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Growth arrest by the cyclin-dependent kinase inhibitor $p27^{Kip1}$ is abrogated by c-Myc

Jaromir Vlach, Silke Hennecke, Konstantinos Alevizopoulos, Daniela Conti and Bruno Amati[1]

Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland

[1]Corresponding author

We show here that c-Myc antagonizes the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$. p27 expressed from recombinant retroviruses in Rat1 cells associated with and inhibited cyclin E/CDK2 complexes, induced accumulation of the pRb and p130 proteins in their hypophosphorylated forms, and arrested cells in G_1. Prior expression of c-Myc prevented inactivation of cyclin E/CDK2 as well as dephosphorylation of pRb and p130, and allowed continuous cell proliferation in the presence of p27. This effect did not require ubiquitin-mediated degradation of p27. Myc altered neither the susceptibility of cyclin E/CDK2 to inhibition by p27, nor the intrinsic CDK-inhibitory activity of p27, but induced sequestration of p27 in a form unable to bind cyclin E/CDK2. Neither Myc itself nor other G_1-cyclin/CDK complexes were directly responsible for p27 sequestration. Retroviral expression of G_1 cyclins (D1-3, E or A) or of the Cdc25A phosphatase did not overcome p27-induced arrest. Growth rescue by Myc required dimerization with Max, DNA binding and an intact transcriptional activation domain, as previously shown for cellular transformation. We propose that this activity is mediated by the product of an as yet unknown Myc–Max target gene(s) and represents an essential aspect of Myc's mitogenic and oncogenic functions.

Keywords: CDK/CKI/cyclin/Myc/p27

Introduction

The c-*myc* proto-oncogene is a central regulator of cell proliferation and apoptosis. Expression of c-*myc* is strictly dependent on mitogenic signals and is suppressed by growth-inhibitory or differentiation signals (reviewed by Marcu *et al.*, 1992; Henriksson and Lüscher, 1996). Induction of c-*myc* is required for mitogenic signalling by growth factor receptors, such as the colony-stimulating factor (CSF)-1 and platelet-derived growth factor (PDGF) tyrosine-kinase receptors (Roussel *et al.*, 1991; Barone and Courtneidge, 1995). Constitutive expression of Myc generally enforces unscheduled cell proliferation, prevents differentiation and sensitizes cells to apoptosis (Marcu *et al.*, 1992; Henriksson and Lüscher, 1996). Activation of a conditional Myc–estrogen receptor fusion protein (MycER) in quiescent fibroblasts induces cell cycle entry in the absence of mitogens, but also apoptosis if survival factors are missing (Eilers *et al.*, 1991; Evan *et al.*, 1992; Harrington *et al.*, 1994). Thus, Myc promotes entry into, and prevents exit from, the cell cycle. However, the mechanisms connecting Myc function to cell cycle control remain unknown.

Myc is a sequence-specific transcriptional activator which must dimerize with a second protein, Max, in order to bind DNA. Myc can also suppress transcription of several genes *in vivo* by as yet unknown mechanisms. The mitogenic, apoptotic and oncogenic functions of Myc depend upon dimerization with Max, DNA binding and transactivation, suggesting that Myc transforms cells by activating genes involved in cell proliferation and/or apoptosis (reviewed by Amati and Land, 1994; Henriksson and Lüscher, 1996; see also Desbarats *et al.*, 1996).

In vertebrate cells, the commitment to complete a mitotic division cycle takes place in the late G_1 phase, at a stage called the restriction (R-) point (Pardee, 1989; Zetterberg *et al.*, 1995). One of the molecular events required for passage through the R-point is the inactivation by hyperphosphorylation of the retinoblastoma protein (pRb), catalysed by cyclin-dependent kinases (CDKs). The activities of CDK4 or CDK6 (associated with D-type cyclins) and CDK2 (associated with cyclins E or A) are required for hyperphosphorylation of pRb (reviewed by Sherr, 1993, 1994, 1995; Weinberg, 1995). The pRb-related proteins p107 and p130 are also CDK substrates (Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995; Xiao *et al.*, 1996). In their active form, pRb family proteins associate with transcription factors of the E2F/DP family and repress transcription of their target genes (reviewed by Müller, 1995; Weinberg, 1995, 1996).

The activity of CDKs is regulated by various mechanisms including association with cyclins, phosphorylation/dephosphorylation and association with a group of inhibitory proteins collectively called CKIs (reviewed by Morgan, 1995; Sherr and Roberts, 1995). Primary sequence homology defines two families of CKIs. The first includes $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which bind to and inhibit all G_1-cyclin/CDK complexes. The second family includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which bind to CDK4 and CDK6 and can interfere with cyclin–CDK interactions (Sherr and Roberts, 1995). Overexpression of both classes of CKIs by transient transfection causes cell cycle arrest in G_1 (Guan *et al.*, 1994; Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; Hirai *et al.*, 1995; Quelle *et al.*, 1995). Indeed, CKIs play key roles in the response of cells to growth-inhibitory signals (reviewed by Sherr and Roberts, 1995; Harper and Elledge, 1996).

Various observations indicate that p27 and Myc have generally opposite roles in growth control by mitogens. First, p27 is induced in Balb/c-3T3 fibroblasts upon serum deprivation, and is required for G_1 arrest (Coats *et al.*,

1996). In fibroblasts, constitutive Myc prevents cell cycle arrest upon withdrawal of mitogens (Armelin *et al.*, 1984; Keath *et al.*, 1984; Evan *et al.*, 1992; Marcu *et al.*, 1992; Henriksson and Lüscher, 1996). Second, activation of MycER in quiescent fibroblasts induces rapid activation of cyclin E/CDK2, accompanied by degradation of p27 (Steiner *et al.*, 1995; Rudolph *et al.*, 1996). Third, p27 is accumulated in stimulated T cells arrested in G_1 by interleukin-2 (IL-2) deprivation. Upon exposure to IL-2, which induces expression of c-*myc* (Minami *et al.*, 1993), p27 is down-regulated, cyclin E/CDK2 complexes are activated and cells enter the cell cycle (Firpo *et al.*, 1994; Nourse *et al.*, 1994). Expression of a v-*myc* gene in T cells abrogates their dependence on IL-2 (Rapp *et al.*, 1985), and thymocytes from p27-deficient mice show an enhanced mitogenic response to IL-2 (Fero *et al.*, 1996). Fourth, Myc overcomes transforming growth factor β (TGFβ)-induced cell cycle arrest (Alexandrow *et al.*, 1995), which is mediated by the combinatorial action of several CKIs including p27, p15 and p21 (Sherr and Roberts, 1995; Harper and Elledge, 1996). In all the above examples, it remains unclear whether Myc and p27 act on a common or on parallel, independent pathways.

In this work, we asked whether Myc could counteract p27 function and addressed the molecular mechanisms by which this might be achieved. We demonstrate that Myc overcomes p27-induced growth arrest by allowing cyclin E/CDK2 function in the presence of elevated, but physiological levels of p27. This effect of Myc is mediated by a non-covalent sequestration of p27 and requires transcriptionally active Myc–Max dimers. We also provide evidence that the CDK-activating phosphatase Cdc25A, recently shown to be encoded by a Myc-induced gene (Galaktionov *et al.*, 1996), is not mediating rescue of p27-induced arrest.

Results

Myc overcomes p27-induced growth arrest

To test whether Myc might interfere directly with the growth-inhibitory function of p27, we expressed both proteins with retroviruses encoding resistance to hygromycin (Hygro) and puromycin (Puro), following the protocol outlined in Figure 1. Briefly, pools of cells infected with Hygro or Hygro-Myc viruses were expanded under hygromycin selection and superinfected with Puro or Puro-p27 viruses. After the second infection, cells were split and seeded at serial dilutions (e.g. 1/20, 1/200, 1/2000) in high serum, puromycin-selective medium. All non-infected cells had detached within 48 h of selection, at which time the denser dishes were used for flow cytometric analysis of cell cycle distribution and for biochemical studies. All infected cell populations, whether growing or arrested, were subconfluent at this time. The more dilute dishes were incubated further to assess colony outgrowth.

Infection of control Rat1 cells with a p27-encoding retrovirus induced accumulation in the G_1 phase of the cell cycle (Figure 2) and suppressed colony outgrowth (Figure 3, Hygro cells: compare Puro and p27). Microscopic examination showed that p27-arrested cells remained isolated or formed sparse colonies of few, flat and enlarged cells, with no signs of apoptosis. In contrast, Myc + p27 cells retained a proliferative cell cycle profile

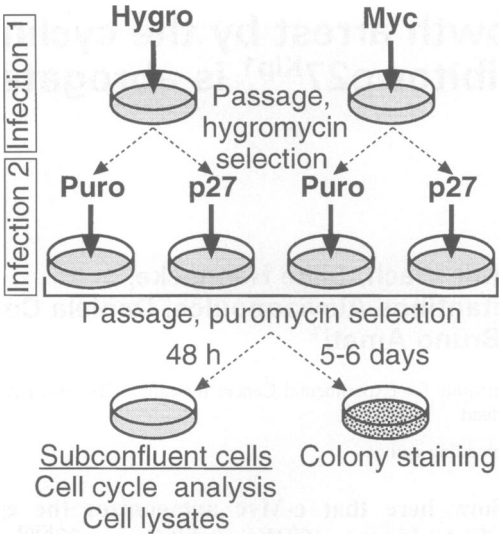

Fig. 1. Schematic representation of our double retroviral infection protocol. See text for explanation.

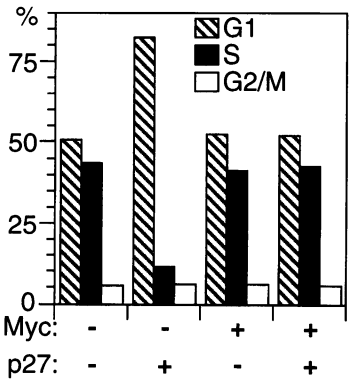

Fig. 2. p27 induces accumulation of control cells in G_1, but does not modify the cell cycle distribution of Myc-expressing cells. Serial infections were performed as shown in Figure 1 with retroviruses expressing Myc and/or p27, as indicated at the bottom. Cells were harvested after 48 h of selection in puromycin, and cell cycle distribution analysed by flow cytometry. The percentage of cells in each phase of the cell cycle is indicated for all cell populations.

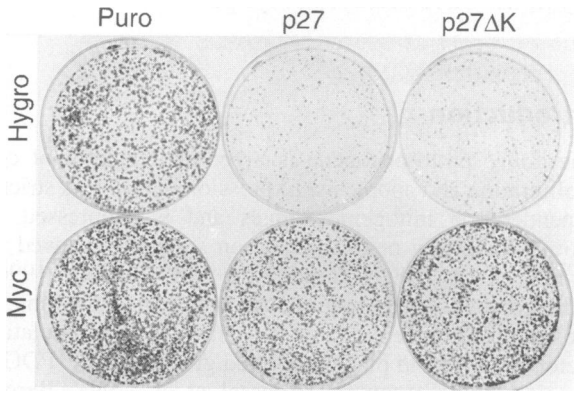

Fig. 3. p27 and the mutant p27ΔK prevent colony outgrowth in control, but not in Myc-expressing cells. Serial infections were performed as shown in Figure 1 and colonies were fixed and stained on dishes after 6 days in puromycin-selective medium. Results with human p27 are shown throughout this work, but identical observations were made with murine p27. p27ΔK is a lysine-free, non-ubiquitinatable mutant of murine p27 (see text).

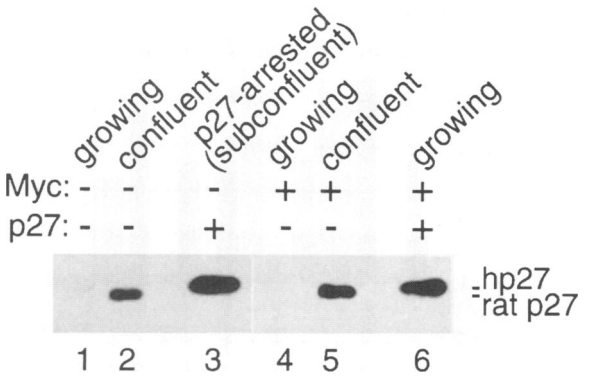

Fig. 4. Retrovirally expressed p27 accumulates to physiological levels in the presence or absence of Myc. An immunoblot experiment is shown, comparing the levels of endogenous rat p27 in growing and confluent control cells with retrovirally expressed human p27 (hp27). The degree of confluence and the exogenous p27 and/or Myc proteins expressed in the cells are indicated at the top.

(Figure 2) and formed colonies with normal efficiency (Figure 3). Thus, Myc cells were resistant to growth arrest by the p27 retrovirus.

p27 is ubiquitinated and degraded through the proteasome pathway (Pagano *et al.*, 1995). To address the role of p27 ubiquitination in growth rescue by Myc, we constructed the non-ubiquitinatable mutant p27ΔK, with conservative substitutions of all lysines (the acceptor residue for ubiquitin). p27ΔK behaved exactly as p27 in the double infection assay (Figure 3). Thus, ubiquitination of p27 was not required for Myc action.

Expression of exogenous p27 was assessed by immunoblot analysis and compared with that of endogenous p27 in growing or contact-inhibited control cells. Two major conclusions were drawn from these experiments. First, the levels of exogenous p27 in arresting, subconfluent cells were similar to those of cellular p27 induced by contact inhibition (Figure 4, lanes 2 and 3). Second, retrovirally expressed Myc had no effect on exogenous or endogenous p27 levels (Figure 4, compare lanes 2–5 and 3–6; Figure 5, top). When expressed at higher levels, exogenous p27 arrested Myc cells as well as controls (Rudolph *et al.*, 1996; data not shown). In conclusion, Myc prevents growth arrest by p27; this effect is only apparent at physiological p27 concentrations and is not mediated by degradation of p27.

p27 arrests cells by suppressing the activity of cyclin E/CDK2 complexes

To understand p27 action in infected cells, we analysed the expression levels of cellular cyclins and CDKs (Figure 5), the interactions among these proteins and with retrovirally expressed p27 (Figure 6), and the associated kinase activities (Figure 7). Results for D-type cyclins/CDK4 and 6, on one hand, and cyclins A and E/CDK2, on the other, are presented separately below.

p27 significantly induced expression of cyclins D1 and D3 and of the respective mRNAs, while levels of their partners CDK4 and CDK6 remained unaltered (Figure 5, lanes 1 and 2 and data not shown). Co-immunoprecipitation analysis showed that the induced cyclins associated with CDK4 and CDK6 in cells. p27 also associated with CDK4, CDK6 and cyclins D1 and D3, but its levels were

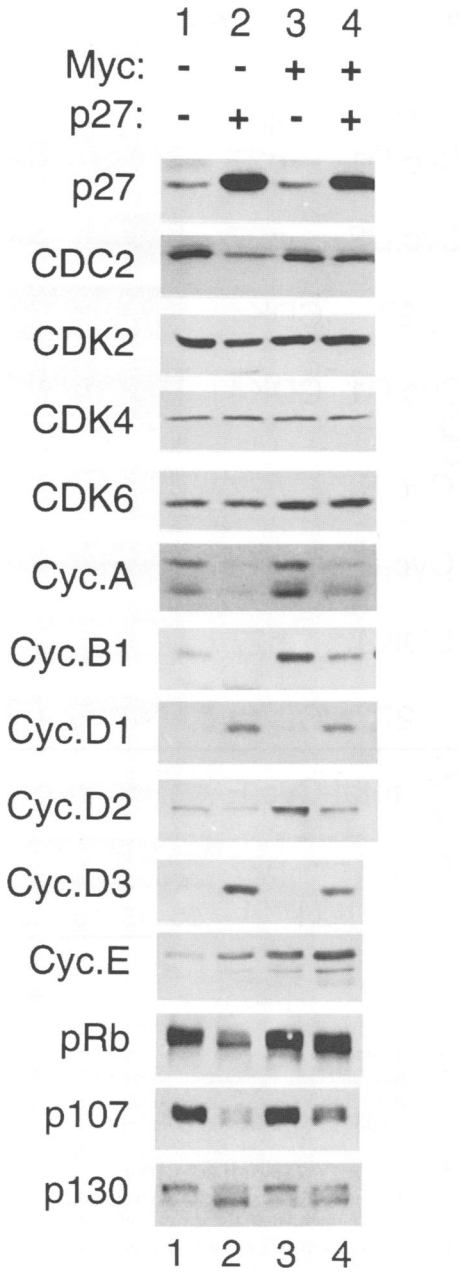

Fig. 5. Immunoblot analysis of cellular proteins in doubly infected Rat1 cells. Cells were infected with retroviruses expressing Myc or p27, as indicated at the top. At the time of analysis (see Figure 1), all populations were subconfluent and p27 cells were arrested, as shown in Figures 2 and 3. The cellular proteins visualized in each panel are indicated to the left.

superseded by the cyclins (Figure 6A, lanes 1 and 2 and data not shown). Most likely as a result of this compensatory loop, the kinase activities associated with CDK4 and CDK6 immunoprecipitates remained invariant in p27-infected cells relative to controls (data not shown).

Cyclin A levels were suppressed by p27, while cyclin E levels were slightly elevated and CDK2 levels remained constant (Figure 5, lanes 1 and 2). Co-immunoprecipitation analysis showed that p27 associated with cyclin E and CDK2, with a concomitant increase in the amounts of cyclin E/CDK2 complexes (Figure 6B, lanes 1 and 2). Cyclin E-, cyclin A- and CDK2-associated kinase activities

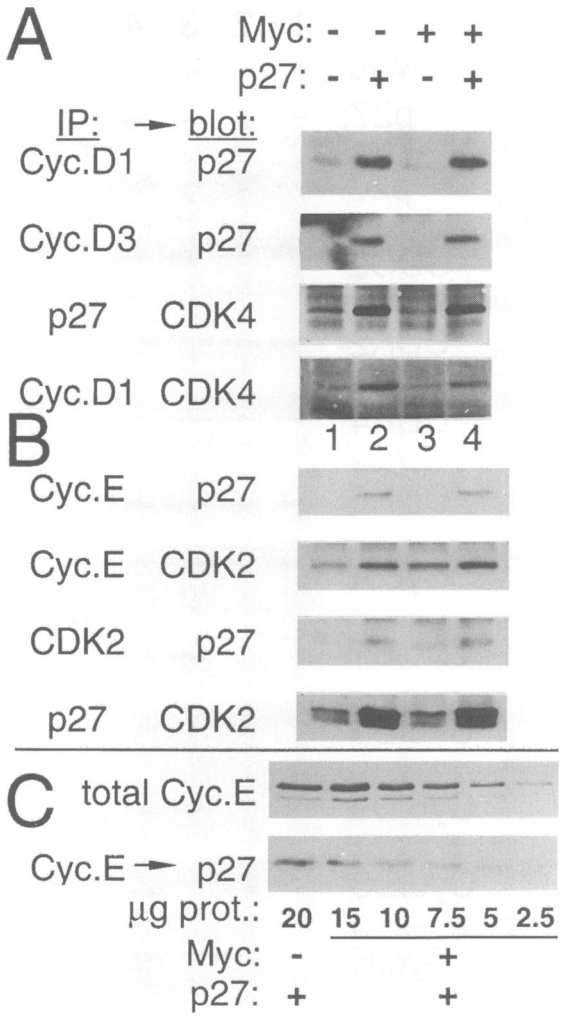

Fig. 6. Composition of cyclin/CDK–p27 complexes in doubly infected cells. Cells were infected as indicated above (A) and below (C). Immunoprecipitations with specific antibodies (IP) from infected cell lysates were followed by immunoblot analysis of co-precipitated proteins (blot), as indicated on the left. (**A**) Protein–protein interactions between cyclins D1, D3, CDK4 and p27. (**B**) Protein–protein interactions between cyclin E, CDK2 and p27. Each IP in (A) and (B) was from equal amounts of total cellular protein. (**C**) Amounts of total cyclin E, and of p27 recovered with cyclin E immunoprecipitates, were compared using varying amounts of p27-only and Myc + p27 cell lysates, as indicated below the panels.

were strongly suppressed in p27-arrested cells (Figure 7, lanes 1 and 2). In conclusion, p27 virtually eliminated CDK2 activity. This was due primarily to the formation of inactive p27–cyclin E/CDK2 ternary complexes, and to the concomitant decrease in cyclin A expression.

The effects of p27 on pRb family proteins were investigated by immunoblotting. pRb and p130 accumulated in their faster migrating, hypophosphorylated forms in p27-arrested cells, but the apparent ratio of hypo- to hyperphosphorylated p107 remained constant (Figure 5). This result is consistent with the requirement for CDK2 for phosphorylation of pRb and p130, but presumably not of p107 (Li *et al.*, 1993; Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995; Weinberg, 1995; Xiao *et al.*, 1996), although changes in p107 phosphorylation that do not alter its electrophoretic mobility cannot be ruled out at the moment.

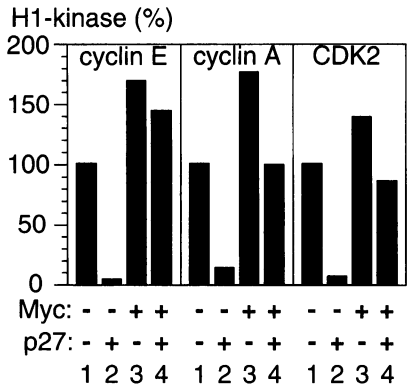

Fig. 7. Effect of Myc and p27 on the kinase activities associated with cyclin E, cyclin A and CDK2. Histone H1 kinase activities were measured in immunoprecipitates of cyclins E, A and CDK2 from doubly infected cells (as indicated at the bottom). Each measurement was from equal amounts of total cellular protein and was normalized to the activity in control cells (lanes 1, 100%).

Myc prevents inactivation of cyclin E/CDK2 by p27

To understand how Myc could suppress p27-induced growth arrest, we compared the molecular effects of p27 in control cells with those in Myc-expressing cells. The most remarkable effect of Myc was to prevent suppression of cyclin E-, cyclin A- and CDK2-associated kinase activities by p27 (Figure 7, lanes 3 and 4). Consistent with the restoration of kinase activities, Myc also restored phosphorylation of pRb and p130 in the presence of p27 (Figure 5). Cyclin A mRNA and protein levels in Myc + p27 cells were comparable with those found in control cells (Figure 5 and data not shown), accounting for the maintenance of cyclin A (and most of CDK2) kinase activity (Figure 7). It is worth remarking here that the mitotic regulators cyclin B1 and CDC2 followed the same pattern as cyclin A (Figure 5), further emphasizing the proliferative status of Myc + p27 cells. Altogether, the above observations are consistent with cyclin A expression requiring cyclin E/CDK2 activity and release of E2F from pRb family proteins (Schulze *et al.*, 1995; Rudolph *et al.*, 1996). Thus, the key parameter to explain Myc action was to understand how it prevented inhibition of cyclinE/CDK2 by p27.

Expression of Myc alone increased the levels of cyclin E (Figure 5, lanes 1 and 3) and its interaction with CDK2 (Figure 6B), probably because Myc induces premature expression of cyclin E in early G_1 (Jansen-Dürr *et al.*, 1993; O.Pusch, G.Bernaschek, M.Eilers and M.Hengstschläger, manuscript submitted). These effects of Myc were independent from, and additive to, the effects of p27 (Figures 5 and 6). Analysis of the changes induced by Myc in the stoichiometries of p27–cyclin E/CDK2 interactions in doubly infected Rat1 cells was complicated by the fluctuations in the levels of cyclins E and A (see Figure 5). For example, association of p27 with CDK2 immunoprecipitates, and vice versa, was similar in p27-only and Myc + p27 cells (Figure 6B, lanes 2 and 4), but was due to a comparatively higher proportion of cyclin A-containing complexes in the latter (data not shown). Equal amounts of p27 were recovered with cyclin E immunoprecipitates from p27-only and Myc + p27 cells (Figure 6B), but corresponded to higher levels of cyclin E in the latter (Figure 5, lanes 2 and 4). In contrast, cyclin E-associated

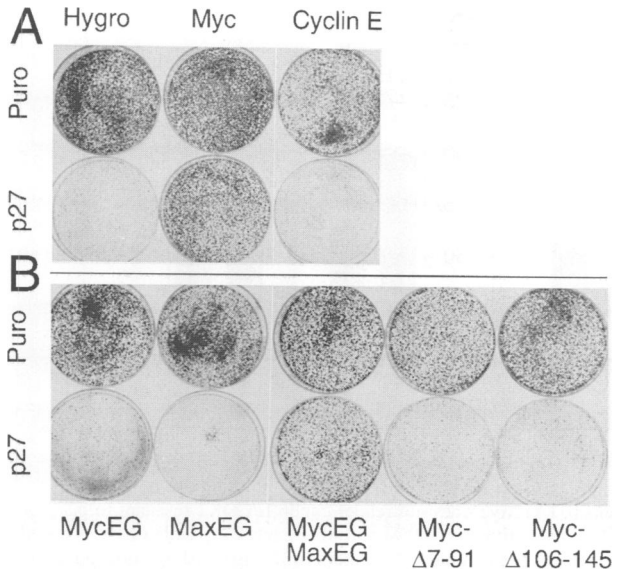

Fig. 8. (**A**) Human cyclin E does not overcome p27-induced growth arrest. (**B**) Overcoming p27-induced arrest by Myc requires dimerization with Max, DNA binding and transactivation. Rat1 cells were infected with retroviruses expressing human cyclin E or the indicated Myc and Max mutants (see text), and superinfected with control (Puro) or p27 viruses as outlined in Figure 1. Colonies were visualized as described in Figure 2.

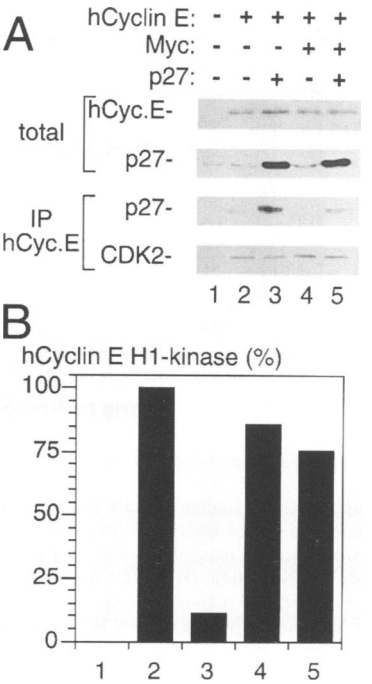

Fig. 9. Expression of human cyclin E together with Myc and p27 in Rat1 cells. Cyclin E was expressed from a retrovirus conferring neomycin resistance. (**A**) Immunoblot analysis of total human p27 and cyclin E (hCyc.E), and of CDK2 and p27 associated with immunoprecipitated human cyclin E (as described in Figure 6). (**B**) H1 kinase activity in immunoprecipitates of human cyclin E (as described in Figure 7). Values were normalized to cells expressing human cyclin E alone (lane 2).

CDK2 was slightly higher in Myc + p27 than in p27-only cells (Figure 6B). Altogether, these data indicated that Myc reduced the stoichiometry of p27 per cyclin E/CDK2 complex. To illustrate this phenomenon more clearly, we performed the experiment shown in Figure 6C. Total cyclin E levels and cyclin E-associated p27 were measured in a p27-arrested cell lysate, and compared with decreasing amounts of a Myc + p27 lysate. At equivalent cyclin E levels (with 20 versus 10–7.5 μg of lysate respectively), less p27 was associated with cyclin E in the presence of Myc. Altogether, we estimate that Myc reduced the levels of cyclin E-associated p27 by 2.5- to 3-fold. As described below, analysis of triple-infected cells expressing human cyclin E, Myc and p27 allowed us to confirm this interpretation.

Enhanced expression of cyclin E does not account for Myc activity

One possible explanation of the above data was that deregulation of cyclin E expression by Myc may be able to overcome p27-induced arrest. This possibility was ruled out by co-expressing human cyclin E with p27 and/or Myc. Infection of Rat1 cells with a cyclin E-expressing retrovirus did not prevent p27-induced growth arrest (Figure 8A). Immunoprecipitation with a human cyclin E-specific antibody showed that exogenous cyclin E associated with endogenous CDK2 and activated it (Figure 9A and B, lanes 1 and 2). Retrovirally expressed p27 associated with and inactivated human cyclin E/CDK2 complexes (lanes 2 and 3). Retrovirally expressed Myc rescued human cyclin E-associated kinase activity in the presence of p27 (Figure 9B, lanes 4 and 5) and restored cell proliferation (data not shown). Association of p27 with human cyclin E was virtually eliminated by Myc, while total p27 levels were unchanged (Figure 9A, compare lanes 3 and 5) and human cyclin E/CDK2 interactions remained constant (lanes 2–5). These data demonstrated that the major effect of Myc was to prevent association of p27 with cyclin E/CDK2.

Myc induces sequestration of p27 away from cyclin E/CDK2

How did Myc prevent association of p27 with cyclin E/CDK2 complexes? Cyclin E and CDK2 were not rendered inherently resistant to p27, as cyclin E-associated kinase activity in lysates of control and Myc cells was equally sensitive to a purified p27 protein (Figure 10A). Consistent with this finding, the CDK activators CAK and Cdc25A appeared to play no causal role. First, neither the specific activity of CAK, nor the ratio of CAK-phosphorylated to unphosphorylated CDK2 was affected by p27 or Myc (Figures 5 and 6B and data not shown; Kato *et al.*, 1994; Steiner *et al.*, 1995). Second, the steady-state levels of Cdc25A mRNA and protein were not increased in Myc-expressing cells (Figure 11A and data not shown). Infection with a retrovirus encoding Cdc25A, resulting in expression of the protein 2- to 3-fold above endogenous levels (Figure 11A, lane 5), did not rescue p27-induced arrest (Figure 11B).

Myc did not affect p27 activity intrinsically. Indeed, p27 released by boiling (Hengst *et al.*, 1994; Polyak *et al.*, 1994a) from either Myc + p27 or p27-only cell lysates could inhibit human cyclin E-associated kinase equally well in a target lysate (Figure 10B). In contrast, the use of non-boiled lysates revealed p27 activity in p27-only, but not in Myc + p27 cells (Figure 10C). This suggests that Myc inactivates p27 by inducing its sequestration.

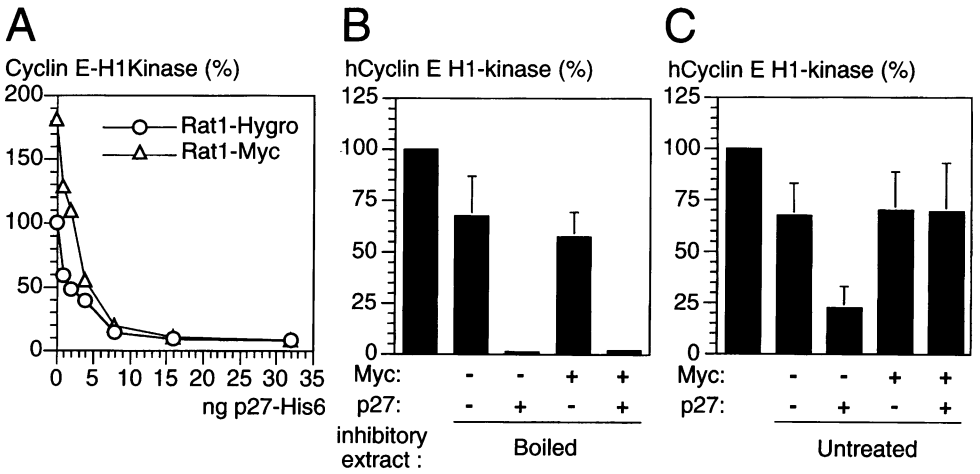

Fig. 10. Myc suppresses the inhibitory activity of p27 by a non-covalent mechanism. (**A**) Myc does not render cyclin E/CDK2 resistant to p27. Cyclin E-associated H1 kinase activity in lysates of control Rat1 Hygro cells (○, initial activity is 100%) and Myc-expressing cells (△) was tested following incubation with increasing amounts of a purified recombinant, hexahistidine-tagged human p27 protein (p27-His6); 10 ng correspond to 8.7 nM p27-His6 in the reaction. (**B** and **C**) Activity of p27 in boiled and untreated lysates from doubly infected cells expressing Myc and/or p27 (as indicated at the bottom). p27 activity was monitored by mixing with a target lysate from Rat1 cells expressing human cyclin E, followed by selective measurement of human cyclin E-associated H1 kinase activity, as shown in Figure 9B. All activities were normalized to that of the target alone (100%).

Immunofluorescence showed p27 in cell nuclei in the presence or absence Myc (data not shown).

What protein might inactivate p27 in Myc-expressing cells? Myc itself did not bind p27 directly, as tested by co-immunoprecipitation and two-hybrid assays. Cyclin D/CDK complexes can sequester p27 away from cyclin E/CDK2 (Polyak *et al.*, 1994a; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996). However, Myc did not increase association of p27 with cyclins D1, D3, CDK4 or CDK6 (Figure 6A, compare lanes 2 and 4; data not shown). Cyclin D2 expression was slightly increased in Myc cells (Figure 5), but its levels remained low and cyclin D2-associated p27 was undetectable. Ectopic expression of cyclins D1, D2, D3 or A (with or without cyclin E) prevented neither p27-induced arrest, nor rescue by Myc (data not shown). Thus neither D-type cyclins nor cyclin A accounted for Myc action.

Rescue of p27-induced arrest requires transcriptionally active Myc–Max dimers

To address the role of Myc–Max dimerization in preventing growth arrest by p27, we expressed in Rat1 cells the leucine zipper mutants MycEG and MaxEG. These mutants have reciprocally altered dimerization specificities; when expressed in cells, MycEG does not bind Max, and MaxEG does not bind Myc, but MycEG and MaxEG together form stable, biologically active dimers (Amati *et al.*, 1993a,b). Cells expressing MycEG or MaxEG alone were arrested by p27, whereas MycEG and MaxEG together, like Myc, prevented growth arrest by p27 (Figure 8B). Three additional Myc mutants were tested in the p27 rescue assay: Myc 364,6,7R/A (a basic region mutant selectively deficient in DNA binding; Amati *et al.*, 1992), MycΔ7–91 and MycΔ106–145 (transactivation domain deletions, deficient in activation of a Myc–Max target gene; Stone *et al.*, 1987; Kato *et al.*, 1990; Desbarats *et al.*, 1996). All Myc mutants were expressed as efficiently as Myc, but were unable to prevent p27-induced growth

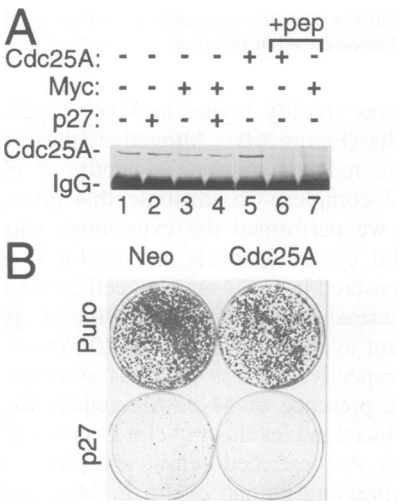

Fig. 11. Cdc25A does not mediate the p27-antagonizing activity of Myc. (**A**) Cdc25A protein levels in infected cells expressing exogenous Myc, p27 or Cdc25A, as indicated at the top. Cdc25A in Rat1 cells was detected by immunoprecipitation followed by immunoblotting, and migrates 15–20 kDa above immunoglobulin heavy chains (IgG), as previously reported (Galaktionov *et al.*, 1996). Specificity of the IP was controlled by blocking of the antibody with its cognate immunogenic peptide (+pep, lanes 6 and 7). (**B**) Colony outgrowth assay (as shown in Figure 1) on cells expressing Cdc25A and/or p27. Cdc25A was expressed from a retrovirus conferring neomycin resistance (Neo).

arrest (Figure 8B; data not shown). Thus, like the oncogenic and apoptotic activities of Myc, suppression of p27 function is mediated by transcriptionally active Myc–Max dimers.

Discussion

Mechanisms of growth arrest by ectopic p27 expression

Expression of p27 by transient transfection arrests cells in G_1 (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994),

a result confirmed here with retroviral expression of p27 in Rat1 fibroblasts. The cyclin/CDK complexes targeted by p27 *in vivo* and *in vitro* are cyclins D1-3 with CDK4 or CDK6, and cyclins E or A with CDK2 (Hengst *et al.*, 1994; Kato *et al.*, 1994; Nourse *et al.*, 1994; Polyak *et al.*, 1994a,b; Slingerland *et al.*, 1994; Toyoshima and Hunter, 1994; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996). Indeed, retrovirally expressed p27, which accumulated to physiological levels in infected cells, associated with both types of G_1-cyclin/CDK complexes.

In p27-arrested Rat1 cells, p27 was stably associated with cyclin E/CDK2 complexes and inhibited their catalytic activity. We observed slight p27-dependent increases in cyclin E levels and cyclin E–CDK2 interactions, which might be due to stabilization of cyclin E in the inactive ternary complexes (Clurman *et al.*, 1996; Won and Reed, 1996). p27 also induced loss of cyclin A/CDK2 activity. This was due primarily to decreased levels of cyclin A mRNA and protein, consistent with the notion that cyclin A expression requires the activity of cyclin E/CDK2 (Rudolph *et al.*, 1996). In contrast to CDK2, the activities of CDK4 and CDK6 were unaltered in p27-arrested cells. Two explanations are possible for this paradox. First, p27 may not *a priori* inhibit cyclin D/CDK4 and 6 complexes upon association. For example, pRb kinase activity can be co-immunoprecipitated with p27 from MANCA cells (Soos *et al.*, 1996). Second, the amount of cyclin D/CDK4 and 6 complexes may exceed p27. In favour of this hypothesis, p27 significantly increased cyclin D1 and D3 mRNA and protein levels in infected cells. The induced cyclins associated with p27 and CDK4 and 6 (but not with CDK2), and were in excess of p27 itself. The mechanism of cyclin D1 and D3 induction by p27 is currently under study. In summary, the major target of p27 in fibroblasts is cyclin E/CDK2.

Cyclin E/CDK2 activity is required for phosphorylation of pRb and p130 (Mayol *et al.*, 1995; Weinberg, 1995). Active pRb and p130 bind to various cellular proteins, in particular to the E2F/DP family of transcription factors, resulting in repression of their target genes (such as cyclin A) and G_1 arrest (Müller, 1995; Weinberg, 1996). Consistent with these facts, pRb and p130 accumulated in their hypophosphorylated, active forms in p27-arrested cells, as assessed by immunoblotting experiments.

Our data on p27-arrested cells are consistent with the mode of action of various anti-mitotic stimuli. For example, IL-2 deprived T cells, TGFβ-arrested epithelial cells or mitogen-deprived fibroblasts arrest with elevated p27 levels and/or inhibited cyclin E/CDK2 (Nourse *et al.*, 1994; Polyak *et al.*, 1994a; Slingerland *et al.*, 1994; Reynisdottir *et al.*, 1995; Coats *et al.*, 1996). Importantly, however, p27 must be redundant with other CKIs in executing growth arrest, since cells derived from p27-deficient mice arrest normally in response to all the aforementioned stimuli (Nakayama *et al.*, 1996).

Myc, in association with its partner Max, prevents inhibition of cyclin E/CDK2 and growth arrest by p27

Upon infection with a p27 retrovirus, Myc-expressing cells continued to proliferate with normal DNA content and cell cycle distribution. p27 was expressed in Myc cells as efficiently as in control p27-arrested cells, but no longer suppressed cyclin E/CDK2 activity. As a result, the ratio of hyper- to hypophosphorylated pRb and p130, as well as expression of cyclin A, cyclin B1, CDC2 and associated activities remained similar to those found in proliferating control cells. To address directly the role of p27 ubiquitination (Pagano *et al.*, 1995), we constructed the lysine-free, non-ubiquitinatable mutant p27ΔK. This mutant arrested cell growth and was antagonized by Myc as efficiently as wild-type p27. In conclusion, our data demonstrate that Myc counteracts inhibition of cyclin E/CDK2 and growth arrest by p27 without requiring its ubiquitination and/or degradation.

All tested biological activities of Myc, including cellular transformation, prevention of cell cycle arrest in low serum and induction of apoptosis, depend upon dimerization with Max, sequence-specific DNA binding, and transcriptional activation (Stone *et al.*, 1987; Amati *et al.*, 1993a,b; Amati and Land, 1994; Henriksson and Lüscher, 1996). Using previously characterized Myc and Max mutants, we have shown that rescue of p27-induced arrest by Myc has exactly the same prerequisites, implying that one or more Myc–Max target genes are mediating this effect. These data also suggest that counteracting p27 function is an essential aspect of Myc's mitogenic and transforming activities.

Like Myc, the E1a oncoprotein of adenovirus functionally antagonizes p27, presumably through a direct molecular interaction (Mal *et al.*, 1996; K.Alevizopoulos and B.Amati, unpublished data). This convergence of function between E1a and Myc further implies that inactivation of p27 is important for mitogenesis, immortalization and/or cellular transformation. Whether the p27 gene is a tumour suppressor is still unclear. p27 mutations occur very infrequently and in few human tumours (Kawamata *et al.*, 1995; Morosetti *et al.*, 1995; Pietenpol *et al.*, 1995; Ponce-Castaneda *et al.*, 1995; Harper and Elledge, 1996). p27 deletion in mice increases cell proliferation and body size, but not the incidence of multiple tumours. However, like *Rb* mutations, p27 deletion increases the frequency of benign pituitary tumours (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996).

Effects of Myc on the function of other CKIs

The gene encoding $p21^{Cip1/Waf1}$ is a transcriptional target of p53 and is required for p53-dependent G_1 arrest in response to DNA damage. p21 is also induced independently of p53 by various signals, such as TGFβ or differentiation inducers (Sherr and Roberts, 1995; Harper and Elledge, 1996). Myc overcomes G_1 arrest by p53 or differentiation stimuli without suppressing induction of p21 (Steinman *et al.*, 1994; Hermeking *et al.*, 1995). However, the G_1 arrest response to p53 and differentiation inducers seems to require other common target genes, such as gadd45, MyD118, gadd34/MyD116 and others (e.g. Zhan *et al.*, 1994). gadd45 and MyD118 encode two related proteins which can both interact with p21 (Kearsey *et al.*, 1995; Vairapandi *et al.*, 1996). In our experiments, p21 or gadd45 expressed individually from retroviruses only marginally affected cell growth. Thus, it remains unclear whether Myc overcomes p53- or differentiation-induced arrest by antagonizing p21, gadd/MyD proteins and/or other growth inhibitors.

CKIs of the INK4 family are also induced in response to growth-inhibitory signals (Hannon and Beach, 1994; Quelle *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995). Using the approach described in this work, we have found that Myc suppresses growth arrest by INK4 proteins, by mechanisms currently under study.

Myc reduces association of p27 with cyclin E/CDK2 by a non-covalent and saturable mechanism

Activation of MycER leads to a loss of cell cycle control on the expression of cyclins E and A, causing premature activation of CDK2, inactivation of pRb and release of active E2F (Jansen-Dürr *et al.*, 1993; O.Pusch, G.Bernaschek, M.Eilers and M.Hengtschläger, manuscript submitted). Conversely, the same events are delayed in cells bearing a heterozygous deletion of c-*myc* (Hanson *et al.*, 1994). In this context, deregulated expression of cyclin A is most likely a consequence of cyclin E activity (Rudolph *et al.*, 1996), and both cyclins may be induced as a consequence of activated E2F (DeGregori *et al.*, 1995; Schulze *et al.*, 1995), thereby constituting a positive autoregulatory loop.

We demonstrated that deregulation of cyclin E (or other cyclins, see below) does not account for rescue of p27-induced arrest by Myc. Rat1 cells constitutively expressing human cyclin E were still arrested by p27, and human cyclin E/CDK2 complexes were repressed by p27 in those cells. Most importantly, infection of human cyclin E-expressing cells with a Myc retrovirus rescued growth in the presence of p27. As for endogenous cyclin E, inhibition of human cyclin E/CDK2 by p27 was prevented by Myc. Co-immunoprecipitation studies showed that this effect was attributable to decreased interaction of p27 with cyclin E/CDK2.

Cyclin E/CDK2 was not the direct target of Myc in our system, since this complex was equally sensitive to inhibition by p27 in lysates of Myc-expressing or control cells. Myc also did not affect the intrinsic CDK-inhibitory activity of p27, as detected by boiling of infected cell lysates. In contrast, CDK-inhibitory activity was detectable in non-boiled lysates from p27-arrested but not from Myc + p27 cells. These data were analogous to those previously obtained from resting versus growing Mv1Lu cells (Polyak *et al.*, 1994a) and demonstrated that p27 in Myc cells was held away from cyclin E/CDK2 by a non-covalent interaction. Consistent with this conclusion, activation of MycER in quiescent cells induced dissociation of p27 from cyclin E/CDK2 and its entry into a heat-labile inactive state analogous to the one shown here (M.Eilers, personal communication; J.Vlach and B.Amati, unpublished). One major prediction for such a sequestration mechanism was that it should be saturable *in vivo*: an excess of p27 should titrate the sequestering protein and impose growth arrest. This prediction was met, since Myc could not overcome growth arrest induced by supraphysiological levels of p27 (Rudolph *et al.*, 1996).

Myc did not associate directly with p27, suggesting that its effects were mediated by a distinct cellular protein(s). Cyclins D1-3 were plausible candidates, since cyclin D/CDK complexes can sequester p27 away from cyclin E/CDK2 *in vitro* or *in vivo* (Polyak *et al.*, 1994a; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996). Cyclin A was another candidate, since it was re-expressed in Myc + p27 compared with p27-arrested cells. However, Myc did not increase association of p27 with cyclins D1-3 or A. In addition, when expressed from retroviruses (alone or in several combinations), none of these cyclins prevented p27-induced arrest. Thus derepression of cyclin E/CDK2 by Myc is not mediated by other G_1 cyclins.

That D-type cyclins are not sufficient, in our experimental system, to prevent p27-induced arrest does not contradict the notion that they may also antagonize p27 *in vivo*. In fact, these cyclins are likely to cooperate with Myc in growth control. For example, induction of Myc and cyclin D1 by the CSF-1 receptor are parallel and essential pathways for mitogenic signalling (Roussel *et al.*, 1995). Additional mechanisms that regulate p27 include translational control (Hengst and Reed, 1996), ubiquitination (Pagano *et al.*, 1995) and possibly others. We have demonstrated that the effect of Myc on p27 can be detached from (although it is sometimes followed by) p27 degradation (Steiner *et al.*, 1995; O.Pusch, G.Bernaschek, M.Eilers and M.Hengtschläger, manuscript submitted). How the various mechanisms regulating p27 are integrated in growth control and perturbed during tumorigenesis remains to be determined.

Recently, the CDK-activating phosphatase Cdc25A was reported to be encoded by a Myc-induced gene (Galaktionov *et al.*, 1996). Several lines of evidence show that release of cyclin E/CDK2 from p27 is not mediated by Cdc25A and requires a distinct, independent effect of Myc. First, cyclin E/CDK2 complexes derepressed by MycER *in vivo* could be activated further by Cdc25A *in vitro* (Steiner *et al.*, 1995), whereas p27-repressed complexes could not (M.Eilers, personal communication). Thus, repression of cyclin E/CDK2 by p27 appears to be dominant over activation by Cdc25A. Second, retrovirally expressed Myc did not increase Cdc25A levels in our experiments. Third, retroviral expression of Cdc25A did not prevent p27-induced arrest.

In summary, Myc induces an as yet unknown cellular activity which leads to the sequestration of p27 and derepression of cyclin E/CDK2. In particular, we predict that a p27-sequestering protein may be encoded by a Myc–Max-induced gene. Probably as a result of cyclin E/CDK2 activation, Myc induces premature expression of cyclins E and A (Jansen-Dürr *et al.*, 1993; Pusch *et al.*, 1996; Rudolph *et al.*, 1996). The result of these activities altogether is premature or permanent phosphorylation of pRb (Steiner *et al.*, 1995; this work) and escape from restriction point control, as recently shown for pRb-deficient cells (Herrera *et al.*, 1996).

Materials and methods

Retroviral expression vectors

The retroviral vectors pBabe-Puro (pBP), -Neo (pBN) and -Hygro (pBH) were described previously (Morgenstern and Land, 1990). pBabe-Neo2 and -Hygro2 (pBN2, pBH2) were constructed by ligating the *NotI–ClaI* fragment of pBN and pBH to the *ClaI–NotI* fragment of pBP. This places the bacterial replication origin outside of the proviral DNA insert (as in pBP), and sometimes increased protein expression from cDNA inserts. Human cyclin E, Myc and p27 cDNAs were subcloned in pBN2, pBH2 and pBP, respectively. The murine p27 mutant p27ΔK was constructed by PCR and subcloned in pBP. In p27ΔK, lysines were

substituted by either arginine (positions 25, 59, 96, 100, 153, 165, 189 and 190), alanine (position 47) or valine (position 68). These changes were determined in part by the residues found at equivalent positions in human p27 or in the related CKIs p21 and p57.

Retroviral infections and cell cycle analysis

High titre retroviral supernatants ($\leq 5\times10^6$/ml) were generated by transient transfection of BOSC23 cells and used to infect Rat1 cells as described (Pear *et al.*, 1993). Serial infections were perfomed as shown in Figure 1. All non-infected cells had detached after 36 h of puromycin selection (2.5 μg/ml), allowing analysis of pure infected cells. For cell lysates, $\sim5\times10^6$ infected cells were seeded onto five 15 cm dishes. This yielded ~100 μg of total cellular protein from p27-arrested cells and 600 μg from all growing populations (scaled up when required). All cells were subconfluent at the time of harvesting to avoid the interfering effects of contact inhibition. For flow cytometric analysis, cells were labelled with 33 μM bromodeoxyuridine (BrdU) for 30 min. Total DNA content and BrdU incorporation (S phase) were determined as described (Renno *et al.*, 1995). Colonies were fixed and stained on culture dishes with crystal violet (Sigma) in 20% methanol.

Biochemical analysis of infected cells

Infected cells (see above) were lysed in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 20 mM NaF. Between 10 and 50 μg of total cellular protein (determined with a Bio-Rad DC protein assay) were used for gel electrophoresis (SDS–PAGE) and immunoblotting, or for immunoprecipitations (IPs). Immunoprecipitates were washed four times in lysis buffer and loaded onto SDS–PAGE for immunoblot analysis. After incubation with primary antibodies (see list below), reactive proteins on immunoblots were detected through peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (DAKO) and enhanced chemiluminescence (ECL, Amersham). For kinase assays, IPs performed as above were washed twice more and resuspended in kinase reaction buffer (final: 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 50 μM ATP, 1 μg of histone H1, 7 μCi of [γ-^{32}P]ATP) and incubated for 30 min at 30°C. Radiolabelled histone H1 was resolved by SDS–PAGE and quantified with a phosphorimager. For *in vitro* p27 inhibition experiments, purified recombinant p27-His6 (Polyak *et al.*, 1994b) was added to 25 μg of target Rat1 or Rat1-Myc cell lysates. For extract mixing experiments, 15 μg of boiled infected cell lysates or 30 μg of untreated lysates were added to 20 μg of a target lysate from Rat1 cells expressing human cyclin E. All target–inhibitor mixes were incubated at 30°C for 30 min prior to immunoprecipitation of cyclin E and kinase assays, as above.

Antibodies

The following antibodies were used: against p27, C-19 (Santa Cruz [sc]-528) or K25020 (Transduction Laboratories); CDK2, M2 (sc-163); CDK4, C22 (sc-260) or H303 (sc-749); CDK6, C21 (sc-177); CDC2(17), (sc-54); cyclin A, H-432 (sc-751); rodent cyclin E, M20 (sc-481); human cyclin E, C19 (sc198); cyclin D1, 72-13G (sc-450); cyclin D2, DCS-5 (from J.Bartek); cyclin D3, C16 (sc-182); cyclin B1, GNS1 (sc-245); pRb, 14001A (Pharmingen); p107, C18 (sc-318); p130, C20 (sc-317); Cdc25A, 144 (sc-97).

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