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Correspondence and requests for materials should be addressed to A.K. (e-mail: LVKIMCHI@WEIZMANN.weizmann.ac.il).

Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins

Charles Swanton*, David J. Mann*, Bernhard Fleckenstein†, Frank Neipel†, Gordon Peters* & Nic Jones*

* *Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK*
† *Institut für Klinische und Molekulare Virologie, Friedrich-Alexander Universität, Loschgestrasse 7, D-8520 Erlangen, Germany*

The passage of mammalian cells through the restriction point into the S phase of the cell cycle is regulated by the activities of Cdk4 and Cdk6 complexed with the D-type cyclins and by cyclin E/Cdk2 (refs 1–3). The activities of these holoenzymes are constrained by CDK inhibitory proteins[4,5]. The importance of the restriction point is illustrated by its deregulation in many tumour cells[6,7] and upon infection with DNA tumour viruses[8]. Here we describe the properties of cyclins encoded by two herpesviruses, herpesvirus saimiri (HVS) which can transform blood lymphocytes[9] and induce malignancies of lymphoid origin in New World primates[9,10], and human herpesvirus 8 (HHV8) implicated as a causative agent of Kaposi's sarcoma and body cavity lymphomas[11,12]. Both viral cyclins form active kinase complexes with Cdk6 that are resistant to inhibition by the CDK inhibitors $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$. Furthermore, ectopic expression of a viral cyclin prevents G1 arrest imposed by each inhibitor and stimulates cell-cycle progression in quiescent fibroblasts. These results suggest a new mechanism for deregulation of the cell cycle and indicate that the viral cyclins may contribute to the oncogenic nature of these viruses.

Because transformation by DNA tumour viruses is accompanied by loss of restriction point control, we investigated the possibility that the HVS- and HHV8-encoded cyclins[13,14] (V- and K-cyclin, respectively) could promote such deregulation and hence contribute to the known and suspected transforming properties of HVS and HHV8. We prepared cell lysates from Sf9 cells infected with recombinant baculoviruses expressing either cyclin together with Cdk6, a CDK subunit which is abundantly expressed in lymphocytes[15] and shown to form active kinase complexes with the viral cyclins[13,14]. The complexes were assayed for their ability to phosphorylate pRb in the absence or presence of bacterially expressed $p21^{Cip1}$ or $p27^{Kip1}$ inhibitor protein. Lysates containing Cdk6 and either of the viral cyclins efficiently phosphorylated pRb *in vitro* (Fig. 1a). Kinase activity was considerably higher than with lysates expressing cyclin D1 and Cdk6 (Fig. 1a), in agreement with previous results[13]. Importantly, both the V-cyclin- and K-cyclin-associated activities were resistant to inhibition by either $p21^{Cip1}$ or $p27^{Kip1}$ over a 100-fold range of inhibitor concentration. This is in contrast to cyclin D1/Cdk6 activity which was sensitive to both inhibitors in a dose-dependent manner. A second family of CDK inhibitors typified by the $p16^{Ink4a}$ protein also plays a significant role in restriction point control[4,5]. This class of inhibitor is specific for Cdk4 or Cdk6 complexes. Both V-cyclin and K-cyclin complexes also showed resistance to a wide range of $p16^{Ink4a}$ levels, whereas the cyclin D1/Cdk6 control was efficiently inhibited (Fig. 1b).

The difference in activity of the cyclin D1 and viral cyclin complexes and their sensitivity to inhibitors could not be explained by differences in the levels of cyclin/CDK binary complexes. ^{35}S-methionine labelling of infected cell extracts followed by immunoprecipitaiton with a Cdk6-specific antibody (Fig. 1c) showed comparable levels of immunoprecipitated cyclin D1 and K-cyclin and slightly higher levels of V-cyclin. We repeated the kinase inhibition assays using these Cdk6 immunoprecipitates. At the highest level of inhibitor, both the K-cyclin/Cdk6 and the V-cyclin/Cdk6 complexes were resistant to inhibition whereas the cyclin D1/Cdk6 complex was completely inhibited by $p16^{Ink4a}$ and $p27^{Kip1}$ and significantly inhibited by $p21^{Cip1}$ (Fig. 1d). Therefore the results with the crude extracts and the immunoprecipitated complexes were directly comparable. To examine the sensitivity of the viral complexes further, we extended the ratio of inhibitor:complex by repeating the kinase assays with the highest level of inhibitor but 1/10 or 1/100 the level of immunoprecipitated Cdk6-containing complex. With a 10-fold decrease, partial inhibition of the K-cyclin complex was observed with $p16^{Ink4a}$ and $p27^{Kip1}$ but there was still little effect on the V-cyclin complex. With 100-fold less complex, although inhibition of the K- and V-cyclin activities was evident, significant activity remained (about 15% in the case of K-cyclin and 30% in the case of V-cyclin). Even at this dilution, however, $p21^{Cip1}$ had negligible effect. Thus these results demonstrate that the viral cyclin complexes are significantly more resistant to inhibition than cyclin D1 complexes.

The efficiency of inhibition is likely to be defined by the binding affinity of the inhibitor for a given cyclin/CDK complex. The $p21^{Cip1}$ and $p27^{Kip1}$ inhibitors have been shown to bind both cyclin and CDK subunits[16–19] and the interactions with both subunits contribute to the inhibition of the holoenzyme. We considered whether the resistance of the viral cyclin/Cdk6 complexes was due to lack of interaction between the viral cyclins and these inhibitors. Cyclins were synthesized by coupled transcription/translation in the presence of ^{35}S-methionine and mixed with unlabelled lysates containing $p21^{Cip1}$, $p27^{Kip1}$ or Cdk6. The unlabelled components were immunoprecipitated and analysed for the presence of the labelled cyclin. Cyclin D1, K-cyclin and V-cyclin were all efficiently co-immunoprecipitated together with Cdk6 (Fig. 2a). Cyclin D1 was also immunoprecipitated with $p21^{Cip1}$ and $p27^{Kip1}$ inhibitors, confirming the direct binding shown previously using this assay (Fig. 2a)[17]. In marked contrast, $p21^{Cip1}$ failed to co-immunoprecipitate either viral cyclin (Fig. 2a). Additionally, V-cyclin failed to interact with $p27^{Kip1}$ (Fig. 2a) and although K-cyclin did show some interaction, it was 15-fold less than that of $p27^{Kip1}$ with cyclin D1. The inefficient interaction was confirmed by co-expressing viral cyclin, Cdk6 and the $p21^{Cip1}$ or $p27^{Kip1}$ inhibitor in Sf9 cells in the presence of ^{35}S-methionine. The infected extracts were subjected to immunoprecipitation with an antibody specific to the inhibitor. Figure 2b shows efficient co-immunoprecipitation of cyclin D1 and Cdk6 with $p21^{Cip1}$ or $p27^{Kip1}$. In contrast, at least 10-fold less viral cyclin was co-immunoprecipitated. These results demonstrate that the viral cyclin/Cdk6 complexes are resistant to inhibition by $p21^{Cip1}$ and $p27^{Kip1}$ because of the inability of these inhibitors to interact efficiently with the cyclin subunits. This conclusion is supported by the lack of conservation of certain key residues predicted to be crucial for the $p27^{Kip1}$ interaction. The structure of cyclin A/Cdk2 bound to the N terminus of $p27^{Kip1}$ has recently been described[20],

illustrating a region of $p27^{Kip1}$ that binds directly to cyclin A through hydrophobic and electrostatic interactions. The surface residues of cyclin A lining the $p27^{Kip1}$-binding pocket are highly conserved among mammalian cyclins known to interact with $p27^{Kip1}$. The viral cyclins show less conservation in this region. Notably, E220 of cyclin A, which forms a hydrogen bond with S27 and a salt bridge with R30 of $p27^{Kip1}$, is not conserved (Fig. 2c). Thus the loss of electrostatic character in the predicted $p27^{Kip1}$ binding surface of the viral cyclins may be responsible for their lack of inhibition by $p27^{Kip1}$. The mechanism behind the resistance of the viral cyclin complexes to $p16^{Ink4a}$ is presently unknown and there is no evidence that this inhibitor interacts directly with the cyclin. We speculate that the resistance could arise from higher affinity of the viral cyclin for the Cdk6 subunit thus preventing its possible displacement by the inhibitor–Cdk6 interaction.

The results described above demonstrate that V-cyclin/Cdk6 and K-cyclin/Cdk6 complexes are resistant to inhibition by both families of inhibitor implicated in regulating the passage of cells from G1 into S. These properties suggest that they may stimulate the progression of cells through the G1 period of the cell cycle even in the presence of high levels of CDK inhibitor activity. We therefore assessed the ability of the K-cyclin to release a G1-arrest imposed by the ectopic expression of $p16^{Ink4a}$, $p21^{Cip1}$ or $p27^{Kip1}$ (Table 1). The human osteosarcoma cell line U2-OS was transiently transfected with a plasmid encoding a CDK inhibitor together with a plasmid encoding the cell-surface marker CD8 to enable specific analysis of the transfected cells. Cells expressing each of the inhibitors accumulated in the G1 phase of the cell cycle. Co-transfection of K-cyclin partially, or in some cases fully, prevented the $p16^{Ink4a}$-imposed arrest whereas cyclin D1 had little effect. In $p21^{Cip1}$-arrested cells, K-cyclin expression not only prevented arrest but significantly decreased the percentage of cells present in the G1 phase compared to the untransfected controls. Overexpression of cyclin D1 largely prevented $p21^{Cip1}$-imposed arrest although there was no reduction in the G1 population compared to the control. The ability of cyclin D1 to overcome a $p21^{Cip1}$-mediated arrest has been previously reported[19]. Overexpression of K-cyclin also efficiently prevented a $p27^{Kip1}$-imposed G1 arrest whereas cyclin D1 was unable to do so. Western analysis of extracts prepared from the transfected population of cells indicated that both cyclin D1 and K-cyclin were efficiently expressed (data not shown).

Accumulating evidence suggests a separate role for both cyclin D1 and cyclin E complexes in promoting the transition of cells from the G1 phase into the S phase[21–24]. It is noteworthy that the K-cyclin

Table 1 Ability of K-cyclin to release G1 arrest

Experiment	Inhibitor:	Increase in G1 (%) p16			p21			p27		
	Cyclin:	–	Cyclin D1	K-cyclin	–	Cyclin D1	K-cyclin	–	cyclin D1	K-cyclin
1		41.2	40.6	11.6	30.0	5.7	–26.0	41.3	35.7	9.8
2		21.6	18.0	–6.5	19.1	6.4	–30.1	21.4	20.8	–1.8
3		45.4	39.0	13.4	13.7	–4.2	–30.3	20.1	17.6	–9.1
4		25.1	23.5	–5.9	21.1	5.8	–24.6	25.0	17.8	4.4
5		55.6	43.3	10.0	23.8	7.4	–17.2	36.4	30.1	2.8

U2-OS cells were transfected with plasmids encoding the cell surface marker CD8, inhibitor protein ($p16^{Ink4a}$, $p21^{Cip1}$ or $p27^{Kip1}$ as indicated) and cyclin (cyclin D1 or K-cyclin as indicated). Data are represented as the percentage change in the number of cells in G1 phase of the cell cycle relative to the G1 population of cells transfected with the plasmid expressing CD8 only. The results from five independent experiments are shown.

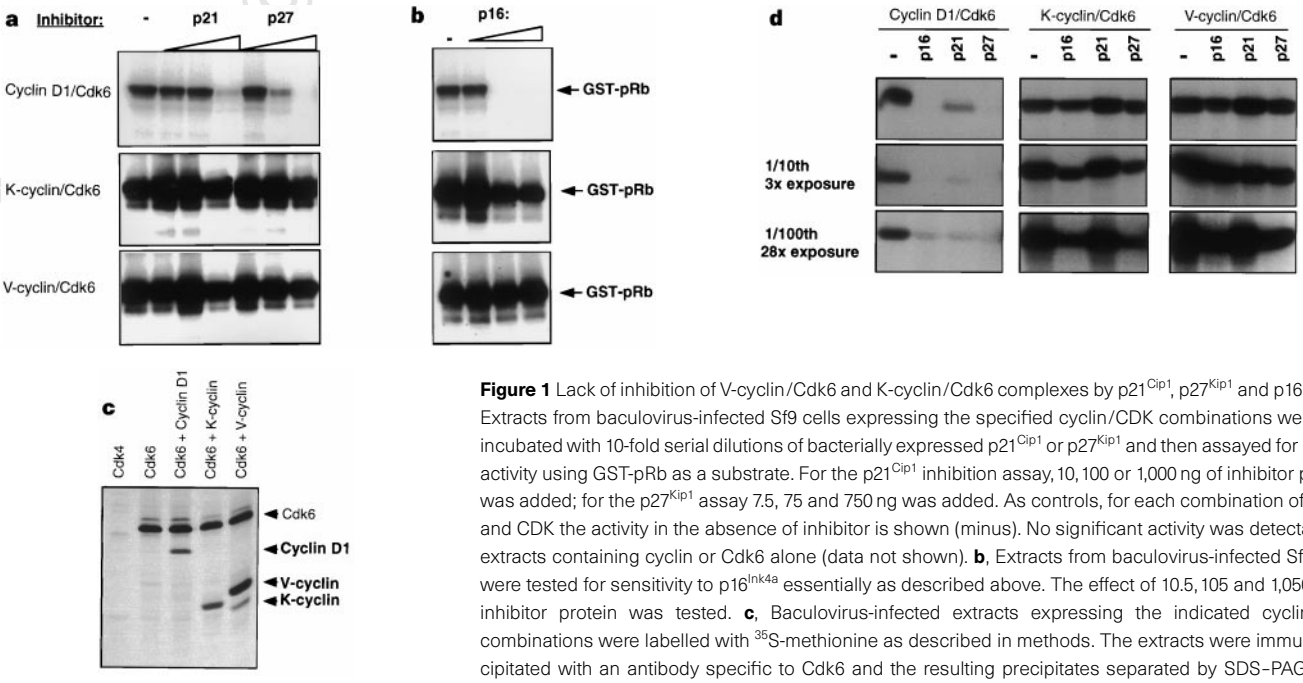

Figure 1 Lack of inhibition of V-cyclin/Cdk6 and K-cyclin/Cdk6 complexes by $p21^{Cip1}$, $p27^{Kip1}$ and $p16^{Ink4a}$. **a**, Extracts from baculovirus-infected Sf9 cells expressing the specified cyclin/CDK combinations were pre-incubated with 10-fold serial dilutions of bacterially expressed $p21^{Cip1}$ or $p27^{Kip1}$ and then assayed for kinase activity using GST-pRb as a substrate. For the $p21^{Cip1}$ inhibition assay, 10, 100 or 1,000 ng of inhibitor protein was added; for the $p27^{Kip1}$ assay 7.5, 75 and 750 ng was added. As controls, for each combination of cyclin and CDK the activity in the absence of inhibitor is shown (minus). No significant activity was detectable in extracts containing cyclin or Cdk6 alone (data not shown). **b**, Extracts from baculovirus-infected Sf9 cells were tested for sensitivity to $p16^{Ink4a}$ essentially as described above. The effect of 10.5, 105 and 1,050 ng of inhibitor protein was tested. **c**, Baculovirus-infected extracts expressing the indicated cyclin/CDK combinations were labelled with ^{35}S-methionine as described in methods. The extracts were immunoprecipitated with an antibody specific to Cdk6 and the resulting precipitates separated by SDS–PAGE and visualized by autoradiography. **d**, Complexes immunoprecipitated with an antibody against Cdk6 were tested for pRb phosphorylation activity in the presence of 1,000 ng $p21^{Cip1}$, 750 ng $p27^{Kip1}$ or 1,050 ng $p16^{Ink4a}$. In lower panels, the amount of complex was reduced 10-fold or 100-fold, respectively, and assayed with the levels of inhibitor indicated above. The autoradiographs were exposed for different times to account for the different levels of kinase activity resulting from the dilutions.

complex can promote entry into S phase in the presence of high levels of each of the three inhibitors tested. This would imply that the viral cyclin/CDK complexes may be able to perform the role of both G1 cyclin complexes.

DNA tumour viruses have evolved mechanisms to force quiescent cells to enter S phase creating an environment more conducive to viral replication. To investigate whether the viral cyclin can perform such a role, an NIH 3T3 cell derivative expressing K-cyclin in an isopropyl-β-D-thiogalactoside (IPTG)-inducible manner was generated. In the absence of inducer, these cells efficiently quiesced upon serum deprivation as judged by BrdU labelling and fluorescence-activated cell sorting (FACS) analysis with roughly 90% of cells in the G0/G1 states (Fig. 3a). However, subsequent addition of IPTG to induce K-cyclin expression resulted in a significant proportion of the cells entering S phase as demonstrated by the incorporation of BrdU into DNA. The increased expression of K-cyclin following induction was confirmed by western analysis (Fig. 3b). Thus, expression of K-cyclin is sufficient to induce S phase entry in quiescent cells.

In this report we highlight important biochemical and functional differences between the viral cyclins and the D-type cyclins, the most significant being their profound resistance to both families of CDK inhibitor proteins. The expression of such cyclins provides a new mechanism for deregulating cell-cycle progression. Such deregulation is an important prerequisite for efficient replication of many DNA tumour viruses and is essential for their tumorigenic potential. Given these parallels, we suggest that the viral cyclins are likely to be important for the replication and transforming properties of these herpesviruses. □

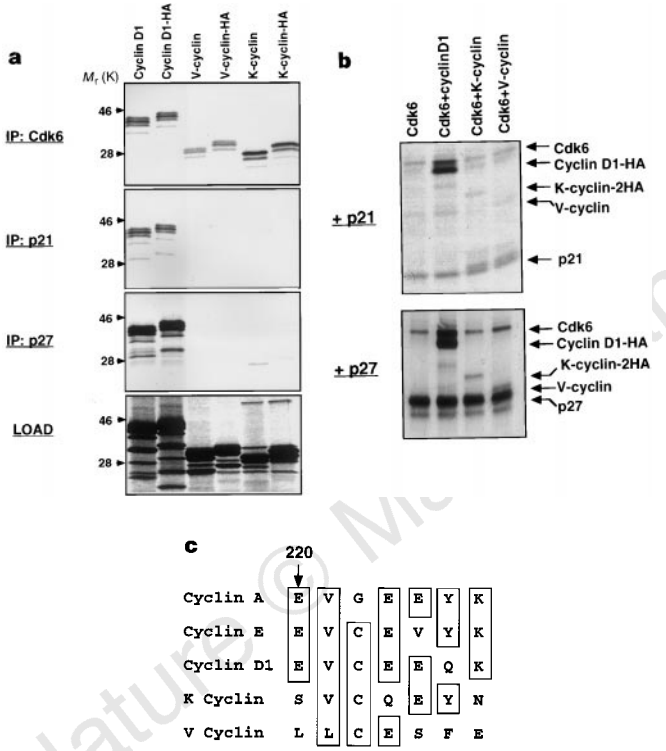

Figure 2 Co-precipitation of cyclins with $p21^{Cip1}$, $p27^{Kip1}$ and Cdk6. ^{35}S-labelled cyclins were synthesized by *in vitro* translation and analysed by SDS–PAGE. For each cyclin, both an epitope-tagged product and an untagged product were analysed. **a**, Equal amounts of the labelled cyclins were mixed with translation lysates containing unlabelled Cdk6, $p21^{Cip1}$ or $p27^{Kip1}$ protein. Immunoprecipitations were performed using antibodies specific for the unlabelled component (as indicated) and the precipitates were analysed by SDS–PAGE and autoradiography to determine the proportion of labelled cyclin which was co-immunoprecipitated. **b**, Baculovirus-infected extracts expressing the indicated cyclin/Cdk combinations together with either $p21^{Cip1}$ or $p27^{Kip1}$ were labelled with ^{35}S-methionine as described in Methods. The extracts were immunoprecipitated with an antibody specific to $p27^{Kip1}$ or $p21^{Cip1}$ and the resulting precipitates separated by SDS–PAGE and visualized by autoradiography. In the case of K-cyclin, a double HA-tagged version was expressed because the mobility of K-cyclin is similar to that of p27. The levels of immunoprecipitated proteins were estimated using Molecular Dynamics phosphoimager analysis taking into account differing methionine content of the cyclin components. **c**, Sequence comparisons of K- and V-cyclins with the cyclins A, E and D1 that interact with $p21^{Cip1}$ and $p27^{Kip1}$. The E220 residue of cyclin A which contributes to the electronegative surface of the cyclin and is involved in hydrogen bonding with $p27^{Kip1}$ (ref. 20) is aligned with residues S60 and L61 of K- and V-cyclin, respectively.

Methods

Plasmid and recombinant baculovirus constructs. Cyclin D1, HHV8 K-cyclin and HVS V-cyclin were cloned as polymerase chain reaction (PCR) fragments into the pRSETA vector (Invitrogen) as *Bam*H1 5′/*Eco*R1 3′ fragments. In the case of HHV8 cyclin, the *Bam*H1 site at +36 was eliminated using a mutated 5′ PCR oligonucleotide; the mutation had no effect on the amino-acid sequence of the cyclin. In some cases, single haemagglutinin (HA) epitopes were added to the C terminus by incorporating the epitope coding region in the 3′ PCR primer. N-terminal 2HA-K-cyclin was made by ligating a 2HA coding oligonucleotide into the *Nco*I site at the 5′ end of K-cyclin. All clones were sequenced.

$p21^{Cip1}$, $p27^{Kip1}$ and $p16^{Ink4a}$ clones in pRSETA and Cdk6 in Bluescript[17] and the mammalian expression constructs containing CD8, $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$ (ref. 22) have been described previously. For mammalian expression constructs, coding sequences of the HA-epitope-tagged V-cyclin and K-cyclin sequences were subcloned from pRSETA vectors into the *Bam*H1/*Eco*R1 sites of

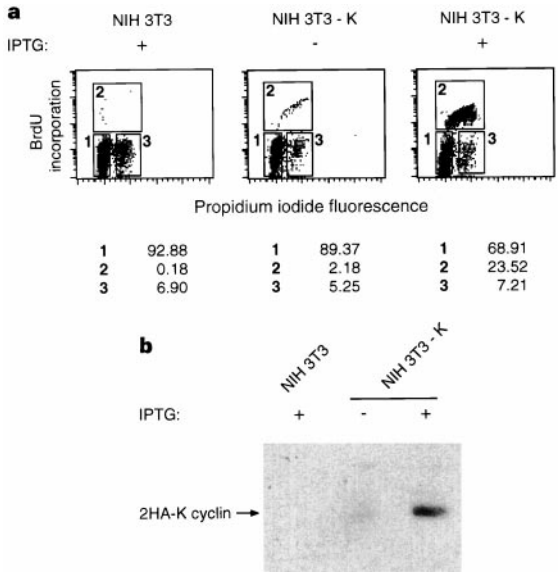

Figure 3 Stimulation of quiescent cells by expression of K-cyclin. An NIH 3T3 cell derivative was generated that expresses K-cyclin upon the addition of the inducer IPTG (NIH3T3-K). **a**, NIH 3T3 or NIH3T3-K cells were quiesced in the absence of IPTG by incubation in medium containing 0.2% serum for 72 h. As appropriate, K-cyclin expression was then induced by addition of 5 mM IPTG. BrdU (10 μM) was added 10 h later and the cells incubated for a further 24 h. The cells were then collected, fixed and stained both with propidium iodide and anti-BrdU-isothocyanate conjugate and analysed by FACS. The percentage of cells in either G1 (box 1), S (box 2) or G2/M (box 3) are indicated. **b**, Cells treated as above were lysed in SDS–PAGE sample buffer and subjected to western blot analysis using an anti-HA antibody.

the mammalian expression vector pcDNA3 (Invitrogen). For recombinant baculovirus construction, the same fragments were subcloned into pvl1393 (Invitrogen). For the construction of the IPTG-inducible K-cyclin, HA-tagged K-cyclin was amplified from the pRSETA vector described above using oligonucleotide primers containing *Not*1 restriction sites. The ensuing fragment was cloned into the *Not*1 site of pOPRSVICAT (Stratagene) to give the plasmid DM179.

Recombinant *Autographa californica* nuclear polyhedrosis virus were produced using the BaculoGold transfection system (Pharmingen) and subsequently amplified. Recombinant baculoviruses expressing cyclin D1 and cyclin E were a gift of C. Sherr, and Cdk6 a gift of M. Meyerson.

Antibodies and immunoblotting. Antisera generated against C-terminal peptides of human $p21^{Cip1}$, $p27^{Kip1}$ and Cdk6 have been described in previous reports[17]. A polyclonal antibody against the C terminus of $p27^{Kip1}$ (SC528; Santa Cruz) was used for ^{35}S immunoprecipitations from Sf9 cells. The 12CA5 anti-haemagglutinin antibody was from Boehringer Mannheim. Immunoblot analysis was performed as described[22].

***In vitro* kinase assays.** Sf9 cells were co-infected with the appropriate recombinant baculoviruses and whole-cell extracts prepared 48 h after infection as described[24]. Phosphorylation reactions (40 μl) were performed in 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA and incubated with 500 ng GST-pRb bound to glutathione–Sepharose beads, 100 μM ATP and 5 μCi [γ-^{32}P]ATP. After 5 min at 30 °C, reactions were terminated by the addition of 500 μl cold NETN (20 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The beads were recovered, boiled in dissociation buffer and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). For inhibition assays, the extracts were pre-incubated with an equal volume of histidine-tagged $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$ (10-fold dilutions) for 10 min at 30 °C.

For the immunoprecipitation kinase assays, extracts were prepared and immunoprecipitated with an antibody directed against Cdk6 essentially as described below. The resulting lysates were washed and resuspended in kinase buffer and kinase assays performed as described in ref. 15.

***In vitro* interaction assay.** pRSET vectors containing the coding sequences of V-cyclin, K-cyclin and cyclin D1 were used to generate ^{35}S-labelled proteins by coupled transcription and translation using the TNT expression system (Promega). The same system was used to generate unlabelled Cdk6, $p21^{Cip1}$ or $p27^{Kip1}$ protein. Samples of each reaction were mixed, incubated for 30 min at 30 °C, diluted using cold 50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% NP40, 3% BSA and precipitated using antiserum against the unlabelled component. The labelled proteins in the immunoprecipitate were analysed by SDS–PAGE.

Cell culture, transfection and FACS analysis. Culture of U2-OS cells, transfection and analysis of the cell cycle distribution of transfected cells have been described[22].

Co-infections of Sf9 cells have been described[25]. For labelling of expressed proteins, culture medium was replaced 48 h after infection with 3 ml Graces medium lacking methionine (Gibco) and containing 10% dialysed fetal calf serum. After 1 h the medium was replaced with a further 5 ml of medium lacking methionine and containing 10% dialysed serum with 140 μCi of ^{35}S-methionine (Amersham). Cells were left for 12 h and resuspended in 1 ml IP buffer (0.1% Tween-20, 50 mM Tris pH 7.5, 50 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT, and protease inhibitors). The Cdk6 antibody LB02 (10 μl) was added to 500 μl of lysate in a total volume of 1 ml. After 1 h at 4 °C protein A-Sepharose beads were added, and the lysates rotated for a further 60 min. Beads were washed 3 times in IP buffer and resuspended in SDS–PAGE loading buffer before analysis on 12% gels. In the case of $p21^{Cip1}$/$p27^{Kip1}$ immunoprecipitations, Sf9 cells were co-infected with viruses expressing inhibitor, cyclin and Cdk either individually or in combination, and labelling was performed as described above. RIPA buffer was used for the immunoprecipitaiton and subsequent washing.

To isolate IPTG-inducible cell lines, NIH3T3 cells were transfected with DM179 and p3′SS (Stratagene) expressing the *lacI* gene. G418- and hygromycin-resistant colonies were isolated and screened for IPTG-dependent K-cyclin expression by western analysis. For FACS analysis, BrdU-labelled cells were trypsinized and fixed in 70% ethanol for 30 min on ice. Detection of BrdU-incorporation with an anti-BrdU-isothyocyanate conjugate (1202 693, Boehringer Mannheim) was carried out according to the manufacturer's protocol. Cells were stained for DNA with propidium iodide (10 μg ml^{-1}) and analysed using bivariate flow cytometry.

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Correspondence and requests for materials should be addressed to N.J. (e-mail jonesn@icrf.icnet.uk).

Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis

Stephen K. Dove*†‡, Frank T. Cooke‡§, Michael R. Douglas‖, Lee G. Sayers§, Peter J. Parker§ & Robert H. Michell*†

* *Centre for Clinical Research in Immunology and Signalling, and Departments of* † *Biochemistry and* ‖ *Rheumatology, University of Birmingham, Birmingham B15 2TT, UK*

§ *Imperial Cancer Research Fund Laboratories, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK*

‡ *These authors contributed equally to this work*

Inositol phospholipids play multiple roles in cell signalling systems. Two widespread eukaryotic phosphoinositide-based signal transduction mechanisms, phosphoinositidase C-catalysed phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) hydrolysis and 3-OH kinase-catalysed PtdIns(4,5)P_2 phosphorylation, make the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) *sn*-1,2-diacylglycerol and PtdIns(3,4,5)P_3 (refs 1–7). In addition,