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A novel function of adenovirus E1A is required to overcome growth arrest by the CDK2 inhibitor $p27^{Kip1}$

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We show here that the adenovirus E1A oncoprotein prevents growth arrest by the CDK2 inhibitor $p27^{Kip1}$ (p27) in rodent fibroblasts. However, E1A neither binds p27 nor prevents inhibition of CDK2 complexes *in vivo*. In contrast, the amount of free p27 available to inhibit cyclin E/CDK2 is increased in E1A-expressing cells, owing to reduced expression of cyclins D1 and D3. Moreover, E1A allows cell proliferation in the presence of supraphysiological p27 levels, while c-Myc, known to induce a cellular p27-inhibitory activity, is only effective against physiological p27 concentrations. E1A also bypasses G_1 arrest by roscovitine, a chemical inhibitor of CDK2. Altogether, these findings imply that E1A can act downstream of p27 and CDK2. Retinoblastoma (pRb)-family proteins are known CDK substrates; as expected, association of E1A with these proteins (but not with p300/CBP) is required for E1A to prevent growth arrest by either p27 or the CDK4/6 inhibitor $p16^{INK4a}$. Bypassing CDK2 inhibition requires an additional function of E1A: the mutant E1A Δ26–35 does not overcome p27-induced arrest, while it binds pRb-family proteins, prevents p16-induced arrest, and alleviates pRb-mediated repression of E2F-1 transcriptional activity (although E1A Δ26–35 fails to restore expression of E2F-regulated genes in p27-arrested cells). We propose that besides the pRb family, E1A targets specific effector(s) of CDK2 in G_1–S control.

Keywords: CDK2/E1A/E2F/$p27^{Kip1}$/retinoblastoma

Introduction

In vertebrate cells, the commitment to complete a round of mitotic division takes place during the initial phase of the cell cycle (G_1), at a stage called the restriction (R-) point, preceding the onset of DNA synthesis (S phase) (Pardee, 1989; Zetterberg *et al*., 1995). The best-characterized molecular event required for passage through the R-point is inactivation of the retinoblastoma protein (pRb) by phosphorylation. pRb and the related proteins p107 and p130 are negative growth-regulators. In their active form, they associate with various cellular targets, and in particular with transcription factors of the E2F/DP family, repressing their target genes, which include regulators of S-phase entry (e.g. B-*myb*, cyclin E, p107) and genes required for DNA replication (e.g. DHFR, DNA polα) (for reviews, see Weinberg, 1995; Helin, 1998).

The enzymes catalyzing phosphorylation and inactivation of pRb-family proteins are cyclin-dependent kinases (CDKs), including CDK4 or CDK6 (associated with D-type cyclins) and CDK2 (associated with cyclins E or A) (reviewed in Sherr, 1994, 1995; Weinberg, 1995; Mittnacht, 1998). The activities of these CDKs are regulated by various mechanisms including association with cyclins, phosphorylation/dephosphorylation, as well as association with two families of inhibitory proteins (CKIs) (Morgan, 1995; Sherr and Roberts, 1995). The Cip/Kip family of CKIs includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which bind to all G_1–cyclin/CDK complexes. The second family includes the INK4 proteins $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which bind to CDK4 and CDK6. Ectopic expression of both classes of CKIs causes cell-cycle arrest in G_1 (Guan *et al*., 1994; Polyak *et al*., 1994; Toyoshima and Hunter, 1994; Hirai *et al*., 1995; Quelle *et al*., 1995; Vlach *et al*., 1996; Alevizopoulos *et al*., 1997). CKIs play key roles in the response of cells to growth-inhibitory signals, such as induction of differentiation, p53 activation (e.g. in response to DNA damage), TGFβ treatment, contact-inhibition, senescence and others (Sherr and Roberts, 1995; Harper and Elledge, 1996).

Several lines of evidence suggest that the major role of cyclin D/CDK4 complexes is to inactivate pRb-family proteins and thereby derepress E2F/DP-dependent transcription. For example, pRb-negative cells become insensitive to expression of p16 or microinjection of antibodies neutralizing cyclin D/CDK4 (Lukas *et al*., 1994, 1995a, 1995b; Koh *et al*., 1995; Medema *et al*., 1995) and ectopic expression of E2F/DP proteins prevents G_1 block by p16, allowing cells to enter S phase (DeGregori *et al*., 1995; Lukas *et al*., 1996; Mann and Jones, 1996; Alevizopoulos *et al*., 1997). Although CDK2 complexes are clearly involved in pRb phosphorylation (Kelly *et al*., 1998; Lundberg and Weinberg, 1998; reviewed in Weinberg, 1995; Mittnacht, 1998), these complexes appear to have at least one additional role in G_1–S progression. First, inducible expression of cyclin E in fibroblasts accelerates G_1–S progression without affecting the kinetics of pRb phosphorylation (Resnitzky and Reed, 1995). Secondly, G_1 arrest induced by dominant-negative (DN-) CDK2 in transfected U2OS cells is not relieved by the SV40 large T antigen (LT), although LT blocks pRb and rescues E2F function in this system (Hofmann and Livingston, 1996). Thirdly, unlike D-type cyclins, cyclin E is essential for cell-cycle progression in pRb-deficient cells (Ohtsubo *et al*., 1995). Fourthly, ectopic expression of E2F-1 bypasses growth arrest by p16, but not by p27, which inhibits cyclin E/CDK2 (Mann and Jones, 1996; Alevizopoulos *et al*., 1997), although elevated E2F1 expression can also bypass p27 (DeGregori *et al*., 1995).

Fifthly, ectopic expression of cyclin E bypasses p16- or pRb-mediated cell-cycle arrest independently of pRb phosphorylation (Alevizopoulos *et al*., 1997; Leng *et al*., 1997; Lukas *et al*., 1997). Sixthly, adenovirally expressed Myc and Ras induce S-phase entry in REF52 cells via activation of cyclin E/CDK2, but without concomitant activation of cyclin D1 or phosphorylation of pRb (Leone *et al*., 1997). Finally, in *Drosophila*, cyclin E and E2F/DP reciprocally activate each other, but each also has a non-redundant function required for S-phase entry (Duronio and O'Farrell, 1995; Duronio *et al*., 1995, 1996, 1998; Du *et al*., 1996), a conclusion which most likely applies also to mammalian cells (for review, see Amati *et al*., 1998).

Early gene products of DNA tumour viruses, such as large T of SV40, E7 of human papilloma viruses and E1A of adenoviruses, are involved in stimulating entry of quiescent or differentiated cells into S phase, allowing replication of viral genomes. These proteins have convergently evolved to interact with key components of cellular growth-regulatory pathways, including pRb-family proteins and, in the case of E1A and LT, the transcriptional coactivators p300 and CBP. Either type of interaction suffices for the induction of S-phase entry by E1A, but both are necessary for cellular immortalization and transformation (for reviews, see Dyson and Harlow, 1992; Moran, 1993; Bayley and Mymryk, 1994; Eckner, 1996).

In this work, we show that the 12S E1A protein of Adenovirus 5 overcomes growth arrest by p27. Strikingly, we find that p27 remains functional and inhibits CDK2 activity in E1A-expressing cells. These and other data indicate that E1A can promote G_1–S progression with low CDK2 activity. This function of E1A requires both its pRb-binding domain and an additional region (residues 26–35), distinct from the p300/CBP-interaction domain. We propose that E1A promotes growth by targeting two sets of cellular proteins which act downstream of cyclin E/CDK2: the pRb-family and an as yet unidentified component(s) of a pRb-independent pathway. Deregulating both pathways may reduce the requirement not only for CDK4 and CDK6, but also for CDK2 in G_1–S progression.

Results

E1A prevents p27-induced cell-cycle arrest, but does not bind p27 in fibroblasts

The ability of E1A to overcome p27-induced growth arrest was investigated in rodent fibroblasts using serial retroviral infections as described previously (Vlach *et al*., 1996; Alevizopoulos *et al*., 1997). Rat1 cells were infected with pBH2 (control vector) or pBH2-E1A viruses (INF.1, Figure 1A), expanded under hygromycin selection, superinfected with pBP or pBP-p27 viruses (INF.2) and seeded in puromycin-selective medium. Expression of p27 in control cells (pBH2) induced G_1 arrest and prevented colony outgrowth (Figure 1A) (Vlach *et al*., 1996). In contrast, E1A-expressing cells entered S phase and formed colonies with similar efficiencies in the presence or absence of p27 (Figure 1A and B). Immunoblot analysis showed that exogenous p27 was expressed at similar levels in control or E1A cells (Figure 1B, bottom) and did not affect E1A levels (data not shown). The same results were obtained with NIH 3T3 cells (Figure 1C) and rat embryo

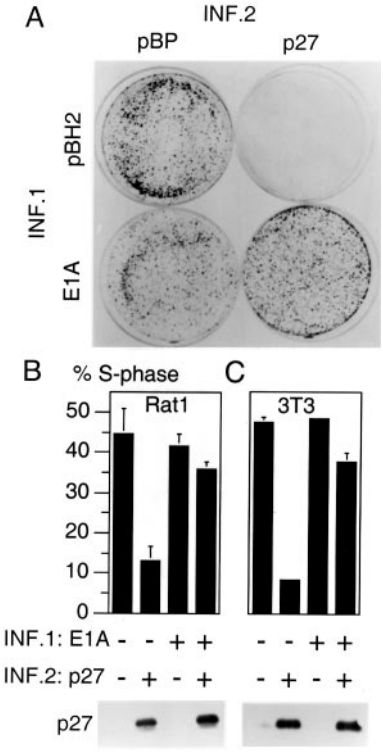

Fig. 1. E1A overcomes growth arrest by p27. (**A**) p27 prevents colony outgrowth in Rat1 cells, but not in E1A-expressing cells. Serial infections were performed as described previously (Vlach *et al*., 1996; Alevizopoulos *et al*., 1997; see text). Cells were infected first (INF.1) with a retrovirus expressing E1A or with the empty control vector (pBH2), followed by a pBP retrovirus with or without p27 (INF.2). Colonies were fixed and stained on dishes after 6 days in puromycin-selective medium. (**B**) Percentage of S phase cells in Rat1 and (**C**) NIH 3T3 populations infected with retroviruses expressing E1A and/or p27 (INF.1, INF.2, as defined in A). A 30 min pulse of BrdU incorporation was applied after 48 h of puromycin selection, and S phase cells were visualized by immunocytochemical detection of incorporated BrdU (Alevizopoulos *et al*., 1997). The decrease in the S phase fraction upon expression of p27 alone was due to accumulation of cells in the G_1 phase (Vlach *et al*., 1996). Lower panels: immunoblot analysis of retrovirally expressed p27. Cells were harvested after 48 h of puromycin selection.

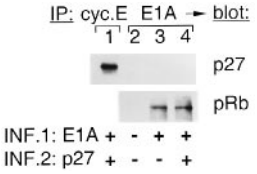

Fig. 2. E1A does not associate with p27 in Rat1 cells. Cells were infected as indicated at the bottom (see Figure 1) and harvested after 48 h of puromycin selection. E1A or cyclin E (cyc.E) were immunoprecipitated (IP) from cell lysates, followed by immunoblot (blot) analysis of co-precipitated p27 or pRb, as indicated.

fibroblasts (data not shown). Thus, E1A prevents growth arrest by p27.

A previous report suggested that E1A may associate with p27 and inactivate it in TGFβ-treated epithelial cells (Mal *et al*., 1996). However, when E1A was immunoprecipitated from Rat1 cells expressing E1A and p27, no p27 was detectable in the precipitate (Figure 2, lane 4). As controls, pRb was associated with E1A (lanes 2–4) and p27 with cyclin E (lane 1). In the reciprocal experiment,

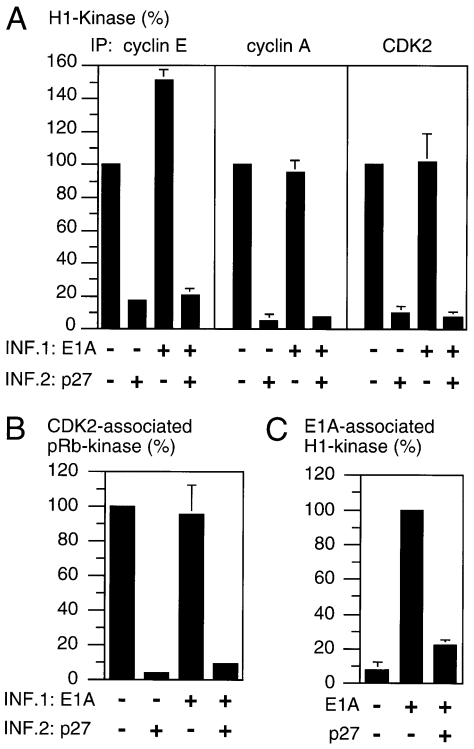

Fig. 3. E1A does not overcome inhibition of CDK2 activity by p27. Cells were infected as indicated below each graph (Figure 1). (**A**) Cyclin E, cyclin A or CDK2 as indicated (IP) were immunoprecipitated from cell lysates. Histone H1-kinase activities were measured in the immunoprecipitates and normalized to the activities in control cells (100%). (**B**) Same as A, using a purified GST pRb protein as substrate in the kinase assay. (**C**) Histone H1-kinase activity was measured in E1A immunoprecipitates. All values are the average of at least three independent experiments.

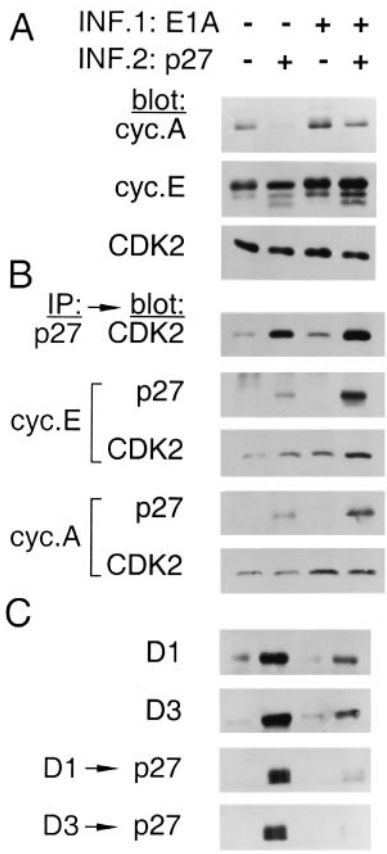

Fig. 4. Expression of endogenous cyclins and CDKs, and composition of cyclin/CDK/p27 complexes in infected cells. Cells were infected as indicated at the top (Figure 1). Cell lysates were analysed by immunoblot (blot), either directly (using 20–40 μg of total cellular proteins), or following immunoprecipitation (IP), as indicated on the left. (**A**) Expression levels of cyclins A, E and CDK2. (**B**) Interactions between p27, CDK2 and cyclins A or E. (**C**) Expression of cyclins D1 and D3 and their interaction with p27.

p27 immunoprecipitates contained CDK2 but not E1A (data not shown). Thus, E1A does not appear to interact with p27 in Rat1 cells.

p27 inhibits CDK2 in the presence of E1A

In Rat1 cells, exogenous p27 associated with cyclin E/CDK2 and cyclin A/CDK2 complexes, and inhibited their kinase activity. In addition, cyclin A levels were decreased, resulting in an overall suppression of CDK2 activity (Vlach *et al*., 1996) (Figures 3A, 4A and B). Since E1A did not associate with p27, it might have indirectly prevented CDK2 inhibition in order to promote cell-cycle progression. Surprisingly, however, p27 still suppressed cyclin E, cyclin A and CDK2-associated histone H1-kinase activities in the presence of E1A as efficiently as in p27-arrested cells (Figure 3A). Similar results were obtained when CDK2 activity was measured using pRb as a substrate (Figure 3B). Although cyclin A expression was restored in E1A+p27 cells (Figure 4A), cyclin A/CDK2 complexes were associated with p27 (Figure 4B). Likewise, p27 associated with cyclin E/CDK2 in E1A+p27 cells, to similar or even greater extents than in p27-arrested cells (Figure 4B). The same observations were made in NIH 3T3 cells, or in Rat1 cells expressing human cyclin E, which forms active complexes with endogenous CDK2 (Vlach *et al*., 1996); in the presence or absence of E1A, p27 associated with human cyclin E/rat CDK2 complexes and inhibited their kinase activity (data not shown). Finally, we measured H1-kinase activity in E1A immunoprecipitates. This activity most likely stems from cyclin A or E/CDK2 complexes associated with E1A via their interaction with p107 and p130 (reviewed by Bayley and Mymryk, 1994). Expression of p27 resulted in suppression of kinase activity (Figure 3C) and concomitant loss of E1A associated CDK2 protein (data not shown). Taken together, our results suggest that E1A does not act by suppressing p27 function or preventing inhibition of CDK2.

p27 expression in Rat1 cells induced accumulation of cyclins D1 and D3, and a large fraction of p27 associated with these cyclins (Vlach *et al*., 1996). E1A counteracted this effect, and thus decreased the fraction of p27 bound to cyclins D1 and D3 (Figure 4C). Although the underlying mechanism remains to be understood, this effect of E1A would be predicted to increase the amount of p27 bound to endogenous cyclin E/CDK2 and cyclin A/CDK2 (as apparent in Figure 4B) as well as the intracellular pool of free p27 (Reynisdottir *et al*., 1995; Reynisdottir and Massagué, 1997). To measure the CDK-inhibitory activity of this pool, we mixed lysates of p27-expressing cells (inhibitory lysate, Figure 5) with a target lysate from cells expressing human cyclin E. In this assay, free p27 associates with human cyclin E/CDK2 and decreases its

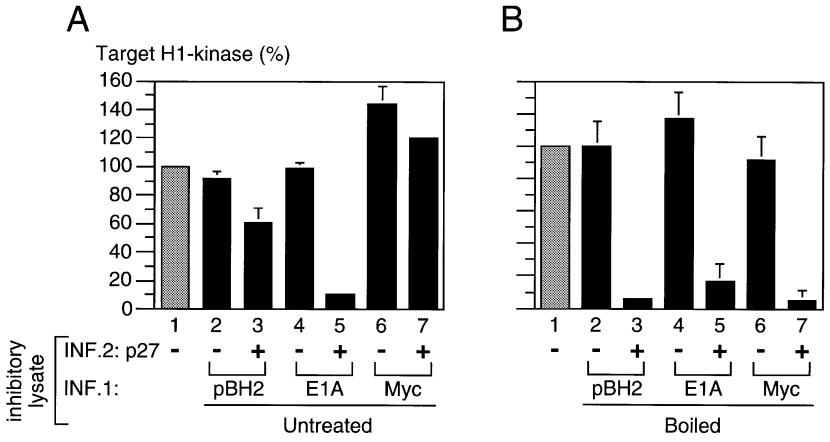

Fig. 5. CDK-inhibitory activity of p27 in native (**A**) and boiled (**B**) lysates from cells expressing E1A, Myc and/or p27. The activity of a target human cyclin E/CDK2 complex (from Rat1 cells expressing human cyclin E) was measured by selective immunoprecipitation of human cyclin E. In parallel, the target was co-incubated prior to immunoprecipitation with either native (A) or pre-boiled (B) inhibitory lysates from infected Rat1 cells expressing E1A or Myc (INF.1), with or without p27 (INF. 2). The kinase activity of the target following the co-incubation step (black bars) was normalized to that of the target alone (100%, grey bars). Note that cells which do not express exogenous p27 contain no significant CDK-inhibitory activity in this assay (A and B, lanes 2). Note also that, although this particular series of experiments showed an apparent increase of the target's activity upon incubation with Myc-cell extracts (A, lane 6), this effect is neither reproducible nor significant (Vlach *et al*., 1996).

kinase activity (Figure 5A, lanes 1–3) (Vlach *et al*., 1996). As predicted above, E1A+p27 cells contained elevated levels of inhibitory activity compared with p27-arrested cells (Figure 5A, lanes 3 and 5). Boiling of the lysates released similar amounts of inhibitory activity in the presence or absence of E1A (Figure 5B, lanes 3 and 5), showing that E1A did not modify the intrinsic activity of p27. In conclusion, the net effect of E1A is to increase the amount of free inhibitory p27 in cells, consistent with the observations that E1A does not bind p27 (Figure 2) and that CDK2 complexes remain associated with p27 and inactive (Figures 3A and B, and 4B).

Collectively, our data indicate that E1A does not overcome cell-cycle arrest by inactivating p27. Instead, E1A allows cell proliferation with sustained inhibition of cyclin E/CDK2 and cyclin A/CDK2 (comparable to the inhibition found in p27-arrested cells), suggesting that p27 can act downstream of CDK2 function.

E1A does not prevent p27-induced dephosphorylation of CDK2 substrates in vivo

Although cells expressing E1A and p27 grow in the absence of detectable E1A-p27 interaction and CDK2 activity, it could still be argued that E1A binds p27 in cells but releases it after lysis, leading to an inhibition of CDK2 that does not reflect the real situation *in vivo*. Thus, as a simple readout of CDK2 inhibition by p27 *in vivo*, we examined the phosphorylation state of two natural CDK2 substrates, pRb and p130, in infected Rat1 cells. Expression of p27 induced loss of the slower-migrating hyperphosphorylated forms, with the ensuing accumulation of these proteins in their faster-migrating hypophosphorylated forms (Figure 6A, lanes 1 and 2). This effect of p27 was not altered by E1A, even though E1A increased pRb and p130 levels in the presence of p27 (Figure 6A, lanes 2–4). Thus, p27 still prevents phosphorylation of pRb and p130 in the presence of E1A. These data confirm that p27 inhibits CDK2 activity in E1A-expressing cells.

It should be noted here that expression of p16 in Rat1 cells prevented phosphorylation of pRb, p130 and p107.

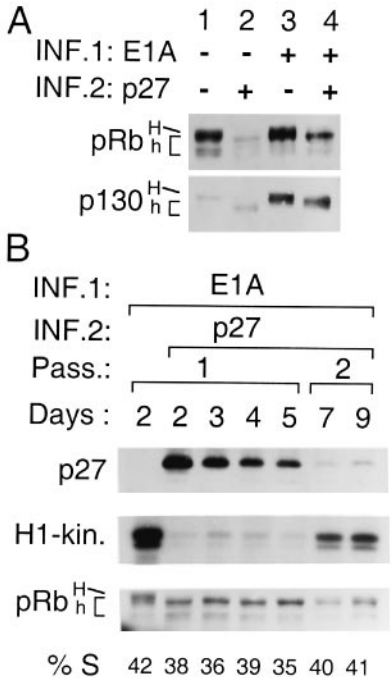

Fig. 6. p27 prevents phosphorylation of pRb in the presence of E1A, and remains active over several division cycles. Cells were infected as indicated and analysed after (**A**) our standard time-point of 2 days or (**B**) after prolonged periods of time. p27, pRb and p130 were analysed by immunoblot. The slower-migrating, hyperphosphorylated forms of pRb and p130 and their faster-migrating, hypophosphorylated forms are indicated (H and h, respectively). CDK2-associated H1-kinase activity (H1-kin.) was measured as in Figure 3A, and the percentage of cells in S phase (%S) as in Figure 1B.

However, p27 did not block phosphorylation of p107 (Alevizopoulos *et al*., 1997; data not shown), which is a substrate of CDK4/6 but most likely not of CDK2 (Beijersbergen *et al*., 1995; Xiao *et al*., 1996). This observation, together with IP-kinase assays (Vlach *et al*., 1996; unpublished data) suggests that p27 acts as a selective inhibitor of CDK2 *in vivo*, and not—at least in

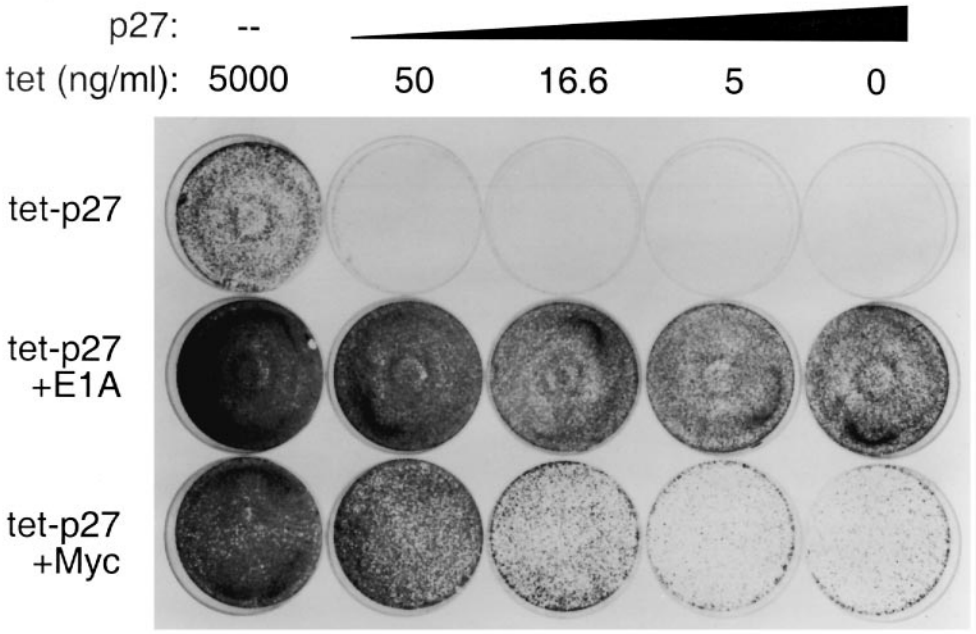

Fig. 7. E1A and Myc confer differential resistance to p27 overexpression. A Rat1 cell clone expressing a tetracycline-regulatable p27 gene (tet-p27; see text) was infected with retroviruses expressing E1A or Myc (as indicated to the left). Cells were seeded in medium containing decreasing concentrations of tetracycline, to induce gradually p27 (as indicated at the top; see text). Colonies were stained after 6 days. Note that in the various tet-p27 clones screened, as well as in cells expressing E1A or Myc, p27 induction never caused any signs of apoptosis, in contrast to what has been proposed recently (Katayose *et al*., 1997; Wang *et al*., 1997).

those cells—of CDK4 and CDK6. Others have reached similar conclusions (Blain *et al*., 1997).

E1A allows long-term proliferation with p27-inhibited CDK2

All the biochemical experiments presented so far were performed 2 days after infection with the p27 retrovirus. To rule out a transient effect, by which E1A-expressing cells might have inactivated p27 shortly after our standard time of analysis, we followed E1A+p27 cell pools for several days. The percentage of cells in S phase, as measured by BrdU incorporation, was comparable at all time points to that of control proliferating cells (Figure 6B, bottom). Over a period of 5 days (or ~6–7 generations) p27 levels were maintained, CDK2 H1-kinase activity remained inhibited and pRb and p130 remained hypophosphorylated (Figure 6B and data not shown). Upon further passaging of the cells, however, p27 levels declined, concomitant with a partial recovery of CDK2 activity and re-appearance of phosphorylated pRb (Figure 6B, pass. 2), indicating that some selective pressure remained against elevated p27 levels in these cell pools. Nevertheless, these results altogether show that E1A allows cell proliferation in the presence of active p27, which inhibits CDK2 over a sustained period of time.

E1A, unlike Myc, confers resistance to supraphysiological p27 levels

The finding that E1A overcomes p27-induced arrest without rescuing CDK2 activity is in sharp contrast with the effect of Myc, which prevents CDK2 inhibition by inducing sequestration of p27 within cells into a form unable to associate with CDK2 (Vlach *et al*., 1996; Amati *et al*., 1998). This can be illustrated with the *in vitro* mixing assay described above (Figure 5): while kinase-inhibitory activity increased in lysates of E1A+p27 cells, it was masked in Myc+p27 cells (Vlach *et al*., 1996; Figure 5A, lanes 4–7; see legend). However, both lysates released active p27 upon boiling (Figure 5B). Based on these observations, one would predict that E1A, but not Myc, should render cells resistant to increased, supraphysiological p27 levels. To test this hypothesis, we subcloned p27 in the tetracycline-regulatable retroviral vector pBPSTR1 (Paulus *et al*., 1996), infected Rat1 cells with this retrovirus and selected clones showing tight growth arrest and elevated p27 levels upon removal of tetracycline from the culture medium (J.Vlach, unpublished data). One of these clones (tet-p27) was infected with retroviruses expressing E1A or Myc. Infected cells were pooled and seeded either with 5000 ng/ml tetracycline (to maintain p27 fully repressed), no tetracycline (to induce maximal p27 levels) or intermediate amounts of the drug (to induce p27 at lower levels; data not shown). Control tet-p27 cells required the maximal amount of tetracycline to form colonies (Figure 7). Myc cells showed a strong dose dependence, growing normally at 50 ng/ml tetracycline, but not at lower concentrations. Although a slight growth inhibitory effect of p27 was still apparent in E1A cells, these cells proliferated well under all conditions. Neither E1A nor Myc affected induction of p27 upon tetracycline removal. Furthermore, in agreement with our previous data, cyclin E/CDK2 was totally inhibited by p27 in the presence of E1A (data not shown). In summary, Myc overcomes p27-induced arrest by a mechanism sensitive to p27 overexpression, while E1A allows cell growth with supraphysiological p27 levels and maximally inhibited CDK2.

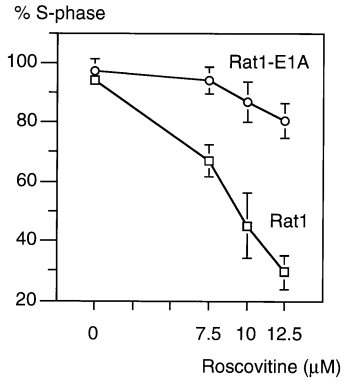

Fig. 8. G_1-arrest induced by roscovitine is bypassed by E1A. Control Rat1 and E1A-expressing cells (Rat1-E1A) were arrested by contact inhibition (2 days), trypsinized and re-seeded in fresh medium containing serum and BrdU, in the presence of increasing concentrations of roscovitine. After 14.5 h, the fraction of S phase cells was visualized by immunocytochemical detection of incorporated BrdU. The values presented are the average of five independent experiments.

E1A bypasses G_1 arrest by a chemical inhibitor of CDK2

If E1A acted downstream of p27 and CDK2, it might be expected to bypass G_1 arrest induced by chemical inhibitors of CDK2. We thus tested whether E1A allows S-phase entry in the presence of roscovitine, a specific inhibitor of CDK2 and CDC2 (Meijer, 1996). To offset the problem of CDC2 inhibition at mitosis, contact-inhibited Rat1 and E1A-expressing cells were trypsinized and re-seeded in fresh medium with or without roscovitine. S-phase entry was monitored over a period of 14.5 h by measuring BrdU incorporation (Figure 8). In the absence of roscovitine, >90% of cells in both populations had entered S phase. In control Rat1 cells, roscovitine suppressed S-phase entry in a dose-dependent manner. In contrast, the drug had very little effect on E1A-expressing cells. Roscovitine was functional in those cells, since it prevented phosphorylation of pRb, indicating that CDK2 was effectively being inhibited *in vivo* (data not shown). This result reinforces the notion that E1A largely reduces the requirement for CDK2 activity in S-phase entry.

Two functional domains of E1A are required for bypassing CDK2 inactivation

In Rat1 cells, retroviral expression of either p27 or p16 induces activation of pRb-family proteins (Vlach *et al*., 1996; Alevizopoulos *et al*., 1997). We thus predicted that the rescue of p27- or p16-induced G_1 arrest by E1A should depend upon its pRb-binding domain. Furthermore, if E1A acted downstream of CDK2, it might also be expected to target the pRb-independent pathway(s) regulated by CDK2 (see Introduction). If this were true, additional domain(s) of E1A might be required for overcoming p27-, but not p16-induced arrest (the latter being only dependent upon binding to pRb-family proteins).

To map the domains of E1A required for rescuing p27- and p16-induced arrest, a series of E1A mutants were expressed in Rat1 cells. These mutants were previously characterized for their ability to interact with cellular proteins, as summarized in Figure 9A (see legend). Mutants 2R→G, Δ2–11 and Δ64–68 are defective in binding to p300/CBP. Mutants 38–44A, 124,135A and the combined

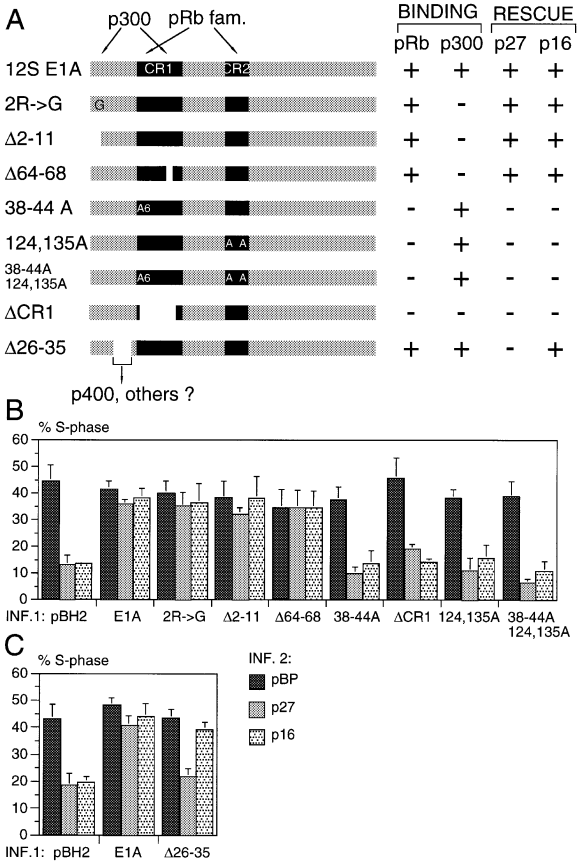

Fig. 9. Two distinct domains of E1A are required for bypassing p27-induced arrest. (**A**) Schematic representation of the E1A mutants used in this work. The mutant ΔCR1 carries a deletion of residues 38–67. 'Binding': summary of the experimentally determined ability of E1A mutants to bind pRb-family proteins (indicated as pRb) and p300 (Howe and Bayley, 1992; Wang *et al*., 1993; Barbeau *et al*., 1994; Bayley and Mymryk, 1994; Trouche and Kouzarides, 1996; data not shown). 'Rescue': summary of the data presented in parts B and C on rescue of p27- and p16-induced growth arrest. (**B** and **C**) Percentage of S phase cells in Rat1 populations infected with retroviruses expressing E1A mutants and p27 or p16, as indicated. Note that the measurements shown in B and C are from different series of experiments, which yielded slightly different efficiencies of cell-cycle arrest. In all experiments, however, cells expressing Δ26–35 were as effectively arrested by p27 as control cells, as also confirmed by colony assays (data not shown). All shown values are averaged from a series of 3–6 independent measurements. E1A proteins were expressed from the pBH2 vector (INF.1), followed by p27 or p16 in pBP (INF.2). BrdU incorporation was measured as in Figure 1B. As for p27, the decrease in S phase induced by p16 was due to accumulation of cells in G_1 (Alevizopoulos *et al*., 1997).

mutant 38–44A/124,135A are defective in their interaction with pRb-family proteins, whereas ΔCR1 is defective in both interactions. All mutants were expressed as efficiently as 12S E1A in Rat1 cells (data not shown). The mutants defective in p300/CBP-binding were capable of overcoming p27- and p16-induced arrest, whereas the pRb-interaction mutants were not, as shown by BrdU incorporation and colony assays (Figure 9A and B; data not shown). Thus, neutralization of pRb-family proteins, but not of p300/CBP, is a prerequisite for E1A to bypass growth arrest by either p27 or p16.

While searching for the existence of additional functional sequences in E1A, we found that the E1A mutant Δ26–35 (Howe and Bayley, 1992; Barbeau *et al*., 1994)

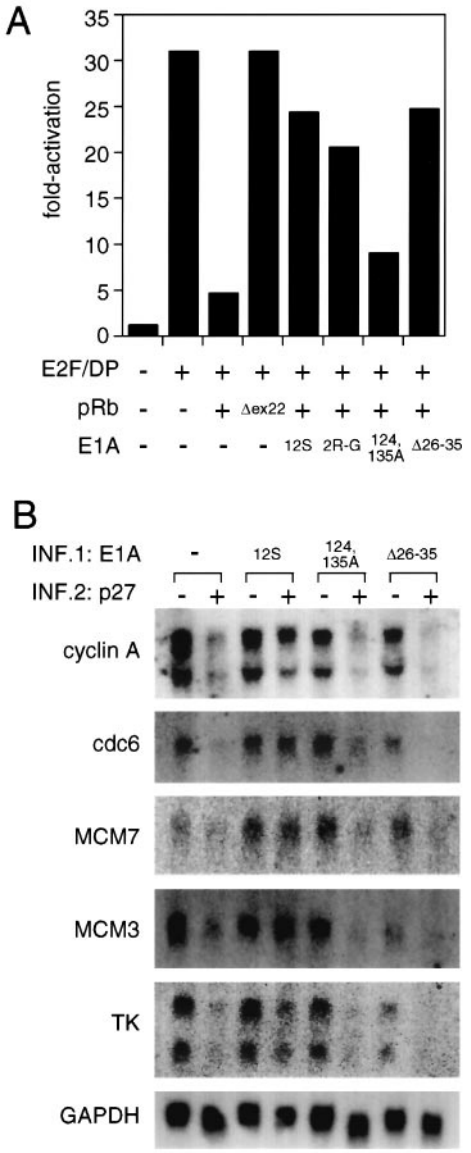

Fig. 10. Effect of E1A Δ26–35 on E2F activity. (**A**) E2F Δ26–35 is functional in relieving pRb-mediated repression of E2F transcriptional activity. 293T cells were transfected with an E2F-responsive cyclin E promoter–luciferase reporter gene (Ohtani *et al*., 1995) together with plasmids encoding E2F-1, DP-1, pRb or E1A proteins (12S or mutants 2R→G, Δ26–35, 124,135A), as indicated. Δex22: a pRb mutant deleted in exon 22, which does not associate with E2F (Qin *et al*., 1992). Relative luciferase units are expressed in fold-activation relative to the reporter alone. The data shown are from one out of six independent experiments, which yielded the same results. (**B**) E2F Δ26–35 fails to re-induce E2F-target genes in p27-arrested cells. mRNA levels for the genes indicated to the left were measured by Northern blot analysis of total RNA from infected Rat1 cells (as indicated at the top; Figure 1). TK: thymidine kinase; GAPDH: glyceraldehyde-phosphate dehydrogenase, used as a loading control.

was defective in preventing p27-induced arrest (Figure 9A and C, see legend; data not shown). This mutant was expressed as well as 12S E1A, associated efficiently with all pRb-family proteins (Howe and Bayley, 1992; Barbeau *et al*., 1994; data not shown) and was capable of overcoming p16-induced arrest. Thus, the p27-antagonizing activity of E1A requires a function distinct from pRb- (or p300-) binding, mediated by residues 26–35.

Effect of E1A Δ26–35 on E2F activity

The above observations suggested that E1A Δ26–35 should be functional in sequestering pRb-family proteins and preventing repression of E2F. To address this question, we transiently transfected 293T cells with an E2F-responsive cyclin E promoter-luciferase reporter gene (Figure 10A). Consistent with previous data (Ohtani *et al*., 1995; Geng *et al*., 1996), co-expression of E2F-1 and DP-1 strongly transactivated this reporter. Activation was suppressed by co-expression of pRb, but not by a mutant defective in binding to E2F (Δex22; Qin *et al*., 1992). Co-expression of 12S E1A or of the p300-interaction mutant 2R→G relieved pRb-mediated suppression. As expected, the pRb-interaction mutant 124,135A was ineffective in reactivating E2F. In contrast, Δ26–35 was active in this assay. All E1A proteins were expressed at comparable levels and none of them affected the basal activity of the reporter (data not shown). We conclude that residues 26–35 are not required for inactivation of pRb and derepression of E2F.

We also monitored expression of several E2F-target genes in infected Rat1 cells using Northern-blot analysis. Cyclin A, Cdc6, thymidine kinase (TK), Mcm3 and Mcm7 mRNA levels, which all respond to E2F (Helin, 1998; Leone *et al*., 1998; Yan *et al*., 1998), were down-regulated in p27-arrested cells (Figure 10B). Expression of 12S E1A prevented this effect while, as expected, the pRb-interaction mutant 124,135A did not. Surprisingly, p27 still suppressed expression of E2F-target genes in the presence of Δ26–35. It remains to be addressed whether this result reflects a specific defect of Δ26–35, or is an indirect consequence of p27-induced arrest in those cells. These data, however, raise the possibility that the pRb-independent function of E1A may be selectively required for E2F function in the presence of p27 (see Discussion).

Discussion

Action of E1A downstream of p27 and CDK2

Using a retroviral expression system, we show that the 12S E1A protein of adenovirus 5 overcomes the proliferative arrest induced by $p27^{Kip1}$ in rodent fibroblasts. Consistent with this finding, DeGregori *et al*. (1995) observed that E1A permits S-phase entry in REF52 cells upon transient transfection with a p27-expressing plasmid. Another report suggested that E1A can associate with p27 and prevent CDK2 inhibition in transfected epithelial cells (Mal *et al*. 1996). Here, we characterize the mechanism underlying growth restoration by E1A. Strikingly, E1A does not interfere with the molecular action of p27 but, instead, allows cell proliferation with inhibited CDK2.

Retrovirally expressed p27 associates with cellular cyclin E/CDK2 and cyclin A/CDK2 complexes and inhibits their activity *in vivo*, preventing phosphorylation of CDK2 substrates (pRb and p130). All of these molecular changes still occur upon introduction of p27 in E1A-expressing cells and are not merely transitory, but instead persist over several division cycles. In contrast with the conclusions of Mal *et al*. (1996), E1A clearly does not interact with p27 in infected Rat1 cells. In fact, there is a larger pool of free p27 in the presence of E1A, owing to decreased expression of cyclins D1 and D3 and to the ensuing release of p27 from these cyclins. Thus, E1A

indirectly increases the amount of p27 capable of inhibiting CDK2. The differences between our findings and those of Mal *et al*. (1996) may be due to the use of distinct cell types, to different E1A and/or p27 expression levels, or to other experimental parameters. In any case, our data unravel a novel level of action for E1A in cell-cycle control and reveal that E1A can promote proliferation with inhibited CDK2 activity. Consistent with this finding, E1A also prevents G_1 arrest by roscovitine, a chemical inhibitor of CDK2. Taken together, these results suggest that E1A can rescue cell growth by acting downstream of CDK2.

Using the same experimental system, we have shown previously that Myc also overcomes p27-induced arrest (Vlach *et al*., 1996). Myc suppressses p27 function by inducing its sequestration into a form unable to associate with CDK2 complexes (Vlach *et al*., 1996; Müller *et al*., 1997; Pérez-Roger *et al*., 1997; reviewed in Amati *et al*., 1998). Therefore, unlike E1A, Myc prevents inhibition of cyclinE/CDK2 and cyclin A/CDK2. We further show here that supraphysiological p27 levels arrest Myc- but not E1A-expressing cells. Thus, the effect of Myc is saturable by p27 *in vivo*, whereas the effect of E1A is not, supporting the notion that E1A acts downstream of CDK2. Finally, in contrast to E1A, Myc-induced cell-cycle entry is blocked by roscovitine (Rudolph *et al*., 1996; Prall *et al*., 1998). In summary, Myc is fully dependent upon CDK2 function in order to induce S-phase entry, whereas E1A can reduce the requirement for CDK2 activity.

Would E1A-expressing cells be resistant to a total loss of CDK2 activity? Two lines of evidence argue against this idea. First, G_1 arrest by DN-CDK2 (van den Heuvel and Harlow, 1993) is not bypassed by E1A in U2OS cells (data not shown). Secondly, microinjection data suggest that the cdc25A phosphatase is required for E1A-induced S-phase entry (Spitkovsky *et al*., 1996). However, DN-CDK2-induced arrest requires overexpression of the protein (e.g. it does not occur with retroviral vectors), which may block other regulatory pathways in a non-specific manner. It also remains unclear whether cdc25A is critical for CDK2 versus CDK4/6 activity *in vivo* (e.g. Terada *et al*., 1995; Iavarone and Massagué, 1997) and whether it has additional cellular targets. Regardless of these reservations, the above results suggest that E1A does not totally bypass the requirement for CDK2 function. In agreement with this, we see a residual growth-inhibitory effect of high p27 expression or roscovitine treatment in the presence of E1A. Finally, E1A may protect a minor fraction of CDK2, which would remain able to phosphorylate critical substrates and induce cell-cycle progression. We show, however, that E1A-associated H1-kinase activity is suppressed by p27 in Rat1 cells. In summary, it remains to be established whether or not CDK2 function is entirely dispensable in the presence of E1A; this will require the availability of a conditional CDK2 knock-out allele. Nevertheless, our data clearly demonstrate that E1A-expressing cells can proliferate with very low levels of CDK2 activity comparable to those found in p27-arrested cells.

Two distinct functions of E1A are required for bypassing p27-induced arrest

E1A is known to bind several cellular proteins through separable, but also overlapping functional domains. The best-characterized ligands of 12S E1A are pRb-family proteins (pRb, p107 and p130) and the transcriptional coactivators p300/CBP. Both types of interactions are involved in the growth-promoting activities of E1A (reviewed by Dyson and Harlow, 1992; Bayley and Mymryk, 1994). We therefore tested mutants of E1A defective in either interaction for their ability to overcome p27-induced arrest. As a control, we also used the CDK4- and CDK6-specific inhibitor $p16^{INK4a}$ (p16), since the ability of E1A to bypass p16-induced arrest was expected to correlate with its ability to neutralize pRb (see Introduction). Our data show that E1A mutants that do not associate with pRb, p107 and p130 are unable to overcome arrest by either p27 or p16 (summarized in Figure 9A), confirming that both inhibitors block the cell cycle via pRb-family proteins. In contrast, E1A mutants defective in interaction with p300/CBP bypass arrest by either inhibitor as efficiently as 12S E1A.

CDK2 is believed to have a pRb-independent function in G_1–S progression (see Introduction). On the other hand, our experiments suggest that E1A can act downstream of CDK2 (see above). On this basis, we postulated that binding to pRb-family proteins might not be sufficient for E1A to prevent p27-induced arrest, and that a distinct activity of E1A might be required. We thus searched for new mutants of E1A capable of binding pRb and bypassing p16-induced arrest, but ineffective against p27. We found that a previously described mutant, E1A Δ26–35 (Jelsma *et al*., 1988; Howe and Bayley, 1992; Barbeau *et al*., 1994), exactly fulfils these expectations. Thus, residues 26–35 of E1A define a novel function that is required to allow cell-cycle progression with low CDK2 activity.

One possible interpretation of the above results was that E1A Δ26–35 might be defective in preventing repression of E2F activity by pRb-family proteins. Compatible with this notion, Δ26–35 fails to restore expression of several E2F-target genes in p27-arrested cells (see also below). However, transient transfection experiments show that Δ26–35 is functional in derepressing E2F-1 activity in the presence of pRb. Although a defect of Δ26–35 in relieving p107- or p130-mediated repression cannot be ruled out at the moment, several lines of evidence render this hypothesis unlikely. E1A Δ26–35 binds avidly to p130, as well as to p107 and pRb (data not shown) and is fully functional in preventing growth arrest by p16, which induces dephosphorylation and activation of all pRb-family proteins (Alevizopoulos *et al*., 1997). Moreover, p107 is not a CDK2 substrate (Beijersbergen *et al*., 1995), does not appear to be activated by p27 in Rat1 cells (Alevizopoulos *et al*., 1997) and is thus probably not involved in p27-induced arrest (see Results). It remains formally possible that residues 26–35 of E1A are specifically required for neutralization of p130 or pRb when these proteins are unphosphorylated at CDK2-specific sites (i.e. in p27-arrested cells). None the less, it appears more likely that amino acids 26–35 of E1A target a cellular protein(s) distinct from pRb-family proteins.

The major question raised by our data is whether the pRb-independent pathway targeted by E1A and CDK2 regulates a rate-limiting step in G_1–S progression distinct from E2F activity, or whether it is required for E2F function. The failure of E1A Δ26–35 to restore expression of E2F-target genes in p27-arrested cells is not conclusive

per se: this result is consistent with a requirement of CDK2 either for E2F activity (independently of pRb-family proteins) or for some other step in the regulation of E2F-target genes. In addition, the failure to re-express E2F-target genes could also be a secondary consequence of p27-induced arrest, not related to the specific defect of E1A Δ26–35. Previous experiments have suggested the existence of an E2F-independent function for CDK2 in G_1–S control. In transiently transfected U2OS cells, DN-CDK2 induces G_1 arrest and suppressses E2F activity; co-expression of SV40 LT, which inactivates pRb, rescues E2F function, but does not alleviate G_1 arrest (Hofmann and Livingston, 1996). In addition, cyclin E overexpression can bypass G_1 arrest by a DN-DP1 mutant which blocks E2F function (Lukas *et al*., 1997). Reciprocally, however, E2F-1 overexpression can promote S-phase entry in the absence of CDK2 activation (DeGregori *et al*., 1995), although moderate expression of E2F-1 cannot bypass p27-induced arrest (and thus CDK2 inhibition) (Mann and Jones, 1996; Alevizopoulos *et al*., 1997). Thus, while it is clear that E2F and cyclin E/CDK2 activities reciprocally up-regulate each other (via pRb/E2F-mediated regulation of *cyclin E* transcription and cyclin-E-mediated phosphorylation of pRb; e.g. Ohtani *et al*., 1995; Geng *et al*., 1996; Herrera *et al*., 1996; Hurford *et al*., 1997; Helin, 1998; Mittnacht, 1998), their precise hierarchical relationship in G_1–S control remains to be elucidated. The best model at present is that both have independent and non-redundant downstream functions, as is evident in *Drosophila* (Duronio and O'Farrell, 1995; Duronio *et al*., 1995, 1996). Finally, CDK3 rather than CDK2 may be required for E2F function in a pRb-independent manner, since LT can restore neither E2F activity nor S-phase entry in the presence of DN-CDK3 (Hofmann and Livingston, 1996). We have been unable to detect CDK3-specific activity in Rat1 cells and ignore whether CDK3 inhibition is involved in p27-induced arrest and/or repression of E2F-target genes. Altogether, it is most likely—but not yet proven—that the pathway targeted by E1A downstream of CDK2 is both pRb- and E2F-independent. It is also possible that multiple pRb-independent functions of E1A are required to prevent G_1 arrest by p27, one of these being the reactivation of E2F; it will thus be interesting to address whether additional mutations in E1A confer the same p27-sensitive phenotype as Δ26–35. More work is needed to unravel the cellular targets by which E1A allows cell-cycle progression with low CDK2 activity, and whether their activities converge on E2F function. In particular, we will need to identify the cellular protein(s) which associate with 12S E1A but not with Δ26–35.

E1A Δ26–35 is defective in interacting with p400, a cellular E1A-binding protein which remains to be cloned and characterized (Howe and Bayley, 1992; Barbeau *et al*., 1994). In addition, a region spanning residues 2–36 of E1A is involved in interaction with the transcriptional regulators Dr1, YY-1 and AP-2, as well as with several uncharacterized cellular proteins (Kraus *et al*., 1994; White *et al*., 1994; Lee *et al*., 1995a, 1995b; Lewis *et al*., 1995; Somasundaram *et al*., 1996; Sang and Giordano, 1997). It remains to be investigated whether any of these interactions is related to the biological activity of E1A uncovered in this work. Finally, it will be interesting to address whether CDK2 substrates such as NPAT (Zhao *et al*., 1998) are targets of E1A.

The inability of E1A Δ26–35 to overcome p27-induced arrest is the most dramatic phenotype attributed so far to this mutant. E1A Δ26–35 is capable of transforming primary baby rat kidney (BRK) cells or rat embryo fibroblasts in cooperation with *ras* (Jelsma *et al*., 1989; data not shown) but is defective in morphologically transforming an established BRK derivative (Dorsman *et al*., 1995). Δ26–35 is also active in induction of DNA synthesis in quiescent BRK cells, but this assay is equally permissive for individual pRb- and p300-interaction mutants (Howe *et al*., 1990). Thus, additional work is required to re-evaluate the specific biological defects of the Δ26–35 mutant. It is worth recalling here that E1A has evolved in the context of the lytic cycle of adenovirus. Its main functions are to regulate transcription of viral genes and promote S-phase entry in infected, quiescent cells (reviewed by Bayley and Mymryk, 1994). The interaction with pRb-family proteins fulfils both functions, since the viral early gene E2 is regulated by E2F and since inactivation of pRb (as well as p300/CBP) contributes to cell-cycle promotion by E1A. We propose that the novel function uncovered in this work can be equally important in the viral lytic cycle. By releasing the strict requirement for CDK2 activity, in addition to CDK4 and CDK6, E1A liberates cells from upstream signals, abrogates R-point control and promotes unrestrained cell-cycle progression.

Materials and methods

Retroviral expression vectors

The retroviral vectors pBabe-Puro (pBP), -Neo2 (pBN2) and -Hygro2 (pBH2) have been described previously (Morgenstern and Land, 1990; Vlach *et al*., 1996), as well as pBPSTR1 (Paulus *et al*., 1996). cDNAs encoding adenovirus 12S E1A, E1A mutants (see legend to Figure 9), Myc and human cyclin E were subcloned in pBH2 or pBN2. cDNAs encoding the human CKIs p16 and p27 were subcloned in pBP (Vlach *et al*., 1996; Alevizopoulos *et al*., 1997).

Retroviral infections, cell-cycle analysis and biochemical analysis

High-titer retroviral supernatants were generated as described before (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) or puromycin (Sigma, 2.5 μg/ml). Serial infections of cell pools, preparation of cell lysates and biochemical analysis (immunoblots, immunoprecipitations, kinase assays, mixing and boiling experiments) were as described previously (Vlach *et al*., 1996). For cell-cycle analysis, cells were labelled with 33 μM bromodeoxyuridine (BrdU) for 30 min. and analysed by immunocytochemical detection of BrdU, as described previously (Alevizopoulos *et al*., 1997).

Northern (RNA) blot analysis

Total RNA was isolated from retrovirally infected Rat1 cells using an RNeasy kit (Qiagen). Fifteen μg of RNA were electrophoresed in 1% agarose-formaldehyde gels and transferred to Immobilon-N nylon membranes. Blots were hybridized with [^{32}P]-labelled random-primed cDNA probes from the indicated genes (Figure 10B). Hybridization was performed overnight at 65°C in 1 mM EDTA, 7% SDS and 0.5 M sodium-phosphate buffer pH 7.2. Blots were washed six times at increasing stringencies, reaching 0.1× SSC at 60°C (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), and exposed to autoradiographic film. Blots were stripped by boiling for 5 min in 0.5% SDS, 1 mM EDTA prior to reprobing.

Transient transfections

293T cells were transiently transfected with a standard calcium-phosphate–DNA precipitation procedure in 12-well plates. Transfection mixes

contained 0.5 μg of a cyclin E promoter–Luciferase reporter (Ohtani *et al*., 1995), 0.5 μg of CMV-E2F-1 and CMV-DP-1 expression plasmids, 0.75 μg of CMV-pRb, 1.25 μg of CMV-E1A (12S E1A or mutants) and 0.2 μg of CMV-LacZ. Where CMV-pRb or CMV E1A were omitted, they were replaced by an equal amout of the empty CMV-promoter vector. Transfected cells were incubated in DMEM (Gibco-BRL 31966-021) with 10% fetal-calf serum and harvested 24 h post-transfection. Cells were lysed and Luciferase activity was measured following standard procedures (Promega) using a Lumac Biocounter M2500. β-galactosidase activity was used to normalize Luciferase activity within each sample.

Antibodies

The following antibodies were used: against E1A: M73 (Santa Cruz, sc025); p27: C19 (sc-528); CDK2: M2 (sc-163); cyclin A: H-432 (sc751); rodent cyclin E: M20 (sc-481); human cyclin E: C19 (sc198); cyclin D1: 72-13G (sc-450); cyclin D3: C16 (sc-182); pRb: 14001A (Pharmingen); p130: C20 (sc-317).

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