

DAP kinase links the control of apoptosis to metastasis

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DAP kinase is a new type of calcium/calmodulin-dependent enzyme that phosphorylates serine/threonine residues on proteins. Its structure contains ankyrin repeats and the 'death' domain, and it is associated with the cell cytoskeleton^{1–3}. The gene encoding DAP kinase was initially isolated as a positive mediator of apoptosis induced by interferon- γ , by using a strategy of functional cloning⁴. We have now tested whether this gene has tumour-suppressive activity. We found that lung carcinoma clones, characterized by their highly aggressive metastatic behaviour and originating from two independent murine lung tumours, did not express DAP kinase, in contrast to their low-metastatic counterparts. Restoration of DAP kinase to physiological levels in high-metastatic Lewis carcinoma cells suppressed their ability to form lung metastases after intravenous injection into syngeneic mice, and delayed local tumour growth in a foreign 'microenvironment'. Conversely, *in vivo* selection of rare lung lesions following injection into syngeneic mice of low-metastatic Lewis carcinoma cells or of DAP kinase transfectants, was associated with loss of DAP kinase expression. *In situ* TUNEL staining of tumour sections revealed that DAP kinase expression from the transgene raised the incidence of apoptosis *in vivo*. DAP-kinase transfectants also showed increased sensitivity *in vitro* to apoptotic stimuli, of the sort encountered by metastasizing cells at different stages of malignancy. We propose that loss of DAP kinase expression provides a unique mechanism that links suppression of apoptosis to metastasis.

Some tumour-suppressor genes (for example, those encoding p53 or APC) and oncogenes (for example, members of the Bcl-2 family) mediate or suppress apoptosis, respectively^{5–13}, and thus establish a link between tumorigenesis and loss of apoptotic responses. We investigated whether genes that were initially identified as positive mediators of apoptosis could have tumour-suppressive activity. One such gene, encoding DAP kinase^{1–3}, was studied because its expression is frequently lost in human carcinoma and B-cell leukaemia cell lines¹⁴. We analysed two independent sets of high- and low-metastatic clones, selected from the murine Lewis (3LL)¹⁵ and CMT64¹⁶ lung carcinoma cell lines, and an intriguing pattern emerged: DAP kinase protein was undetectable in the two high-metastatic derivatives, whereas the low-metastatic counterparts were positive for DAP kinase (Fig. 1a). No DAP kinase messenger RNA could be detected in the high-metastatic clones (not shown). These initial observations prompted us to test whether the introduction of a functional DAP kinase gene into one of the high-metastatic clones might reduce its tumorigenic and metastatic capacity.

The Flag-tagged wild-type DAP kinase gene was transfected into the 3LL clone-D122 cells. Stable DAP-kinase-positive clones, which displayed normal growth kinetics in culture, were isolated (Fig. 1b). The transgene was expressed at physiological levels, as evaluated by comparison with the endogenous DAP kinase protein levels in the A9-F low-metastatic clone. Expression ranged between 0.7 (clone 6-DAPk), 1.5 (clone 48-DAPk), 1.8–3 (28-DAPk) and 3–6 (42-DAPk) times endogenous levels (calculated from Fig. 1b and other immu-

noblots; results not shown). *In vitro* kinase assays² proved that the DAP kinase protein, expressed from the transgene, was catalytically active (not shown). The ectopic expression of DAP kinase in clone 42-DAPk did not evoke any signs of chromatin condensation or fragmentation (Fig. 3c, bottom left panel), indicating that DAP kinase by itself, even in the clone that displayed the highest expression levels, did not trigger apoptosis under normal growth conditions.

C57BL/6 syngeneic mice were then injected with the transfected D122 cells using two different protocols¹⁵. One group of mice received intravenous (i.v.) injections and experimental metastases in the lungs were monitored. A second group received intrafootpad (i.f.p.) injections to investigate local tumour growth in the foreign microenvironment of footpad muscles, and to monitor post-surgical spontaneous metastasis in the lungs. The experiments were repeated three times with reproducible results.

In the protocol measuring experimental metastasis, the lungs were examined 30–32 days after i.v. injection. Whereas the parental D122- and G418-resistant control clones were highly metastatic, the metastatic activity of DAP kinase transfectants was strongly suppressed (Fig. 2a, b). Both the average lung weight (Fig. 2a) and the number of lung lesions (Table 1) were reduced. The extent of suppression was proportional to the levels of exogenously expressed DAP kinase, and even the low-expressor clone 6-DAPk had a significant reduction in its metastatic activity (Table 1). Mice injected with the 42-DAPk clone remained viable for at least 112 days post-injection; their lungs at this time contained only 0–2 nodules.

In the second protocol, which consisted of intrafootpad injections, the effect of DAP kinase was milder, being restricted to clones that expressed the highest DAP kinase levels (28- and 42-DAPk). The effect was manifested by a delay in growth of local tumours, which was directly proportional to the levels of exogenously expressed DAP kinase (Fig. 2c and inset). Once tumours emerged, however, the kinetics of increase in total tumour mass were similar to those observed in the control clones. Moreover, surgical removal of the late-appearing tumours, generated by clones 28- or 42-DAPk, gave rise to spontaneous lung metastases which eventually killed the mice. These observations further suggested that revertants, in which the transgene was lost or inactivated, might have been selected *in vivo* after i.f.p. injection. Indeed, examination of tumour cells, recovered in culture from the post-surgical lung metastases,

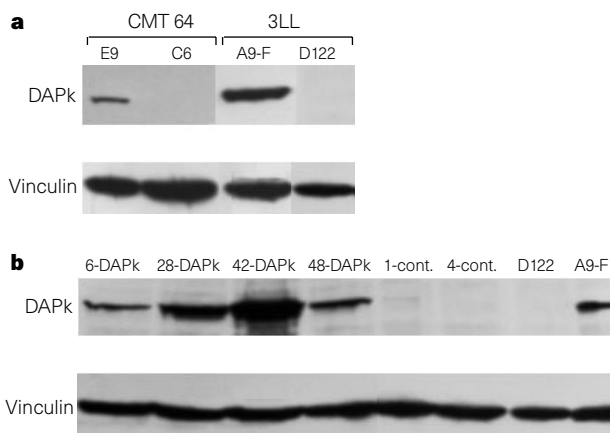


Figure 1 Immunoblot analysis of DAP kinase expression. **a**, DAP kinase protein was measured in low- (A9-F and E9) and high- (D122 and C6) metastatic clones derived from the 3LL and CMT64 tumours. **b**, Immunoblot analysis of parental D122 cells, and of the different G418-resistant derivative clones transfected with pcDNA control vector (-cont.) or with pcDNA-DAP kinase (-DAPk). The low-metastatic A9-F clone is used as a reference.

revealed that these cells had lost exogenous DAP kinase expression (Fig. 2d). Treatment of these recovered tumour cells with 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation, completely restored DAP kinase protein expression (Fig. 2d), suggesting that attenuation of expression from the transgene was caused by DNA methylation.

A second *in vivo* selection was applied to the A9-F low-metastatic clone in an attempt to assess the status of the endogenous DAP kinase in the rare macroscopic lung lesions appearing late after its i.v. injection. The development of these metastatic lesions correlated with loss of DAP kinase expression (an example of three lung lesions appearing in a single mouse is shown in Fig. 2e). Altogether, the calculated value of loss of DAP kinase expression in these *in vivo* selections was 0.54 ($n = 11$). In contrast, random subcloning in culture of the A9-F cells showed that, without selection, the frequency of DAP kinase low expressors was 0.04 ($n = 48$) (the difference from the *in vivo* selections is significant at $P < 5 \times 10^{-4}$). Treatment of cells recovered from one of the lung lesions lacking DAP kinase, with 5-aza-2'-dioxycytidine restored protein expression to the normal levels of A9-F cells (Fig. 2e). DNA methylation was therefore also responsible for silencing the endogenous DAP kinase gene in the *in vivo*-selected A9-F cells, as previously documented for various tumour-suppressor genes¹⁷. Yet, this may not be an exclusive mechanism for suppressing DAP kinase expression

because demethylation failed to restore DAP kinase expression in the parental D122 high-metastatic clone. Moreover, in the CMT64 high-metastatic clone, gross DNA rearrangements of the DAP kinase gene could be identified by Southern blot analysis (not shown). We suggest that loss of DAP kinase expression (either from the transgene or from the endogenous gene) provides a positive selective advantage during the formation of lung metastases.

Next, the mechanisms underlying the suppressive effects of DAP kinase on metastasis and local tumour growth were studied. *In situ* TUNEL staining was performed on histological sections of footpad tumours. The apoptotic index in the slow-growing local tumours, formed by DAP-kinase-transfected cells, was higher than that in the tumour mass formed by the control clone (Fig. 3a). The mean values were $6.3\% \pm 1.13$ and $1.9\% \pm 0.35$ for 42-DAPk and the control clone, respectively ($P \ll 0.001$), reflecting a tremendous difference in total cell death in view of the rapid elimination of apoptotic cells in tissues¹⁸. These results implicated the DAP kinase gene in the augmentation of the threshold sensitivity of the tumour cells to apoptotic signals. We therefore exposed the transfected cells *in vitro* to two types of apoptotic stimuli, tumour-necrosis factor- α (TNF- α) and anchorage-independent cell growth. The Flag-tagged DAP kinase protein in clone 42-DAPk was tightly regulated by TNF- α , as reflected by its conversion into a form that rapidly migrates on gels (Fig. 3b, inset). At the phenotypic level, these transfectants displayed

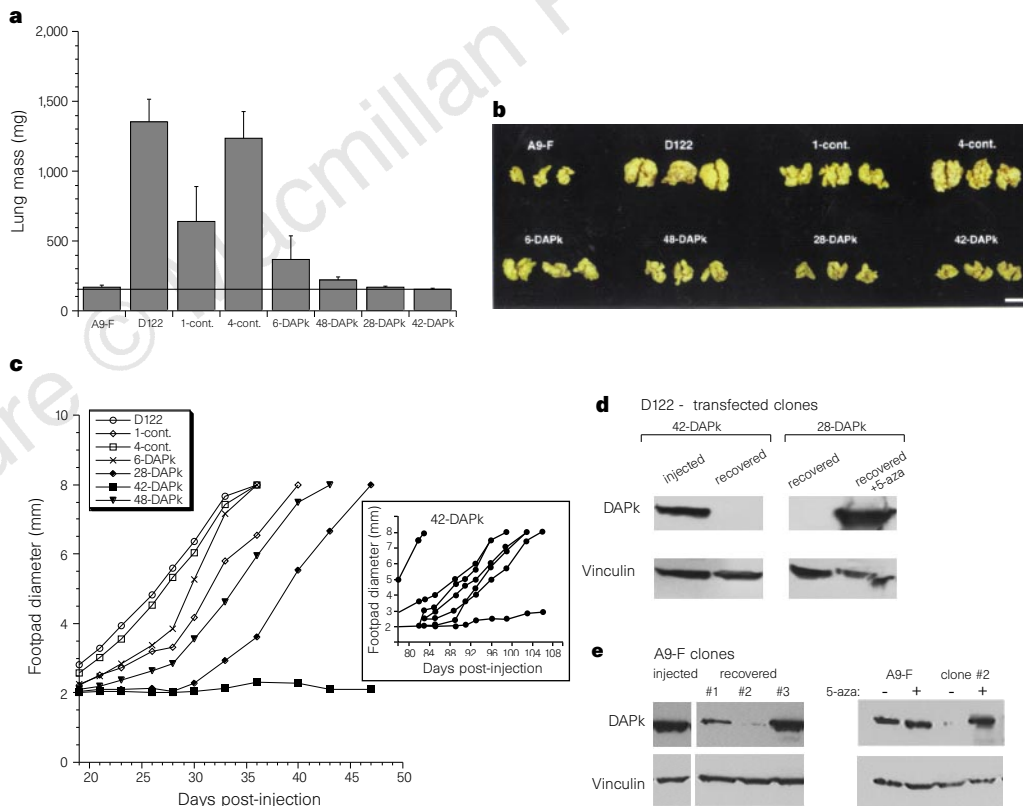


Figure 2 Inverse relationships between DAP kinase expression and metastatic activity. **a**, Transfection with DAP kinase strongly suppresses experimental metastasis. Values are mean lung weight \pm s.d. of 5 individuals in each group. The solid line indicates the average lung weight of uninjected mice. Differences between the less aggressive 1-cont. clone and each one of the DAP kinase transfectants were significant at $P < 0.001$ for 48-, 28- and 42-DAPk clones, and at $0.025 < P < 0.05$ for the low-expressing clone 6-DAPk (the last clone differed from 4-cont. clone and parental D122 cells at $P < 0.001$). **b**, Three representative lungs from each group of mice as in **a**. Scale bar, 1 cm. **c**, Transfection with DAP-kinase delays the growth of local tumours. Values present the mean pad diameter of eight individuals in each group. The size difference between tumours formed by the slowest-growing control clone and by 28-DAPk clone was significant at

$P < 0.001$. The outburst of clone 42-DAPk after day 80 in each of 7 injected mice is shown in the inset. **d**, *In vivo* selections of DAP kinase revertants. Clone 42-DAPk was tested for protein expression both before the i.f.p. injections, and after the recovery in culture of spontaneous lung lesions appearing on day 35 post-surgery. Clone 28-DAPk was tested after recovery in culture from the spontaneous lung lesions without or with treatment with 5-aza-2'-deoxycytidine. **e**, *In vivo* selections of A9-F cells. DAP kinase expression was assessed in the original A9-F cells used for injection, and in cells recovered in culture from three independent lung lesions which appeared on day 61. Two out of three lesions display attenuated levels of DAP kinase, one of which is below detection limits. The latter clone was also treated with 5-aza-2'-deoxycytidine.

Table 1 Experimental metastasis of DAP kinase transfectants

Clone	D122	1-cont.	4-cont.	6-DAPk	48-DAPk	28-DAPk	42-DAPk
Number of mice that developed lung metastasis	5/5	5/5	5/5	5/5	5/5	4/5	0/5
Mean number of metastatic lesions per mouse*	>100	>100	>100	34 ± 27	12 ± 7	3 ± 2	0

*The number of metastatic lesions was determined by counting surface nodules under a binocular microscope.

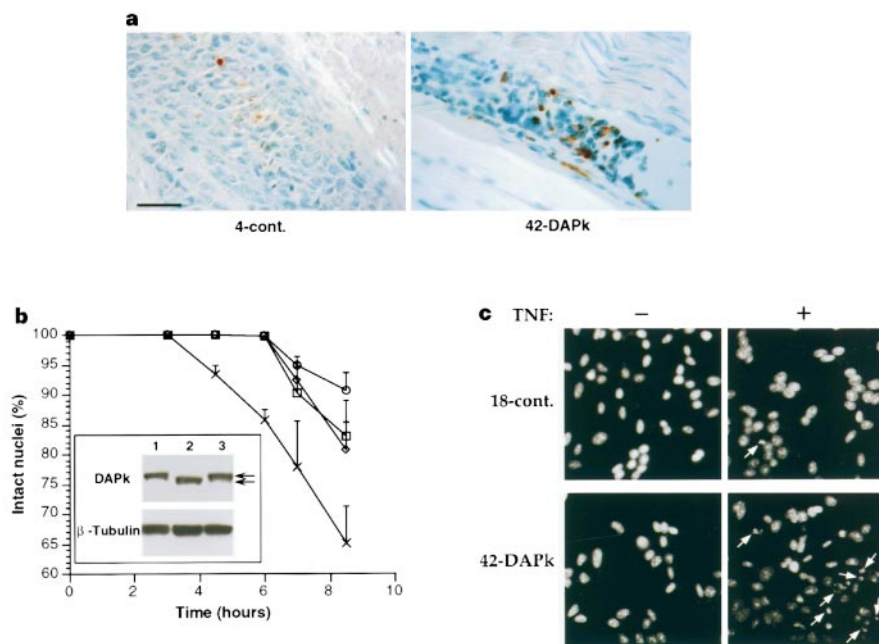


Figure 3 a, *In situ* TUNEL staining of footpad sections on five days after local injection of 4-cont. cells (left) or 42-DAPk cells (right). Scale bar, 100 μ m. **b**, Kinetics of killing by TNF- α plus cycloheximide. The 42-DAPk transfectants (cross) were compared to the parental D122 cells (circle) and to 4-cont. (square) and 18-cont. (diamond). The values are the mean of the percentage of intact nuclei \pm s.d. The inset shows immunoblot analysis of DAP kinase protein, from clone 42-DAPk, incubated for 4 h with no treatment, treatment with TNF- α and cycloheximide, or treatment with cycloheximide alone (lanes 1-3, respectively). **c**, DAPI staining of nuclei before and after treatment with TNF- α . The 18-cont. and 42-DAPk transfectants were treated with a combination of TNF- α and cycloheximide (right panels, marked by a plus), or with cycloheximide alone (left panels, marked by a minus). DAPI staining was done after 6 h. Arrows indicate apoptotic nuclei.

higher sensitivity to the apoptotic effects of TNF- α . Fragmented nuclei appeared much faster and total cell death was greater compared to the control D122 clones that lacked DAP kinase (Fig. 3b, c). A second type of apoptotic stress was induced by growing cells under anchorage-independent conditions in soft agar. In contrast to the parental D122 cells and control clones which formed large viable colonies in semi-solid medium, the various DAP-kinase-transfected clones formed small colonies of dying cells (Fig. 4A and B, a, b). Most of the cells in these colonies had an apoptotic morphology, and contained fragmented nuclei (compare Fig. 4B, c and d and their insets). DAP kinase expression, therefore, also mediates cell death that is induced by anchorage-independent growth¹⁹⁻²². Interestingly, the revertants of clone 42-DAPk, which formed lung lesions and did not express the transgene, lost their increased sensitivity to TNF- α (Fig. 4C, left) as well as the cell-death responses to detachment from extracellular matrix (Fig. 4C, right). Based on these experiments, we suggest that DAP-kinase-mediated suppression of metastasis results, at least in part, from increased apoptotic sensitivity to various death-inducing stimuli.

We have excluded a direct involvement of cytotoxic T cells in the DAP-kinase-mediated suppression of metastasis. DAP-kinase-transfected clones did not display increased sensitivity to killing by cytotoxic T cells induced against immunogenic K^b D122 transfectants²³, and the cell-surface major histocompatibility complex (MHC) class I expression remained low, as previously reported for the parental D122 cells¹⁵ (not shown).

In conclusion, we have made two important findings. First, we have demonstrated the existence of inverse relationships between DAP kinase expression and the ability of carcinoma cells to metastasize to the lungs. Loss of DAP kinase expression therefore may convey selective advantage during the transition of lung carcinoma tumour cells from a low- to a more aggressive high-metastatic phenotype. Second, our results suggest a mechanism for this selective process, by coupling the loss of DAP kinase expression

to resistance to various apoptotic stimuli encountered by tumour cells, particularly during the multiple stages of metastasis. These findings strongly support the notion that loss of apoptotic control is an important factor in metastasis. Further support came from the finding that the dormancy of micrometastases is caused by indirect stimulation of apoptosis by angiogenic inhibitors²⁴, and from proof of a link between experimental metastasis and loss of p53 (ref. 25) or CC3 (ref. 26). We propose that DAP kinase may control apoptosis at early stages of metastasis, such as detachment from the original tumour (for example, cell death that is induced by loss of cell-matrix interactions, named anoikis) and transport in the circulation. In the bloodstream, metastasizing cells may be killed by interactions with monocytes, natural killer cells, and neutrophils, by exposure to cytokines, to nitric oxide anions, or by different mechanical stresses²⁷⁻³⁰. This high number of apoptotic stimuli may explain why restoration of DAP kinase was much more effective in suppressing metastasis after the intravenous injections than in reducing local tumour growth in the foreign environment of footpad muscles. We suggest that loss of DAP kinase expression may act with the many other genetic alterations affecting cellular motility, immunity or invasion in the multistep process of metastasis. □

Methods

Growth conditions, transfections and immunoblot analysis. The 3LL clone-D122 and the CMT64 clone -C6 are the high-metastatic clones and clones A9-F, and E9 are the low-metastatic counterparts, respectively. All cells were grown in DMEM medium supplemented with 10% fetal calf serum (Gibco BRL). D122 cells were stably transfected with pcDNA3-DAP kinase and selected with G418 (800 μ g ml⁻¹) as described². Cell lysates, always prepared from exponentially growing cells, were analysed on immunoblots with anti-DAP-kinase monoclonal antibodies, anti-vinculin, and anti- β -tubulin antibodies (Sigma) as described². Clones 1-DAPk and 21-DAPk expressed exogenous DAP-kinase protein at levels similar to clone 28-DAPk (data not shown).

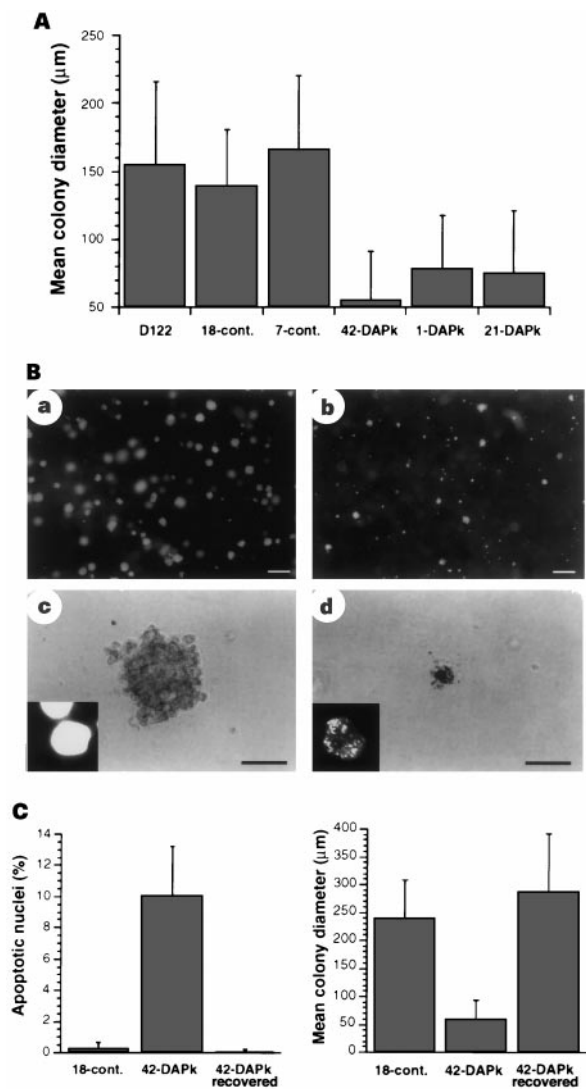


Figure 4 The growth of the D122 transfectants in semi-solid medium. **A**, Values are the mean colony diameter of 100 clones from each group \pm s.d. The difference between the controls (such as 18-cont.) and the DAP kinase transfectants (such as 1-DAPk) was significant at $P \ll 0.001$. **B**, Light microscopy of the clones cultured in soft agar for 7 days, comparing the parental D122 cells (left: **a**, **c**) to H2-DAPk cells (right: **b**, **d**). Bars correspond to 350 μ m in the upper panels (**a**, **b**) and to 80 μ m in the lower panels (**c**, **d**). The insets in **c** and **d** show DAPI staining of nuclei from the corresponding cells. **C**, *In vivo* selection for attenuated DAP kinase expression ablates the increased sensitivity of clone 42-DAPk to TNF- α (left) and to death by detachment from extracellular matrix (right). Responses to TNF- α were assayed as for Fig. 3b at 6h. The original 42-DAPk clone was compared to the cultures recovered from the spontaneous lung metastases.

Tumour development and metastasis. All mice injected were 10–12-week-old C57BL/6 females. For experimental metastasis, the different D122-transfected clones were injected into tail veins (5×10^5 cells per mouse). Mice were killed 30–32 days later (when 50% of mice injected with parental D122 cells had died from lung metastasis), and their lungs were removed, weighed and fixed in Bouin's solution. Diameters of tumour-bearing feet, after intrafootpad injections (2×10^5 cells per mouse), were measured using calipers every 1–3 days. When tumour diameter reached 8–9 mm, tumour-bearing feet were amputated below the knee and the day of death resulting from spontaneous lung metastasis was scored for each individual mouse, in some cases, the tumour cells were recovered in culture from dissected lung lesions. For the *in vivo* selections, A9-F cells were injected (10^6 cells per mouse) into the tail veins of γ -irradiated mice (500 rad for 10 min), and killed after 2 months. Individual

lung lesions were removed, and the tumour cells were recovered in culture. Treatment of cell cultures with 5-aza-2'-deoxycytidine (10 μ M) (Sigma) was done by exposing the cells to the drug for 24 h during the exponential phase of growth. Protein expression was measured 72 h later.

In situ TUNEL assay. Fragments of mouse footpads were fixed for 12 h in 4% buffer formaldehyde (Frutarom), embedded in paraffin and sectioned (4 μ m thick). TUNEL assay on these sections was performed according to the manufacturer's instructions (ApopTag Plus Peroxidase kit; Oncor, Gaithersburg). Six different sections were scored; in each case 500–1,000 tumour cells were counted and the mean apoptotic index was calculated.

TNF- α assays. Exponentially growing cells were treated with a combination of murine TNF- α (100 ng ml $^{-1}$; R&D systems, Minneapolis) and cycloheximide (5 μ g ml $^{-1}$; Sigma), or with cycloheximide alone, and assessed for apoptotic nuclei by DAPI staining as in ref. 2. Five different fields, each consisting of 100 nuclei, were scored for every time point.

Soft agar assays. The different clones were cultured in a semi-solid medium containing 0.33% soft agar (Bacto-agar; Difco) at an initial count of 5×10^3 cells per 6-cm plate, on top of a layer containing 0.5% agar. The diameters of the clones that appeared on day 7 were measured under a light microscope. Cells were recovered from the upper agar layer by dissolving the agar with 6M sodium iodide (10 min at 37 $^{\circ}$ C). The washed cells were assessed for apoptotic nuclei by DAPI staining.

Statistics. Unpaired one-tailed Student's *t*-test was performed in all statistical analyses except for the *in vivo* selection experiments of the low-metastatic A9-F cells, in which both the χ^2 test for two independent samples and the Fisher exact probability test were used to calculate the *P*-value.

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

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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 viruses⁸. Here we describe the properties of cyclins encoded by two herpesviruses, herpesvirus saimiri (HVS) which can transform blood lymphocytes⁹ 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 lymphocytes¹⁵ 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 results¹³. 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. ³⁵S-methionine labelling of infected cell extracts followed by immunoprecipitation 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 ³⁵S-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 ³⁵S-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 described²⁰,