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Cyclin E and c-Myc promote cell proliferation in the presence of p16^{INK4a} and of hypophosphorylated retinoblastoma family proteins

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Retroviral expression of the cyclin-dependent kinase (CDK) inhibitor p16^{INK4a} in rodent fibroblasts induces dephosphorylation of pRb, p107 and p130 and leads to G_1 arrest. Prior expression of cyclin E allows S-phase entry and long-term proliferation in the presence of p16. Cyclin E prevents neither the dephosphorylation of pRb family proteins, nor their association with E2F proteins in response to p16. Thus, cyclin E can bypass the p16/pRb growth-inhibitory pathway downstream of pRb activation. Retroviruses expressing E2F-1, -2 or -3 also prevent p16-induced growth arrest but are ineffective against the cyclin E–CDK2 inhibitor p27^{Kip1}, suggesting that E2F cannot substitute for cyclin E activity. Thus, cyclin E possesses an E2F-independent function required to enter S-phase. However, cyclin E may not simply bypass E2F function in the presence of p16, since it restores expression of E2F-regulated genes such as cyclin A or CDC2. Finally, c-Myc bypasses the p16/pRb pathway with effects indistinguishable from those of cyclin E. We suggest that this effect of Myc is mediated by its action upstream of cyclin E–CDK2, and occurs via the neutralization of p27^{Kip1} family proteins, rather than induction of Cdc25A. Our data imply that oncogenic activation of c-Myc, and possibly also of cyclin E, mimics loss of the p16/pRb pathway during oncogenesis.

Keywords: CDK/cyclin/Myc/p16/retinoblastoma

Introduction

The activities of the cyclin-dependent kinases CDK4 and CDK6, which associate with D-type cyclins, and of CDK2, which associates with cyclins E or A, are rate-limiting for progression through G_1 and into S-phase of the vertebrate cell cycle (reviewed by Sherr, 1994, 1995). Besides association with cyclins, the activity of CDKs is controlled by site-specific phosphorylation or dephosphorylation and by association with a group of inhibitory proteins collectively called CKIs (reviewed by Morgan, 1995; Sherr and Roberts, 1995). CKIs of the Kip/Cip family, p21$^{Cip1/Waf1}$, p27^{Kip1} and p57^{Kip2}, can associate with and inhibit all known G_1 cyclin–CDK complexes. CKIs of the INK4 family, p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} bind CDK4 and CDK6 and can interfere with cyclin–CDK interactions. Ectopic expression of CKIs induces G_1 arrest (Guan *et al.*, 1994; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Hirai *et al.*, 1995; Quelle *et al.*, 1995; Vlach *et al.*, 1996), and numerous observations link CKIs to growth arrest and/or differentiation in diverse cell types (reviewed by Sherr and Roberts, 1995; Assoian, 1997).

The best characterized substrates of cyclin–CDK complexes are the retinoblastoma family proteins pRb, p107 and p130 (or 'pocket proteins'), which negatively regulate cell cycle progression and are inactivated through phosphorylation by CDKs (Weinberg, 1995). In their active, hypophosphorylated forms, pocket proteins bind to and negatively regulate the activities of several targets, such as the heterodimeric E2F–DP transcription factors. Five E2F (E2F-1 to -5) and three DP proteins (DP-1 to -3) have been identified. E2F-1, -2 and -3 bind predominantly pRb, E2F-4 binds p107 and p130, and E2F-5 binds predominantly p130 (reviewed by Cobrinik, 1996). Depending on the target promoters, E2F complexes can function as either transcriptional activators or repressors (in association with pocket proteins). E2F target genes include regulators of S-phase entry (e.g. B-*myb*, CDC2, cyclins E and A) and genes required for DNA replication (e.g. DHFR, DNA polα, thymidine kinase) (DeGregori *et al.*, 1995a; reviewed by Nevins, 1992; Zwicker and Müller, 1997).

When ectopically expressed, E2F-1 is sufficient to induce entry of quiescent cells into S-phase (but also apoptosis) (Johnson *et al.*, 1993; Qin *et al.*, 1994; Shan and Lee, 1994; Lukas *et al.*, 1996), a property shared by other E2F proteins (Lukas *et al.*, 1996; reviewed by Adams and Kaelin, 1996). E2F-1 and E2F-4 can override pRb- and p130-induced G_1 arrest, respectively (Zhu *et al.*, 1993; Qin *et al.*, 1995; Vairo *et al.*, 1995). E2F-1 also prevents G_1 arrest imposed either by antibodies neutralizing cyclin D–CDK4 function or by p16, in spite of the p16-induced dephosphorylation of cellular pRb (DeGregori *et al.*, 1995b; Lukas *et al.*, 1996; Mann and Jones, 1996). Expression of p16 or neutralization of cyclin D–CDK4 also fail to induce G_1 arrest in cells lacking functional pRb (Lukas *et al.*, 1994, 1995a,b; Koh *et al.*, 1995; Medema *et al.*, 1995). Thus, the essential role of cyclin D–CDK4 is to inactivate pRb, thereby allowing E2F function. In summary, p16, cyclin D1–3, CDK4/6, pRb and E2F can be ordered within a single G_1-regulatory pathway (the p16/pRb pathway).

The p16/pRb pathway plays a critical role in suppressing tumorigenesis. Several of its components are genetically altered in various human malignancies, resulting either in oncogenic activation (cyclins D1, D2 and CDK4) or loss of function (pRb and p16) (reviewed by Hall and Peters, 1996). p16-null mice are viable, but develop malignancies at increased rates (Serrano *et al.*, 1996). Strikingly, E2F-1-null mice also have a tumour-prone phenotype (Field *et al.*, 1996; Yamasaki *et al.*, 1996). Thus, the major role

of E2F-1 *in vivo* might be to relay the repressive action of pRb, rather than its own activating function. It should be noted here that pRb has other targets in cells, which may also be critical for its growth- and tumour-suppressive functions (Weinberg, 1996; Wang, 1997).

CDK2 and its partners, cyclins E and A, also phosphorylate and inactivate pocket proteins (Weinberg, 1995). For example, in transfected SAOS-2 cells, the G_1 arrest induced by pRb is relieved by cyclins A or E, and this correlates with hyperphosphorylation of pRb (Hinds *et al.*, 1992). Moreover, rapid activation of cyclin E–CDK2 upon induction of Myc (see below) coincides with pRb phosphorylation, whereas activation of cyclin A–CDK2 and cyclin D–CDK4 follow later in this particular system (Steiner *et al.*, 1995). Cyclin E–CDK2 appears to have additional function(s) in G_1–S progression, distinct from pRb phosphorylation and E2F activation. First, inducible expression of cyclin E in Rat1 fibroblasts accelerates G_1–S progression upon serum stimulation, without affecting the kinetics of pRb phosphorylation (Resnitzky and Reed, 1995). Second, G_1 arrest induced by dominant-negative CDK2 in U2OS cells is not relieved by the SV40 large T antigen (LT), although LT rescues E2F function in this system (Hofmann and Livingston, 1996). Third, unlike D-type cyclins, cyclin E is essential for cell cycle progression in pRb-deficient cells (Ohtsubo *et al.*, 1995).

The functional hierarchy between the pRb/E2F pathway and cyclin E is complicated further by the finding that the cyclin E gene is itself an E2F target. Cyclin E is induced by ectopic expression of E2F-1 (DeGregori *et al.*, 1995a; Soucek *et al.*, 1997), and functional E2F-binding sites in the cyclin E promoter are activated by E2F-1 and repressed by pRb (Ohtani *et al.*, 1995; Geng *et al.*, 1996). Furthermore, pRb-deficient fibroblasts express enhanced levels of cyclin E (Herrera *et al.*, 1996). These observations suggest the existence of a positive feedback regulatory loop, in which cyclin E is not only an upstream regulator of E2F (via the phosphorylation of pRb), but also its downstream effector. If the latter were true, deregulated cyclin E expression might be expected to bypass growth arrest by p16 or pRb, exactly as E2F.

Myc, the transcription factor encoded by the c-*myc* proto-oncogene, was also linked to the activation of cyclin–CDK complexes in G_1. Myc expression is strictly controlled by mitogens, and is required for cell cycle entry and continued proliferation. Constitutive Myc expression prevents growth arrest by a variety of growth-inhibitory signals, and activation of a conditional Myc–estrogen receptor chimera (MycER) induces entry into the cell cycle (but also apoptosis) in the absence of mitogens (Eilers *et al.*, 1991; Alexandrow *et al.*, 1995; Hermeking *et al.*, 1995; reviewed by Marcu *et al.*, 1992; Henriksson and Lüscher, 1996). Since antimitogenic stimuli are mediated by CKIs, we investigated whether Myc could overcome the growth-inhibitory function of these proteins. We previously reported that constitutive expression of Myc abrogates growth arrest by retrovirally expressed p27, by preventing its association with cyclin E–CDK2 (Vlach *et al.*, 1996). Myc also induces transcription of the CDK-activating phosphatase Cdc25A (Galaktionov *et al.*, 1996), which appears not to be involved in rescue of p27-induced arrest (Vlach *et al.*, 1996). Moreover, MycER activation in quiescent fibroblasts rapidly activates cyclin E–CDK2 complexes (Steiner *et al.*, 1995), and this also occurs through the suppression of p27 function (Perez-Roger *et al.*, 1997; J.Vlach and B.Amati, unpublished data). However, no link was yet established between Myc and p16 function.

In this study, we addressed whether cells infected with retroviruses expressing G_1 cyclins, Cdc25A, E2F proteins or Myc could escape growth arrest induced by p16, and compared the effects of p16 and p27 in those cells. Our data demonstrate that cyclin E promotes cell proliferation in the presence of elevated p16 levels, without inducing phosphorylation of pocket proteins. We suggest that cyclin E cannot simply substitute for E2F function, but rather rescues it in the presence of p16. Reciprocally, the requirement for cyclin E in G_1–S progression cannot be substituted by E2F. Finally, we demonstrate that Myc also bypasses the p16/pRb pathway, and argue that this effect is mediated by the positive action of Myc on cyclin E.

Results

Myc and cyclin E prevent growth arrest by INK4 proteins

The ability of Myc and various cell cycle regulators to suppress the growth-inhibitory function of INK4 family proteins (p15, p16 or p18) was investigated in Rat1 fibroblasts as previously described for Myc and p27 (Vlach *et al.*, 1996). First, cells were infected with retroviruses expressing Myc, Cdc25A, cyclins A and E, or D1–3. Serial infections using the pBabe series of retroviral vectors conferring resistance to neomycin (pBNeo), hygromycin (pBHygro) or phleomycin (pBBleo) (Morgenstern and Land, 1990) allowed co-expression of up to three exogenous proteins prior to INK4 proteins, which were expressed with a puromycin resistance vector (pBPuro). Following infection, cells were 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 36 h of selection. Subconfluent populations of infected cells were harvested at 48 h after a 30 min pulse with bromodeoxyuridine (BrdU) for analysis of cell cycle distribution. Biochemical studies were performed at the same time. Dilute dishes were incubated further to assess long-term proliferation and colony outgrowth.

Expression of p16 induced accumulation of Rat1 cells in G_1 and suppressed colony outgrowth (Figure 1A and B). Most cells in these p16-arrested populations remained isolated or formed sparse colonies, with no signs of apoptosis (data not shown). Retroviruses expressing cyclins A, D1, D2, D3 or Cdc25A did not prevent p16-induced cell cycle arrest, although all exogenous proteins were detectable by immunoblotting (data not shown and Table I). In contrast, cells expressing Myc or cyclin E entered S-phase and formed colonies efficiently in the presence or absence of p16 (Figure 1A and B, and Table I). Similar results were obtained with NIH-3T3 cells (data not shown). As for p16, Myc and cyclin E bypassed cell cycle arrest by p15 and p18 in Rat1 cells (Figure 1C). Immunoblot analysis revealed that Myc and cyclin E did not suppress expression of p16, p15 or p18 (Figure 2A), and had no significant effect on the expression of the cellular p16 targets CDK4, CDK6 and D-type cyclins, apart for a slight increase in cyclin D2 levels in Myc cells

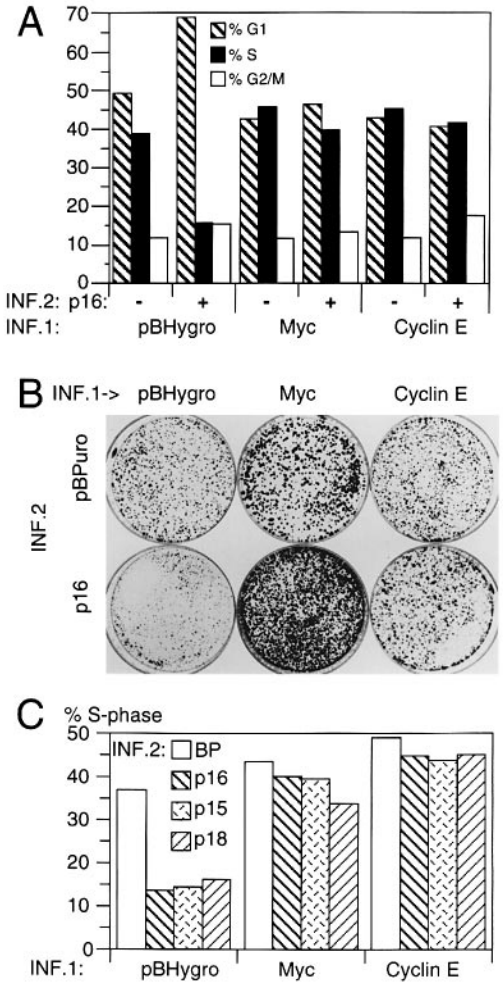

Fig. 1. Myc and cyclin E overcome growth arrest by INK4 family proteins. (**A**) p16 induces accumulation of control cells in G_1, but does not modify the cell cycle distribution of cells expressing Myc or cyclin E. Serial infections were performed as described in the text. Cells were infected first (INF.1) with retroviruses expressing Myc or cyclin E or with the empty control vector (pBHygro), followed by a pBPuro retrovirus with or without p16, as indicated (INF.2). Cells were harvested after 48 h of puromycin selection, and cell cycle distribution was analysed by flow cytometry. The percentage of cells in each phase of the cell cycle is indicated for all cell populations. (**B**) p16 prevents colony outgrowth in control, but not in Myc- or cyclin E-expressing cells. Infections were performed as above. Colonies were fixed and stained on dishes after 6 days in puromycin-selective medium. (**C**) Percentage of S-phase cells in populations infected with retroviruses expressing Myc or cyclin E (INF.1), and p15, p16 or p18 (INF.2). S-phase cells were visualized by immunocytochemical detection of incorporated BrdU.

(Figure 2B). In conclusion, Myc and cyclin E overcome growth arrest by INK4 proteins.

Myc and cyclin E do not prevent p16-induced dephosphorylation of pocket proteins

To investigate the effects of Myc or cyclin E on INK4 function, we examined the relative phosphorylation state of pRb, p107 and p130 by immunoblot analysis. In INK4-arrested Rat1 cells, pocket proteins were found exclusively in their hypophosphorylated, faster migrating form (Figure 3A), consistent with the induction of G_1 arrest. Although pRb levels were decreased consistently in p16-arrested cells in comparison with controls, the remaining pRb was

Table I. Summary of proliferation data with doubly infected Rat1 cells

	INF.2	
	p16	p27
INF.1		
vector	–	–
Myc	+	+
cyclin A	–	–
cyclins D1–3	–	–
cyclin E	+	–
Cdc25A	–	–
E2F-1–3	+	–
E2F-4, 5	–	–
DP-1	–	–
E2F-4 + DP-1	+	n.d.

Data from this work are compiled together with data from Vlach *et al.* (1996), for a direct comparison between p16 and p27. + and – indicate proliferating and arrested cells, respectively, according to BrdU incorporation and colony outgrowth assays. All cells proliferated after the first infection (INF. 1) as efficiently as control, vector-infected cells. n.d., not determined.

hypophosphorylated (Figure 3A and B, lane 2). p107, in particular, was present as a doublet of both hyper- and hypophosphorylated forms in p27-arrested cells (Vlach *et al.*, 1996 and Figure 3A), whereas INK4-arrested cells contained only the lower band. Strikingly, Myc and cyclin E did not reverse the effect of p16 on pRb family proteins, which were also mostly hypophosphorylated in proliferating Myc+p16 or cyclin E+p16 cells (Figure 3B, lanes 4 and 6). This observation was reproduced for pRb and p107 in NIH-3T3 cells (Figure 3C) in which p16 induced no changes in p130 mobility (data not shown). Thus, pocket proteins were hypophosphorylated in growing Myc+p16 or cyclin E+p16 cells, unlike in control growing cells. This was not merely a transient phenomenon. Indeed, when passaged and cultured for longer periods of time, Rat1 cells co-expressing Myc or cyclin E with p16 proliferated with elevated p16 levels and maintained hypophosphorylated pRb, p107 and p130 (Figure 4). At all time points, the percentage of S-phase cells was equivalent to those of control growing cells (data not shown). Altogether, these observations suggested that Myc and cyclin E allowed cell proliferation by bypassing pRb activation, and that p16 remained functional in inhibiting its targets, CDK4 and CDK6. Indeed, p16–CDK6 interactions were not altered by Myc or cyclin E, as detected by CDK6 immunoprecipitation followed by immunoblotting of p16 (Figure 2C). Retroviral expression of CDK4 (wild-type or a kinase-defective mutant) or CDK6 also prevented p16-induced arrest (data not shown), supporting the notion that these kinases were the p16 targets in cells. However, we were unable to demonstrate directly that p16 suppressed the kinase activities associated with CDK4, CDK6 or D-type cyclins. In summary, Myc and cyclin E promote cell growth in the presence of p16 and hypophosphorylated pocket proteins.

Retrovirally expressed pRb cooperates with p16 and is bypassed by Myc or cyclin E

If Myc and cyclin E bypass pRb activation by p16, they might rescue growth in the presence of elevated pRb levels. To address this question, we constructed retroviruses

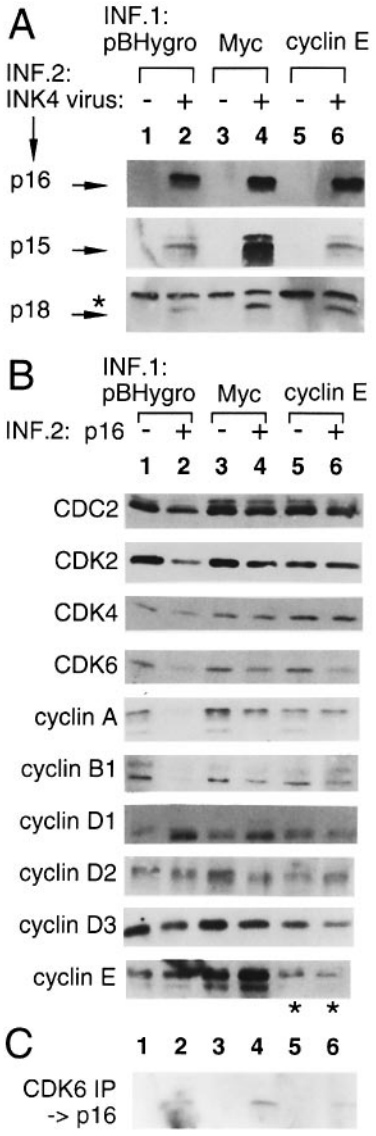

Fig. 2. Expression of exogenous INK4 proteins, of cellular cyclins and CDKs, and association of exogenous p16 with cellular CDK6. (**A**) Immunoblot analysis of retrovirally expressed INK4 proteins in doubly infected cells, as indicated (INF.1 and INF.2). The asterisk beside the p18 panel represents a non-specific band cross-reacting with the p18 antibody. (**B**) Immunoblot analysis of cellular proteins (indicated on the left of each panel) in doubly infected Rat1 cells expressing Myc, cyclin E and/or p16, as indicated at the top. The asterisks below lanes 5 and 6 of the cyclin E panel indicate that only the endogenous, rodent cyclin E is visualized in this blot. (**C**) CDK6 was immunoprecipitated from the same cells as above, and the co-precipitated p16 protein revealed by immunoblotting.

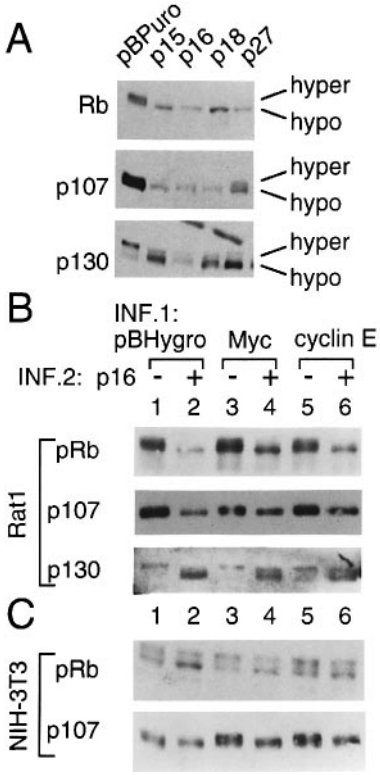

Fig. 3. Immunoblot analysis of expression and relative phosphorylation states of pRb family proteins. Rat1 cells were infected with (**A**) viruses expressing p15, p16, p18 or p27 alone, as indicated, or (**B**) with the indicated combination of viruses. (**C**) The same experiment as in (B) was performed with NIH-3T3 cells. The slower migrating, hyperphosphorylated forms of pRb, p107 or p130 and their faster migrating, hypophosphorylated forms are indicated on the right (hyper, hypo) in (A).

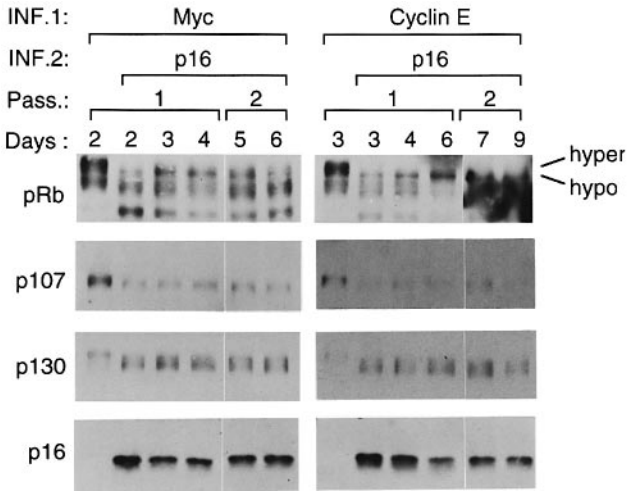

Fig. 4. p16 levels are maintained, and pRb, p107 and p130 remain hypophosphorylated in growing cells expressing exogenous Myc or cyclin E together with p16. Cells infected with the indicated combinations of viruses were allowed to grow for the indicated number of passages (Pass.) and days. All cells were proliferating continuously throughout the experiment, and were subconfluent at each harvesting time point. Immunoblotting of p16 (lower panels) shows that its expression is maintained. Pocket proteins (indicated to the left) are found in their hypophosphorylated forms in all cells expressing p16, but not in control cells. The faster migrating pRb bands seen in the presence of p16 represent further dephosphorylated forms, as previously seen (Chen *et al.*, 1989).

expressing full-length, hemagglutinin (HA)-tagged murine pRb (HApRb) or a derivative mutated in several potential CDK phosphorylation sites (HAΔp34) (Hamel *et al.*, 1992). Although HApRb and HAΔp34 accumulated in cells at higher levels than endogenous pRb (Figure 5A), they were insufficient to affect cell cycle progression or proliferation on their own (Figure 5B and data not shown). However, HApRb slightly, albeit reproducibly, enhanced the efficiency of p16-induced arrest, as shown by the lower percentage of S-phase cells in HApRb+p16 cells compared with p16-expressing cells (Figure 5C). Furthermore, HApRb+p16 cells showed a marked increase in

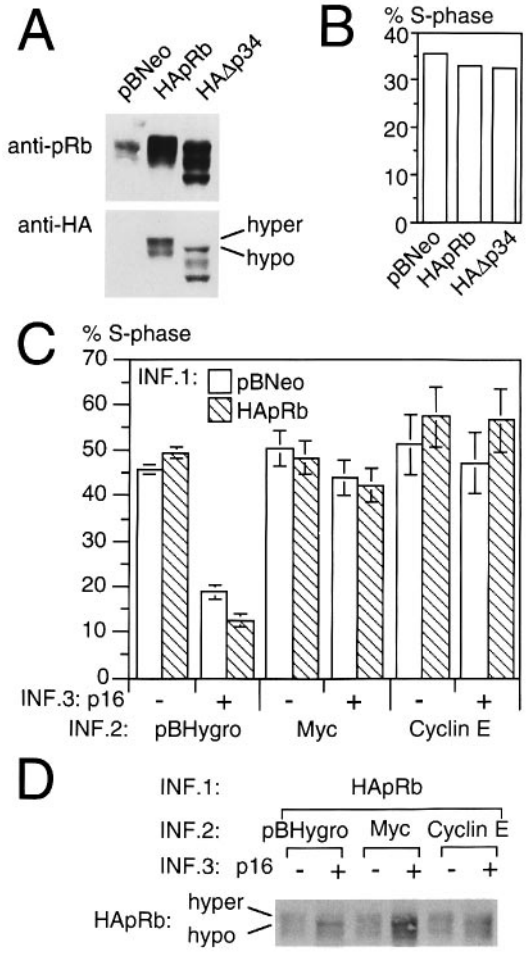

Fig. 5. Retrovirally expressed pRb enhances p16-induced arrest, but does not prevent rescue by Myc or cyclin E. (**A**) Immunoblot analysis of retrovirally expressed HApRb of the HAΔp34 mutant with anti-pRb and anti-HA antibodies, as indicated. Hyper, hypo: forms of pRb as defined in Figure 3A. (**B**) Percentage of cells in S-phase 48 h post-infection with the same retroviruses as in (A). (**C**) Percentage of S-phase cells following serial infections with the indicated viruses (INF.1, INF.2, INF.3). The data presented here represent the average of three independent experiments. (**D**) Immunoblot analysis of HApRb in the same cells.

the flattened, senescent-like phenotype, reminiscent of that seen in pRb-arrested SAOS-2 cells (Templeton *et al.*, 1991; Hinds *et al.*, 1992) (data not shown). Co-expression of Myc or cyclin E with HApRb prior to p16 fully prevented cell cycle arrest (Figure 5C) and the appeareance of the 'flat' phenotype (data not shown). Although the differentially phosphorylated forms of HApRb were difficult to resolve on immunoblots, a significant fraction of the protein was found in its faster-migrating form in all p16-expressing cells (Figure 5D). These data confirmed two important points: first, that pRb levels are limiting for p16-induced arrest; second, that Myc and cyclin E rescue cell growth without preventing p16-induced dephosphorylation of either endogenous or exogenous pRb.

Pocket proteins are functional in growing Myc+p16 or cyclin E+p16 cells

The data presented so far suggested that Myc and cyclin E promote cell growth in the face of p16-induced activation of pRb, p107 and p130. At this stage, however, inactivation of pocket proteins by a specific cyclin E-mediated phosphorylation event could not be ruled out. To address the functional status of pRb, we sought to analyse its interaction with E2F-1, -2 or -3. Because these proteins were undetectable in Rat1 cells, we co-expressed Myc and cyclin E with an E2F variant (HA-E2F1eco) which interacts with pRb but does not bind DNA (Johnson *et al.*, 1993). In spite of a slight residual activity of E2F1eco (see Figure 9C), growth of E2F1eco cells was suppressed by p16 and restored by Myc or cyclin E (data not shown). Following superinfection with the p16 retrovirus, the interactions between HA-E2F1eco and pRb were monitored by immunoprecipitation with anti-E2F-1 antibodies, followed by pRb immunoblotting (Figure 6A, panels ii). Total and immunoprecipitated HA-E2F1eco levels were constant throughout the experiment (panels iii and iv), and no pRb was detectable in anti-E2F-1 immunoprecipitates from cells lacking HA-E2F1eco (panels ii, lanes 0). The results showed that p16 increased the association of pRb with HA-E2F1eco in control cells (Figure 6A, lanes 1 and 2), as well as in cells expressing Myc (lanes 3 and 4) or cyclin E (lanes 5 and 6), even though total pRb levels were decreased by p16 in all cells (Figure 6A, panels i; Figure 3B). The same experiment was repeated in cells expressing exogenous HApRb together with HA-E2F1eco, and similar controls were provided (Figure 6B). The result was identical, showing that the association of pRb (endogenous plus exogenous) with HA-E2F1eco was induced by p16 in all cells (panels ii). Taken together, these experiments demonstrate that Myc and cyclin E do not interfere with p16-induced activation of pRb.

Similar experiments were performed to assess the functional status of p130. As HA-tagged E2F-4 is not functional in the p16 rescue assay (see below, Figure 9C), we co-expressed it together with Myc or cyclin E and monitored its interaction with endogenous p130. As above, we controlled the amounts of total p130 (Figure 7, panel i) and of total and immunoprecipitated E2F-4 (panels iii and iv). No p130 was detectable in HA immunoprecipitates in the absence of HA-E2F-4 (lane 0). The data (panel ii) showed that p16 induced binding of p130 to E2F-4 in control, Myc and cyclin E cells, analogous to the results with pRb. The same experiment did not yield a clear answer for the interaction between p107 and HA-E2F-4. This interaction was detectable in growing Rat1 cells, but was not increased further upon expression of p16 (data not shown).

To analyse E2F–pocket protein complexes further and to assess the amount of 'free' E2F activity, we performed gel retardation assays with retrovirally infected cells. As detection of E2F–DNA complexes was very difficult in Rat1 cells, we used NIH-3T3 cells, which showed similar responses to p16 (Figure 3C and data not shown). Three DNA-binding complexes specific for E2F sites were identified with oligonucleotide competition studies (complexes A, B and C; Figure 8). Complex A corresponds to 'free' E2F–DP dimers, as treatment of the extracts with deoxycholate (DOC) resulted in loss of B and C and a simultaneous increase in the amount of complex A. The E2F species in complex A was most likely E2F-4, since it co-migrated with the major E2F–DNA complex seen in

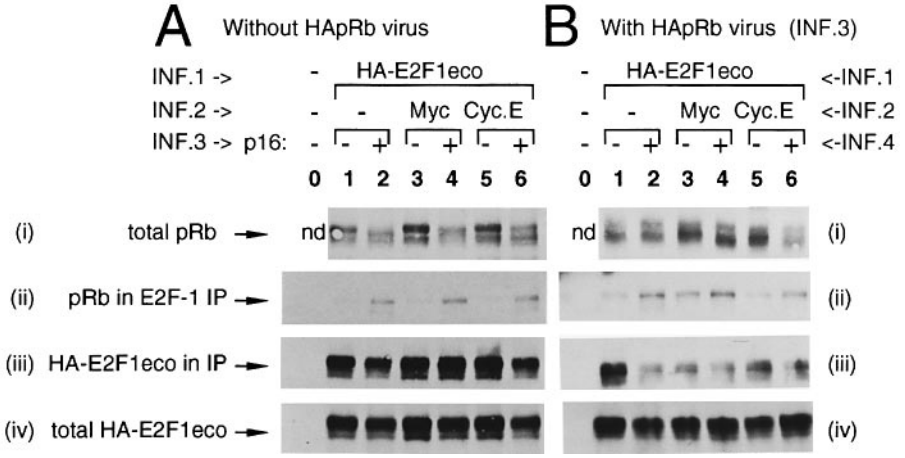

Fig. 6. Interaction of pRb with the E2F-1eco protein in the absence (**A**) or presence (**B**) of exogenous pRb. Cells were serially infected with the indicated combinations of viruses. The E2F-1 mutant HA-E2F1eco (see text) was immunoprecipitated with anti-E2F-1 antibodies. Immunoblotting was performed to visualize the immunoprecipitated HA-E2Feco with anti-HA antibodies (panels iii) and the associated pRb (endogenous and exogenous) with anti-pRb antibodies (panels ii). Total levels of pRb (panels i) and HA-E2F1eco (panels iv) were also measured. nd: not determined.

NIH-3T3 cells expressing recombinant E2F-4, but not E2F-1, -2, -3 or -5 (data not shown). The composition of complex B, which was not supershifted by any tested antibody, is unknown. Complex C could be supershifted by p107 or CDK2 antibodies (data not shown) and presumably also contained cyclins E or A (Lees *et al.*, 1992; Schwarz *et al.*, 1993; Cobrinik, 1996). No other pocket protein–E2F complexes were detected in growing NIH-3T3 cells, as previously described (Lam and Watson, 1993; Schwarz *et al.*, 1993).

Two major points can be made concerning the effects of Myc, cyclin E and/or p16 on E2F DNA-binding complexes (Figure 8). First, p16 alone reduced the amount of 'free' E2F-4–DP (complex A) present in control cells. This decrease was minimized in the presence of Myc or cyclin E. Second, complex C was increased in response to p16 in Myc or cyclin E cells. The lack of complex C induction in control cells may be due to the formation of alternative complexes not resolved here and, in part, to slightly lower p107 levels in p16-arrested cells (Figure 3C). Regardless of this, our data show that p16 activates the E2F-binding activity of p107 in Myc and cyclin E cells. In summary, the results from co-immunoprecipitation and gel retardation studies show that Myc and cyclin E prevent growth arrest by p16 without interfering with the ability of pRb, p107 and p130 to associate with their respective E2F targets.

E2F proteins bypass p16-, but not p27-induced arrest: implications for an E2F-independent function of cyclin E

The data presented so far may be interpreted in two alternative ways. First, cyclin E or Myc might allow cell growth in the absence of E2F function. Second, cyclin E or Myc might rescue the transcriptional activity of some E2F complexes in the presence of active pRb, p107 and p130. Unfortunately, we could not detect significant regulation of stably or transiently transfected E2F-responsive reporter genes by retrovirally expressed p16. However, cyclin A and CDC2 protein levels were suppressed by p16 in control cells, but not in cells expresing cyclin E or Myc (Figure 2B). The simplest interpretation of these

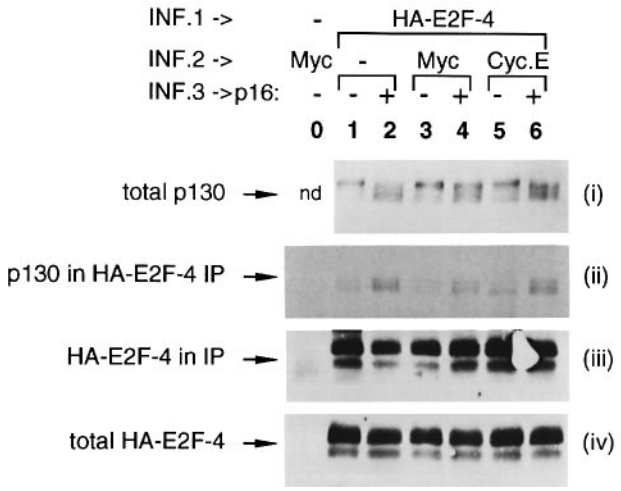

Fig. 7. Interaction of p130 with E2F-4. Cells were serially infected with the indicated combinations of viruses. HA-E2F-4 was immunoprecipitated with anti-HA antibodies. Immunoblotting was performed to visualize the immunoprecipitated HA-E2F-4 with anti-HA antibodies (panel iii), or the associated p130 (panel ii). Total p130 (panel i) and HA-E2F-4 (panel iv) were also visualized. nd: not determined.

results is that cyclin E and Myc restore activity of some E2F complexes (see Discussion), although alternative routes to restore expression of E2F target genes cannot be ruled out.

If cyclin E acted solely through E2F, E2F proteins would be expected to bypass G_1 arrest not only by p16, as previously reported (DeGregori *et al.*, 1995b; Lukas *et al.*, 1996; Mann and Jones, 1996), but also by p27, which inhibits cyclin E–CDK2 function. To address this question, we infected Rat1 cells with retroviruses expressing HA-tagged versions of E2F-1 to -5, as well as E2F-1Δ5 and E2F-4Δ4 (two mutants which are not bound by pocket proteins; Krek *et al.*, 1994; Vairo *et al.*, 1995), the DNA-binding mutant E2F-1eco and a T7-tagged form of DP-1. Expression of all these proteins was detected readily by immunoblot (Figure 9A and B and data not shown). E2F-1, -2, -3 and E2F-1Δ5 prevented growth arrest by p16, whereas E2F-4, E2F-5 and DP-1 were

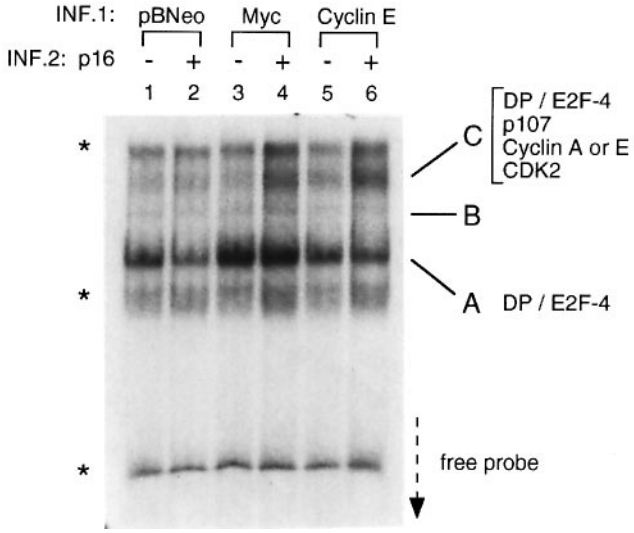

Fig. 8. Gel retardation analysis of E2F DNA-binding activities in NIH-3T3 cells. Cells were infected with the indicated viruses and complexes binding to a radiolabeled E2F-binding oligonucleotide were resolved by native gel electrophoresis. Three specific complexes were identified (A, B and C). Where known, the proteins comprising these complexes are indicated on the right (see text). The asterisks indicate non-sequence-specific complexes, as determined by competition studies.

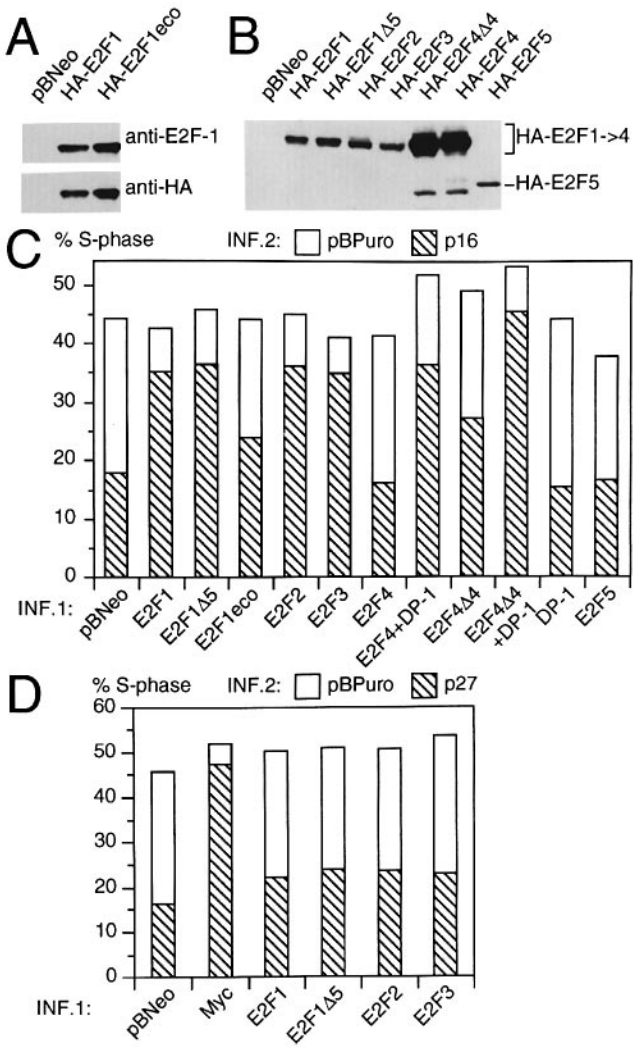

Fig. 9. Retrovirally expressed E2F-1 to -3 overcome p16-, but not p27-induced cell cycle arrest. Cells were infected with viruses expressing the indicated tagged E2F and DP proteins and mutant derivatives (see text). (A and B) Immunoblot analysis of retrovirally expressed E2F proteins (indicated at the top), with anti-E2F-1 or anti-HA in (**A**), and anti-HA in (**B**). Given that the HA-tag is N-terminal, the faster migrating band in the E2F-4 and E2F-4Δ4 lanes is probably a degradation product. (**C**) Percentage of cells in S-phase in pools infected with viruses expressing HA-tagged E2F proteins and p16, as indicated (INF.1, INF.2). The values for p16 cells (shaded bars) are superimposed on those for control cells (white bars). (**D**) Same as above with p27 instead of p16. Myc was used as a positive control to overcome p27-induced arrest.

inactive (Figure 9C and Table I), consistent with previous reports (Lukas *et al.*, 1996; Mann and Jones, 1996). The lack of activity of E2F-4 and E2F-5 may be due to the combination of several parameters, including interaction with pocket proteins and/or inefficient dimerization. Indeed, E2F-4Δ4 had a weak rescuing activity, and co-expression with DP-1 allowed E2F-4 and E2F-4Δ4 to fully rescue p16-induced arrest (Figure 9; Lukas *et al.*, 1996). In contrast to the p16 results, none of the E2F proteins prevented growth arrest by p27 (Figure 9D and data not shown, and Table I). Association of E2F-1 with pocket proteins did not account for its inability to bypass p27 arrest, since cells expressing E2F-1Δ5 were also arrested by p27. Thus, E2F-1 to -3 in our system behaved like cyclin E, which rescued growth arrest by p16 (this work), but not by p27 (Vlach *et al.*, 1996) (Table I). In summary, although E2F-1, -2 and -3 were expressed at levels sufficient to bypass pRb activation in response to p16, they could not bypass repression of cyclin E–CDK2 by p27. This implies that cyclin E has a distinct function, for which its targets lie outside of the pRb/E2F pathway.

Discussion

Cyclin E bypasses activation of the p16/pRb growth-inhibitory pathway

Previous observations suggested that cyclin E has a function distinct from pRb phosphorylation required for G_1–S progression (see Introduction; Ohtsubo and Roberts, 1993; Resnitzky and Reed, 1995; Hofmann and Livingston, 1996). We demonstrate here that cyclin E is sufficient to bypass growth arrest by p16, and allows cell proliferation in the presence of active pRb family proteins. Infection with a p16-expressing retrovirus arrested Rat1 cells in G_1, but had no effect on cells expressing exogenous cyclin E. p16 remained active in these cells since it bound its target CDK6 and induced activation of pocket proteins and their association with E2Fs. This effect was not transient since cells expressing cyclin E and p16 sustained long-term proliferation with hypophosphorylated, active pocket proteins. Cyclins A, D1, D2 and D3 were inactive in bypassing p16-induced arrest in this system (Table I).

In normal cells, cyclin E expression is under the control of E2F and pRb. The promoter of the cyclin E gene contains E2F-responsive elements and is repressible by pRb (Ohtani *et al.*, 1995; Botz *et al.*, 1996; Geng *et al.*, 1996). Consistent with this, pRb-deficient cells showed enhanced expression of cyclin E (Herrera *et al.*, 1996), and overexpression of E2F-1 induced cyclin E (DeGregori *et al.*, 1995a; Soucek *et al.*, 1997). Since cyclin E–CDK2

complexes also contribute to pRb phosphorylation and inactivation, these observations led to the proposal that a positive feedback loop may operate in late G_1 (see Introduction). According to this model, the increase in cyclin E–CDK2 activity would contribute to further activation of E2F, and thus to enhanced transcription of the cyclin E gene. The outcome of such a loop would be to boost activation of both E2F and cyclin E. However, such a model does not predict which of E2F or cyclin E, or both, performs the rate-limiting downstream function in S-phase entry.

Ectopic activation of an essential downstream effector may bypass the presence of active pRb. This is the case for E2F-1, which suppresses both pRb- (Zhu *et al.*, 1993; Qin *et al.*, 1995) and p16-induced G_1 arrest, in conditions in which cellular pRb remains hypophosphorylated (Lukas *et al.*, 1996; Mann and Jones, 1996; this work and unpublished data). Moreover, adenovirus-mediated gene transfer of E2F-1 suppressed G_1 arrest and allowed completion of a mitotic cycle in the presence of either p16 or p27, leading to the suggestion that E2F-1 allows S-phase entry in the absence of cyclin E function (DeGregori *et al.*, 1995b). In those studies, however, E2F-1 was strongly overexpressed, which may have led to non-physiological effects. Indeed, we and others (Mann and Jones, 1996) found that p27 is dominant over E2F-1. In fact, E2F proteins behaved similarly to cyclin E, which rescued p16-, but not p27-induced arrest (Table I), owing to the inhibition of cyclin E by p27 (Vlach *et al.*, 1996). These observations imply that E2F activity cannot bypass inhibition of cyclin E–CDK2 by p27, even though it can bypass activation of pRb by p16. Two conclusions can be drawn from these studies: first, that E2F is unable to promote growth in the absence of cyclin E activity; second, that cyclin E has at least one E2F-independent function required for S-phase entry. Such a function of cyclin E–CDK2 was also implied by the observation that the SV40 LT restored E2F activity, but not cell cycle progression, in the presence of transiently expressed dominant-negative CDK2 (Hofmann and Livingston, 1996).

If cyclin E has a function downstream of E2F, could it be sufficient for S-phase entry in the absence of E2F activity? A direct test of this question would be to block E2F activity in cells expressing cyclin E. We attempted this with a dominant-negative mutant of DP-1 (DN-DP-1) which blocks E2F function and arrests cells in G_1 in transient transfections (Wu *et al.*, 1996). However, retroviral expression of DN-DP-1 (or a variant tagged with a nuclear localization sequence) had no effect on cell cycle progression. Thus DN-DP-1 may effectively block E2F activity *in vivo* only if overexpressed, which may have non-specific side effects. A direct test of the requirement for E2F in the presence of cyclin E awaits alternative ways to ablate E2F activity. In spite of this, several observations suggest that cyclin E does not bypass, but rather rescues E2F activity by an alternative route in the presence of p16 and active pRb. In transient transfections, cyclin E relieved p16 (but not p27)-mediated repression of the cyclin A promoter (Zerfass-Thome *et al.*, 1997). In our experiments, cyclin E rescued the expression of cellular cyclin A and CDC2 in the presence of p16. Zerfass-Thome *et al.* (1997) proposed that cyclin E–CDK2 binding to E2F-4/DP/p107 results in the formation of activating complexes. Compatible with, but not proving, this hypothesis, our gel retardation assays showed that p16 in cyclin E-expressing cells induced formation of E2F-4/DP/p107/cyclin–CDK2 DNA-binding complexes. On the other hand, cyclin E also favored the maintenance of 'free' E2F-4–DP complexes. However, whether and how E2F contributes to cyclin E function in overcoming the p16 block remains to be investigated.

The functional relationship of cyclin E and E2F is highlighted further by studies in *Drosophila* embryos. E2F- or cyclin E-deficient embryos fail to enter S-phase of embryonic cycle 17 in the endocycling cell compartment (Knoblich *et al.*, 1994; Duronio *et al.*, 1995). In this tissue, the limiting downstream target of E2F for S-phase entry is cyclin E. Ectopic E2F–DP expression requires cyclin E function to trigger S-phase entry, although the transcriptional function of E2F itself does not require cyclin E (Duronio *et al.*, 1995, 1996). Conversely, ectopic expression of cyclin E can trigger S-phase entry, but requires expression of additional E2F target genes. Cyclin E expression induces E2F-dependent transcription (Duronio and O'Farrell, 1995), most likely through phosphorylation of the pRb homolog RBF (Du *et al.*, 1996). In the central nervous system, cyclin E is expressed by an E2F-independent mechanism, and is required upstream of E2F function (Duronio *et al.*, 1995). Thus, the dependence of one gene upon the other can be reversed in different tissues or stages, depending on which gene product becomes rate-limiting. In summary, in *Drosophila* as in mammalian cells, cyclin E is both a downstream effector and an activator of E2F. In addition to this cross-regulation, both E2F and cyclin E have at least one independent downstream function required for S-phase entry.

What might be the E2F-independent function of cyclin E? *In vitro* experiments suggest that it might be linked to the initiation of DNA replication. In *Xenopus* extracts, which replicate in the absence of transcription (and thus of E2F activity), addition of p21 blocked DNA replication, and cyclins A or E could overcome this block (Strausfeld *et al.*, 1994). In addition, G_1-phase HeLa cell nuclei initiated DNA replication when co-incubated with S-phase nuclear and cytoplasmic extracts. The nuclear extract could be replaced by cyclin E–CDK2 or cyclin A–CDK2 (Krude *et al.*, 1997). A direct involvement of cyclin–CDK activity in triggering the onset of DNA replication has also been demonstrated in yeast (reviewed by Stillman, 1996).

Rescue of p16-induced arrest by Myc is most likely a cyclin E-mediated activity

We show here that Myc bypasses p16/pRb-induced growth arrest. Like other biological activities of Myc (Henriksson and Lüscher, 1996; Vlach *et al.*, 1996), this effect depends upon the formation of transcriptionally active Myc–Max dimers (unpublished data). As for cyclin E, cells expressing Myc and p16 proliferate with hypophosphorylated, active pocket proteins. Thus, Myc and cyclin E rescue p16-induced arrest by indistinguishable mechanisms. The main rationale for these findings is provided by the notion that Myc can act as an upstream activator of cyclin E–CDK2. Constitutive expression of Myc prevented inactivation of cyclin E–CDK2 and cell cycle arrest by retrovirally expressed p27, through the sequestration of p27 into heat-

labile complexes (Vlach *et al.*, 1996) (Table I). The same mechanism was involved in activation of cyclin E–CDK2 by MycER in quiescent fibrobasts (Steiner *et al.*, 1995; Perez-Roger *et al.*, 1997; J.Vlach and B.Amati, unpublished data). In some cell lines, p27 has a role in limiting the rate of G_1–S progression during proliferation (Coats *et al.*, 1996), whereas constitutive expression of cyclin E (Ohtsubo and Roberts, 1993) or Myc (Karn *et al.*, 1989) can accelerate it. These observations suggest that Myc, by suppressing p27 function, can accelerate the activation of cyclin E during G_1. This, through the positive cyclin E–E2F feedback loop discussed above, should further enhance cyclin E expression. Consistent with this view, MycER activation induced expression of E2F target genes (Jansen-Dürr *et al.*, 1993; Pusch *et al.*, 1997b), and in particular of cyclin E (Perez-Roger *et al.*, 1997; Pusch *et al.*, 1997a), although there is no evidence that any of these genes is a direct transcriptional target of Myc. The *cdc25A* gene, on the other hand, is a Myc target (Galaktionov *et al.*, 1996). However, overexpression of Cdc25A rescued neither p16- nor p27-induced arrest (Vlach *et al.*, 1996; Table I), suggesting that additional Myc/Max target genes are involved.

In addition to its upstream action, Myc may also be a downstream target of pocket proteins. First, the c-*myc* gene was proposed to be an E2F target; however, unlike other E2F targets, it was not induced upon adenovirus expression of E2F-1 (DeGregori *et al.*, 1995a). Second, p107 can associate with the Myc protein and suppress its transactivation potential in transient transfections (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994; Hoang *et al.*, 1995). In these assays, Myc bypassed cell cycle arrest by p107, but not by pRb (Beijersbergen *et al.*, 1994). In contrast, microinjected GST–Myc proteins prevented cell cycle arrest by pRb (Goodrich and Lee, 1992). Since the mechanisms underlying these effects were not studied, these discrepancies remain unexplained and illustrate the potential dangers of overexpression. Although we cannot rule out that the Myc–p107 interaction plays a role in overcoming p16 (or p27)-induced arrest by Myc, it is unlikely to account for our findings. First, in our system, we did not detect the Myc–p107 interaction. Second, cells expressing Myc and p16 proliferate in the presence of not only active p107, but also of pRb and p130. Third, Myc was expressed at low levels in our studies, making titration of p107 by Myc a very unlikely mechanism.

In summary, we propose that the overcoming of p16-induced growth arrest by Myc is mainly the consequence of its upstream action on cyclin E–CDK2: Myc suppresses p27 function, allowing an elevation of cyclin E activity above the threshold required to bypass the p16/pRb block. In situations in which p16 is not overexpressed, the same activity of Myc accelerates hyperphosphorylation of pRb by cyclin E–CDK2 in concert with cyclin D–CDK4.

Relevance of bypassing p16/pRb function for tumorigenesis

The p16/pRb pathway has a fundamental role in suppressing tumorigenesis. Both p16 and pRb are encoded by tumor suppressor genes, as indicated by loss of function in different tumor types. On the other hand, oncogenic activation in human tumors has been reported for the genes encoding cyclins D1, D2 and CDK4 (reviewed by Hall and Peters, 1996). These observations strongly suggest that other genetic lesions resulting in the functional suppression of the p16/pRb pathway should also be tumorigenic.

Recently, aberrant expression of cyclin E has been linked to tumorigenesis (see, for example, Keyomarsi *et al.*, 1994; Porter *et al.*, 1997). It was suggested that cyclin E–CDK2 replaces CDK4 function in tumor cells that express high p16 levels by phosphorylating pRb (Gray-Bablin *et al.*, 1996). However, our data show that cyclin E bypasses p16/pRb function through a different pathway. Although cyclin E was also shown to have a mild oncogenic potential in transgenic mice (Bortner and Rosenberg, 1997), its causal involvement in human tumors remains to be investigated.

The widespread oncogenic potential of *myc* family genes and their involvement in numerous malignancies in both humans and animals are well established (reviewed by Marcu *et al.*, 1992; Garte, 1993). As discussed above, Myc antagonizes p27 function, but loss of p27 is not *per se* a tumorigenic event (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). This suggests that Myc may have other direct targets, which may include the p27-related CKIs p21 and p57, as well as p53 (Hermeking *et al.*, 1995). Independently of this aspect, bypassing the tumour suppressor function of the p16/pRb pathway may be an important, if not the essential, aspect of Myc's oncogenic activity. In support of this hypothesis, fibroblasts isolated from p16-null mouse embryos are transformed by *ras* alone, whereas wild-type cells undergo premature senescence (Serrano *et al.*, 1996, 1997). On the other hand, transformation of wild-type cells requires cooperation of *ras* with immortalizing oncogenes, such as c-*myc* (Land *et al.*, 1983). Thus, loss of p16 mimics *myc* activation, and vice versa. Based on this, we predict that *myc* activation may partly release the pressure to mutate components of the p16/pRb pathway during tumorigenesis.

Materials and methods

Retroviral expression vectors

The retroviral vectors pBabe-Puro (pBP), -Bleo (pBB), - Neo2 (pBN2) and- Hygro2 (pBH2) were described previously (Morgenstern and Land, 1990; Vlach *et al.*, 1996). cDNAs encoding human cyclins A, D1–3 and E, as well as Myc, HApRb, HAΔp34, HA-E2F-1 to -3 and HA-E2F1eco proteins were subcloned in pBN2, pBH2 and/or pBB, as required. HA-E2F-4 and HA-E2F-4Δ4 were expressed from the pRcCMVneo retroviral vector (a kind gift from W.Krek). cDNAs encoding the human CKIs p15, p16, p18 and p27 were subcloned in pBP.

Retroviral infections, cell cycle analysis and biochemical analysis

High-titer retroviral supernatants ($\leqslant 5\times10^6$/ml) were generated as described (Vlach *et al.*, 1996). Infected Rat1 or NIH-3T3 cells were selected with the appropriate drug: G418 (Calbiochem, 1000 μg/ml), hygromycin (Calbiochem, 150 μg/ml), phleomycin (Cayla, 50 μg/ml) or puromycin (Sigma, 2.5 μg/ml). Serial infections of cell pools, preparation of cell lysates and biochemical analysis (immunoblots, immunoprecipitations) were as previously described (Vlach *et al.*, 1996). Gel retardation assays were performed from infected cells as previously described (Beijersbergen *et al.*, 1995). For cell cycle analysis, cells were labeled with 33 μM BrdU for 30 min, and analyzed by either two-dimentional flow cytometry (Vlach *et al.*, 1996), or by immunocytochemical detection of BrdU. For the latter, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) (5 min), permeabilized with acetone at –20°C (30 s), treated with 1.5 M HCl (10 min), blocked in PBS with 1% bovine serum albumin (BSA) (1–2 min), incubated for 1 h with the

primary antibody (undiluted mouse anti-BrdU+Nuclease, Amersham No. RPN202), and for 1 h with the secondary antibody (goat anti-mouse Fab–FITC, Sigma, dil. 1/200 in PBS + 1% BSA) with Hoechst (0.5 μg/ml, Calbiochem), and mounted with FluorSave™ Reagent (Calbiochem). All steps were performed at room temperature and separated by several washes in PBS (with 1% BSA after the blocking step).

Antibodies

The following antibodies were used: against CDK2, M2 (Santa Cruz 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); E2F-1, C20 (sc-193); p15, C20 (sc-612); HA-tag, HA11(Babco); T7-tag, T7 (Novagen); p16, DCS 50.2 (from G.Peters); p18, 11256 (from Y.Xiong).

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