E1A Can Provoke G1 Exit That Is Refractory to p21 and Independent of Activating Cdk2

Saïd Akli, Song Zhan, Maha Abdellatif, Michael D. Schneider

Abstract—E1A can evoke G1 exit in cardiac myocytes and other cell types by displacing E2F transcription factors from tumor suppressor “pocket” proteins and by a less well-characterized p300-dependent pathway. Bypassing pocket proteins (through overexpression of E2F-1) reproduces the effect of inactivating pocket proteins (through E1A binding); however, pocket proteins associate with a number of molecular targets apart from E2F. Hence, pocket protein binding by E1A might engage mechanisms for cell cycle reentry beyond those induced by E2F-1. To test this hypothesis, we used adenoviral gene transfer to express various E2F-1 and E1A proteins in neonatal rat cardiac myocytes that are already refractory to mitogenic serum, in the absence or presence of several complementary cell cycle inhibitors—p16, p21, or dominant-negative cyclin-dependent kinase-2 (Cdk2). Rb binding by E2F-1 was neither necessary nor sufficient for G1 exit, whereas DNA binding was required; thus, exogenous E2F-1 did not merely function by competing for the Rb “pocket.” E2F-1–induced G1 exit was blocked by the “universal” Cdk inhibitor p21 but not by p16, a specific inhibitor of Cdk4/6; p21 was permissive for E2F-1 induction of cyclins E and A, but prevented their stimulation of Cdk2 kinase activity. In addition, E2F-1–induced G1 exit was blocked by dominant-negative Cdk2. Forced expression of cyclin E induced endogenous Cdk2 activity but not G1 exit. Thus, E2F-1–induced Cdk2 function was necessary, although not sufficient, to trigger DNA synthesis in cardiac muscle cells. In contrast, pocket protein–binding forms of E1A induced G1 exit that was resistant to inhibition by p21, whereas G1 exit via the E1A p300 pathway was sensitive to inhibition by p21. Both E1A pathways—via pocket proteins and via p300—upregulated cyclins E and A and Cdk2 activity, consistent with a role for Cdk2 in G1 exit induced by E1A. However, p21 blocked Cdk2 kinase activity induced by both E1A pathways equally. Thus, E1A can cause G1 exit without an increase in Cdk2 activity, if the pocket protein–binding domain is intact. E1A also overrides p21 in U2OS cells, provided the pocket protein–binding domain is intact; thus, this novel function of E1A is not exclusive to cardiac muscle cells. In summary, E1A binding to pocket proteins has effects beyond those produced by E2F-1 alone and can drive S-phase entry that is resistant to p21 and independent of an increase in Cdk2 function. This suggests the potential involvement of other endogenous Rb-binding proteins or of alternative E1A targets. (Circ Res. 1999;85:319-328.)

Key Words: adenovirus ■ cell cycle ■ cyclin-dependent kinase ■ E1A ■ p21

Irreversible cycle exit (the “postmitotic” phenotype) poses a barrier to restoring functional cardiac mass by myocyte proliferation, to compensate for cell loss via infarction or apoptosis in ventricular muscle.1,2 Although it remains controversial whether this barrier is absolute or not,3,4 even optimistic accounts of mitosis in failing hearts cite the relatively meager frequency of roughly one cell per ten thousand.5 In general, cell cycle control, downstream from mitogenic signaling cascades, is mediated by a number of multigene families6,7—cyclins, which are expressed cyclically during the cell cycle; their targets, cyclin-dependent protein kinases (Cdks); inhibitors of the kinases; and tumor suppressor pocket proteins (the retinoblastoma gene product, Rb, p107, and p130), which are substrates for Cdk4/6, Cdk2, and Cdc2 (Cdk1),8–10 with some differences among the pocket protein family members.11,12 Disinhibition of E2F transcription factors that are bound to the “pocket” is a principal effect of the Cdks that phosphorylate pocket proteins,13,14 activating the transcription of cyclins E and A, Cdk2, and other proteins that are required for S-phase entry. Although cyclin A once was thought to be chiefly involved in mitosis or progression through S phase, cyclin A/Cdk2 kinase activity also has been reported to be rate-limiting for S-phase entry.8,15–18 By contrast, entry into M phase requires cyclins A and B, activating Cdc2; the essential substrates for Cdk2 and Cdc2 remain a subject of speculation. The postmitotic phenotype in skeletal muscle and replicative senescence more generally have been ascribed to the developmentally regu-
labeled increase in Cdk inhibitors.19–22 Observational studies have confirmed that similar changes occur in cardiac muscle soon after birth,23–25 perhaps in concert with a shift from p107 to Rb as the predominant pocket protein,2 but such evidence cannot by itself assert the functional importance of these events.

To identify and override cell cycle constraints in cardiac muscle cells, we and others26–30 have used adenoviral gene transfer to dissect growth control pathways in primary cell culture, without the immortalizing events, clonal variance, and fortuitous mutations that may confound the use of permanent cell lines. Moreover, no permanent cardiac cell line has had adequate fidelity to differentiated ventricular myocytes themselves,31 although cardiac myocytes differentiated from clonal bone marrow stromal cells are promising in this regard.32 By using point mutations of the viral cell cycle regulator E1A, cardiac myocytes, like other cell types, were shown to possess dual growth inhibitory pathways, one dependent on binding the transcriptional coactivator, p300, and its homologue, CREB-binding protein (CBP).26,29,30 dependent on binding the transcriptional coactivator, p300, and its homologue, CREB-binding protein (CBP).26,29,30 Forced expression of E2F-1, the prototype for E2F proteins that are the best understood endogenous occupants of the “pocket,” was sufficient by itself to reproduce the effect of pocket protein binding by E1A on G1 exit in cardiac muscle.26,27 a finding also seen in other cell backgrounds.13,33,34 Thus, G1 exit could be provoked by overexpression of a mammalian protein that is native to the cells, not merely by the viral protein E1A. However, multiple forms of E2F exist, which may be distinct functionally.35,36 and pocket proteins also associate physically with numerous factors apart from E2F.37 For these reasons, despite apparent similarities, it is ambiguous if G1 exit provoked by E2F-1 can replicate all aspects of G1 exit provoked by Rb-binding E1A proteins.

To test the hypothesis that pocket protein binding by E1A might engage mechanisms beyond those used by E2F-1, we expressed E2F-1 versus E1A proteins in cardiac myocytes in the absence or presence of 3 complementary cell cycle inhibitors: p16 (INK4a), p21 (Cip1/Waf1/Sdi1), or dominant-negative Cdk2. Unlike E2F-1, E1A was found to trigger G1 exit that is both refractory to p21 and independent of inducing Cdk2 function. This mechanism specifically requires the ability of E1A to bind pocket proteins but not its ability to bind p300/CBP.

Materials and Methods

Cell Culture and Recombinant Viruses

Ventricular myocytes from 2-day-old Sprague-Dawley rats (Charles River Laboratories) were purified by Percoll density gradient centrifugation, incubated overnight in DMEM/Ham’s nutrient mixture F-12 (1:1) and 5% horse serum, and then cultured in serum-free medium containing 5 μg/mL transferrin, 1 mmol/L NaSeO₃, 1 mmol/L LiCl, and 25 μg/mL ascorbic acid; details of the cell isolation and myocyte purification were reported previously.38 Animals were cared for and euthanized in accordance with AAALAC guidelines.

U2OS osteosarcoma cells (American Type Culture Collection, Manassas, Va) were propagated in DMEM containing 10% FBS (Hyclone Laboratories) and were growth-arrested using serum reduced to 0.5% for 48-hours before viral gene transfer.

AdE2F-1, AdE2F-1-VP16, and AdcycE, driven by the cytomegalovirus immediate-early promoter, were obtained from J. Nevins (Duke University Medical Center, Durham, NC),13,39 Adp21 from G. Nabel (University of Michigan, Ann Arbor),40 and Adp16 from V. Sandig (Max-Delbrück-Zentrum, Berlin-Buch, Germany).41 AdE2F-1-VP16 contains E2F-1 residues 1 to 368 fused to the activation domain of herpes simplex virus VP16. AdE2F-1 E1382 was constructed by cloning E2F-1 E1382 into pAE1sp1LMCMV,43 which was then cotransfected with pMJ17 into 293 cells. Adenovirus encoding dominant-negative Cdk2 was constructed analogously, by first cloning Cdk2-D145N44 into pAE1sp1LMCMV. AdHCMVsp1LaE2 into E1A (M2, 2 μg/mL; Santa Cruz Biotechnology) and rabbit polyclonal antibody to p21 (C-19, 2 μg/mL; Santa Cruz Biotechnology) were used, followed by rhodamine-conjugated anti-mouse IgG (10 μg/mL; Boehringer Mannheim) and FITC-conjugated anti-rabbit IgG (10 μg/mL; Boehringer Mannheim); the primary and secondary antibody incubations were for 60 minutes each. Nuclei were stained with 2.5 μg/mL DAPI (Figure 1) or 0.2 μg/mL Hoechst dye 33258 (Figure 6). Cells were photographed using a Zeiss Axioplan 2 microscope.

Flow Cytometry

Cardiac myocytes were seeded at 10⁵ cells per well in Falcon Primaria 6-well plates (Becton Dickinson) and infected with recombinant viruses as above. MF20 antibody was purified by affinity chromatography (MABTrapG1, Pharmacia), concentrated by centrifugation using Microcon-50 filters (Amicon), and directly conjugated to FITC (200 μg/reaction) using the FluoReporter FITC protein labeling kit (Molecular Probes). Cardiac myocytes were harvested 36 to 48 hours after infection by trypsinization, fixed in 70% ethanol at −20°C, washed in PBS containing 1% BSA, labeled with 2 μg/mL FITC-MF20 at 4°C, and then labeled with 50 μg/mL propidium iodide (PI) in the presence of 50 μg/mL RNase A. FITC and PI fluorescence were analyzed by two-color flow cytometry (Coulter): total populations were gated to remove doublets and debris, and >5000 MF20-positive cells were assessed for each DNA histogram, using MODFIT 2.0 software. U2OS cells were labeled in 500 μL of PBS and 1% BSA, containing 10 μg/mL of PI and 250 μg/mL of RNase A, for 30 minutes at 37°C.
illustrated in the Western blot montages was corroborated using appropriate molecular size markers (not shown).

Immune complex kinase assays for Cdk4 and Cdk2 were performed 24 hours after infection, using anti-Cdk4 (C-22AC) and anti-Cdk2 (M2AC) antibody-agarose conjugates (Santa Cruz Biotechnology) for the immunoprecipitations (30 μL; 2 hours at 4°C) and 500 or 200 μg of cell lysate, respectively, as previously detailed.13 For Cdk2 activity, immune complexes were washed twice in 20 mmol/L Tris-HCl (pH 8.0), 250 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% NP-40, twice in the same buffer containing 100 mmol/L NaCl, and once in kinase buffer (50 mmol/L Tris-HCl, pH 7.5), 10 mmol/L MgCl2, and 1 mmol/L DTT). Kinase activity was assayed for 30 minutes at 30°C, using 5 μg of histone H1 (Boehringer Mannheim) as substrate, 1 μmol/L ATP, and 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), in a final volume of 50 μL. For Cdk4 activity, immune complexes were washed four times with 50 mmol/L HEPES (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L DTT, 0.1% Tween-20, 10% glycerol, 0.1 mmol/L PMSF, 1 mmol/L NaF, and 0.1 mmol/L Na3VO4 and once with Rb kinase buffer (50 mmol/L HEPES [pH 8.0], 10 mmol/L MgCl2, and 1 mmol/L DTT). Kinase activity was assayed for 30 minutes at 30°C, using 2 μg of GST-Rb(769) (Santa Cruz Biotechnology) as substrate, 2.5 mmol/L [γ-32P]ATP in a final volume of 30 μL.

Statistics

Results, shown as mean±SE, were compared by ANOVA and Dunnett’s one-tailed t test, with a significance level of P<0.05.

Results

Rb Binding by E2F-1 Was Neither Necessary nor Sufficient for G1 Exit, Whereas DNA Binding Was Required

To confirm the efficacy of adenoviral gene transfer and ensure appropriate nuclear localization of the exogenous E2F-1, we performed epifluorescence microscopy for E2F-1, labeling the ventricular myocytes simultaneously with MF20 antibody to sarcomeric myosin heavy chains. Homogeneous delivery of E2F-1 was seen at a multiplicity of infection (MOI) of 20, with appropriate targeting to the cell nucleus (Figure 1A). By Western blot analysis, E2F-1 expression was maximal within 24 to 36 hours and was similar after coinfection with p21 (Figure 1B).

To monitor the cell cycle in authenticated myocytes after gene transfer with virus encoding E2F-1 proteins, in the absence or presence of p21, we used two-color flow cytometry. PI was used to define DNA content, in concert with FITC-conjugated MF20 antibody to sarcomeric myosin heavy chains as a marker of cell type. A representative two-color plot is shown in Figure 2A; MF20-positive cells (quadrants 1 and 2) are selected as the region of interest for constructing the DNA histograms (Figure 2B). Numbers presented adjacent to each histogram in the figure correspond to the single histograms shown; numbers presented in the text below correspond to the mean values of replicate studies (Figure 2C). In uninfected cells or LacZ-infected cells, the predominant myocyte population comprised a single peak, with DNA content corresponding to G0/G1, the percentage of myocytes with greater DNA content (S phase plus G2/M) was 16.4±0.53% for uninfected cells and 17.4±0.8% for LacZ-infected cells. As seen previously with the more limited approach of immunostaining for BrdU—a measure of DNA synthesis not content—efficient G1 exit was provoked in ventricular myocytes by wild-type E2F-1 (S+G2/M: 37.1±3.7%). E2F-1–induced G1 exit was potentiated by E1B (not shown), a viral homologue of Bcl-2, which inhibits cell loss from apoptosis in this setting.27 However, E2F-1 did not elicit the hypodiploid sub–G1 peak associated with apoptosis from other causes.47 The lack of a hypodiploid population at this early a time point is consistent with published results for E2F-1 in other cell types, where a sub–G1 peak required 4 to 5 days,35 perhaps because this mode of apoptosis and the associated DNA fragmentation occur preferentially in the S+G2/M cells.

Table 1. DNA content of cardiac myocytes infected with adenoviral E1A- and E2F-1-containing vectors

<table>
<thead>
<tr>
<th>Virus</th>
<th>MOI</th>
<th>G0/G1 (%)</th>
<th>S phase (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F-1</td>
<td>40</td>
<td>60.8 ± 2.3</td>
<td>18.2 ± 0.8</td>
<td>21.0 ± 1.7</td>
</tr>
<tr>
<td>E2F-1</td>
<td>20</td>
<td>64.2 ± 2.1</td>
<td>17.5 ± 0.9</td>
<td>21.4 ± 1.5</td>
</tr>
<tr>
<td>E2F-1</td>
<td>10</td>
<td>66.3 ± 2.4</td>
<td>16.7 ± 0.6</td>
<td>21.0 ± 1.3</td>
</tr>
</tbody>
</table>

To exclude the possibility that an E1A-like mechanism (competition for the pocket, at high levels of expression) might explain the activity of E2F-1 in cardiac muscle cells, we made use of an E2F-1/VP16 chimera: in this hybrid protein, the Rb-binding and transactivation domains were replaced with an activation domain that does not bind Rb.13 Notably, the chimeric E2F-1 increased the prevalence of ventricular myocytes in S phase plus G2/M to a similar extent as did wild-type E2F-1 (S+G2/M: 27.6±1.3%; Figure 2B). Conversely, G1 exit was not elicited by E2F-1 E138, containing a point mutation that prevents DNA binding42 (S+G2/M: 12.3±1.2%). Thus, as in other systems, Rb binding was neither sufficient nor necessary for E2F-1 to drive the G1/S transition, whereas an intact DNA-binding site was obligatory.48 Together, these findings therefore suggest a
requirement for the induction of one or more E2F-dependent genes.

As anticipated by this conclusion, forced expression of E2F-1 resulted in the induction of cyclins E and A, demonstrated by Western blot analysis (Figure 3A). To test whether the induction of cyclins E and A was accompanied by an increase in the activity of Cdk2, the target of these cyclins, in vitro immune complex kinase assays were performed (Figure 3B). E2F-1 was sufficient to evoke a marked increase in Cdk2 activity; by contrast, Cdk4 function was not increased. (The upper and lower panels were exposed for 15 minutes and 12 hours, respectively, for the Cdk4 kinase activity to be visualized.)

Cdk2 Kinase Activity Was Necessary, but Not Sufficient, for E2F-1–Induced G1 Exit

To ascertain whether this increase in Cdk2 function was merely an epiphenomenon or instead a prerequisite for G1 exit provoked by E2F-1, cardiac myocytes were cointfected with viruses encoding either of two Cdk inhibitors—p16, which is selective for Cdk4/6, versus p21, which also inhibits Cdk2. Coinfection with p21 had no adverse effect on E2F-1 expression (Figure 1B) but prevented G1 exit completely, whether wild-type E2F-1 was used (S1G2/M: 12.1±0.5%; Figure 2B and 2C) or the E2F-1/VP16 chimera (S1G2/M: 11.1±0.6%). p21 was highly effective, even when E1B was present (not shown). Despite the arrest of cells in G0/G1, p21 did not prevent upregulation of the E2F-inducible proteins cyclins E and A (Figure 3A). By contrast, despite the induction of these endogenous cyclins, p21 did completely block activation of their target, Cdk2 (Figure 3B). Although these results do not exclude the possibility that diminished induction of the cyclins contributed, in part, to the effect of p21, the all-or-none effect on Cdk2 function is more readily reconciled with the known effect of p21 on Cdk2 itself.

As one criterion to test whether this block to G1 exit by p21 was contingent on the inhibition of Cdk2 activity, parallel studies were performed with p16; Western blots, to corroborate expression, and two-color flow cytometry are shown in Figure 4A. Consistent with the expected specificity of p16, like other INK4 proteins, for Cdk4/6, adenoviral delivery of p16 failed to suppress the observed increase in Cdk2 activity induced by E2F-1 (Figure 3B) and had no effect on G1 exit induced by E2F-1 (E2F-1, S+G2/M: 49.9±9.2%; E2F-1+p16, 49.6±8.8%; Figure 4B).

Taken together, these results for E2F-1 suggest, as in other preparations, that p21 can block E2F-dependent G1 exit, whereas p16 cannot. This ability of Cip/Kip Cdk inhibitors to override E2F-1 has been observed by some authors34,49,50 but not others,13,33 possibly because of levels of expression or
activation of endogenous cell cycle proteins differing between experimental systems. Wherever this block has been shown to exist, it has been ascribed to the ability of p21 or p27 to inhibit Cdk2, not merely Cdk4/6, as do p16 and other INK4 proteins. Consequently, as a second criterion to support this inference in the present study, we coinfected cardiac myocytes with E2F-1 plus dominant-negative Cdk2 itself (Figure 5). As expected, the dominant-interfering form of Cdk2 largely inhibited G1 exit (S
\[\rightarrow\] G2/M: 32.8 ± 2.4% versus E2F-1 alone: 42.8 ± 5.1%). Conversely, an increase in Cdk2 activity, comparable to that seen with E2F-1, could be induced by overexpression of exogenous cyclin E but was not accompanied by G1 exit (S
\[\rightarrow\] G2/M: 25.2 ± 2.5%; Figure 5).

Thus, in cardiac myocytes, an increase in Cdk2 function was required for G1 exit provoked by E2F-1, but it was not sufficient for this response, unlike several other cell backgrounds.

The Pocket Protein Binding Form of E1A Provokes G1 Exit That Is Refractory to Inhibition by p21

To isolate the pocket protein versus p300–dependent actions of E1A, we used 2 well-characterized point mutations, R2G (which disrupts binding to p300/CBP) and Y47H, C124G (which disrupts pocket protein binding). Thus, these mutations elicit the pocket protein- and p300/CBP-dependent effects of E1A, respectively. Each of these three E1A viruses, driven by the native E1A promoter, also expressed E1B to avert apoptosis. By indirect immunofluorescence, all three E1A proteins were widely expressed in cardiac myocytes and were appropriately targeted to the nucleus, even on coinfec-

Figure 4. G1 exit provoked by E2F-1 is resistant to p16. A, Western blot analysis confirming p16 expression after viral delivery. Cardiac myocytes infected with the viruses shown were analyzed at the indicated times after infection. B and C, DNA content by two-color flow cytometry. Cardiac myocytes infected with the viruses shown were analyzed 48 hours after infection (n=3 to 4; *P<0.05 vs LacZ-infected cells).

Figure 5. Cdk2 function was necessary but not sufficient for G1 exit. A, Western blot analysis confirming the expression of dominant-negative Cdk2 (left) and cyclin E (right). B, Immune complex kinase assays, demonstrating the induction of endogenous Cdk2 activity to comparable levels by cyclin E and E2F-1. C and D, Dominant-negative Cdk2 inhibited G1 exit induced by E2F-1; cyclin E was not sufficient for G1 exit. Cardiac myocytes infected with the viruses shown were analyzed 36 hours after infection (n=5 to 7; *P<0.05 vs LacZ-infected cells).

and evoked comparably efficient G1 exit, by two-color flow cytometry (Figure 7). A 3-fold-reduction of myocytes in G0/G1, with a preponderant accumulation of cells in G2/M, was seen (S
\[\rightarrow\] G2/M: wild-type 12S E1A, 77.6 ± 6.0%; R2G, 81.1 ± 2.8%; and Y47H, C124G, 71.1 ± 7.8%; Figure 7, bottom). Because the relative contribution of p300/CBP versus pocket protein binding has previously been questioned, as a trigger for G1 exit by E1A in cardiac myocytes, the use of flow cytometry is technically advantageous compared with immunostaining for BrdU or other S-phase markers, given the very large number of cells in each sample (>5000). In addition, this method confirms not only S-phase entry but also its completion. Under these conditions, however, neither E2F-1 nor E1A yields mitotic figures or increases cardiac muscle cell number, suggesting the need for additional signals for M-phase entry.

To determine if either or both of the E1A-dependent pathways were sensitive to disruption by Cdk inhibitors, as was forced expression of E2F-1, coinfection was performed with the virus encoding p21 (Figure 7). G1 exit via the p300/CBP pathway (by Y47H, C124G E1A) was blocked by p21 (S
\[\rightarrow\] G2/M: 20.1 ± 4.6%), yet G1 exit caused by wild-type 12S E1A or R2G E1A was not (S
\[\rightarrow\] G2/M: 78.2 ± 7.2%; R2G, 77.2 ± 8.2%). Thus, although the three E1A viruses were comparable in their effect on the DNA
content of the myocytes, their sensitivity to block by this Cip/Kip Cdk inhibitor was not.

E1A Does Not Override the p21 Block to Cdk2 Function

To compare the effect of the pocket protein and p300/CBP pathways on endogenous cell cycle regulators, Western blot analysis was performed, as shown earlier for E2F-1. Each form of E1A was sufficient to induce both cyclins E and A (Figure 6B). Each of the three E1A proteins also induced the expression of NPAT, a recently described substrate for cyclin E-Cdk2, which accumulates at the G1/S boundary and promotes S-phase entry.46 p21 had little or no effect on cyclin A and NPAT induction by E1A via pocket proteins and did not prevent cyclin E induction by any form of E1A. However, p21 did prevent the induction of both cyclin A and NPAT by E1A via the p300/CBP pathway, responses that may contribute to the observed growth arrest. Whether this interference is mediated here by transcriptional mechanisms, posttranscriptional mechanisms, or both has not been established. However, illustrated by results presented earlier (Figure 3A), E2F-1 can upregulate cyclin A in the presence of p21, despite a complete block to G1 exit. Thus, growth arrest per se cannot explain the failure of cyclin A to be induced by E1A via p300/CBP, when delivered together with p21. One mechanism consistent, in theory, with these results is a block to the phosphorylation of p300/CBP by Cdk2, affecting its histone acetyltransferase activity.53,54 By contrast, induction of cyclin A by E1A via the pocket protein pathway was refractory to inhibition by p21 and presumably independent of Cdk2 activity.

In the presence of exogenous p21, both E2F-1 and pocket protein–binding forms of E1A can cause the accumulation of cyclins A and E (Figures 3A and 6B), despite their markedly differing sensitivity to growth arrest by this Cdk inhibitor. Hence, an alternative explanation is required, such as differential sensitivity of Cdk2 to p21 after E2F-1 versus E1A, respectively. To test this candidate mechanism directly, Cdk2 activity was compared after infection of cardiac myocytes with viruses encoding E1A or E2F-1 versus coinfection of
these in turn with virus encoding p21. Surprisingly, given the marked differences that occurred in its effect on G1 exit, p21 blocked induction of Cdk2 activity to a comparable extent after delivery either of R2G E1A, Y47H, C124G E1A, or E2F-1: whereas each of these genes increased Cdk2 activity at least 12-fold in the absence of exogenous p21, little or no increase was detected in the presence of exogenous p21 (Figure 8). Thus, G1 exit caused by the pocket protein–binding form of E1A was independent of increasing Cdk2 function beyond that seen in the quiescent cells, distinct from G1 exit provoked by E2F-1 or the p300/CBP pathway.

To test the generality of our observations, another cell type was used, U2OS osteosarcoma cells, with similar results for both flow cytometry and Cdk2 activity (Figure 9): G1 exit produced by E1A via the p300/CBP pathway was sensitive to inhibition by p21 (%S
\text{G2/M}: 35.6 versus 55.7), whereas G1 exit mediated by pocket protein binding was refractory to inhibition by p21 (%S
\text{G2/M}: 72.4 versus 74.0). In U2OS cells as in cardiac myocytes, the inhibition of Cdk2 activity by p21 was comparable for both forms of E1A.

One remaining concern was that levels of Y47H, C124G E1A were consistently diminished in the presence of exogenous p21 (Figure 6B), raising the theoretical possibility that differences between this virus and R2G E1A might merely be dose related. That is, in principle at least, downregulation of Y47H, C124G E1A might itself contribute to the p21-induced block of G1 exit. However, downregulation of E1A is a foreseeable consequence of cell cycle arrest, mediated by relative transcription of the native E1A promoter: in transgenic mice, the E1A promoter is active in proliferative tissue but is poorly expressed in postmitotic tissue, including the heart. Therefore, to exclude this possibility, we compared cardiac myocytes that were subjected to 3 different doses of the R2G and Y47H, C124G viruses in the absence or presence of exogenous p21 (Figure 10, top and middle). The level of E1A accumulation in myocytes infected with Y47H, C124G E1A at the MOI of 5 was inherently sufficient for G1 exit, but this form of E1A could not override the p21 block, eg, compare results of p21+Y47H, C124G with MOI of 10, with results of p21+R2G, with MOI of 5.

**Discussion**

The ability to coerce G1 exit at basal levels of Cdk2 kinase activity, comparable to those in growth-arrested cells, highlights an unsuspected difference between the pocket protein- and p300-dependent pathways for cell cycle reactivation by E1A. Although contingent on the pocket protein–binding site, this consequence of E1A also differed from G1 exit.
produced by exogenous E2F-1, a principal occupant of the "pocket." Thus, our investigations demonstrate that bypassing Rb with E2F-1 does not suffice for all effects of these pocket protein–binding forms of E1A. The failure of p16 to suppress E2F-1–induced G1 exit in our studies is consistent with the known ability of E2F-1 to override even Rb, whose phosphorylation sites have been mutated. Exogenous p21 can confer growth arrest, despite forced expression of E2F-1 in other settings. However, the ability of exogenous p21 to override E2F-1 in the present study departs from findings reported in some serum-responsive systems after viral gene transfer and certain transfected cell lines, perhaps because of differences among endogenous cell cycle regulators.

A seemingly essential role for activation of Cdk2 in the G1/S transition has been inferred from dominant-negative Cdns, mutagenesis of p21, DNA replication in vitro, and on the basis of these findings collectively, it has been inferred that Cdk2 supports a unique function that cannot be complemented by other Cdns. Despite these several lines of evidence that Cdk2 itself is necessary, E2F-1 itself was reported to override the requirement for Cdk2 in other circumstances, when E2F-1 was used in the presence of mitogenic serum. This counter example does not provide a plausible mechanism for the findings in the present study, however, given that E1A, but not exogenous E2F-1, could overcome the p21-dependent growth arrest. Our findings do not exclude the possibility that basal levels of Cdk2 function play an obligatory, permissive effect, in concert with other events, that must be activated by E1A. Because p21 was equally effective at inhibiting Cdk2 activity induced by either the pocket protein– or p300-binding form of E1A, the suggested binding of E1A to p21 is unlikely to explain the differential sensitivity of these two E1A-dependent pathways to p21-induced growth arrest.

What E1A-induced (or E1A-modulated) target proteins might account for escape from p21 and for the independence from an increase in Cdk2 function? One possibility is that alternative cell cycle–dependent kinases could be operative, such as Cdk3 or more distantly related G1/S kinases. However, the ability of exogenous p21 to override E2F-1 in the present study departs from findings reported in some serum-responsive systems after viral gene transfer and certain transfected cell lines, perhaps because of differences among endogenous cell cycle regulators.

Highlighted by the model shown in Figure 11, one attractive theoretical possibility is that E1A might, directly or indirectly, substitute for Cdk2 and activate a Cdk2 substrate (pathway 1). Two possibilities exist to explain the ability of p21 to inhibit Y47H, C124G but not R2G E1A. More simply, the activation of this substrate might be impaired by the Y47H, C124G mutation. Alternatively, this activation might not suffice for G1 exit by itself. Suggesting the likelihood of this latter possibility, Cdk2 activity was necessary but not sufficient for G1 exit induced by E2F-1, implying that other effectors of E2F-1 must also function, in concert (pathway 2). In an effort to define a biochemical basis for this novel action of E1A, Rb-binding proteins other than E2F-1 are especially attractive as a focus for future effort (pathway 3), especially those that modulate or complement E2F-1, such as Myc and brahma-like transcription factors, in addition to the functionally distinct E2F isoform E2F-3. However, although the pocket protein–binding site was essential to override p21, a role for alternative or additional E1A targets, acting in concert with this site, is not excluded (pathway 4).

Since the submission of our manuscript to this journal, other investigators have reported the analogous ability of E1A to override p27 and the requirement for increased Cdk2 activity in various cell backgrounds—Rat1 fibroblastic cells, NIH 3T3 cells, and rat embryo fibroblasts—and have suggested that a requirement exists for a second E1A domain, amino acids 26 to 35, concurrent with the Rb-binding domain. This region of E1A is required for interaction with p400 and postulated to be a homologue of p300 on the basis of peptide mapping, and perhaps for interaction with other cellular proteins. Consistent with the interpretation that a second region of E1A may be required in addition, it is noteworthy that pocket protein binding by SV40 T antigen did not induce S phase in the presence of dominant-negative Cdk2. Given that p21 is highly abundant and Cdk2 is poorly expressed in the adult ventricle—two developmentally regulated events that are thought to underlie the postmitotic phenotype—this p21-resistant pathway may possibly offer inherent advantages for the manipulation of cardiac growth.

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