mutants were expressed at equivalent levels to the wild type v-cyclin.

γHV68 v-cyclin:cdk2 complexes phosphorylate cyclin E:cdk2 substrates

Since the γHV68 v-cyclin binds and presumably activates cdks, we first tested the ability of v-cyclin complexes to phosphorylate histone H1. When overexpressed in Cos-1 cells, in conjunction with cdk2, the v-cyclin-containing immune complexes strongly phosphorylate histone H1 (Fig. 4A). This activity was not simply due to autophosphorylation of part of the v-cyclin complex since no signal was detected without the addition of histone H1 substrate or in the absence of v-cyclin (Fig. 4A). Additionally, the cdk-binding mutant E133V was unable to phosphorylate histone H1, presumably due to the lack of cdk binding. Notably, the γHV68 v-cyclin immunoprecipitated from Cos-1 cells is capable of phosphorylating H1 without overexpression of cdk2, although H1 phosphorylation was enhanced upon overexpression of cdk2 (Fig. 4A).

It has been previously shown that γHV68 viral cyclin:cdk2 complex can phosphorylate pRb (Card et al., 2000). To confirm this, we assessed pRb phosphorylation (Fig. 4B). Immunoprecipitates from Cos-1 cells containing FLAG-v-cyclin strongly phosphorylate a pRb-GST fusion

protein, and these complexes do not phosphorylate GST alone (Fig. 4B). As above, the v-cyclin E133V mutant was unable to phosphorylate pRb above background levels (Fig. 4B). To further assess which endogenous cdks play a role in v-cyclin phosphorylation of histone H1 and pRb in the in
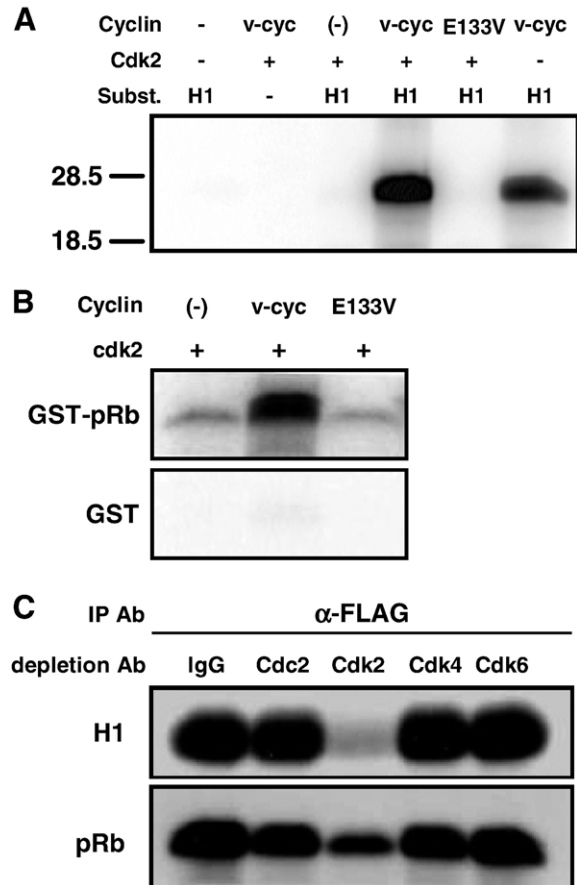

Fig. 4. γHV68 v-cyclin complexes phosphorylate histone H1 and pRb in vitro. (A) Cos-1 cells were transfected with pCMV-TAG2B vector control (lanes 1 and 3), FLAG-v-cyclin (lanes 2, 4, and 6), FLAG-v-cyclin.E133V (lane 5), and pCMV-cdk2 (lanes 2–6). 48 h post-transfection, cells were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibodies (Sigma). Immunoprecipitated complexes were resuspended in kinase reaction buffer with histone H1 (2 μg) and 10 μCi γATP, and the reaction allowed to proceed for 30 min at 37 °C. Reactions were subjected to 15% SDS-PAGE followed by autoradiography. Gels were dried and exposed to phosphor-screen for 10 min. (B) Cos-1 cells were transfected with pCMV-TAG2B vector control (lane 1), FLAG-v-cyclin (lane 2), or FLAG-v-cyclin.E133V (lane 3) and pCMV-cdk2 (lanes 1–3). 48 h post-transfection, all samples were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibodies (Sigma). Immunoprecipitated complexes were resuspended in kinase reaction buffer with 2 μg GST-Cterm-pRb (top panel) or GST (bottom panel) and 10 μCi γATP. The reaction was allowed to proceed for 30 min at 37 °C. Reactions were subjected to 15% SDS-PAGE followed by autoradiography. Gels were dried and exposed to phosphor-screen for 10 min. (C) Cos-1 cells transfected FLAG-v-cyclin were harvested 48 h post-transfection. Equivalent lysate aliquots were depleted of the indicated cdk by 3 rounds of immunoprecipitation with agarose-conjugated cdk-specific antibodies. Following the third IP depletion, lysates were split equally, subjected to overnight immunoprecipitation with anti-FLAG-agarose beads, and used for in vitro kinase assays using histone H1 (top panel) or GST-Cterm pRb (lower panel) as exogenous substrate.
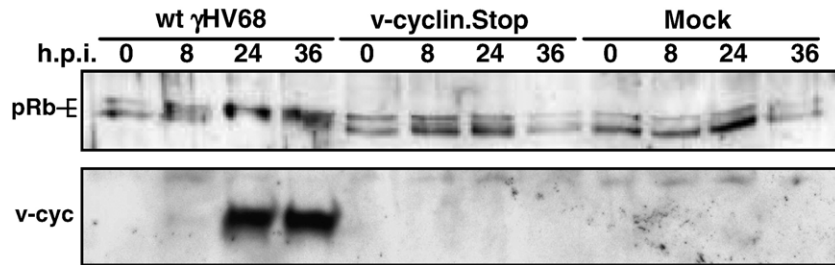
Fig. 5. γHV68 v-cyclin complexes phosphorylate pRb during lytic infection. NIH3T12 cells were serum-starved for 48 h then infected with wild type γHV68, v-cyclin.Stop, or mock-infected in serum-free media for 1 h at 37 °C. Cells were harvested at the indicated times, and lysates separated by 7.5% (top panel) or 15% (bottom panel) SDS-PAGE and Western blotted for pRb (top panel) and γHV68 v-cyclin (bottom panel).

vitro kinase assays, extracts were pre-cleared of specific cdks prior to immunoprecipitation of the v-cyclin. Notably, depletion of cdk2 resulted in a significant decrease in phosphorylation of histone H1 and a somewhat more moderate decrease in the observed phosphorylation of pRb (Fig. 4C). Depletion of cdk1/cdc2 appeared to have little impact on histone H1 phosphorylation but did appear to modestly decrease pRb phosphorylation—suggesting that it might play a role along with cdk2 in targeting v-cyclin phosphorylation of pRb (Fig. 4C).

To determine whether the v-cyclin mediates pRb phosphorylation in vivo, we assessed pRb phosphorylation during virus infection (Fig. 5). NIH3T12 cells were serum-starved for 72 h, then infected with wild type γHV68, v-cyclin.Stop (van Dyk et al., 2000), or mock-infected. Cells were harvested at the indicated times post-infection and subjected to Western blotting to determine the extent of pRb phosphorylation, as determined by mobility shift (Fig. 5). Infection with wild type γHV68 demonstrated by 24 h that the majority of pRB is found in the slower migrating hyperphosphorylated form, while in cells infected with v-cyclin.Stop or mock-infected showed little change in the phosphorylation status of pRb (Fig. 5, top panel). Importantly, the kinetics of pRb phosphorylation closely parallel the onset of v-cyclin
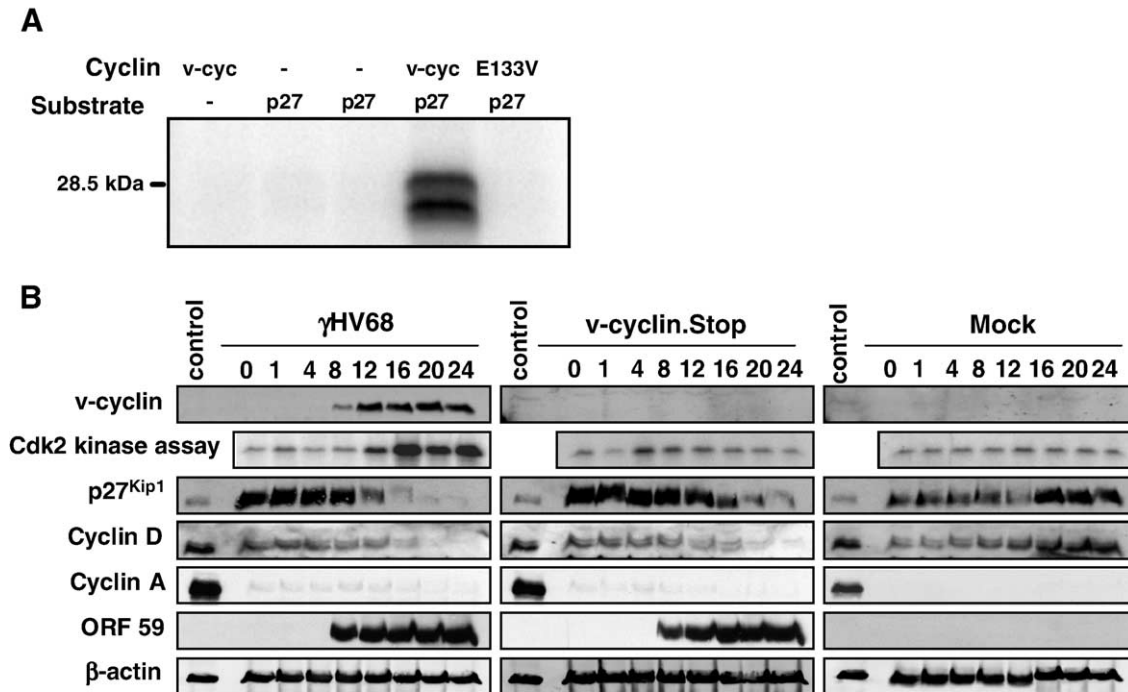

Fig. 6. γHV68 v-cyclin complexes phosphorylate the p27[Kip1] CKI and target it for degradation. (A) Cos-1 cells were transfected with pCMV-TAG2B vector control (lanes 2 and 3), FLAG-v-cyclin (lanes 1 and 4), or FLAG-v-cyclin.E133V (lane 5) and pCMV-cdk2 (lanes 3–5). 48 h post-transfection, cells were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibody (Sigma). Immunoprecipitated complexes were resuspended in kinase reaction buffer with p27[Kip1] (2 μg) and 10 μCi γATP, and the reaction allowed to proceed for 30 min at 37 °C. Reactions were subjected to 15% SDS-PAGE followed by autoradiography. Gels were dried and exposed to phosphor-screen for 30 min. (B) Contact-inhibited serum-starved NIH3T3 cells were infected at MOI = 10.0 with wild type γHV68, v-cyclin.Stop, or mock-infected in serum-free media. Cells were harvested at the indicated times, and 100 μg total protein per sample was separated by 12.5% SDS-PAGE and analyzed by Western blot with the indicated antibody or 300 μg total protein was subjected to immunoprecipitation with anti-cdk2–agarose and used for in vitro kinase assays directed against GST-Cterm-pRb. Control lanes contain 100 μg total protein from uninfected actively dividing NIH3T3 cells.

expression (Fig. 5, bottom panel). Taken together, with the observed lack of increase in pRb hyperphosphorylation upon infection with v-cyclin.Stop virus, these data argue strongly that the observed increase in pRb hyperphosphorylation is due to expression of the γHV68 v-cyclin.

The CKI p27^{Kip1} is a negative regulator of Cdks and cell cycle progression. Cyclin E:cdk2 is able to phosphorylate p27^{Kip1}, targeting it for proteosomal degradation, thus facilitating cell cycle progression (Morisaki et al., 1997; Sheaff et al., 1997). The KSHV v-cyclin:cdk6 complex has also been shown to possess the ability to phosphorylate p27^{Kip1}, targeting it for degradation and thus bypassing p27^{Kip1} imposed growth arrest (Ellis et al., 1999; Mann et al., 1999). To determine if the γHV68 v-cyclin is able to mediate phosphorylation of p27^{Kip1}, we immunoprecipitated FLAG-v-cyclin-containing complexes from transfected Cos-1 cells for kinase assays, using p27^{Kip1} as an exogenous substrate. Fig. 6A shows that wild type γHV68 v-cyclin strongly phosphorylates p27^{Kip1} in vitro and the phosphorylated substrate resolved as a doublet, as is commonly seen (Montagnoli et al., 1999). Additionally, the γHV68 v-cyclin E133V mutant did not mediate phosphorylation of p27^{Kip1}, providing further evidence that the mutation introduced precludes cdk binding and activation.

To further assess whether p27^{Kip1} is modulated during the course of γHV68 infection, we infected serum-starved murine NIH 3T3 fibroblasts with either wild type virus, v-cyclin.Stop, or mock-infected cells, and followed the levels of several cell cycle-associated gene products (Fig. 6B). Notably, a significant increase in cdk2-associated kinase activity was observed in wild type virus-infected cells that was not apparent in either mock-infected or cells infected with the v-cyclin.Stop mutant (Fig. 6B). Furthermore, the increase in cdk2 kinase activity correlated precisely with the rise in v-cyclin expression during the infection time course (Fig. 6B). Concomitant with the increase in v-cyclin expression and cdk2-associated kinase activity, there was a sharp decrease in p27^{Kip1} levels, consistent with the v-cyclin targeting p27^{Kip1} for degradation. Notably, there was also a drop in p27^{Kip1} levels in v-cyclin.Stop-infected that was not observed in mock-infected cells. However, this decrease in p27^{Kip1} in v-cyclin.Stop-infected cells was delayed compared to wild type virus-infected cells (Fig. 6B). Similarly, in both wild type and v-cyclin.Stop-infected cells, there was a decrease in cellular cyclin D expression that was not observed in mock-infected cells (Fig. 6B). Taken together, these data suggest that there is, in addition to v-cyclin directed degradation of p27^{Kip1}, a v-cyclin independent shutoff of host gene expression during the course of virus replication. We failed to detect a rise in cyclin A levels over the time course analyzed (Fig. 6B). However, expression of the early gene product of ORF 59 was readily detected with the same kinetics in both wild type and v-cyclin.Stop-infected cells (Fig. 6B). Levels of cdk2 and cdc2 did not change over the course of the experiment, and kinase activity was not detected from cdc2 IP kinase assay (data not shown).

γHV68 v-cyclin-mediated phosphorylation of cyclin A:cdk substrates

The KSHV v-cyclin has been shown to bind cdk6 and promote phosphorylation of a number of cell-cycle-related proteins (Ellis et al., 1999; Godden-Kent et al., 1997; Laman et al., 2001; Mann et al., 1999; Ojala et al., 2000; Swanton et al., 1997). Recent work has also demonstrated that expression of the KSHV v-cyclin in primary fibroblasts leads to genomic instability and centrosome amplification, underscoring the importance of proper checkpoint regulation and the tumor suppressor p53 in preventing KSHV v-cyclin-mediated tumorigenesis (Verschuren et al., 2002, 2004a, 2004b). Because we observed that the γHV68 v-cyclin interacts with both cdk2 and cdc2, we investigated whether γHV68 v-cyclin might play a role in later cell cycle stages by phosphorylating components of the cell cycle machinery associated with S and G2 phases of the cell cycle. Fig. 7 shows that γHV68 v-cyclin-containing complexes can phosphorylate p21^{Cip1}, a known cyclin A:cdk substrate (Bornstein et al., 2003; Jaumot et al., 1997), while the γHV68 v-cyclin E133V mutant exhibits only background levels of p21^{Cip1} phosphorylation. Furthermore, the addition of roscovitine, an inhibitor of cdc2 and cdk2, to the kinase
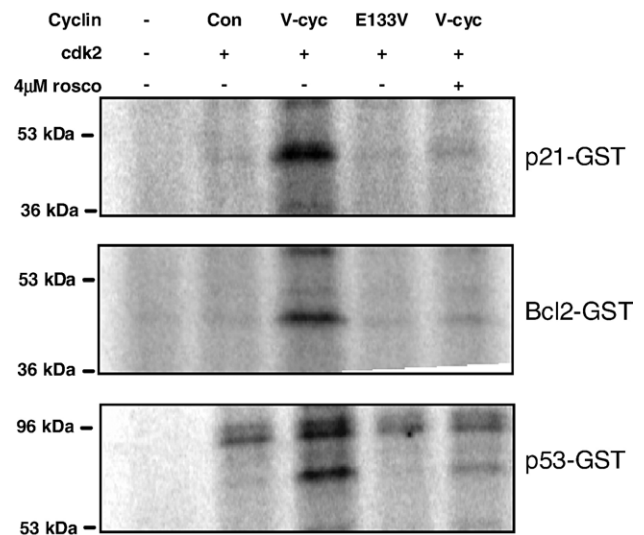

Fig. 7. γHV68 v-cyclin complexes phosphorylate both cdk2 and cdc2 substrates. Cos-1 cells were transfected with pCMV-TAG2B vector control (lanes 1 and 2), FLAG-v-cyclin (lanes 3 and 5), or FLAG-v-cyclin.E133V (lane 4) and pCMV-cdk2 (lanes 2–5). 48 h post-transfection, cells were harvested and immunoprecipitated with agarose-conjugated anti-FLAG antibodies (Sigma). Immunoprecipitated complexes were resuspended in kinase reaction buffer with p27^{Kip1} (2 μg), 10 μCi γATP, and either 16 μM roscovitine (+) or equivalent volume DMSO vehicle control (−). The reactions were allowed to proceed for 30 min at 37 °C. Reactions were subjected to 12.5% SDS-PAGE followed by autoradiography. Gels were dried and exposed to phosphor-screen for 12 h.

reaction resulted in a significant reduction in the phosphory-lation of p21^{Cip1}.

To extend this analysis, we assessed two other substrates, Bcl-2 and p53, as potential targets for the γHV68 v-cyclin:cdk complexes. Bcl-2 is phosphorylated by cdc2 during G2/M, modulating its cell cycle regulatory properties without affecting its anti-apoptotic function (Furukawa et al., 2000). Also of note, the KSHV v-cyclin:cdk6 complex has been shown to phosphorylate, and inactivate the anti-apoptotic functions of Bcl-2 (Ojala et al., 2000). The tumor suppressor p53 has also been shown to be a substrate for S/G2 cyclin:cdk complexes (Addison et al., 1990; Bischoff et al., 1990; Blaydes and Hupp, 1998; Luciani et al., 2000; Wang and Prives, 1995). We found that γHV68 v-cyclin complexes immunoprecipitated from Cos-1 cell were able to phosphorylate both Bcl-2 and p53. As above, phosphor-ylation was dependent on cdk binding since no activity was observed with the v-cyclin E133V mutant. Furthermore, phosphorylation was sensitive to the addition of roscovitine to the kinase reaction (Fig. 7).

Discussion

All γ2-herpesviruses encode a v-cyclin that is conserved in both sequence and position. We have previously shown that the v-cyclin encoded by γHV68 promotes cell cycle progression and tumorigenesis when expressed as a trans-gene in mice and is a critical regulator of reactivation from latency (van Dyk et al., 1999, 2000). Here, we extend the analysis of the function of the γHV68 v-cyclin to show that it binds multiple cdks and mediates phosphorylation of a number of cell-cycle-related proteins in vitro.

Specific biochemical properties of the KSHV v-cyclin have been a topic of intense research to determine how the KSHV v-cyclin contributes to tumorigenesis (Verschuren et al., 2004a, 2004b). The KSHV v-cyclin binds both G1 and S-phase cdks but shows a marked preference for cdk6 and allows for an expanded repertoire of phosphorylation substrates for the kinase, including a number of critical cell cycle genes. While it is known that γHV68 v-cyclin interacts with cdk2, we sought to clarify which cdks the γHV68 v-cyclin binds and activates in light of the seemingly promiscuous activity of the KSHV v-cyclin. We determined that the γHV68 v-cyclin binds cdk2 and cdc2, but not cdk4 or cdk6 (Figs. 1, 2). This is in agreement with previous reports that the v-cyclin interacts with cdk2, and not the G1-associated cdk4 or cdk6 (Card et al., 2000). However, there has previously been no description of a herpesvirus v-cyclin binding cdc2. This makes the inter-action between γHV68 v-cyclin and cdc2 a novel finding among herpesvirus v-cyclins, although the implications of cdc2 binding remain unclear. Since substrate specificity for cyclin:cdk complexes is likely due to determinants imparted by the cyclin (Goda et al., 2001; Horton and Templeton, 1997; Sarcevic et al., 1997; Schulman et al., 1998; Zarkowska

and Mittnacht, 1997), it will be interesting to determine whether γHV68 v-cyclin promotes an expanded substrate range for either or both cdk2 and cdc2 or whether binding of the v-cyclin to each cdk allows for kinase activity only toward traditional substrates associated with each.

Autographica californica nucleopolyhedrovirus encodes a structural protein, ODV-EC27, that is a multi-functional cyclin, binding to and activating cdc2 and cdk6 (Belyavskyi et al., 1998). Although both complexes can phosphorylate histone H1 and pRb, specific functions of each complex are different; the cdc2:ODV-EC27 complex exhibits cyclin B-like activity, while cdk6:ODV-EC27 is able to bind proliferating cell nuclear antigen (PCNA), presumably to aid establishment of an S-phase like cellular environment (Belyavskyi et al., 1998). Whether γHV68 v-cyclin imparts differential substrate preference upon its different binding partners like ODV-EC27 or expands the substrate range of each binding partner like the KSHV v-cyclin will be an important point to investigate.

The herpes simplex virus-1 polymerase processivity factor, UL-42, has been shown to directly interact with, and activate, cdc2 to recruit topoisomerase IIα for the expression of a subset of late genes (Advani et al., 2000, 2001, 2003). While it is tempting to speculate that the γHV68 v-cyclin:cdc2 interaction may mediate similar functions, it should be noted that γHV68 also encodes a protein kinase, ORF36, which shares homology to kinases ascribed cdc2-like functions in other herpesviruses (Kawa-guchi and Kato, 2003; Kawaguchi et al., 2003). However, ORF36 of γHV68 has not been characterized biochemically or genetically, and, as such, it remains to be determined whether it exhibits cdc2-like functions and/or whether there is a role of the γHV68 v-cyclin:cdc2 complex in the activation of late genes.

As discussed above, the structure of γHV68 v-cyclin in complex with cdk2 has been determined (Card et al., 2000) and, in addition to a number of novel interactions between v-cyclin and cdk2, the γHV68 viral cyclin possesses a number of conserved sequence and structural features indicating that the v-cyclin binds to and activates cdk2 in a manner similar to cellular cyclins. Based on these findings, we hypothesized that mutation of residues absolutely conserved among cyclins, both viral and cellular, would abolish interaction between the v-cyclin and cdks. A similar approach was taken in the biochemical character-ization of the HVS v-cyclin, although that study focused on multiple combinatorial mutations (Jung et al., 1994). We chose two residues within the cyclin box of γHV68 that are conserved in all known cyclins for substitutional mutation. Side chains of the lysine residue at amino acid 104 and the glutamate at amino acid 133 of the γHV68 v-cyclin both participate in critical bonds between the γHV68 v-cyclin and cdk2 (Card et al., 2000). We substituted a glutamate for the lysine at position 104 (K104E) and, in independent mutant, substituted a valine for the glutamate at position 133 (E133V). When assayed for interaction with cdk2 in

immunoprecipitation experiments from transfected Cos-1 cells, we observed that both the K104E and E133V mutations severely impaired the ability of γHV68 v-cyclin to bind cdk2. We chose to use the E133V mutant in further experiments, and it was found to abolish kinase activity of v-cyclin immunoprecipitates in vitro. Additionally, these mutations abolish binding of cdc2 to the γHV68 v-cyclin, further supporting the hypothesis that the γHV68 v-cyclin engages cdks in a manner similar to cellular cyclins. Mutation of the conserved lysine residue has been previously characterized for cyclin D1 and was found to cause a defect in the ability of cyclin D1 to transform cells (Hinds et al., 1994). Similarly, mutation of OrfA (rv-cyclin) of walleye dermal sarcoma virus at the conserved lysine and glutamate residues within the defined cyclin box abrogates its ability to localize to the nucleus, a property potentially mediated by additional components of the cyclin:cdk complex (Rovnak et al., 2001). However, recent work showed that mutation of the corresponding conserved lysine of the KSHV v-cyclin does not appreciably affect binding to cdk6 (Kaldis, 2005), although binding to other cdks was not assessed. This characteristic appears specific to the KSHV v-cyclin as these mutations in other cellular and viral cyclins result in significant loss of function. How the previously described novel features of the v-cyclin:cdk2 interaction affect function of the complex remains to be determined, although functions such as CKI resistance have been proposed (Card et al., 2000).

KSHV and γHV68 v-cyclins have been shown, with their cognate cdk, to phosphorylate pRb, and it is believed that this phosphorylation results in cell cycle progression necessary for the viral life cycle. Another γ-herpesvirus, Epstein–Barr virus (EBV), although it does not encode a v-cyclin, uses a similar mechanism to push cells into cycle by inducing the expression of cyclin D2 (Arvanitakis et al., 1995). An obvious consequence of such direct cell cycle manipulation is tumorigenesis, and both the KSHV v-cyclin and γHV68 v-cyclin have been shown to be oncogenic when expressed as lymphocyte-specific transgenes in mice (van Dyk et al., 1999; Verschuren et al., 2002, 2004a, 2004b), although the specific role of pRb phosphorylation and inactivation in tumorigenesis by v-cyclins has not been directly assessed. In the context of lytic virus infection, the γHV68 v-cyclin is necessary for the hyperphosphorylation of pRb characteristic of cell cycle progression since a recombinant virus lacking v-cyclin fails to induce a similar level of pRb phosphorylation. However, it should be noted that the γHV68 v-cyclin is not required for virus replication, in vitro or in vivo (Hoge et al., 2000; van Dyk et al., 2000), and thus pRb phosphorylation is likely not required for the completion of the lytic program. The γHV68 v-cyclin is required for efficient reactivation from latency. While little is known about the role of pRb in viral latency, it is tempting to speculate that pRb phosphorylation may be a critical step in the process of reactivation from a quiescent cell population.

Cyclin E:cdk2 and the KSHV v-cyclin:cdk6 complexes have both been shown to phosphorylate p27^{Kip1}, targeting it for proteosomal degradation (Ellis et al., 1999; Mann et al., 1999; Montagnoli et al., 1999; Morisaki et al., 1997; Shirane et al., 1999; Sutterluty et al., 1999; Vlach et al., 1997). Phosphorylation and eventual degradation of CKIs by v-cyclins represents another mechanism by which v-cyclins alter the normal host cell cycle. More recently, the KSHV v-cyclin was shown to bind to p27^{Kip1} in primary effusion lymphoma (PEL) cells, which have been shown to express high levels of the CKI (Jarviluoma et al., 2004), underscoring the potential importance of v-cyclins in modulating p27^{Kip1} activity. p27^{Kip1} is an in vitro substrate for the γHV68 v-cyclin:cdk complex, and expression of v-cyclin during infection correlates directly with accelerated degradation of p27^{Kip1}. It is interesting to note that p27^{Kip1} levels also decline in cells infected with a v-cyclin null virus, although with delayed kinetics. Levels of D-type cyclins also gradually decrease in both wild type and mutant-infected cells with strikingly similar kinetics. It is likely that other virally encoded mechanisms enhance ubiquitination and proteosomal degradation of cellular targets as both p27^{Kip1} and D-type cyclins are regulated by these processes (Diehl et al., 1997; Shirane et al., 1999; Sutterluty et al., 1999). However, the presence of v-cyclin during infection enhances the rate of p27^{Kip1} degradation, while D-type cyclin levels are unaffected. Taken together, these findings indicate that p27^{Kip1} is a substrate for v-cyclin:cdk complexes, which target it for destruction.

Establishment of an S-phase environment is hypothesized to be necessary for viral replication. While expression of the γHV68 v-cyclin induces markers of the G1 to S transition (i.e. pRB hyperphosphorylation and p27^{Kip1} degradation), it is not known whether v-cyclin expression can drive entry into S-phase during infection. Cyclin A is the canonical marker for, and is necessary for progression through, S-phase. Surprisingly, cyclin A was not appreciably upregulated in infected samples, similar to mock infection and in stark contrast to uninfected actively dividing cells. While this indicates that contact inhibition and serum starvation were sufficient to arrest cells in G0/early G1, it also suggests that an S-phase conducive to cellular replication is not initiated. It appears that other viral factors may be responsible for this phenomenon since wild type and v-cyclin.Stop-infected cells are similar in this respect, despite the ability of v-cyclin to affect cellular processes that would normally induce S-phase and cyclin A expression. One possibility is that γHV68 v-cyclin is necessary to enhance specific cdk activities, while other viral factors subvert normal cell cycle progression. Cyclin A:cdk complexes have been shown to associate with and phosphorylate p21^{Cip1} (Bornstein et al., 2003; Jaumot et al., 1997). Since cyclin A is not induced during infection, it is possible that the γHV68 v-cyclin performs functions usually ascribed to cyclin A. We have shown here that v-cyclin:cdk complexes are able to phosphorylate p21^{Cip1} in vitro in a

roscovitine-sensitive manner. The functional consequences of p21^{Cip1} phosphorylation by cdks, whether targeting it for degradation or enhancing G2 cdk activity, remain controversial (Cayrol and Ducommun, 1998; Dash and El-Deiry, 2005), and it will be interesting to determine whether v-cyclin-mediated phosphorylation of p21^{Cip1} has specific effects.

Co-expression of KSHV v-cyclin and cdk6 in U2-OS osteosarcoma cells induces apoptosis mediated by phosphorylation and inactivation of Bcl-2, which can be reversed by expression of the KSHV v-Bcl-2 (Ojala et al., 1999). Since γHV68 also encodes a v-Bcl-2 and because Bcl-2 is phosphorylated during G2/M phase by cdc2 (Furukawa et al., 2000), we assessed Bcl2 phosphorylated and demonstrated that the γHV68 v-cyclin:cdk complex can phosphorylate Bcl-2 in vitro (Fig. 7) and determined that this phosphorylation is sensitive to inhibition by the cdc2/cdk2 inhibitor roscovitine. Whether the γHV68 v-cyclin phosphorylates Bcl-2 while in complex with cdk2 or with cdc2 and the in vivo consequences remain to be determined.

Another cellular factor intimately involved in cell cycle regulation and survival is the tumor suppressor p53. It is subject to a myriad of post-translational modifications with varying effects on its function (Xu, 2003). p53 possesses a cdk phosphorylation site and is phosphorylated by both cdc2 and cdk2 in vitro (Addison et al., 1990; Bischoff et al., 1990; Blaydes and Hupp, 1998; Luciani et al., 2000; Wang and Prives, 1995). However, the in vivo effects of phosphorylation by cdks are poorly understood. We determined that p53 can be phosphorylated in vitro by v-cyclin-containing complexes. Whether this activity is biologically relevant remains to be determined. There has been no report of the KSHV v-cyclin interacting with p53, although loss of p53 function uncovers the oncogenic potential of the KSHV v-cyclin in transgenic mice (Verschuren et al., 2004a, 2004b). Notably, the oncogenic potential of the γHV68 v-cyclin was readily observed in normal mice (e.g., did not require crossing the v-cyclin transgenic mice onto a p53 null background), perhaps suggesting that a distinguishing feature of the γHV68 and KSHV v-cyclins is the ability of the γHV68 v-cyclin to target and inactivate p53. However, in the context of a latent KSHV infection, KSHV latent nuclear antigen (LANA) expression leads to p53 inactivation (Friborg et al., 1999; Wong et al., 2004), further indicating that suppression of the p53 pathway is important during virus infection. Finally, cyclin A:cdk2 complexes have been shown to regulate protein–protein interactions of MDM2 by phosphorylation (Zhang and Prives, 2001), which functions to downregulate p53 protein levels and activity (Momand et al., 2000). Further studies will be required to determine whether p53 function is regulated during latent γHV68 infection and whether modulation of p53 activity through phosphorylation by v-cyclin:cdk complexes occurs.

In summary, we have shown that the γHV68 v-cyclin binds cdc2 and cdk2, activates cdks in a manner similar to cellular cyclins, and directs them in the phosphorylation of a number of components of the cell cycle machinery. In the context of ectopic expression in transgenic mice, the γHV68 v-cyclin is an oncogene, while in the context of virus infection, it is a critical regulator of reactivation from latency. It is likely that these functions are intertwined in the signaling pathways affected by the γHV68 v-cyclin, and careful dissection of these pathways may lead to a better understanding of the link between the cell cycle, γ-herpesvirus tumorigenesis, and γ-herpesvirus reactivation.

Materials and methods

Cell culture

NIH3T12 (ATCC CCL-164), NIH3T3 (ATCC CCL-163), and Cos-1 cells (ATCC CLR-1650) were grown and maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin/ml, 100 mg streptomycin/ml, and 2 mM L-glutamine.

Reagents

To generate an expression vector encoding a FLAG epitope-tagged v-cyclin (FLAG-v-cyclin), the γHV68 v-cyclin open reading frame was amplified by PCR from γHV68 genomic DNA with primers incorporating *Bam*H1 and *Xho*1 restriction sites using Vent polymerase (New England Biolabs). PCR products were sub-cloned into pCR-Blunt (Invitrogen) and the sequence confirmed. The v-cyclin was then shuttled into the *Bam*H1/*Xho*1 sites of pCMV-TAG2B (Stratagene), providing an in-frame amino-terminal FLAG-tag. K104E and E133V mutations were generated by 2-part overlapping PCR, introducing the indicated mutation and silent mutations generating unique restriction sites at or near the site of the coding mutation, and cloned as described above. Primers used to generate the K104E mutant were juK104ES (5′-CTCCATATC *GCCGGC*GAGGTCAGGGCCTACATG-3′) and juK104-EC (5′-CATGTAGG CCCTGACCTC*GCCGGC*GATATG-GAG-3′). Primers used to generate E133V mutant were juE133VS (5′-GCTGACAAACTATTGAC*ACTAGT*AG-TCAAAAGTCTG GATACCCTCTCGTGGG-3′) and juE133VC (5′-CCCACGAGAGGGTATCCA GACTTTT-GACT*ACTAGT*GTCAATAGTTTGTCAGC-3′). Bold letters represent silent mutations to generate the unique restriction sites depicted in italics (*Nae*I for K104E and *Spe*I for E133V), and underlined letters produce the coding mutations indicated by each name designation. Cyclin E and cyclin D3 expression constructs were generated by RT-PCR amplification from murine fibroblast RNA, using gene specific primers incorporating *Bam*H1 and *Xho*1 restriction sites at the 5′ and 3′ ends of the cDNA, respectively. Cyclin D1 cDNA (38a) was amplified in the same fashion. PCR

products were shuttled into pCR-Blunt (Invitrogen), sequenced, and cloned in frame into pCMV-TAG2B as described above.

pCMV-cdk6 and pCMV-cdk2 were kind gifts from Matt Meyerson (Dana Farber Cancer Institute). Cdk4 was PCR amplified from cDNA prepared from mouse embryonic fibroblasts with gene specific primers incorporating a Kozak consensus translation initiation sequence (Kozak, 1987). The PCR product was blunt-cloned into pCMV-Script (Strategene) and confirmed by sequencing. pCMV-cdc2 was constructed by shuttling the BamH1/EcoR1 fragment of pSAF10-cdc2 (a gift from Helen Piwnica-Worms, Washington University School of Medicine) into the same unique sites of pCMV-Script (Strategene). Antibodies used in this study were: rabbit polyclonal anti-cdc2 (C-19, Santa Cruz Biotechnology), goat polyclonal anti-cdk2 (M2, Santa Cruz Biotechnology), goat polyclonal anti-cdk4 (C-22, Santa Cruz Biotechnology), goat polyclonal anti-cdk6 (C-21, Santa Cruz Biotechnology), rabbit polyclonal anti-pRb (C-15, Santa Cruz Biotechnology), mouse monoclonal anti-p27^{Kip1} (F-8, Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin D3 (H292, Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin A (C-19, Santa Cruz Biotechnology), mouse monoclonal anti-FLAG-HRP (M2, Sigma), mouse monoclonal anti-β-actin-FITC conjugate (Sigma), and rabbit anti-γHV68 v-cyclin antisera (van Dyk et al., 1999). Affinity-purified chicken polyclonal anti-ORF59 antibodies (Gallus Immunotech) were generated against the peptides GKKTRGGNKASDSGT and KRPPP-KKDREPTTKRPKL, corresponding to amino acids 210–224 and 372–388, respectively, of the predicted ORF59 protein of γHV68 (Genbank:AAF19323). Donkey anti-mouse, anti-goat, and anti-rabbit antibodies conjugated with horseradish peroxidase (Jackson Immunochemicals) and donkey anti-chicken–HRP (Gallus Immunotech) were used as secondary antibodies in Western analysis. Normal rabbit IgG–, rabbit anti-cdk2 (M2)–, anti-cdk4 (C-22)–, anti-cdk6 (C-21)–, and mouse anti-cdc2 (17)–agarose conjugates were purchased from Santa Cruz Biotechnology. Recombinant proteins p27^{Kip1}, GST-Cterm-pRb, GST-p21^{Cip1}, GST-Bcl-2, GST-p53, GST (Santa Cruz), and histone H1 (Upstate) were used as exogenous substrates for in vitro kinase assays.

Co-immunoprecipitation and immunoblotting

10^6 Cos-1 cells were plated in 10 cm dishes and allowed to adhere overnight. Cells were then transfected with 10 μg FLAG-v-cyclin and 10 μg of cdk expression vectors using the LT-1 transfection reagent (Mirus) according to the manufacturer's instructions. 48 h post-transfection, cells were washed with phosphate-buffered saline and lysed in 500 μl ELB buffer (50 mM HEPES pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.1% NP-40 substitute, 1 mM NaF, 1 mM Na$_3$VO$_4$, and 0.01% protease inhibitor cocktail (Sigma)) for 20 min at 4 °C. Cells were then scraped into Eppendorf tubes

and cleared of cellular debris by centrifugation. Lysates were precleared with normal mouse IgG-Agarose (Santa Cruz) for 1 h at 4 °C with agitation. Lysates were once again cleared by centrifugation and incubated overnight with 25 μl anti-FLAG (M2) agarose beads (Sigma). Beads were washed 4 times in cold ELB, boiled for 10 min in SDS loading buffer, and subjected to SDS-PAGE. Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences) and immunoblotted with the indicated antibody. Some membranes were stripped with 0.2 M NaOH for 5 min, washed with de-ionized water, re-blocked, and re-immunoblotted, as indicated. Membranes were visualized by enhanced chemiluminescence using the ECL Western Blotting Detection System (Amersham Biosciences).

Kinase assays

Cos-1 cells were transfected with 10 μg FLAG-v-cyclin and 10 μg pCMV-cdk2 and harvested as described above. Lysates from 2 independent transfections were pooled, aliquoted into 3 samples, and then immunoprecipitated with anti-FLAG beads as described above. Beads were washed 4 times with cold ELB, 2 times with cold 50 mM Tris pH 7.4, 10 mM MgCl$_2$, and each sample divided into two parts. Each pellet was re-suspended 25 μl in kinase reaction buffer (50 mM Tris 7.4, 10 mM MgCl$_2$, 1 mM DTT, 250 μM ATP) with 2 μg of exogenous substrate and 10 μCi γ^{32}P-ATP. In experiments assessing chemical inhibition of kinase complexes, 4 μl of a 100 μM stock of roscovitine (Calbiochem) or vehicle control (DMSO, Sigma) was added to the reaction where indicated (for a final concentration of 16 μM) prior to incubation. Reactions were allowed to proceed for 30 min at 37 °C, terminated with the addition of an equal volume of 2× SDS loading buffer, boiled for 10 min, and subjected to SDS-PAGE. Gels were dried and visualized by autoradiography. For kinase assays following cdk depletion, 5×10^6 Cos-1 cells were transfected with 50 μg FLAG-v-cyclin. 48 h post-transfection, cells were harvested, and equivalent lysates were subjected to three rounds of immunodepletion of indicated cdks using 30 μl of agarose-conjugated cdk-specific antibodies. Following the final immunodepletion, lysates were immunoprecipitated with 25 μl anti-FLAG (M2) agarose beads and processed as described above.

Infections and viruses

NIH 3T12 cells were seeded in 10 cm dishes and serum-starved for 72 h in low serum media (DMEM supplemented with 0.5% FCS, 100 U ml/penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine). Cells were then infected at MOI = 5.0 with wild type γHV68 clone WUMS (ATCCVR1465), v-cyclin.Stop (van Dyk et al., 2000), or mock-infected in a volume of 2 ml (with serum concentration not exceeding 0.5%) for 1 h at 37 °C with occasional rocking. Following infection, 8 ml of serum low media was added for the remainder of the infection. Cells were then

harvested and lysed in ELB at the indicated times. Protein concentrations were calculated using the DC Protein Assay (Bio-Rad). 50 μg of total protein per lane was subjected to SDS-PAGE and Western blotting. NIH3T3 cells were seeded and allowed to grow to confluence. Media was then replaced with low serum media for 72 h to allow for serum starvation. Cells were infected as described above at MOI = 10.0 in low serum media for 1 h, washed twice in PBS, and replenished with low serum media until indicated harvest times. Cells were harvested, and protein determined, as described above. 100 μg of total protein was subjected to SDS-PAGE and Western blot analysis. Infected cell kinase assays were performed on lysates containing 300 μg total protein in a total volume of 500 μl ELB as described above using 25 μl of rabbit anti-cdk2 (M2)–agarose slurry (Santa Cruz) for immunoprecipitation and GST-Cterm-pRb as exogenous substrate.

Acknowledgments

References

Addison, C., Jenkins, J.R., Sturzbecher, H.W., 1990. The p53 nuclear localisation signal is structurally linked to a p34cdc2 kinase motif. Oncogene 5 (3), 423–426.

Advani, S.J., Weichselbaum, R.R., Roizman, B., 2000. The role of cdc2 in the expression of herpes simplex virus genes. Proc. Natl. Acad. Sci. U.S.A. 97 (20), 10996–11001.

Advani, S.J., Weichselbaum, R.R., Roizman, B., 2001. cdc2 cyclin-dependent kinase binds and phosphorylates herpes simplex virus 1 U (L)42 DNA synthesis processivity factor. J. Virol. 75 (21), 10326–10333.

Advani, S.J., Weichselbaum, R.R., Roizman, B., 2003. Herpes simplex virus 1 activates cdc2 to recruit topoisomerase II alpha for post-DNA synthesis expression of late genes. Proc. Natl. Acad. Sci. U.S.A. 100 (8), 4825–4830.

Arellano, M., Moreno, S., 1997. Regulation of CDK/cyclin complexes during the cell cycle. Int. J. Biochem. Cell Biol. 29 (4), 559–573.

Arvanitakis, L., Yaseen, N., Sharma, S., 1995. Latent membrane protein-1 induces cyclin D2 expression, pRb hyperphosphorylation, and loss of TGF-beta 1-mediated growth inhibition in EBV-positive B cells. J. Immunol. 155 (3), 1047–1056.

Belyavskyi, M., Braunagel, S.C., Summers, M.D., 1998. The structural protein ODV-EC27 of *Autographa californica* nucleopolyhedrovirus is a multifunctional viral cyclin. Proc. Natl. Acad. Sci. U.S.A. 95 (19), 11205–11210.

Bischoff, J.R., Friedman, P.N., Marshak, D.R., Prives, C., Beach, D., 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. U.S.A. 87 (12), 4766–4770.

Blaydes, J.P., Hupp, T.R., 1998. DNA damage triggers DRB-resistant phosphorylation of human p53 at the CK2 site. Oncogene 17 (8), 1045–1052.

Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., Hershko, A., 2003. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J. Biol. Chem. 278 (28), 25752–25757.

Card, G.L., Knowles, P., Laman, H., Jones, N., McDonald, N.Q., 2000. Crystal structure of a gamma-herpesvirus cyclin–cdk complex. EMBO J. 19 (12), 2877–2888.

Cayrol, C., Ducommun, B., 1998. Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21. Oncogene 17 (19), 2437–2444.

Chang, Y., Moore, P.S., Talbot, S.J., Boshoff, C.H., Zarkowska, T., Godden, K., Paterson, H., Weiss, R.A., Mittnacht, S., 1996. Cyclin encoded by KS herpesvirus. Nature 382 (6590), 410.

Child, E.S., Mann, D.J., 2001. Novel properties of the cyclin encoded by Human Herpesvirus 8 that facilitate exit from quiescence. Oncogene 20 (26), 3311–3322.

Dash, B.C., El-Deiry, W.S., 2005. Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. Mol. Cell. Biol. 25 (8), 3364–3387.

Diehl, J.A., Zindy, F., Sherr, C.J., 1997. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin–proteasome pathway. Genes Dev. 11 (8), 957–972.

Ellis, M., Chew, Y.P., Fallis, L., Freddersdorf, S., Boshoff, C., Weiss, R.A., Lu, X., Mittnacht, S., 1999. Degradation of p27 (Kip) cdk inhibitor triggered by Kaposi's sarcoma virus cyclin–cdk6 complex. EMBO J. 18 (3), 644–653.

Friborg Jr., J., Kong, W., Hottiger, M.O., Nabel, G.J., 1999. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature 402 (6764), 889–894.

Furukawa, Y., Iwase, S., Kikuchi, J., Terui, Y., Nakamura, M., Yamada, H., Kano, Y., Matsuda, M., 2000. Phosphorylation of Bcl-2 protein by CDC2 kinase during G2/M phases and its role in cell cycle regulation. J. Biol. Chem. 275 (28), 21661–21667.

Goda, T., Funakoshi, M., Suhara, H., Nishimoto, T., Kobayashi, H., 2001. The N-terminal helix of *Xenopus* cyclins A and B contributes to binding specificity of the cyclin–CDK complex. J. Biol. Chem. 276 (18), 15415–15422.

Godden-Kent, D., Talbot, S.J., Boshoff, C., Chang, Y., Moore, P., Weiss, R.A., Mittnacht, S., 1997. The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J. Virol. 71 (6), 4193–4198.

Harbour, J.W., Dean, D.C., 2000. The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. 14 (19), 2393–2409.

Harbour, J.W., Luo, R.X., Dei Santi, A., Postigo, A.A., Dean, D.C., 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98 (6), 859–869.

Hinds, P.W., Dowdy, S.F., Eaton, E.N., Arnold, A., Weinberg, R.A., 1994. Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. U.S.A. 91 (2), 709–713.

Hoge, A.T., Hendrickson, S.B., Burns, W.H., 2000. Murine gammaherpesvirus 68 cyclin D homologue is required for efficient reactivation from latency. J. Virol. 74 (15), 7016–7023.

Horton, L.E., Templeton, D.J., 1997. The cyclin box and C-terminus of cyclins A and E specify CDK activation and substrate specificity. Oncogene 14 (4), 491–498.

Jarviluoma, A., Koopal, S., Rasanen, S., Makela, T.P., Ojala, P.M., 2004. KSHV viral cyclin binds to p27KIP1 in primary effusion lymphomas. Blood.

Jaumot, M., Estanol, J.M., Casanovas, O., Grana, X., Agell, N., Bachs, O., 1997. The cell cycle inhibitor p21CIP is phosphorylated by cyclin A–CDK2 complexes. Biochem. Biophys. Res. Commun. 241 (2), 434–438.

Jeffrey, P.D., Tong, L., Pavletich, N.P., 2000. Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. Genes Dev. 14 (24), 3115–3125.

Jung, J.U., Stager, M., Desrosiers, R.C., 1994. Virus-encoded cyclin. Mol. Cell. Biol. 14 (11), 7235–7244.

Kaldis, P., 2005. The N-terminal peptide of the Kaposi's sarcoma-associated herpesvirus (KSHV)-cyclin determines substrate specificity. J. Biol. Chem. 280 (12), 11165–11174.

Kaldis, P., Ojala, P.M., Tong, L., Makela, T.P., Solomon, M.J., 2001. CAK-independent activation of CDK6 by a viral cyclin. Mol. Biol. Cell 12 (12), 3987–3999.

Kawaguchi, Y., Kato, K., 2003. Protein kinases conserved in herpesviruses potentially share a function mimicking the cellular protein kinase cdc2. Rev. Med. Virol. 13 (5), 331–340.

Kawaguchi, Y., Kato, K., Tanaka, M., Kanamori, M., Nishiyama, Y., Yamanashi, Y., 2003. Conserved protein kinases encoded by herpesviruses and cellular protein kinase cdc2 target the same phosphorylation site in eukaryotic elongation factor 1delta. J. Virol. 77 (4), 2359–2368.

Kozak, M., 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. 196 (4), 947–950.

Laman, H., Mann, D.J., Jones, N.C., 2000. Viral-encoded cyclins. Curr. Opin. Genet. Dev. 10 (1), 70–74.

Laman, H., Coverley, D., Krude, T., Laskey, R., Jones, N., 2001. Viral cyclin–cyclin-dependent kinase 6 complexes initiate nuclear DNA replication. Mol. Cell. Biol. 21 (2), 624–635.

Lees, E., 1995. Cyclin dependent kinase regulation. Curr. Opin. Cell Biol. 7 (6), 773–780.

Lees, E.M., Harlow, E., 1993. Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. Mol. Cell. Biol. 13 (2), 1194–1201.

Li, M., Lee, H., Yoon, D.W., Albrecht, J.C., Fleckenstein, B., Neipel, F., Jung, J.U., 1997. Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. J. Virol. 71 (3), 1984–1991.

Luciani, M.G., Hutchins, J.R., Zheleva, D., Hupp, T.R., 2000. The C-terminal regulatory domain of p53 contains a functional docking site for cyclin A. J. Mol. Biol. 300 (3), 503–518.

Mann, D.J., Child, E.S., Swanton, C., Laman, H., Jones, N., 1999. Modulation of p27 (Kip1) levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. EMBO J. 18 (3), 654–663.

Mittnacht, S., Boshoff, C., 2000. Viral cyclins. Rev. Med. Virol. 10 (3), 175–184.

Momand, J., Wu, H.H., Dasgupta, G., 2000. MDM2—Master regulator of the p53 tumor suppressor protein. Gene 242 (1–2), 15–29.

Montagnoli, A., Fiore, F., Eytan, E., Carrano, A.C., Draetta, G.F., Hershko, A., Pagano, M., 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. 13 (9), 1181–1189.

Morisaki, H., Fujimoto, A., Ando, A., Nagata, Y., Ikeda, K., Nakanishi, M., 1997. Cell cycle-dependent phosphorylation of p27 cyclin-dependent kinase (Cdk) inhibitor by cyclin E/Cdk2. Biochem. Biophys. Res. Commun. 240 (2), 386–390.

Nicholas, J., Cameron, K.R., Honess, R.W., 1992. Herpesvirus saimiri encodes homologues of G protein-coupled receptors and cyclins. Nature 355 (6358), 362–365.

Ojala, P.M., Tiainen, M., Salven, P., Veikkola, T., Castanos-Velez, E., Sarid, R., Biberfeld, P., Makela, T.P., 1999. Kaposi's sarcoma-associated herpesvirus-encoded v-cyclin triggers apoptosis in cells with high levels of cyclin-dependent kinase 6. Cancer Res. 59 (19), 4984–4989.

Ojala, P.M., Yamamoto, K., Castanos-Velez, E., Biberfeld, P., Korsmeyer, S.J., Makela, T.P., 2000. The apoptotic v-cyclin–CDK6 complex phosphorylates and inactivates Bcl-2. Nat. Cell Biol. 2 (11), 819–825.

Rovnak, J., Casey, J.W., Quackenbush, S.L., 2001. Intracellular targeting of walleye dermal sarcoma virus Orf A (rv-cyclin). Virology 280 (1), 31–40.

Sarcevic, B., Lilischkis, R., Sutherland, R.L., 1997. Differential phosphorylation of T-47D human breast cancer cell substrates by D1–, D3–, E–, and A-type cyclin–CDK complexes. J. Biol. Chem. 272 (52), 33327–33337.

Schulman, B.A., Lindstrom, D.L., Harlow, E., 1998. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. Proc. Natl. Acad. Sci. U.S.A. 95 (18), 10453–10458.

Schulze-Gahmen, U., Jung, J.U., Kim, S.H., 1999. Crystal structure of a viral cyclin, a positive regulator of cyclin-dependent kinase 6. Struct. Fold Des. 7 (3), 245–254.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M., Clurman, B.E., 1997. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev. 11 (11), 1464–1478.

Sherr, C.J., 1993. Mammalian G1 cyclins. Cell 73 (6), 1059–1065.

Sherr, C.J., Roberts, J.M., 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 13 (12), 1501–1512.

Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., Nakayama, K., Kitagawa, M., 1999. Down-regulation of p27 (Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J. Biol. Chem. 274 (20), 13886–13893.

Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., Krek, W., 1999. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. 1 (4), 207–214.

Swanton, C., Jones, N., 2001. Strategies in subversion: de-regulation of the mammalian cell cycle by viral gene products. Int. J. Exp. Pathol. 82 (1), 3–13.

Swanton, C., Mann, D.J., Fleckenstein, B., Neipel, F., Peters, G., Jones, N., 1997. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature 390 (6656), 184–187.

van Dyk, L.F., Hess, J.L., Katz, J.D., Jacoby, M., Speck, S.H., Virgin, H.I., 1999. The murine gammaherpesvirus 68 v-cyclin gene is an oncogene that promotes cell cycle progression in primary lymphocytes. J. Virol. 73 (6), 5110–5122.

van Dyk, L.F., Virgin, H.W.t., Speck, S.H., 2000. The murine gammaherpesvirus 68 v-cyclin is a critical regulator of reactivation from latency. J. Virol. 74 (16), 7451–7461.

Verschuren, E.W., Klefstrom, J., Evan, G.I., Jones, N., 2002. The oncogenic potential of Kaposi's sarcoma-associated herpesvirus cyclin is exposed by p53 loss in vitro and in vivo. Cancer Cell 2 (3), 229–241.

Verschuren, E.W., Hodgson, J.G., Gray, J.W., Kogan, S., Jones, N., Evan, G.I., 2004a. The role of p53 in suppression of KSHV cyclin-induced lymphomagenesis. Cancer Res. 64 (2), 581–589.

Verschuren, E.W., Jones, N., Evan, G.I., 2004b. The cell cycle and how it is steered by Kaposi's sarcoma-associated herpesvirus cyclin. J. Gen. Virol. 85 (Pt. 6), 1347–1361.

Virgin, H.W.t., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K.E., Dal Canto, A.J., Speck, S.H., 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. 71 (8), 5894–5904.

Vlach, J., Hennecke, S., Amati, B., 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. 16 (17), 5334–5344.

Wang, Y., Prives, C., 1995. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. Nature 376 (6535), 88–91.

Wong, L.Y., Matchett, G.A., Wilson, A.C., 2004. Transcriptional activation by the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen is facilitated by an N-terminal chromatin-binding motif. J. Virol. 78 (18), 10074–10085.

Xu, Y., 2003. Regulation of p53 responses by post-translational modifications. Cell Death Differ. 10 (4), 400–403.

Zarkowska, T., Mittnacht, S., 1997. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. 272 (19), 12738–12746.

Zhang, T., Prives, C., 2001. Cyclin a–CDK phosphorylation regulates MDM2 protein interactions. J. Biol. Chem. 276 (32), 29702–29710.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., Dean, D.C., 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101 (1), 79–89.