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Modulation of $p27^{Kip1}$ levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus

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DNA tumour viruses have evolved a number of mechanisms by which they deregulate normal cellular growth control. We have recently described the properties of a cyclin encoded by human herpesvirus 8 (also known as Kaposi's sarcoma-associated herpesvirus) which is able to resist the actions of $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$ cdk inhibitors. Here we investigate the mechanism involved in the subversion of a G_1 blockade imposed by overexpression of $p27^{Kip1}$. We demonstrate that binding of K cyclin to cdk6 expands the substrate repertoire of this cdk to include a number of substrates phosphorylated by cyclin–cdk2 complexes but not cyclin D1–cdk6. Included amongst these substrates is $p27^{Kip1}$ which is phosphorylated on Thr187. Expression of K cyclin in mammalian cells leads to $p27^{Kip1}$ downregulation, this being consistent with previous studies indicating that phosphorylation of $p27^{Kip1}$ on Thr187 triggers its downregulation. K cyclin expression is not able to prevent a G_1 arrest imposed by $p27^{Kip1}$ in which Thr187 is mutated to non-phosphorylatable Ala. These results imply that K cyclin is able to bypass a $p27^{Kip1}$-imposed G_1 arrest by facilitating phosphorylation and downregulation of $p27^{Kip1}$ to enable activation of endogenous cyclin–cdk2 complexes. The extension of the substrate repertoire of cdk6 by K cyclin is likely to contribute to the deregulation of cellular growth by this herpesvirus-encoded cyclin.

Keywords: cyclin//cdk/$p27^{Kip1}$/human herpesvirus 8/ Kaposi's sarcoma

Introduction

The critical period in cell-cycle control occurs late in the first growth phase (G_1), when cells must assess whether to irrevocably commit themselves to divide or to enter a quiescent state. This transition is determined by the balance between positive and negative factors: the D-type cyclins (D1, D2 and D3) complexed to cyclin-dependent kinases (cdk) 4 or 6 and cyclin E–cdk2 promote cell cycling whilst cdk inhibitors such as $p16^{Ink4a}$ and $p27^{Kip1}$ retard cell division (reviewed in Sherr and Roberts, 1995; Weinberg, 1995; Bartek *et al*., 1996). The central role of these molecules in the regulation of proliferation is emphasized by the frequency of anomalies in their expression and activities in human cancer (reviewed in Hall and Peters, 1996).

Elevations in the levels of $p27^{Kip1}$ are implicated in the maintenance of quiescence in a number of cell types (Kato *et al.*, 1994; Nourse *et al.*, 1994; Coats *et al.*, 1996; Rivard *et al.*, 1996). Although mutations in the $p27^{Kip1}$ gene are rare (Kawamata *et al.*, 1995; Pientenpol *et al.*, 1995; Ponce-Castaneda *et al.*, 1995), it is becoming increasingly clear that aberrant levels of $p27^{Kip1}$ can have a significant bearing on prognosis in a number of human cancers. For example, low levels of $p27^{Kip1}$ correlate with poor rates of survival for breast and colorectal carcinoma patients (Catzavelos *et al*., 1997; Porter *et al*., 1997) and aggressive colorectal carcinomas often exhibit elevated levels of $p27^{Kip1}$ degradation (Loda *et al*., 1997). It is evident, therefore, that the cellular mechanisms which regulate the levels of this cdk inhibitor must be stringently controlled. This has been demonstrated experimentally in T lymphocytes and serum-deprived fibroblasts where elevated $p27^{Kip1}$ levels appear to be the primary determinant in the maintenance of the quiescent state (Nourse *et al.*, 1994; Coats *et al.*, 1996; Rivard *et al.*, 1996).

One apparent mechanism by which $p27^{Kip1}$ levels can be modulated is through ubiquitin-mediated proteolysis (Pagano *et al*., 1995). Regulation of ubiquitin-mediated proteolysis is often achieved by phosphorylation of the target protein making it more susceptible to degradation (for example, see Chen *et al*., 1995; Lanker *et al.*, 1996). This may also be the case with $p27^{Kip1}$ since its downregulation is enhanced through cyclin E–cdk2-mediated phosphorylation on Thr187 (Muller *et al.*, 1997; Sheaff *et al*., 1997; Vlach *et al.*, 1997), although a causal link to ubiquitin-mediated degradation has not been demonstrated.

Much of our understanding of growth control in eukaryotes stems from the study of its dysregulation by viral proteins. For example, the ability of the simian virus 40 (SV40) T antigen, adenovirus E1A and human papilloma Virus E7 to displace proteins sequestered by the retinoblastoma protein (pRb) led to the realization of the central role of pRb in preventing S phase entry (reviewed in Ewen, 1994). We have recently described a novel mechanism by which viral proteins can subvert normal growth control: cyclins encoded by certain γ-herpesviruses can confer resistance to their associated catalytic cdk subunit against the cdk inhibitor proteins (Swanton *et al.*, 1997). One such cyclin, K cyclin, is encoded by human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma-associated herpesvirus. This human tumour virus is strongly implicated as a causative agent for Kaposi's sarcoma (Schulz *et al.*, 1998) and a number of lymphoproliferative disorders including body cavity-based lymphomas (Cesarman *et al.*, 1995). K cyclin forms

an active complex with cdk6 (Chang *et al.*, 1996). The resistance of this cyclin–cdk complex to inhibition by $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$ enables efficient subversion of G_1 arrests imposed by elevated levels of these cdk inhibitors (Swanton *et al.*, 1997).

Here we describe experiments designed to address the mechanism by which K cyclin overcomes a $p27^{Kip1}$-imposed growth arrest. We show that the K cyclin–cdk6 complex can phosphorylate $p27^{Kip1}$ on Thr187, thus triggering the degradation of this cdk inhibitor by the proteasome. The phosphorylation of $p27^{Kip1}$ results from the ability of K cyclin to extend the range of protein targets that can be phosphorylated by cdk6. The combination of resistance to inhibition and modulation of cdk substrate specificity by K cyclin is likely to be important for HHV8-induced deregulation of normal growth control.

Results

Overexpression of $p27^{Kip1}$ in mammalian cells leads to arrest in G_1 due to the action of this cdk inhibitor on G_1-specific cyclin–cdk complexes (Kato *et al.*, 1994; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). We have recently demonstrated that K cyclin can efficiently bind to and activate cdk6 and that K cyclin overexpression can bypass a $p27^{Kip1}$-imposed G_1 arrest (Swanton *et al.*, 1997). A $p27^{Kip1}$-imposed G_1 blockade is achieved by inhibition of not only cdk4/6-containing complexes, but also inhibition of cyclin E–cdk2. Thus, we sought to address the mechanism by which this effect was achieved with a view to further understanding the role of K cyclin in subversion of G_1/S control. A number of mechanisms could explain the ability of K cyclin overexpression to circumvent $p27^{Kip1}$-mediated G_1 arrest: (i) K cyclin may complex with cdk2 (as well as with cdk6) to functionally complement cyclin E and form active $p27^{Kip1}$-resistant holoenzymes with cdk2; (ii) K cyclin–cdk6 complexes may exhibit an extended substrate repertoire such that they can functionally substitute for cyclin–cdk2 complexes; (iii) K cyclin expression may facilitate activation of the endogenous cyclin–cdk2 complexes by, for example, promoting the phosphorylation and subsequent downregulation of $p27^{Kip1}$.

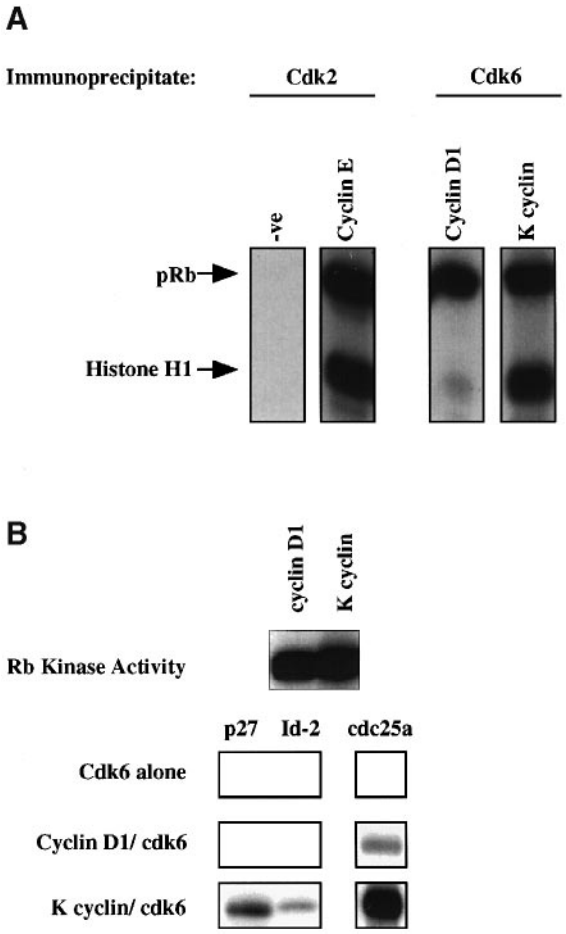

Fig. 1. Characterization of the substrate specificity of K cyclin–cdk6 complexes. (**A**) Lysates of Sf9 cells co-infected with recombinant baculovirus directing expression of either K cyclin and cdk6 or cyclin E and cdk2 were immunoprecipitated through the cdk subunit and assayed for their ability to phosphorylate pRb or histone H1. (**B**) Lysates of Sf9 cells co-infected with recombinant baculovirus directing expression of cdk6 and either cyclin D1 or K cyclin were immunoprecipitated through the cdk subunit. Immunoprecipitates were normalized for pRb kinase activity (upper panel). Equivalent amounts of cyclin D1–cdk6 and K cyclin–cdk6 pRb kinase activity were assayed for their ability to phosphorylate the cdk2 substrates $p27^{Kip1}$, Id-2 and cdc25a.

K cyclin extends the substrate repertoire of cdk6

To test the substrate range of specific holoenzymes, we co-infected Sf9 cells with recombinant baculovirus directing the expression of a cyclin and a cdk. Lysates from these cells were immunoprecipitated through the cdk component and the immune complexes were tested for their ability to phosphorylate various substrates. Figure 1A demonstrates that all cyclin–cdk combinations tested generated immunoprecipitable kinase activity against the C-terminus of pRb synthesized in bacteria as a glutathione *S*-transferase (GST) fusion protein. Immunoprecipitations from control lysates did not yield detectable kinase activity demonstrating the specificity of the assay (Figure 1A). Histone H1 was also included in these assays and, in confirmation of previous observations (Chang *et al.*, 1996), K cyclin–cdk6 complexes could efficiently phosphorylate this substrate to significantly greater levels than observed with cyclin D1–cdk6. Indeed, with similar levels of pRb kinase activity, K cyclin–cdk6 was as efficient as cyclin E–cdk2 in phosphorylating histone H1.

We next tested the activity of these kinases against a panel of cdk2 substrates (cdc25a, Id-2 and $p27^{Kip1}$; Hoffmann *et al.*, 1994; Hara *et al.*, 1997; Muller *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). Baculovirus-infected Sf9 cell lysates were immunoprecipitated through cdk6 and the kinase activity of each immune complex was assessed against pRb (Figure 1B). Then, using equivalent amounts of pRb kinase activity, each immunoprecipitate was tested for its ability to phosphorylate $p27^{Kip1}$, Id-2 and cdc25a. As shown in Figure 1B, K cyclin–cdk6 was able to phosphorylate each of these cdk2 substrates whereas cyclin D1–cdk6 was a very inefficient kinase against these proteins. Thus, K cyclin–cdk6 displays an extended substrate repertoire when compared with cyclin D1–cdk6 and more closely resembles cyclin E–cdk2.

K cyclin can activate cdk2

We next tested the ability of K cyclin to bind to and activate cdk2. Cdks were *in vitro* transcribed and translated in the presence of ^{35}S methionine and mixed with an unlabelled *in vitro* transcribed and translated cyclin. After 30 min incubation, the samples were immunoprecipitated through the cyclin and the products resolved by SDS–PAGE (Figure 2A). Autoradiography revealed that K cyclin complexed efficiently with cdk6 and more weakly with cdk2 and cdk3. A similar pattern of interaction was seen with cyclin D1. In contrast to the strong binding of cyclin D1 to cdk4, interaction of K cyclin with cdk4 was weak although co-infection of insect cells with K-cyclin- and cdk4-expressing viruses does result in an active cdk complex (C.Swanton and N.Jones, unpublished observations).

To assess whether K cyclin–cdk2 complexes were active kinases, Sf9 cells were co-infected with recombinant baculovirus directing expression of cdk2 and either cyclin E or K cyclin. Lysates from these cells were tested for their ability to phosphorylate the C-terminus of pRb. Both cyclin–cdk2 complexes readily phosphorylated pRb (Figure 2B). Lysates from Sf9 cells infected with either a cyclin- or a cdk-expressing baculovirus did not yield significant pRb kinase activity (data not shown). We next tested the sensitivities of these activities to the cdk inhibitors p21^{Cip1} and p27^{Kip1}. p21^{Cip1} and p27^{Kip1} produced in bacteria were included in the kinase assays at increasing concentrations. Cyclin E–cdk2 activity was rapidly abolished by addition of either inhibitor, whereas K cyclin–cdk2 activity was at least 10-fold less sensitive to p21^{Cip1} and p27^{Kip1} than cyclin E–cdk2, with activity only fully abolished at the highest concentration of inhibitor tested. The K cyclin–cdk2 complexes were, however, more sensitive to p21^{Cip1} and p27^{Kip1} than K cyclin–cdk6 complexes (Swanton *et al.*, 1997). Thus, K cyclin can bind to and activate cdk2 to generate a holoenzyme that is partially resistant to the inhibitory action of p21^{Cip1} and p27^{Kip1}. K cyclin–cdk2 complexes, like K cyclin–cdk6 complexes, fail to bind stably to either p21^{Cip1} or p27^{Kip1} (results not shown; Swanton *et al.*, 1997).

The results described above showed that K cyclin could associate with and activate different cdks *in vitro*. We determined whether this was also the case *in vivo* by transfecting U2OS cells with an expression vector containing a flag-epitope tagged version of K cyclin. The cyclin was subsequently immunoprecipitated and associated proteins analyzed by Western blotting. Cells transfected with epitope-tagged cyclin D1 were similarly assayed. As shown in Figure 2C, K cyclin was found to be associated with cdk6, cdk4 and cdk2. In contrast, cyclin D1 associated efficiently with cdk4, poorly with cdk6 and not at all with cdk2. Thus, the *in vivo* data corroborated the pattern of cdk interaction seen with K cyclin *in vitro*. These results are at variance with the results of Godden-Kent *et al.* (1997) who demonstrated efficient interaction with cdk6 only. It is not clear why the two studies differ in this regard but may result from the use of different cell lines. The immunoprecipitated K cyclin complexes were active in phosphorylation of pRb and were insensitive to inhibition with p27^{Kip1} (data not shown).

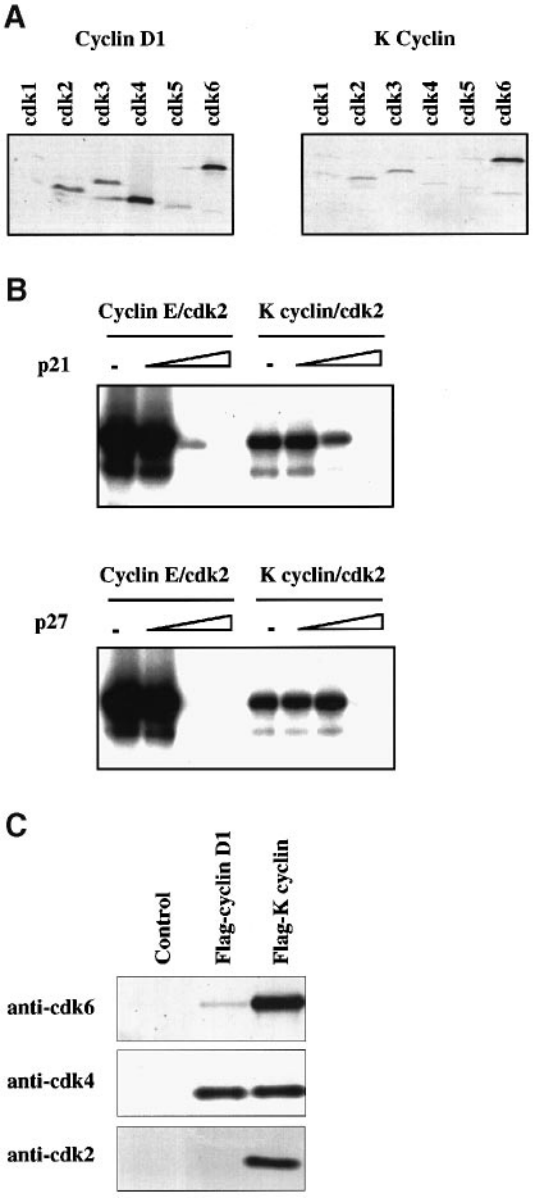

Fig. 2. K cyclin forms active complexes with cdk2. (**A**) cdk subunits were synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine and mixed with either Flag-tagged cyclin D1 or Flag-tagged K cyclin also synthesized by coupled-transcription/translation but with unlabelled methionine. After 30 min incubation, samples were immunoprecipitated through the epitope tag of the cyclin and products resolved by SDS–PAGE. Gels were dried and subjected to autoradiography to reveal the presence or absence of interacting labelled cdk subunits in the cyclin immunoprecipitate. (**B**) Lysates of Sf9 cells co-infected with recombinant baculovirus directing expression of either K cyclin and cdk2 or cyclin E and cdk2 were assayed for their ability to phosphorylate pRb in the absence or presence of the cdk inhibitors p21^{Cip1} (10, 100 or 1000 ng) or p27^{Kip1} (7.5, 75 or 750 ng). Reaction products were resolved by SDS–PAGE and visualized by autoradiography. (**C**) U2OS cells were transiently transfected with 10 μg of either empty pcDNA3 vector (control) or vector containing Flag-tagged K cyclin or cyclin D1. Lysates were prepared and subjected to immunoprecipitation with anti-flag antibody and the immunoprecipitated proteins subjected to immunoblot analysis with the indicated antibodies.

Phosphorylation and downregulation of p27^{Kip1} by K cyclin expression

Downregulation of p27^{Kip1} has been reported to be triggered by phosphorylation of the cdk inhibitor on

Thr187 by cyclin E–cdk2 complexes (Muller *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). Given that K cyclin–cdk6 complexes can phosphorylate $p27^{Kip1}$ (Figure 1B) we investigated whether this kinase can phosphorylate $p27^{Kip1}$ on this specific threonine. Sf9 cells were co-infected with baculoviruses directing the expression of cdk6 and K cyclin or cdk2 and cyclin E. Three days post-infection, cells were lysed and extracts used to phosphorylate the C-terminus of $p27^{Kip1}$ (amino acids 91–198 fused to GST) in the presence of [γ-^{32}P]ATP. Reaction products were resolved by SDS–PAGE, transferred to nylon membrane and digested with trypsin. Peptides were resolved by two-dimensional peptide mapping and phosphorylated products detected by autoradiography. GST–$p27^{Kip1}_{(91–198)}$ phosphorylated by either cyclin E–cdk2 or K cyclin–cdk6 generated maps with a single major phosphopeptide (Figure 3A and B, respectively). Mixing equal amounts of radioactivity from each tryptic digestion gave rise to a single phosphopeptide spot after two-dimensional mapping (Figure 3C), illustrating that these two cyclin–cdk holoenzymes phosphorylated GST–$p27^{Kip1}_{(91–198)}$ on peptides with identical electrophoretic and chromatographic properties. Phosphoamino acid analysis of this phosphopeptide from samples labelled with either K cyclin–cdk6 or cyclin E–cdk2 demonstrated that it contained only phosphothreonine (Figure 3G).

We next performed similar experiments using full-length $p27^{Kip1}$ fused to GST together with a mutant version of full-length $p27^{Kip1}$ in which Thr187 is replaced with Ala [GST–$p27^{Kip1}_{(T187A)}$]. Two-dimensional phosphopeptide mapping of the full-length wild-type $p27^{Kip1}$ fusion protein treated with K cyclin–cdk6 generated a single major phosphopeptide (Figure 3D) which was indistinguishable by mixing experiments with that from phosphorylated C-terminal $p27^{Kip1}$ (not shown) and contained only phosphothreonine (Figure 3G). Surprisingly, GST$p27^{Kip1}_{(T187A)}$ also generated a single major phosphopeptide after phosphorylation with K cyclin–cdk6 (Figure 3E). However, this phosphopeptide did not co-migrate with that from K cyclin–cdk6-treated wild-type $p27^{Kip1}$ (Figure 3F) and contained only phosphoserine (Figure 3G). Taken together, these results demonstrate that K cyclin–cdk6, like cyclin E–cdk2, can phosphorylate $p27^{Kip1}$ on Thr187. In addition, upon mutation of Thr187 to Ala, there is an increase in susceptibility of a Ser residue to phosphorylation by the K cyclin–cdk6 holoenzyme.

We next examined the effects of K cyclin expression on $p27^{Kip1}$ in terms of both phosphorylation status and protein abundance in mammalian cells. For these experiments, we used a cell line derived from NIH 3T3 cells in which K cyclin expression is under negative control of the bacterial *lacI* gene product and thus can be induced by the addition of isopropyl-β-D-galactopyranoside (IPTG) (NIH 3T3-K cells, Swanton *et al.*, 1997). Cells were quiesced by culture for 72 h in serum-depleted medium. K cyclin expression was then induced in the absence of serum stimulation and $p27^{Kip1}$ status analysed during the subsequent 24 h. K cyclin expression was clearly detectable by 8 h after addition of IPTG (Figure 4A). This induction correlates with an increase in the proportion of $p27^{Kip1}$ present in a slower migrating form; this slower mobility form of $p27^{Kip1}$ could be converted to the faster migrating form by treatment with calf intestinal alkaline

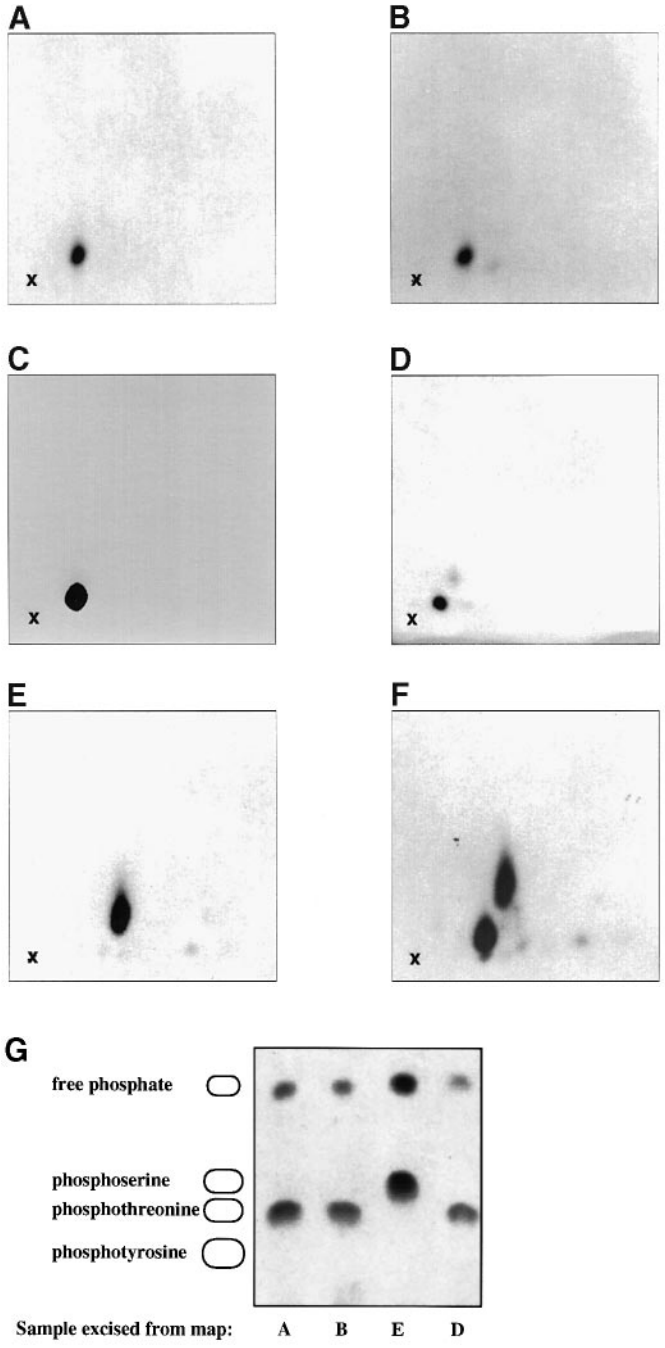

Fig. 3. Analysis of site of phosphorylation of $p27^{Kip1}$ by K cyclin–cdk6. A C-terminal fragment of $p27^{Kip1}$ synthesized as a GST fusion protein in bacteria was bound to glutathione–Sepharose beads and phosphorylated by either K cyclin–cdk6 (**A**) or cyclin E–cdk2 (**B**) produced in Sf9 cells via infection with recombinant baculovirus. Sepharose-bound C-terminal $p27^{Kip1}$ was washed with PBS plus 0.1% Tween 20 and resolved by SDS–PAGE. Radioactive bands were excised and subjected to tryptic digestion prior to two-dimensional phosphopeptide analysis. Mixing equal counts per minute from C-terminal $p27^{Kip1}$ phosphorylated by either K cyclin–cdk6 or cyclin E–cdk2 prior to phosphopeptide analysis yielded a single radioactive spot (**C**). Phosphorylation of full length, wild-type (**D**) or T187A mutant (**E**) $p27^{Kip1}$ fused to GST by K cyclin–cdk6 generated a single major phosphopeptide. Mixing equal counts per minute from K cyclin–cdk6 phosphorylated wild-type and T187A mutant $p27^{Kip1}$ generated two distinct phosphopeptides (**F**). Radioactive phosphopeptides from maps shown in (A, B, D and E) were recovered from cellulose plates and hydrolysed prior to phosphoamino acid analysis (**G**).

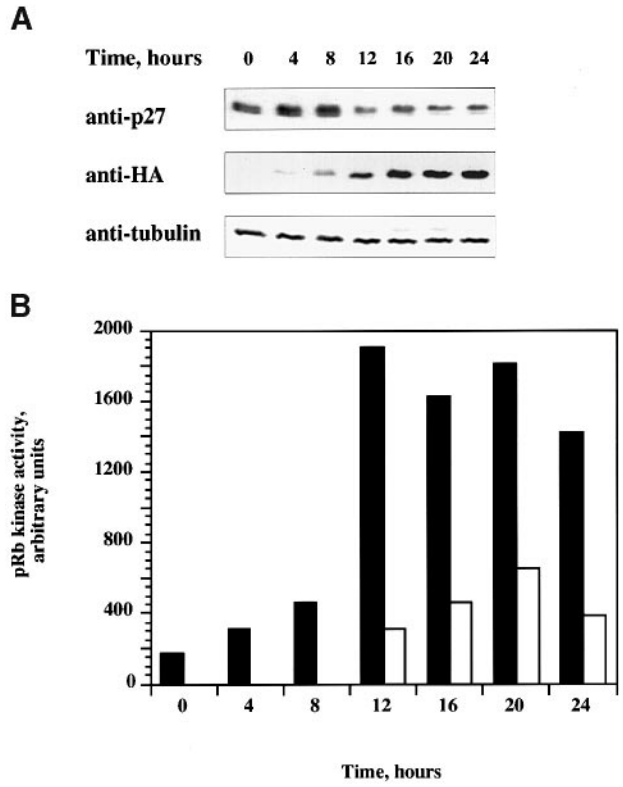

Fig. 4. K cyclin expression leads to $p27^{Kip1}$ hyperphosphorylation and downregulation. NIH 3T3-K cells were quiesced by maintenance in 0.2% serum for 72 h. Cultures were then washed with fresh Dulbecco's modified Eagle's medium (DMEM) plus 0.2% serum and incubated in the presence of 5 mM IPTG for the time indicated. Equal amounts of protein from extracts of these cells were immunoblotted with the indicated antibodies. The $p27^{Kip1}$ blot was reprobed sequentially with anti-HA antibody to detect K cyclin and then with anti-α-tubulin as a loading control (**A**). Equal amounts of protein from extracts of quiescent NIH 3T3-K cells treated for the indicated time with 5 mM IPTG were immunoprecipitated with the antibodies to HA epitope tag of K cyclin (solid bars) or to cyclin E (open bars) and assayed for kinase activity towards pRb (**B**).

phosphatase (not shown) as previously described (Muller *et al.*, 1997). Thus, this retarded gel mobility clearly reflects the increased phosphorylation status of $p27^{Kip1}$. The site of phosphorylation responsible for this mobility shift is Ser10 of $p27^{Kip1}$ rather than Thr187 (D.J.Mann and N.Jones, unpublished observations); in the *in vitro* assays using GST–$p27^{Kip1}$ fusions as substrates, Ser10 is phosphorylated very inefficiently, probably due to the proximity of this amino acid residue to the fusion site. Within 12 h of induction of K cyclin expression, all of the detectable $p27^{Kip1}$ had the mobility of the slower migrating phospho-form and the abundance of $p27^{Kip1}$ had decreased by >50% (Figure 4A). Parallel analysis of NIH 3T3-K cells in the absence of K cyclin expression indicated no change in the phosphorylation status or abundance of $p27^{Kip1}$ (results not shown). In addition, we measured the kinase activity associated with both K cyclin and cyclin E by immunoprecipitation kinase assays. K cyclin-associated kinase activity (in anti-HA epitope tag immunoprecipitations) was low in uninduced cells and increased in parallel to increasing levels of K cyclin protein (Figure 4A and B). Cyclin E-associated kinase activity was undetectable at early time points but increased as $p27^{Kip1}$ levels declined (Figure 4B). Thus, the phosphorylation and downregulation of $p27^{Kip1}$ correlated precisely with the increase in K cyclin-associated kinase activity. The lack of cyclin E-associated kinase activity at early time points indicates that $p27^{Kip1}$ phosphorylation and downregulation are unlikely to be due to the cyclin E–cdk2 holoenzyme. In addition, preliminary estimates place the half life of $p27^{Kip1}$ in the presence of K cyclin at ~80 min compared with 270 min when K cyclin expression is repressed (D.J.Mann, unpublished results). Taken together, these data are consistent with a model in which K cyclin-dependent phosphorylation of $p27^{Kip1}$ on Thr187 triggers the subsequent downregulation of the cdk inhibitor leading to enhanced cyclin E-associated kinase activity.

To assess whether $p27^{Kip1}$ downregulation was a specific event in cells expressing K cyclin, subconfluent growing cultures of NIH 3T3-K cells were maintained in the absence or presence of IPTG for 48 h. Cells in which K cyclin expression was induced contained significantly lower levels (3- to 4-fold) of $p27^{Kip1}$ than parallel cultures of NIH 3T3-K cells in the absence of K cyclin expression (Figure 5A). Control cultures of parental NIH 3T3 cells contained invariant levels of $p27^{Kip1}$ irrespective of the absence or presence of IPTG (results not shown). Compared with serum-deprived quiescent cultures, asynchronous dividing cultures have significantly reduced levels of $p27^{Kip1}$. Thus, K cyclin expression caused the further downregulation of this already depressed basal level of cellular $p27^{Kip1}$. Induction of K cyclin expression did not significantly affect the levels of other proteins involved in cell-cycle control (cyclins D1, D3 or E or cdks 4 or 6, see Figure 5A), demonstrating the specificity of the K cyclin-dependent downregulation of $p27^{Kip1}$. Downregulation of cyclins D1 and E has, like $p27^{Kip1}$, been demonstrated to involve ubiquitin-mediated proteolysis (Clurman *et al.*, 1996; Won and Reed, 1996; Diehl *et al.*, 1997). Indeed, the downregulation of $p27^{Kip1}$ by K cyclin induction could be blocked by LLnL, an inhibitor of cysteine proteases and the proteasome but not by the cysteine protease inhibitor E64 (Figure 5B). These observations indicate that the phosphorylation and downregulation of $p27^{Kip1}$ by K cyclin expression is a specific event involving the proteasome and not a general property of K cyclin on elevating such cellular degradation.

K cyclin-mediated downregulation of $p27^{Kip1}$ requires cdk6

The data presented thus far indicate that K cyclin expression is associated with phosphorylation and downregulation of $p27^{Kip1}$. We have also demonstrated that K cyclin can bind to and activate not only cdk6 but also cdk2, albeit with less efficiency *in vitro* (Figure 2). In order to dissect the role of these two distinct cdk subunits in the downregulation of $p27^{Kip1}$, we utilized dominant negative versions of each cdk in which a key catalytic Asp residue (D145 in cdk2 and D163 in cdk6) is mutated to Asn. These mutant cdks are catalytically inactive but can still bind to their cognate cyclins and thus can be used as dominant negative proteins (van den Heuval and Harlow, 1993). We transfected NIH 3T3-K cells with plasmids directing the expression of green fluorescent protein (GFP) and dominant negative versions of either cdk2 or cdk6. IPTG or vehicle was added to the cells

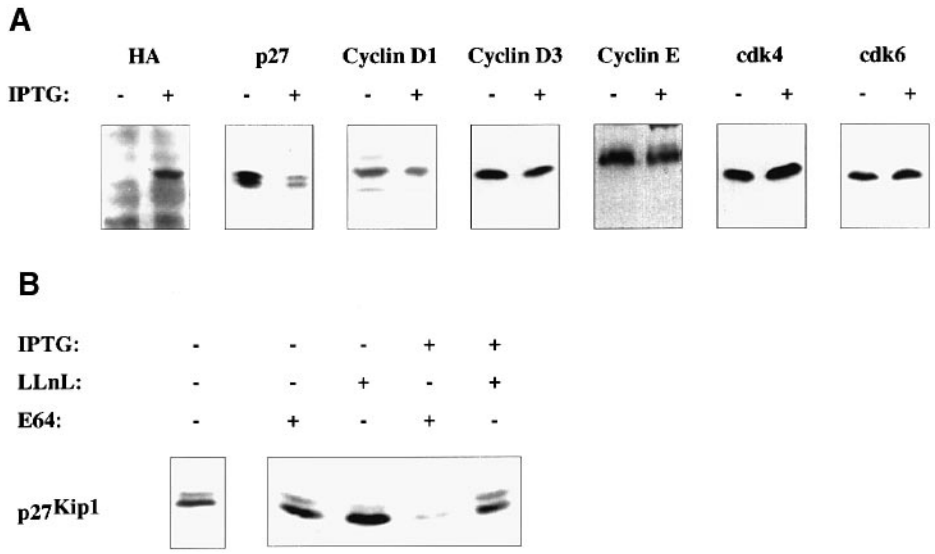

Fig. 5. Specificity of K cyclin-dependent $p27^{Kip1}$ downregulation and involvement of the proteasome in this process. NIH 3T3-K cells were maintained in DMEM plus 10% serum in the absence or presence of 5 mM IPTG for 48 h prior to cell lysis. Equal amounts of protein from extracts of these cells were immunoblotted with the indicated antibodies (**A**). To investigate whether the downregulation of $p27^{Kip1}$ upon K cyclin expression was sensitive to inhibitors of the proteasome, NIH 3T3-K cells were maintained in DMEM plus 10% serum in the absence or presence of 5 mM IPTG for 48 h. LLnL (which inhibits cysteine proteases and the proteasome) or E64 (which inhibits non-proteasomal cysteine proteases) were then added to 50 μM final concentration and the cells harvested 4 h later. Equal amounts of protein from extracts of these cells were immunoblotted with anti-$p27^{Kip1}$ antisera (**B**).

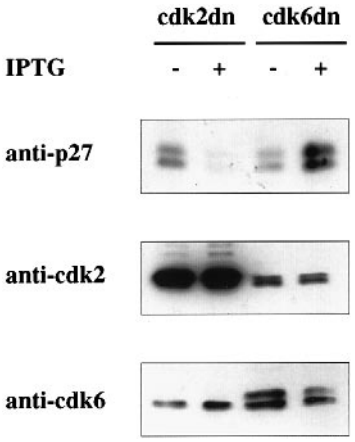

Fig. 6. K cyclin–cdk6 is responsible for $p27^{Kip1}$ downregulation. NIH 3T3-K cells were transfected with plasmids directing expression of GFP and dominant negative versions of either cdk2 or cdk6. Forty-eight hours after transfection, IPTG (5 mM) or vehicle was added to the cells and 14 h later the cells were trypsinized and collected. Fluorescence-activated cell sorting was then used to separate the GFP-positive transfected cells from the bulk untransfected cell population. Cells were lysed and protein from 1×10^5 cells was resolved by SDS–PAGE and subjected to immunoblotting with the antibody indicated. Dominant negative cdk6 runs with a retarded mobility due to the presence of an epitope tag.

48 h after transfection and 14 h later the cells were trypsinized. Fluorescence-activated cell sorting was then used to separate the GFP-positive transfected cells from the bulk untransfected cell population. Equal numbers of GFP-positive cells expressing either dominant negative cdk2 or dominant negative cdk6 in the absence or presence of K cyclin were lysed and subjected to immunoblot analysis to determine the levels of $p27^{Kip1}$ (Figure 6). In the presence of K cyclin expression, dominant negative cdk6 but not dominant negative cdk2 effectively blocked the K cyclin-mediated downregulation of $p27^{Kip1}$. These data are consistent with a model in which K cyclin–cdk6 but not K cyclin–cdk2 is responsible for $p27^{Kip1}$ downregulation. In the absence of K cyclin expression, cells expressing dominant negative cdk2 contained approximately twice as much $p27^{Kip1}$ as cells expressing dominant negative cdk6, indicating that $p27^{Kip1}$ levels are responsive to inhibition of cyclin–cdk2 complexes, as demonstrated previously (Sheaff *et al.*, 1997; Vlach *et al.*, 1997).

K cyclin-dependent escape from $p27^{Kip1}$-mediated G_1 arrest relies on Thr187 of $p27^{Kip1}$

We next addressed the importance of $p27^{Kip1}$ phosphorylation and downregulation to the ability of K cyclin to circumvent the $p27^{Kip1}$-imposed G_1 arrest by utilizing the Thr187 to Ala (T187A) mutant version of $p27^{Kip1}$. This mutant was tested for its ability to exert a G_1 arrest. Transient transfection of wild-type and T187A mutant $p27^{Kip1}$ into U2OS cells demonstrated that both forms of $p27^{Kip1}$ efficiently arrested cells in G_1 phase (Figure 7); both proteins were expressed at similar levels as judged by immunoblotting, although the T187A mutant $p27^{Kip1}$ was ~25% more abundant than wild-type $p27^{Kip1}$ (results not shown). We next repeated these experiments but cotransfected K cyclin and again examined the increase in the G_1 cell population. As shown in Figure 7, K cyclin expression enabled cells to efficiently bypass a G_1 arrest imposed by wild-type $p27^{Kip1}$. However, overexpression of T187A mutant $p27^{Kip1}$ caused a G_1 arrest which was resistant to K cyclin expression. These results demonstrate that the release from a $p27^{Kip1}$-mediated G_1 blockade by K cyclin expression is dependent on the presence of Thr187. Taken together with the other evidence presented, these data imply that the ability of K cyclin to overcome a $p27^{Kip1}$-mediated G_1 arrest is linked to the ability of K cyclin–cdk6 complex to phosphorylate $p27^{Kip1}$ and thereby facilitate its downregulation.

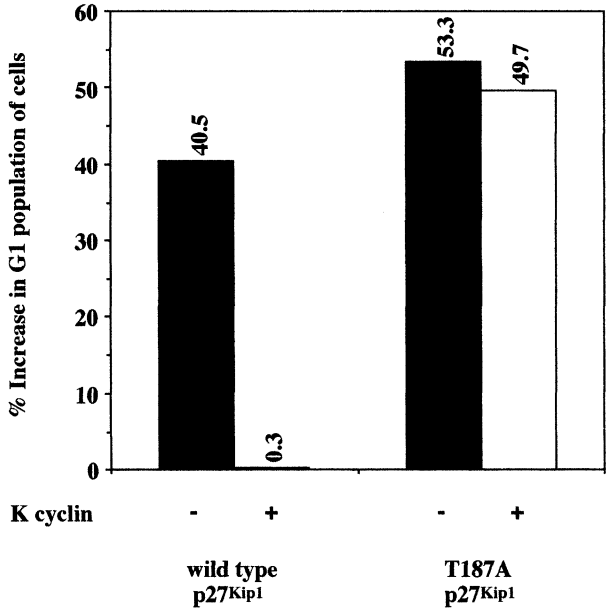

Fig. 7. K cyclin expression cannot prevent G_1 arrest imposed by mutant $p27^{Kip1}$. U2OS cells were transfected with plasmids directing the expression of the cell surface marker CD8, either wild-type or T187A mutant $p27^{Kip1}$ and either pcDNA3 (solid bars) or pcDNA3-K cyclin (white bars). Seventy-two hours after transfection cells were harvested and the cell-cycle distribution of the CD8 positive cells determined. Data are represented as the percentage change in the number of cells in the G_1 phase of the cell cycle relative to the G_1 population of cells transfected with cell surface marker only. The bars represent the mean of duplicate transfections from a single experiment and are representative of three separate experiments each performed in duplicate. At least 15 000 cells were gated for each sample. Untransfected cells displayed the following cell-cycle distribution: G_1, 47%; S, 21%; G_2/M, 32%.

Discussion

By restricting cyclin–cdk activity, the cdk inhibitors provide a powerful means of preventing cell proliferation. Overexpression of $p16^{Ink4a}$ or $p27^{Kip1}$ in mammalian cells leads to arrest in G_1 due to the action of these cdk inhibitors on G_1-specific cyclin–cdk complexes, $p16^{Ink4a}$ inhibiting the cdks associated with the D-type cyclins (that is, cdk4 and cdk6) and $p27^{Kip1}$ inhibiting both cyclin D- and cyclin E-dependent activities (Serrano *et al.*, 1993; Kato *et al.*, 1994; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Lukas *et al.*, 1995).

We have recently demonstrated that the cyclin encoded by ORF 72 of HHV8 (K cyclin) is able to circumvent G_1 arrests imposed by both the $p16^{Ink4a}$ and $p27^{Kip1}$ cdk inhibitors and allow S phase entry in the presence of these growth repressors (Swanton *et al.*, 1997). K cyclin-mediated suppression of the G_1 arrest imposed by overexpression of $p16^{Ink4a}$ can be readily explained by the preference of the viral cyclin for binding to and activation of the endogenous cdk6 (Chang *et al.*, 1996), the K cyclin–cdk6 complex being resistant to $p16^{Ink4a}$-mediated inhibition (Swanton *et al.*, 1997). Thus, K cyclin can functionally substitute for the D-type cyclins in activating cdk6. However, the G_1 arrest imposed by $p27^{Kip1}$ is enforced by blocking the action of both the D-type cyclin–cdks and cyclin E–cdk2 complexes. Therefore, the expression of K cyclin must overcome the requirement for both cyclin–cdk complexes in promoting the $G_1 \rightarrow S$ transition. In this report we have explored the mechanism of this phenomenon. We show that it is due to an expanded repertoire of substrates that can be phosphorylated by K cyclin–cdk6 when compared with cyclin D1–cdk6. One of these substrates is the $p27^{Kip1}$ inhibitor itself, which upon phosphorylation is downregulated. By promoting such downregulation, K cyclin overcomes a $p27^{Kip1}$-imposed G_1 arrest.

Previous studies have demonstrated that both K cyclin and the structurally similar V cyclin (encoded by the related herpesvirus saimiri; Jung *et al.*, 1994), when complexed to cdk6, generated efficient pRb kinases (Jung *et al.*, 1994; Chang *et al.*, 1996; Swanton *et al.*, 1997). When we compared the preference of K cyclin–cdk6 with that of cyclin D1–cdk6 for phosphorylation of pRb or histone H1, the viral cyclin-directed kinase showed little discrimination between these two standard substrates whereas cyclin D1–cdk6 showed a strong preference for pRb (Figure 1). This result suggested that the virally encoded cyclin may broaden the substrate specificity of the associated cdk6 subunit. We tested the ability of K cyclin–cdk6 complexes to phosphorylate three cdk2 substrates: the cdk inhibitor $p27^{Kip1}$ (Muller *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997), the basic helix–loop–helix transcription factor Id-2 (Hara *et al.*, 1997) and the dual specificity phosphatase cdc25a (Hoffmann *et al.*, 1994). In all three cases, efficient phosphorylation of the cdk2 substrate was observed by K cyclin–cdk6 but not, or very weakly, by cyclin D1–cdk6 (Figure 1). These results strongly support the idea that K cyclin binding can extend the substrate range of cdk6 to include at least a subset of cdk2 substrates (although our results indicate that K cyclin–cdk6 cannot phosphorylate all cdk2 substrates *in vivo*, see below). The mechanism of this extended substrate repertoire is unknown at present. The parameters that dictate cdk substrate specificity are poorly defined so that analysis of this property of the viral cyclins should prove to be informative. We are currently trying to distinguish between two possibilities: (i) that cyclin–substrate interactions dictate cdk specificity or (ii) that cyclin-imposed constraints on cdk structure determine cdk specificity. This second model is attractive in that it may account for the more efficient activation of cdk6 by K cyclin (when compared with cyclin D1) and also the ability of K cyclin–cdk6 complexes to resist inhibition by the $p16^{Ink4a}$ cdk inhibitor (Swanton *et al.*, 1997).

The identification of $p27^{Kip1}$ and cdc25a as substrates for K cyclin–cdk6 suggests the possibility that their phosphorylation plays an important role in K cyclin-mediated stimulation of S phase entry. Phosphorylation of cdc25a has been shown to stimulate its phosphatase activity *in vitro* (Hoffmann *et al.*, 1994), implying that cdc25a has greater potential to activate endogenous cdks by catalysing the removal of inhibitory threonine and tyrosine phosphorylations. Phosphorylation of the cdk inhibitor $p27^{Kip1}$ on Thr187 leads to the downregulation of $p27^{Kip1}$ protein (Muller *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). We have provided evidence that K cyclin–cdk6 can phosphorylate $p27^{Kip1}$ on this threonine residue *in vitro* (Figure 3) and *in vivo* (Figure 6) and that K cyclin induction in NIH 3T3 fibroblasts leads to a reduction in the endogenous $p27^{Kip1}$ levels through proteasome-mediated degradation (Figure 4). The

importance of this downregulation is clearly demonstrated by the inability of K cyclin to overcome a G_1 arrest imposed by $p27^{Kip1}$ when Thr187 is changed to Ala (Figure 7).

The resistance of the G_1 blockade imposed by the Thr187→Ala substitution mutant of $p27^{Kip1}$ to K cyclin expression indicates that K cyclin is not sufficient to enforce DNA synthesis when $p27^{Kip1}$ levels remain elevated. These data imply that S phase entry requires the recruitment of cellular factors that are sensitive to elevated $p27^{Kip1}$ levels. One likely candidate for this role is cyclin E. Our data are compatible with a model in which the viral cyclin is able to fulfil only part of the role of cyclin E–cdk2 *in vivo* and that by reducing the levels of $p27^{Kip1}$, K cyclin expression can lead to the generation of an environment in which cyclin E–cdk2 activity is favoured enabling co-operation between the viral cyclin and the endogenous cyclin E to facilitate S phase entry.

The ability to override normal growth control and force cells to cycle is a common characteristic of DNA tumour viruses. This property is essential to viral propagation since it ensures the appropriate cellular environment for viral replication. To achieve S phase entry, both cyclin D- and cyclin E-dependent activities must be stimulated or their actions mimicked. DNA tumour viruses have evolved systems by which to accomplish this feat. In the case of HHV8, we have shown that this is likely to derive from the production of cyclin–cdk complexes which are resistant to inhibition and which display broader target specificity. Other DNA tumour viruses achieve similar ends by different means. For example, adenovirus 5 overcomes G_1 arrest largely through the action of the E1A oncoprotein which displaces cellular factors from pRb sequestration (Bagchi *et al.*, 1990; Bandara and La Thangue, 1991; Chellappan *et al.*, 1991), thus negating the requirement for cyclin D–cdk activity (Lukas *et al.*, 1995), and binds to and inactivates $p27^{Kip1}$ thereby freeing cdk2-containing complexes (Mal *et al.*, 1996).

K cyclin not only has the capacity to interact with cdk6 (Chang *et al.*, 1996) but also weakly interacts with cdk2, cdk3 and cdk4 (this report). In each case, the resulting complexes are able to phosphorylate pRb *in vitro* (this study; C.Swanton and N.Jones, unpublished observations). These observations highlight important differences between K cyclin and D type cyclins, especially cyclin D1, with which K cyclin shows greatest similarity. Cyclin D1 fails to activate any cdk other than cdk4 or cdk6 (C.Swanton and N.Jones, unpublished observations). At present it is not clear whether the ability of K cyclin to form active complexes with these other kinase partners is physiologically relevant, although both cdk2 and cdk3 appear to have essential roles in the G_1→S transition since overexpression of dominant negative mutants of each leads to G_1 arrest (van den Heuval and Harlow, 1993; Hofmann and Livingston, 1996). However, of all of the binary combinations of K cyclin and cdk subunits, the K cyclin binds to cdk6 most efficiently and the resulting complex appears to be the most active and exhibits the greatest resistance to cdk inhibitors. It is likely, therefore, that activation of cdk6 is the critical function of K cyclin. This conclusion is substantiated by the observation that cdk6 is an abundant cdk in lymphocytes (Meyerson and Harlow, 1994), the target cell type of human herpesvirus 8. Given that lymphocytes are largely maintained in G_0/G_1 by high levels of $p27^{Kip1}$ (Nourse *et al.*, 1994; Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Luo *et al.*, 1996; Nakayama *et al.*, 1996), the properties of K cyclin which we describe are likely to be of fundamental importance to viral-mediated deregulation of normal growth control.

Materials and methods

Plasmids and antibodies

The following plasmids have been described previously: CD8, pJ7Ω-$p27^{Kip1}$ (Mann and Jones, 1996), pRSET-$p21^{Cip1}$, pRSET-$p27^{Kip1}$, pRSET-K cyclin (Swanton *et al.*, 1997), all pKS-cdk constructs and pKS-cyclin D1 (Parry *et al.*, 1995). Epitope-tagged dominant negative cdk2 and cdk6 (van den Heuval and Harlow, 1993) were subcloned into pEFcx (a gift from Dr R.Treisman, Imperial Cancer Research Fund, UK).

K cyclin and cyclin D1 were epitope tagged at their N-termini with two FLAG epitopes by ligating a double-stranded oligonucleotide into the unique *Nco*I site of both cyclins (this restriction site includes the initiating methionine codon) in the pRSET constructs (Swanton *et al.*, 1997). The oligonucleotide sequences were 5′-CATGGACTACAAGGACGAC-GACGACAAGGACTACAAGGACGACGACGACAAGGC-3′ (coding) and 5′-CATGGCCTTGTCGTCGTCGTCCTTGTAGCCCTTGTCGTC-GTCGTCCTTGTAGTC-3′. The tagged cyclins were transferred to pvl1393 (Invitrogen) via *Bam*HI–*Eco*RI digestion. Baculovirus directing the expression of cyclin E was a gift from C.Sherr, cdk2 from D.Morgan and cdk6 from M.Meyerson. For expression in mammalian cells, the fragments containing the tagged cyclins were transferred into the pcDNA3 vector (Invitrogen).

GST–cdc25a and GST–Id-2 were gifts from H.Okayama and E.Hara, respectively. GST–$p27^{Kip1}$ was created by subcloning the $p27^{Kip1}$ coding sequence from pJ7Ω (Mann and Jones, 1996) into pGEX-KG. To generate the $p27^{Kip1}$ C-terminus as a GST fusion, PCR was performed using GST–$p27^{Kip1}$ as template and oligonucleotides 5′-GCGCCC-ATGGAGCCCCCGCGGCCCCCAAAGGTGCCTGC-3′ and 5′-GC-GCCTCGAGTTACGTTTGACGTCTTCTGAGGCCAGGCTTC-3′. The resulting fragment was digested with *Nco*I and *Xho*I and ligated into pGEX-KG cut with the same enzymes. For transfection experiments, $p27^{Kip1}$ was subcloned into pcDNA3 (Invitrogen) from pRSET-$p27^{Kip1}$ using *Bam*HI and *Eco*RI. The Thr187→Ala substitution mutant was created by PCR using pcDNA3-$p27^{Kip1}$ as template and oligonucleotides and 5′-GCGCGGATCCATGTCAAACGTGCGAGTGTC-3′ and 5′-GCGCGAATTCTACGTTTGACGTCTTCTGAGGCCAGGCTTCT-TGGGCGCCTGC-3′. The PCR product was digested with *Bam*HI and *Eco*RI and subcloned into pcDNA3 cut with the same enzymes. The DNA sequence of all PCR products was verified.

The following antibodies were used: anti-HA (12CA5, Boehringer Mannheim, 1538 816), anti-$p27^{Kip1}$ (Santa Cruz, sc-528 and sc-527), anti-cyclin E (Santa Cruz, sc-481), anti-cyclin D3 (Santa Cruz, sc-182), anti-cyclin D1 (a gift from Dr G.Peters, Imperial Cancer Research Fund), anti-cdk4 (SantaCruz, sc-260), anti-cdk6 (Santa Cruz, CB02), anti-FLAG (Sigma, M2) and anti-α-tubulin (TAT-1, Imperial Cancer Research Fund antibody service).

Cells and baculovirus

NIH 3T3-K cells were isolated, cultured and quiesced as described (Swanton *et al.*, 1997). K cyclin expression was induced as follows: cells were washed twice with phosphate-buffered saline (PBS) and re-fed with DMEM containing either 0.2% or 10% fetal bovine serum (as appropriate) and 5 mM IPTG. When necessary, LLnL or E64 (Sigma) were added directly to the culture medium at 50 μM final concentration. Culture of U2OS cells, transfection, analysis of the cell-cycle distribution of transfected cells and fluorescence-activated cell sorting have been described (Mann and Jones, 1996). Recombinant baculovirus were produced using the BaculoGold system (PharMingen). Co-infection of Sf9 cells with recombinant baculoviruses has been described (Kato *et al.*, 1993).

In vitro binding assays and immunoblotting

In vitro binding assays were performed as described (Swanton *et al.*, 1997), cDNAs being transcribed and translated using the TNT system (Promega).

For immunoblotting, cells were washed twice with PBS, lysed *in situ*

in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) and collected by scraping. After normalizing for protein content, samples were boiled in 1× SDS–PAGE sample buffer and subjected to SDS–PAGE using 10% gels to resolve cyclins and cdks and 12.5% gels for $p27^{Kip1}$ analysis. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell). Individual proteins were detected using specific antisera, the immunoreactive bands being visualized by using appropriate horseradish peroxidase-conjugated secondary antibody and subsequent detection by enhanced chemiluminescence (Amersham).

In vitro kinase assays, phosphopeptide mapping and phosphoamino acid analysis

To generate extracts for use in *in vitro* kinase assays, Sf9 cells infected with appropriate recombinant baculoviruses were lysed as described (Kato *et al.*, 1993; Parry *et al.*, 1995). Extracts were either used directly or immunoprecipitated with an appropriate antibody. Immunoprecipitation kinase assays were performed as described (Matsushime *et al.*, 1994; Swanton *et al.*, 1997) using [γ-^{32}P]ATP (ICN), GST–pRb and/or histone H1 (Boehringer Mannheim). Inhibition experiments were performed as described (Swanton *et al*., 1997) using $p21^{Cip1}$ and $p27^{Kip1}$ isolated from bacteria harbouring the appropriate pRSET plasmid (kindly provided by M.Hall and G.Peters). GST fusion proteins were isolated from bacteria and bound to glutathione–Sepharose according to the manufacturer's instruction (Pharmacia).

For phosphopeptide mapping, GST-bound products from *in vitro* kinase assays (Parry *et al.*, 1995) were washed with PBS containing 0.1% Tween 20, resolved by SDS–PAGE and transferred to PVDF membrane (Du Pont). Radioactive bands were identified by autoradiography, excised, digested with trypsin (Sigma) and peptides resolved in two dimensions on cellulose thin layer chromatography plates (Kodak) as described (Boyle *et al.*, 1991). Peptide resolution was by electrophoresis at pH 1.9 in the first dimension followed by chromatography using 3:10:12:15 acetic acid:pyridine:water:butan-1-ol in the second dimension. Phosphoamino acid analysis was performed as described (Neufeld *et al.*, 1989).

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References

Bagchi,S., Raychaudhuri,P. and Nevins,J.R. (1990) Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A *trans*-activation. *Cell*, **62**, 659–669.

Bandara,L.R. and La Thangue,N.B. (1991) Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature*, **351**, 494–497.

Bartek,J., Bartkova,J. and Lukas,J. (1996) The retinoblastoma protein pathway and the restriction point. *Curr. Opin. Cell Biol.*, **8**, 805–814.

Boyle,W.J., van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Catzavelos,C. *et al*. (1997) Decreased levels of the cell-cycle inhibitor $p27^{Kip1}$ protein—prognostic implications in primary breast cancer. *Nature Med.*, **3**, 227–230.

Cesarman,E., Chang,Y.A., Moore,P.S., Said,J.W. and Knowles,D.M. (1995) Kaposi's sarcoma-associated herpesvirus-like DNA-sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.*, **332**, 1186–1191.

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

Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M. and Nevins,J.R. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell*, **65**, 1053–1061.

Chen,Z.J., Hagler,J., Palombella,V.J., Melandri,F., Scherer,D., Ballard,D. and Maniatis,T. (1995) Signal-induced site specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.*, **9**, 1586–1597.

Clurman,B.E., Sheaff,R.J., Thress,K., Groudine,M. and Roberts,J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.*, **10**, 1979–1990.

Coats,S., Flanagan,W.M., Nourse,J. and Roberts,J.M. (1996) Requirement of $p27^{Kip1}$ for restriction point control of the fibroblast cell cycle. *Science*, **272**, 877–880.

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

Ewen,M.E. (1994) The cell cycle and the retinoblastoma protein family. *Cancer Metastasis Rev.*, **13**, 45–66.

Fero,M.L. *et al.* (1996) A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis and female sterility in $p27^{Kip1}$-deficient mice. *Cell*, **85**, 733–744.

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

Hall,M. and Peters,G. (1996) Genetic alterations of cyclins, cyclin-dependent kinases and cdk inhibitors in human cancer. *Adv. Cancer Res.*, **68**, 67–108.

Hara,E., Hall,M. and Peters,G. (1997) Cdk2-dependent phosphorylation of Id-2 modulates activity of e2a-related transcription factors. *EMBO J.*, **16**, 332–342.

Hoffmann,I., Draetta,G. and Karsenti,E. (1994) Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G_1/S transition. *EMBO J.*, **13**, 4302–4310.

Hofmann,F. and Livingston,D.M. (1996) Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G_1 exit. *Genes Dev.*, **10**, 851–861.

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

Kato,J., Matsushime,H., Hiebert,S.W., Ewen,M.E. and Sherr,C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene-product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase cdk4. *Genes Dev.*, **7**, 331–342.

Kato,J.Y., Matsuoka,M., Polyak,K., Massague,J. and Sherr,C.J. (1994) Cyclic AMP-induced G_1 phase arrest mediated by an inhibitor (p27) of cyclin-dependent kinase 4 activation. *Cell*, **79**, 487–496.

Kawamata,N. *et al*. (1995) Molecular analysis of the cyclin-dependent kinase inhibitor gene $p27^{Kip1}$ in human malignancies. *Cancer Res.*, **55**, 2266–2269.

Kiyokawa,H. *et al*. (1996) Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{Kip1}$. *Cell*, **85**, 721–732.

Lanker,S., Valdivieso,M.H. and Wittenberg,C. (1996) Rapid degradation of the G_1 cyclin Cln2 induced by cdk-dependent phosphorylation. *Science*, **271**, 1597–1601.

Loda,M., Cukor,B., Tam,S.W., Lavin,P., Fiorentino,M., Draetta,G.F., Jessup,J.M. and Pagano,M. (1997) Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nature Med.*, **3**, 231–234.

Lukas,J., Parry,D., Aagard,L., Mann,D.J., Strauss,M., Peters,G. and Bartek,J. (1995) Retinoblastoma protein-dependent cell cycle inhibition by the tumour suppressor p16. *Nature*, **375**, 503–506.

Luo,Y., Marx,S.O., Kiyokawa,H., Koff,A., Massague,J. and Marks,A.R. (1996) Rapamycin resistance tied to defective regulation of $p27^{Kip1}$. *Mol. Cell. Biol.*, **16**, 6744–6751.

Mal,A., Poon,R.Y.C., Howe,P.H., Toyoshima,H., Hunter,T. and Harter,M.L. (1996) Inactivation of $p27^{Kip1}$ by the viral E1a oncoprotein in TGF-β-treated cells. *Nature*, **380**, 262–265.

Mann,D.J. and Jones,N.C. (1996) E2F-1 but not E2F-4 can overcome p16-induced G_1 cell-cycle arrest. *Curr. Biol.*, **6**, 474–483.

Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J. and Kato,J.Y. (1994) D-type cyclin-dependent kinase-activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.

Meyerson,M. and Harlow,E. (1994) Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.*, **14**, 2077–2086.

Muller,D., Bouchard,C., Rudolph,B., Steiner,P., Stuckmann,I., Saffrich,R., Asoroe,W., Huttner,W. and Eilers,M. (1997) Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. *Oncogene*, **15**, 2561–2576.

Nakayama,K., Ishida,N., Shirane,M., Inomata,A., Inoue,T., Shishido,N.,

Hori,I., Loh,D.Y. and Nakayama,K. (1996) Mice lacking $p27^{Kip1}$ display increased body-size, multiple organ hyperplasia, retinal dysplasia and pituitary tumors. *Cell*, **85**, 707–720.

Neufeld,E., Goren,H.J. and Boland,D. (1989) Thin-layer chromatography can resolve phosphotyrosine, phosphoserine and phosphothreonine in a protein hydrolysate. *Anal. Biochem.*, **177**, 138–143.

Nourse,J., Firpo,E., Flanagan,W.M., Coats,S., Polyak,K., Lee,M.H., Massague,J., Crabtree,G.R. and Roberts,J.M. (1994) Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*, **372**, 570–573.

Pagano,M., Tam,S.W., Theodoras,A.M., Beerromero,P., Delsal,G., Chau,V., Yew,P.R., Draetta,G.F. and Rolfe,M. (1995) Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, **269**, 682–685.

Parry,D., Bates,S., Mann,D.J. and Peters,G. (1995) Lack of cyclin D-cdk complexes in Rb-negative cells correlates with high-levels of $p16^{Ink4/MTS1}$ tumor-suppressor gene-product. *EMBO J.*, **14**, 503–511.

Pietenpol,J.A. *et al*. (1995) Assignment of the human $p27^{Kip1}$ gene to 12p13 and its analysis in leukemias. *Cancer Res.*, **55**, 1206–1210.

Polyak,K., Lee,M.H., Erdjument,B.H., Koff,A., Roberts,J.M., Tempst,P. and Massague,J. (1994) Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, **78**, 59–66.

Ponce-Castaneda,M.V. *et al*. (1995) $p27^{Kip1}$-chromosomal mapping to 12p12–12p13.1 and absence of mutations in human tumors. *Cancer Res.*, **55**,1211–1214.

Porter,P.L., Malone,K.E., Heagerty,P.J., Alexander,G.M., Gatti,L.A., Firpo,E.J., Daling,J.R. and Roberts,J.M. (1997) Expression of cell-cycle regulators $p27^{Kip1}$ and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nature Med.*, **3**, 222–230.

Rivard,N., L'Allemain,G., Bartek,J. and Pouyssegur,J. (1996) Abrogation of $p27^{Kip1}$ by cDNA antisense suppresses quiescence in fibroblasts. *J. Biol. Chem.*, **271**, 18337–18341.

Serrano,M., Hannon,G.J. and Beach,D. (1993) A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/cdk4. *Nature*, **366**, 704–707.

Schulz,T.F., Chang,Y. and Moore,P.S. (1998) Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8). In McCance,D.J. (ed.) *Human Tumor Viruses*. ASM Press, Washington DC, pp. 87–132.

Sheaff,R.J., Groudine,M., Gordon,M., Roberts,J.M. and Clurman,B.E. (1997) Cyclin E-cdk2 is a regulator of $p27^{Kip1}$. *Genes Dev.*, **11**, 1464–1478.

Sherr,C.J. and Roberts,J.M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163.

Swanton,C., Mann,D.J., Fleckenstein,B., Neipel,F., Peters,G. and Jones,N. (1997) Herpes viral cyclin/cdk6 complexes evade inhibition by cdk inhibitor proteins. *Nature*, **390**, 184–187.

Toyoshima,H. and Hunter,T. (1994) p27, a novel inhibitor of G_1 cyclin-cdk protein kinase activity, is related to p21. *Cell*, **78**, 67–74.

van den Heuvel,S. and Harlow,E. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*, **262**, 2050–2054.

Vlach,J., Hennecke,S. and Amati,B. (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. *EMBO J.*, **16**, 5334–5344.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.

Won,K.A. and Reed,S.I. (1996) Activation of cyclin E/cdk2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.*, **15**, 4182–4193.

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