p21 and Retinoblastoma Protein Control the Absence of DNA Replication in Terminally Differentiated Muscle Cells

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Abstract. During differentiation, skeletal muscle cells withdraw from the cell cycle and fuse into multinucleated myotubes. Unlike quiescent cells, however, these cells cannot be induced to reenter S phase by means of growth factor stimulation. The studies reported here document that both the retinoblastoma protein (Rb) and the cyclin-dependent kinase (cdk) inhibitor p21 contribute to this unresponsiveness. We show that the inactivation of Rb and p21 through the binding of the adenovirus E1A protein leads to the induction of DNA replication in differentiated muscle cells. Moreover, inactivation of p21 by E1A results in the restoration of cyclin E–cdk2 activity, a kinase made nonfunctional by the binding of p21 and whose protein levels in differentiated muscle cells is relatively low in amount. We also show that restoration of kinase activity leads to the phosphorylation of Rb but that this in itself is not sufficient for allowing differentiated muscle cells to reenter the cell cycle. All the results obtained are consistent with the fact that Rb is functioning downstream of p21 and that the activities of these two proteins may be linked in sustaining the postmitotic state.

Key words: C2C12 cells • E1A • cell cycle • p21 • DNA replication

Introduction

The process of skeletal muscle differentiation is distinguished by at least three orderly events: permanent withdrawal from the cell cycle, phenotypic differentiation, and the fusion of cells into multinucleated myotubes (Lassar et al., 1994). The regulatory factors required for these biological processes include the four transcription factors MyoD, myogenin, Myf-5, and MRF4 (Weintraub, 1993). Each of these myogenic proteins can induce myogenesis in a variety of nonmuscle cell types, and as a family, they act in concert with the MEF2 family of transcription factors to activate muscle-specific genes, among which are myosin heavy chain (MHC)[1] and muscle creatine kinase (Lassar et al., 1994; Olson et al., 1996). As a rule, most myoblasts in culture express MyoD or Myf5 (Weintraub, 1993), and it is only after these cells are deprived of serum do these factors then begin to initiate temporal events (Andres and Walsh, 1996), which ultimately end in the formation of postmitotic myotubes. Therefore, it would appear that proliferating myoblasts have operative mechanisms that continually restrain the activity of these myogenic regulators. Considerable effort has been directed at defining the nature of this restraint, and it now appears that cyclin D1, the partner of cyclin-dependent kinase (cdk)4/6, may be important in maintaining MyoD in a functionally inactive state (Skapek et al., 1995, 1996). Indeed, forced expression of cdk inhibitors such as p16 or p21 in proliferating myoblasts apparently results in the activation of MyoD function (Skapek et al., 1995), suggesting, therefore, that phosphorylation may be important to its activity.

In order for skeletal muscle cells to terminally differentiate in vivo or in vitro, they must first irreversibly withdraw from the cell cycle (Lassar et al., 1994), and in cultured cells this process usually occurs in mid–late G1 (Thorburn et al., 1993). The coupling of cell cycle arrest to myogenic differentiation may be based on the induction of the cdk inhibitors p18 and p21 (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995; Missero et al., 1996; Franklin

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[1]*Abbreviations used in this paper:* BrdU, 5-bromodeoxyuridine; C2, C2C12 murine skeletal muscle cell line; cdk, cyclin-dependent kinase; DM, differentiating medium; GM, growth medium; GST, glutathione *S*-transferase; MCM, mini chromosome maintenance; MHC, myosin heavy chain; PCNA, proliferating cell nuclear antigen; pre-RC, prereplication complex; Rb, retinoblastoma protein.

and Xiong, 1996). Historically, p21 is perhaps best known for its importance to several processes requiring cell cycle arrest, particularly in cells having sustained DNA damage by genotoxic agents (Sherr and Roberts, 1995). Evidence indicates that p21 may also be important for maintaining cell cycle arrest in differentiated muscle cells, since its expression occurs subsequently to that of myogenin, an early muscle differentiation marker (Andres and Walsh, 1996). In most cases, p21 is upregulated by the tumor suppressor protein p53, but in cultured myoblasts its induction appears to be mediated by MyoD (Halevy et al., 1995; Otten et al., 1997). In fact, forced expression of MyoD in nonmuscle cells causes p21 to accumulate in relatively high amounts (Halevy et al., 1995).

The result of increased levels of p21 protein in differentiated muscle cells is clearly seen by a decrease in cdk2 activity (Guo et al., 1995; Missero et al., 1995). In proliferating cells, p21 preferably inhibits cdks that are involved in promoting cells into S phase and in augmenting the phosphorylation of the retinoblastoma protein (Rb) (Sherr, 1994). In the early stages of the myogenic program, Rb assumes a hypophosphorylated or active form, and evidence indicates that p21 may be taking an active role in this process. Indeed, neither p21 nor p16 is able to prevent Rb-negative myocytes from entering S phase when ectopically expressed in these cells (Novitch et al., 1996). Earlier studies cast Rb in the role of regulating the transition between cell cycle progression and muscle cell differentiation (Lassar et al., 1994). However, it now appears that Rb may be more connected to pathways that prohibit the replication of DNA in differentiated muscle cells. For example, differentiated myotubes with a functionally inactive Rb are fully capable of reentering S phase (Gu et al., 1993), and although Rb-deficient myocytes can successfully differentiate in culture, they are powerless in preventing DNA synthesis when stimulated with serum (Schneider et al., 1994; Novitch et al., 1996). One of the major activities of Rb in undifferentiated cells is to negatively regulate the E2F family of transcriptional factors, especially E2F1 that prefers to activate S phase genes (Dyson, 1998). However, although this particular function of Rb is presumably operative in proliferating myoblasts, it may not be in differentiated cells, since these cells express very little, if any E2F1 (Wang et al., 1995; Tiainen et al., 1996). Moreover, it is intriguing that forced expression of E2F1 in differentiated muscle cells does not result in the reactivation of DNA synthesis (Wang et al., 1995; Tiainen et al., 1996), whereas in quiescent cells it does (Kowalik et al., 1995). This may be attributed to the fact that although exogenous E2F1 increases the levels of cyclin E in differentiated muscle cells, the kinase activity of cdk2 remains unaffected (Pajalunga et al., 1999). Furthermore, unlike E2F1, the E2F4 protein is produced in differentiated muscle cells and is found predominantly in association with p130, an Rb family member. In fact, the E2F4–p130 complex appears to be the most abundant E2F–DNA binding species in these cells (Puri et al., 1997, 1998).

Such observations raise questions concerning the type of mechanisms that prevent differentiated muscle cells from reentering S phase, particularly since viral proteins such as simian virus 40 (SV40) large T antigen and the viral oncoprotein E1A can act alone in inducing DNA synthesis in these cells (Iujvidin et al., 1990; Gu et al., 1993; Crescenzi et al., 1995). It would seem, therefore, that differentiated muscle cells may be fully equipped to undergo DNA synthesis, and because these viral proteins share the ability to bind Rb (Herwig and Strauss, 1997), then conceivably, Rb could be acting as a guard against the initiation of such a process. However, since p21-negative mononucleated myocytes can still synthesize their DNA after undergoing the differentiation process (Andres and Walsh, 1996), it is possible that p21 may be involved in this activity as well. Thus, given the likely importance of p21 and Rb in maintaining the postmitotic state of differentiated muscle cells, we analyzed the effect of E1A on these proteins to further define their role in preventing these cells from reentering S phase. We find that E1A has the capacity to neutralize the effect of p21 in differentiated muscle cells and that this activity correlates with its ability to induce DNA synthesis in these cells. Moreover, our data suggest that there may be a link between p21 and Rb in maintaining the absence of DNA replication in differentiated muscle cells and that both proteins may be equally important to this activity.

Materials and Methods

Cell Culture, Transfections, and Monitoring of DNA Synthesis

Monolayers of C2C12 (C2) mouse myoblasts (kindly provided by Nadia Rosenthal, Massachusetts General Hospital, Boston, MA) were maintained in growth medium (GM) consisting of DME and 20% FBS. To prepare differentiated myotubes, myoblasts were cultured in differentiating medium (DM) (DME, 2% heat-inactivated horse serum, and 10 μg/ml of insulin) for 12 h. Afterwards, cytosine arabinoside (10 μM) was added for a period of 36 h to eliminate proliferating, nondifferentiating myoblasts (Guo et al., 1995). As reported previously (Neville et al., 1997), this particular clone of C2 begins to fuse within 24 h when cultured under these conditions. The extent of differentiation was routinely monitored by MHC immunofluorescence (see below) and visual examination estimated >95% of the nuclei to be present in multinucleated cells. For transfection, differentiated myotubes grown on acid-washed glass coverslips or 10-cm plates were transfected with the appropriate plasmid by the Lipofectamine PLUS (GIBCO BRL) method. The transfection efficiency under our conditions was ~15–20%, as reported previously (Wolff et al., 1992). To monitor for DNA synthesis, myotubes cultured on glass coverslips and transfected with pCMV-E1A$_{12S}$, pCMV-E1A.2-36, pCMV-E1A.928, or pCMV (control vector) were labeled with 25 μM 5-bromodeoxyuridine (BrdU) for 12 h, 24 h after transfection. Afterwards, the cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Trition X-100 as described previously (Cardoso et al., 1993). Primary antibodies used for immunofluorescence were the mAb to E1A (M73) (Harlow et al., 1985), a gift from E. Harlow (Massachusetts General Hospital, Boston, MA), and the anti-BrdU mAb conjugated to FITC (Boehringer). The secondary antibody for E1A staining was Texas red–conjugated rabbit anti–mouse IgG (Jackson Laboratory). Specimens for immunofluorescence were examined using a Nikon Optiphot-2 fluorescence microscope and then digitally captured (Oncor Video Imaging System).

Expression Plasmids and Purification of Protein

The mammalian expression vector pcDNA3 (Invitrogen) containing either E1A$_{12S}$, E1A.928, or E1A.2-36 downstream of the CMV promoter was described previously (Lundblad et al., 1995; Mal et al., 1996). pGEM4 plasmids (Promega) containing the coding sequence of wild-type or mutant E1As were kindly provided by P. Raychaudhuri (University of Illinois, Chicago, IL) (Raychaudhuri et al., 1991). The plasmid expressing glutathione *S*-transferase (GST)-p21 and GST versions of p21 Δ1-37, p21 Δ1-80, p21 Δ1-119, p21 Δ157-164, p21 Δ150-164, p21 Δ138-164, p21 Δ120-164, p21 Δ87-164, p21 Δ72-164, and p21 Δ58-164 were cloned into pGEX-

KG (Poon and Hunter, 1998). Expression of GST-p21 fusion proteins was performed in *Escherichia coli* strain BL21 (DE3). Induction of these proteins by isopropyl-β-D-thiogalactopyranoside and their purification using glutathione-Sepharose beads (Pharmacia) has been described previously (Mal et al., 1996). Once purified, the GST fusion proteins were quantitated by using the Bradford assay (Bio-Rad) and analyzed by SDS-PAGE before use. An authentic E1A 243R amino acid as well as a mutant E1A.928 protein produced and purified from bacteria was reported elsewhere (Wang et al., 1992).

Cell Extractions

Myoblasts or myotubes were extracted for 1 h at 4°C in lysis buffer B containing 50 mM Hepes, pH 7.0, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM EDTA, 10% glycerol, 5 mM NaF, 1 mM sodium orthovanadate, 5 mM Na-pyrophosphate, 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 0.5 mM PMSF. Afterwards, extract in buffer B was passed 10 times through a 22-gauge needle and then centrifuged for the removal of cell debris.

Antibodies, Immunoblotting, and Immunoprecipitations

Anti–cyclin E (M20), anti-cdk2 (M2), anti-p21 (C19), anti-p18 (M-168), and anti-Rb (C15) polyclonal antibodies were from Santa Cruz Biotechnology. Anti-cdk2 mouse mAb was from Transduction Laboratories. The mAbs MF20 (Bader et al., 1982) and M73 (Harlow et al., 1985) were used to identify MHC and the E1A protein, respectively. Anti-p27 was generously provided by J. Massague (Polyak et al., 1994). For immunoblot analysis, whole-cell extracts (50 μg) were separated on 7.5 or 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membranes were blocked in blocking buffer (4% dry milk, 0.1% Tween 20, 1× TBS) and incubated with primary antibodies in immunoblotting buffer (1× TBS, 0.1% Tween 20, 1% BSA). Dilutions of primary antibodies were according to the manufacturer's instructions. After four washes in immunoblot buffer, the blots were incubated with peroxidase-coupled secondary antibodies and then developed by using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech). Lysis of cells and immunoprecipitation conditions were described previously (Mal et al., 1996). For determining the amount of p21 bound to E1A, 1.5 mg of transfected cell lysate was immunoprecipitated with anti-E1A (M73) and the precipitated products resolved on a 10% SDS-polyacrylamide gel. Bound p21 was visualized by immunoblot analysis (described above) using antibodies against p21.

In Vitro Protein Interactions

Binding of equal molar amounts of GST fusion proteins to glutathione agarose beads, incubation with purified bacterially produced E1A 243R protein, and E1A detection by immunoblotting after the elution of beads have been described previously (Lundblad et al., 1995; Mal et al., 1996). Vectors (pGEM) containing the coding sequence of wild-type or mutant E1As were subject to in vitro transcription/translation (Promega) to produce ^{35}S-labeled proteins. Equal amounts of the labeled E1A proteins, as judged by SDS-PAGE, were incubated with GST or GST-p21 as described previously (Mal et al., 1996). GST and GST-p21 were captured by glutathione-agarose beads, which were then washed accordingly. Afterwards, the beads were boiled in 2× Laemmli buffer and the products were then resolved on a 10% SDS-polyacrylamide gel. The [^{35}S]methionine-labeled proteins were analyzed by autoradiography.

Kinase Activity Assays

For restoration of kinase activity by purified E1A, whole cell extract (100 μg) was incubated with or without wild-type or mutant E1A (100 ng) on ice for 30 min. Afterwards, the mixtures were incubated at 30°C for 30 min. cdk2 was then recovered by immunoprecipitation and assayed for associated kinase H1 activity as described previously (Mal et al., 1996). In brief, immune complexes of cdk2 were washed twice in buffer B containing 10% glycerol and twice with kinase buffer containing 20 mM Hepes, pH 7.9, 1 mM DTT, 10 mM MgCl, and 10% glycerol. Afterwards, the beads were resuspended in 20 μl kinase buffer containing histone H1 (0.2 μg), 25 μM cold ATP, and 5 μCi [γ-^{32}P]ATP. After incubating for 30 min at 30°C, the reactions were terminated by 2× sample buffer. Phosphorylated products were analyzed on an 8% SDS-polyacrylamide gel and visualized by autoradiography. Exposure time in all experiments was usually <30 min. To monitor for restoration of kinase activity in myotubes after transfection with wild-type or mutant E1As, 100 μg of transfected whole cell extract was immunoprecipitated with anti-cdk2. The recovered immune complexes were then assayed for associated kinase activity as described above. For the inhibitory reaction, purified E1A (200 ng) was incubated for 30 min at 4°C in extracts of myotubes and recovered by immunoprecipitation by anti-E1A (M73). Proteins released from the immune complexes, as described previously (Mal et al., 1996), were mixed with extracts (100 μg) of proliferating myoblasts and afterwards this mixture was assayed for p21-associated histone H1 activity after immunoprecipitation.

Results

Correlation Kinetics of cdk2 Activity and Timing of p21 Induction during Muscle Cell Differentiation

In general, cdk2 activity requires cyclin binding, and to be fully effective, it needs to be phosphorylated by the cdk-activating kinase (CAK) (Morgan, 1997). In proliferating cells, cdk2 forms a complex with cyclin E near the G1–S boundary, and throughout this interval, it ostensibly acts at origins of replication to induce the initiation of DNA synthesis (Ohtsubo et al., 1995; Mumberg et al., 1996). p21 is an important mediator of differentiative and/or antiproliferative signals, and as a rule, it inhibits cyclin–cdk2 complexes that are important to the induction of DNA synthesis (Harper et al., 1995). In differentiation, this is best illustrated by the fact that myogenic cells devoid of p21 can now synthesize their DNA when restimulated by serum (Andres and Walsh, 1996). Normally, differentiated myotubes display high levels of p21 mRNA and protein compared with proliferating myoblasts (Guo et al., 1995; Halevy et al., 1995; Missero et al., 1995; Tiainen et al., 1996). Moreover, p21 has also been shown to contribute to the reduction of cdk activity in these cells (Jahn et al., 1994; Guo et al., 1995), suggesting therefore that p21 may be in control of inhibiting cdks that might otherwise force differentiated cells into S phase. To extend these results, we analyzed in detail the kinetics of cdk2 activity and the timing of p21 expression during the process of differentiation. In these experiments, we used a well-characterized murine skeletal muscle cell line, C2C12 (hereafter referred to as C2), which can be induced to differentiate in vitro by serum deprivation (Yaffe and Saxel, 1977). For the analysis of cdk2 activity, immune complexes of cdk2 from lysates of C2 myoblasts cultured in GM and in DM for different intervals of time were assayed for associated histone H1 kinase activity. As shown in Fig. 1 A, cdk2-associated kinase activity was detected in lysates of proliferating C2 cells, but not in lysates of C2 cells cultured in DM after 36 or 96 h, a time when myotubes are completely formed (data not shown). Lysates from these experiments were also subjected to Western analysis to assess the levels of p21 protein in proliferating myoblasts and in myoblasts induced to differentiate (Fig. 1 B). C2 myoblasts cultured in GM displayed low amounts of p21, but after switching to DM the levels of p21 in these cells increased linearly over time, peaking at ~36 h, coincidently with the time in which cdk2 activity is dramatically reduced (Fig. 1 A). Thus, the loss of cdk2 activity observed during the differentiation of C2 cells can be attributed, at least in part, to the accumulation of p21, an amount (at least three- to fourfold) which in the end should suffice to inhibit all cdk2 activity (see below). However, the exact stoichiometry of this interaction remains to be established.

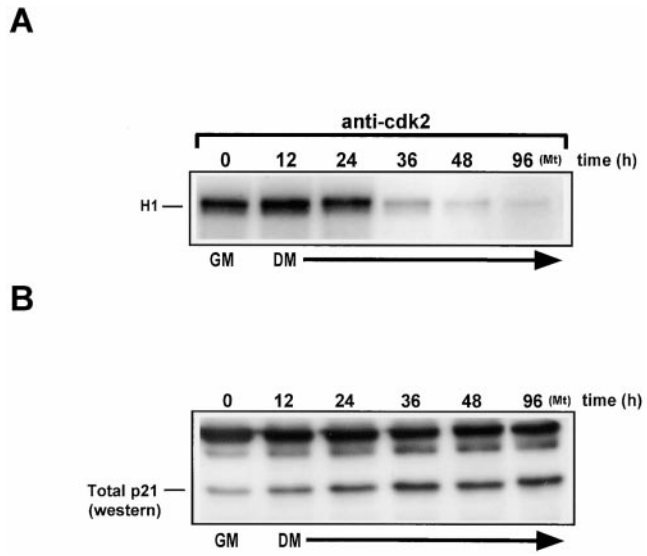

Figure 1. Inactivation of Cdk2 activity correlates with the timing of p21 induction during myogenic differentiation. (A) Whole-cell extracts from C2 cells cultured in GM or in DM at the indicated times were immunoprecipitated with anti-cdk2 antibodies. Cdk2-associated kinase activity in the immune complexes was determined using histone H1 as substrate. (B) Expression of p21 in extracts used above in A was visualized by immunoblot analysis using antibodies against p21.

Inactive Cyclin E–cdk2 Complexes Persist in Differentiated C2 Cells

We next compared the expression levels of cyclin E, cyclin A, and cdk2 in proliferating myoblasts to that of myoblasts cultured in DM. Consistent with previous reports (Jahn et al., 1994; Guo et al., 1995), differentiated muscle cells showed very little expression if any of cyclin A protein (data not shown), but only a modest reduction in the expression of cdk2 (Fig. 2 A). As expected, the expression of MHC, a specific marker for muscle cell differentiation, was significantly increased in these cells (Fig. 2 A). Reportedly, the level of cyclin E protein does not detectably change during differentiation (Jahn et al., 1994; Guo et al., 1995), but we found the amounts of cyclin E in differentiated myotubes compared with that of proliferating myoblasts to be significantly lower and of only one form (Fig. 2 A). As such, this form corresponds in size to a previously reported unphosphorylated form of cyclin E (Koff, 1991; Koff et al., 1992; Dulic et al., 1992) that is resistant to degradation if assembled into complexes with inactive cdk2 (Clurman et al., 1996). Thus, the cyclin E that we find to be present in differentiated C2 cells may not only be associated with cdk2, but with inhibitory amounts of p21 as well. As shown in Fig. 2 B, immunoprecipitates of cyclin E retrieved from extracts of differentiated myotubes do contain cdk2 and p21, and both of these proteins are noticeably absent in precipitates obtained with the control antibody. Perhaps more importantly, the cyclin E precipitates recovered from these extracts did not contain the cdk inhibitor p27 or the cdk inhibitor p18 (Fig. 2 C). (While this manuscript was under review, a similar study showing only the presence of p21 in cyclin E–cdk2 complexes from myotubes was published by Pajalunga et al. [1999]). Taken together, the results indicate that there are low amounts of inactive cyclin E–cdk2 complexes in differentiated C2 cells and that the reason they are nonfunctional is because of the binding of p21.

Purified E1A Restores cdk2-associated Kinase Activity in Differentiated Myotubes

We recently showed that E1A could restore kinase activity to cdk2 complexes that have been inactivated by the cdk inhibitor p27 (Mal et al., 1996), which is closely related to p21. If E1A could inactivate p21, this could partially explain the ability of an adenovirus (Crescenzi et al., 1995) or transiently expressed E1A to force differentiated muscle cells into S phase (see Fig. 6). To explore this possibil-

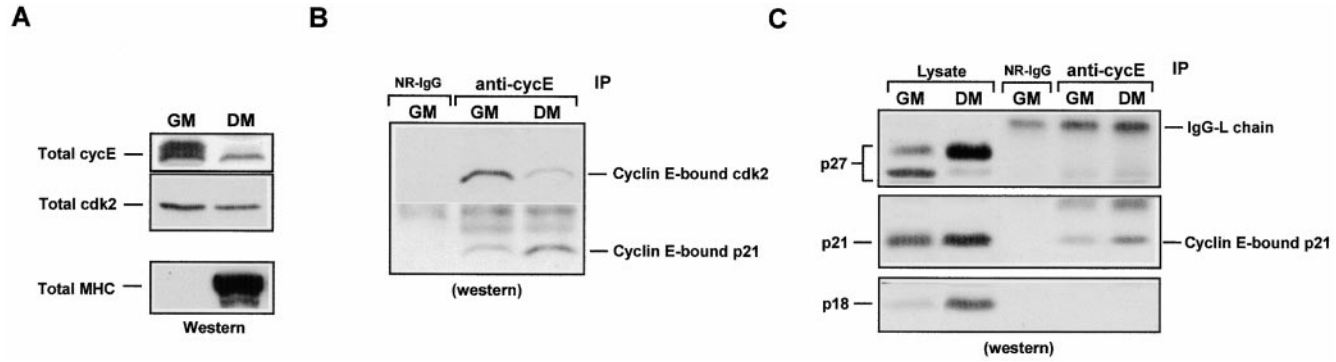

Figure 2. Inactive cyclin E-cdk2-p21 complexes are present in differentiated C2 cells. (A) Levels of cyclin E and cdk2 in C2 cells cultured in GM or DM for 96 h were determined by immunoblot analysis using antibodies specific for cyclin E or cdk2. C2 cells cultured in DM for 96 h showed >95% of the nuclei to be present in myotubes, and expressed MHC as visualized by immunoblot analysis using antibodies against MHC. (B) Whole-cell extracts of C2C12 cells cultured in GM or DM for 96 h were immunoprecipitated with antibodies against cyclin E or nonimmune rabbit IgG. The contents of the precipitated samples were analyzed by immunoblotting using antibodies specific for cdk2 or p21. (C) Immune complexes of cyclin E, recovered from whole cell extracts of C2 cells cultured in GM or DM for 96 h, were resolved alongside straight lysate (100 μg) and molecular weight markers on a 12% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. Afterwards, the membrane was cut into three strips and separately probed with antibodies specific for p27, p21, or p18. As control, extracts of C2 cells cultured in GM were separately precipitated with normal rabbit IgG.

ity, we first determined whether E1A could in fact restore kinase activity to cdk2 in differentiated C2 myotubes. We began by incubating bacterially purified wild-type or mutant E1A proteins in extracts prepared from these cells. The mixtures, with or without E1A, were then immunoprecipitated with anti-cdk2, and the resulting immune complexes were assayed for histone H1 kinase activity. As before, immune complexes of cdk2 from extracts of differentiated myotubes had very little associated histone H1 activity (Fig. 3 A, lane 2). However, after separately adding wild-type E1A or an E1A mutant (E1A.928) failing to bind Rb (Wang et al., 1993) to analogous extracts, we found that immune complexes of cdk2 could now phosphorylate histone H1 (Fig. 3 A, lanes 3 and 4) to levels almost similar to that of cdk2 immune complexes from proliferating myoblasts. To confirm that extracts from myotubes contained an inhibitory activity before exposure to E1A, we mixed this extract with proliferating myoblast extract (1:1) and found that the kinase activity in association with cdk2 complexes was greatly reduced. Moreover, E1A alone did not inhibit the activity of cdk2 in extracts of proliferating myoblasts (data not shown). It is noteworthy that cdk-activating kinase, an enzyme that gives cdks full kinase activity (Morgan, 1997), was found to be enzymatically active in the extract of differentiated muscle cells (data not shown). As such, cdk-activating kinase activity is not regulated by the cell cycle in undifferentiated cells and cdk activation is therefore controlled by the accessibility of the substrates (Morgan, 1995). Collectively, we conclude that inactive cyclin E-cdk2-p21 complexes, which persist in differentiated C2 cells (Fig. 2 C), retain the potential to undergo a restoration in activity, at least in vitro. However, an additional effect by E1A on cyclin D3–cdk2 complexes in association with p21 in extracts of C2 myotubes (Tedesco et al., 1997; Cenciarelli et al., 1999) cannot be excluded, and this could also be contributing to the restoration of kinase activity observed in Fig. 3 A, lanes 3 and 4.

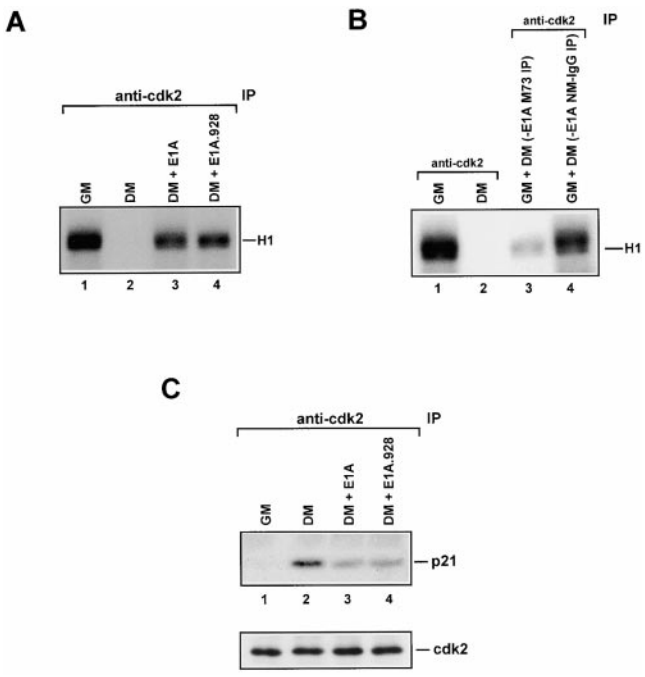

Figure 3. Purified E1A restores cdk2 activity in differentiated C2 cells. (A) Whole cell extracts of C2 cells cultured in DM for 96 h with or without the addition of an authentic wild-type E1A (200 ng) or mutant E1A.928 (200 ng) of 243 amino acids were incubated in parallel with whole-cell extracts of proliferating C2 cells cultured in GM. Cdk2 was then recovered by immunoprecipitation and assayed for associated kinase H1 activity (lanes 1–4). Lane 5: same assay, except cdk2 activity was determined after mixing GM extracts (100 μg) with DM extracts (100 μg). (B) Whole cell extracts were prepared from C2 cells cultured in GM or in DM for 96 h. Lanes 1 and 2: cdk2-associated histone H1 kinase activity was determined in GM or DM extracts, respectively, after immunoprecipitation with antibodies against cdk2. Lanes 3 and 4: wild-type E1A (200 ng) was incubated in extracts of DM and recovered by immunoprecipitation with an antibody specific for E1A (M73) or normal mouse IgG, respectively. The immune complexes were treated accordingly (see Materials and Methods) to release proteins from protein A–Sepharose beads. These products were then mixed with extracts of GM, and afterwards, this mixture was assayed for cdk2-associated histone H1 kinase activity after immunoprecipitation. (C) Same experiment as in A except this time immune complexes of cdk2 were subject to immunoblot analysis and enhanced chemiluminescence, using anti-p21 as a probe. The same membrane was also probed with anti-cdk2, and this served as a loading control.

E1A Restores Kinase Activity to cdk2 by Targeting an Inhibitory Activity, p21

Restoration of kinase activity to cdk2 in extracts of differentiated C2 myotubes appeared to correlate to the titrating out of an inhibitory activity, since cdk2 activity increased linearly after incremental amounts of E1A were added to fixed amounts of myotube extract (data not shown). To investigate whether E1A was physically interacting with this inhibitory activity, we incubated E1A in extracts of myotubes and then recovered it by immunoprecipitation with an antibody specific to E1A (M73) (Harlow et al., 1985). Immune complexes were then treated with sodium deoxycholate (DOC), which has been previously proven to release only those proteins that may have associated with the respective antigen (Lees et al., 1992; Mal et al., 1996), in this case E1A. As a negative control, extracts of myotubes containing E1A in parallel were also immunoprecipitated with normal mouse IgG, and likewise, the resulting immune complexes were disrupted with DOC. Eluates of both were then separately mixed with extracts of proliferating myoblasts. After incubation, the mixtures were immunoprecipitated with an antibody specific for cdk2, and the ensuing immune complexes assayed for associated kinase activity using histone H1 as substrate. As shown in Fig. 3 B, the products released from E1A by M73 contained an inhibitor of cdk2-associated kinase activity (Fig. 3 B, lane 3), as judged by comparing the activity associated with cdk2 from the original extract of proliferating myoblasts (Fig. 3 B, lane 1). In contrast, products released from immune complexes derived from E1A containing extracts with the use of normal mouse IgG had no effect on cdk2-associated kinase activity (Fig. 3 B, lane 4). Furthermore, the extract to which E1A was added and then removed by M73 had no inhibitory effect on cdk2 activity when mixed with an extract from proliferating myoblasts (data not shown). These findings strongly imply that E1A

can directly interfere with an inhibitory activity of cyclin E–cdk2 in differentiated muscle cells, and because this complex does not appear to contain p27 or p18, then this activity is more than likely from p21. With this in mind, we determined whether E1A could reduce the amount of p21 in association with cyclin E–cdk2 complexes. As before, the amount of p21 in association with cyclin E–cdk2 complexes from differentiated cells (Fig. 3 C, lane 2) was considerably more than that found in immune complexes of cdk2 from undifferentiated cells (Fig. 3 C, lane 1). However, the amount of p21 was greatly reduced in cdk2 immune complexes recovered from extracts of differentiated cells with the addition of either wild-type E1A or the E1A.928 mutant (Fig. 3 C, lanes 3 and 4, respectively).

E1A and p21 Can Physically Interact with Each Other

The implication that E1A was targeting p21 in extracts of differentiated muscle cells led us to explore whether E1A could bind directly to this protein, and if so, to what part of the molecule. By incubating purified E1A with a GST-p21 fusion protein, we were able to determine that E1A could bind specifically to p21 but not to GST (Fig. 4 A). We were also able to identify a site on p21 with which E1A interacts by using a set of p21 mutants containing deletions at the NH_2- or COOH-terminal end of the molecule. Coding sequences for these particular mutants were expressed as GST fusion proteins, and after purification, each was separately incubated with purified E1A. The results obtained from these experiments are shown in Fig. 4 A and schematized in Fig. 4 B. In effect, an NH_2-terminal deletion containing amino acid residues 80–164 bound very efficiently to E1A as did a COOH-terminal deletion containing amino acid residues 1–137 (Fig. 4 A). We conclude that E1A can directly interact with p21 and that the region lying between amino acids 80 and 120 of p21 is the major region responsible for this interaction.

Given that E1A can bind directly to p21, it was equally important to ascertain the site on E1A to which p21 can interact. To do so, we challenged GST-p21 fusion proteins against equal amounts of radioactively labeled E1A mutants generated by an in vitro transcription/translational system. In analyzing the bound material, we discovered that an E1A mutant containing a deletion between amino acids 2–36 (E1A.dl2-36) could not bind to p21 when compared with wild-type or the other E1A mutants (Fig. 4 C).

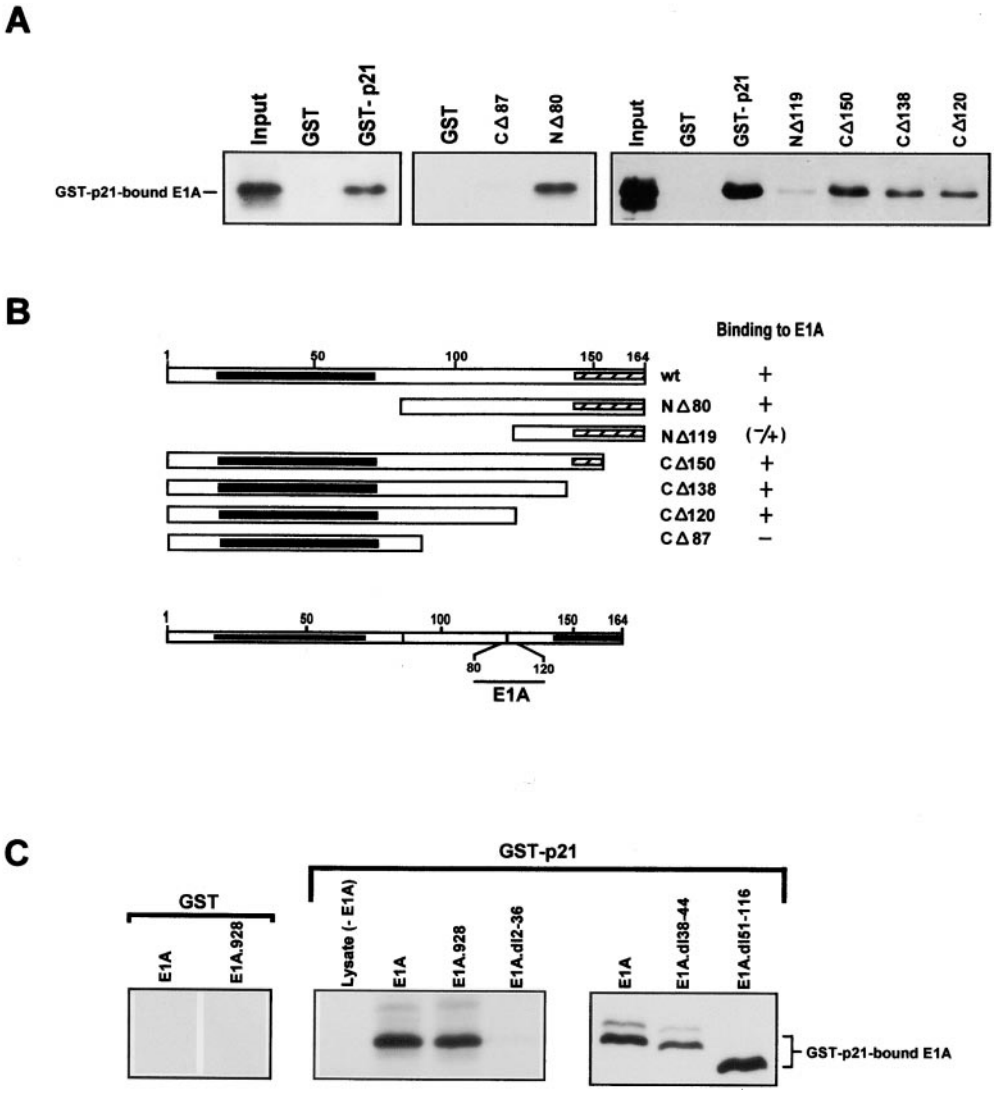

Figure 4. E1A binds directly to p21. (A) GST, GST-p21, or GST-21 deletion mutants, immobilized on glutathione beads, were incubated with purified E1A (75 ng). After elution of the beads, E1A was detected by immunoblot analysis using the anti-E1A mAb M73. Input refers to 100% of the protein used in the binding assay. (B) Schematic representation of p21 and p21 deletion mutants, a summary of their binding activity to E1A, and an illustration of the putative region of p21 to which E1A may interact. The solid box within the p21 drawings represent the binding motifs for cyclin and cdk2. The binding motif for PCNA, as well as a second cyclin binding motif, are shown as hatched boxes. (C) [S^{35}]methionine-labeled wild-type or mutant E1As were produced by in vitro transcription/translation and then mixed with GST-p21 or unfused GST. Complexes after glutathione-Sepharose affinity chromatography were analyzed by SDS-polyacrylamide gels and fluorography.

To be noted is that the E1A.dl2-36 mutant has been previously characterized in its ability to induce DNA synthesis in quiescent cells, and although it can still bind Rb, its efficiency in this activity is severely impaired compared with wild-type E1A (Stein et al., 1990).

E1A Expressed in Differentiated Myotubes Associates with p21 and Restores Kinase Activity to cdk2

Our in vitro results suggest that purified E1A can bind to and disable the cdk inhibitory activities of p21 when added to an extract of differentiated muscle cells. The question that remains, however, is whether E1A can perform these acts when expressed in differentiated C2 cells. To address this issue, parallel plates containing the same number of differentiated C2 myotubes were transfected with plasmids encoding either wild-type E1A (pCMV-E1A$_{12S}$), or mutant E1A failing to bind Rb (pCMV-E1A.928) or p21 (pCMV-E1A.dl2-36). It is important to note that transfection occurred under conditions that allowed ~15–20% of the cells to take up exogenous DNA (Wolff et al., 1992). After 36 h, transfected myotubes were harvested and extracts were then prepared accordingly (Mal et al., 1996). Each of the extracts, in parallel, was immunoprecipitated for the isolation of cdk2. Immune complexes of cdk2 were then tested for associated kinase activity using histone H1 as substrate. As shown in Fig. 5 A, plasmids containing either wild-type E1A or the E1A mutant failing to bind Rb (928) increased histone H1 kinase activity to cdk2–cyclin complexes, whereas the E1A mutant (dl2-36) failing to bind p21 and the control plasmid did not. As expected, the cdk2 immune complexes from extracts of proliferating myoblasts had associated histone H1 kinase activity. The fact that equivalent expression of the wild-type E1A and the two E1A mutants were observed in the myotubes after transfection (Fig. 5 A) strengthens the preceding results. Incidentally, transient expression of an E1A mutant (E1A.RG2), which can bind to p21 but not to p300, was equally efficient as wild-type E1A in restoring cdk2 activity in myotubes (Mal, A., and M.L. Harter, manuscript in preparation).

The above-mentioned experiments support the contention that when E1A is forcibly expressed in myotubes, it can reactivate cdk2 activity. In this situation, it is quite probable that E1A may be directly affecting the cdk inhibitory activity of p21 since E1A can bind to this protein in vitro (Fig. 4). This speculation is supported by the fact that the transcripts and protein levels of p21, p27, and cdk2 remain unchanged after E1A is expressed in myotubes (Tiainen et al., 1996; Cenciarelli et al., 1999; Pajalunga et al., 1999; Mal, A., and M.L. Harter, unpublished results), and although E1A does increase the level of cyclin E in these cells (Tiainen et al., 1996b), the elevation of cyclin E alone does not result in the restoration of cdk2 activity (Pajalunga et al., 1999). For these reasons, we examined whether a transiently expressed E1A could associate with p21 in differentiated myotubes. As can be seen in Fig. 5 B, p21 was present in immune complexes of E1A recovered from extracts of differentiated myotubes transfected with the wild-type E1A vector, but not the control vector or the plasmid encoding the E1A.dl2-36 mutant, which is consistent with our in vitro results.

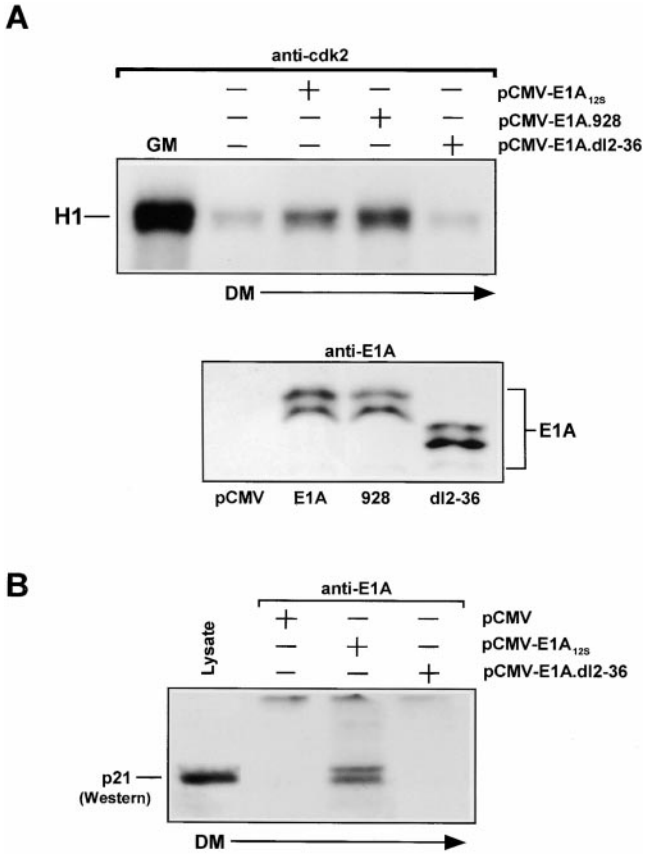

Figure 5. E1A expressed in differentiated C2 cells associates with p21 and restores kinase activity to cyclin–cdk2 complexes. (A) C2 cells cultured in DM for 48 h were transfected in parallel with pCMV-E1A$_{12S}$, pCMV-E1A.928, pCMV-E1A.dl2-36, or control plasmid pCMV. After 36 h, cells were collected and whole cell extracts prepared. Equal fractions of each transfection (representing protein recovered from the same number of cells) were analyzed in all experiments. Cdk2-associated kinase histone H1 activity in transfected myotubes as well as in cells cultured in GM was determined as for Fig. 3. In the lower panel, extracts of transfected myotubes were subject to immunoblot analysis and enhanced chemiluminescence, using anti-E1A as a probe. (B) The same experiment as in A, except this time immune complexes of E1A were subject to immunoblot analysis and enhanced chemiluminescence, using anti-p21 as a probe.

The E1A.dl2-36 Mutant Fails to Induce DNA Synthesis in Differentiated Myotubes

To determine whether E1A's ability to restore kinase activity to cyclin E–cdk2 complexes in differentiated C2 cells was of functional importance, we compared wild-type E1A with mutant E1As for their ability to induce DNA synthesis in these cells. To reiterate, after proliferating C2 myoblasts develop into multinucleated myotubes (Fig. 6 a), they can no longer synthesize their DNA, not even in response to serum stimulation (Skapek et al., 1995). However, DNA replication in myotubes (Fig. 6, b and e) can be induced after transfection with wild-type E1A or the E1A mutant (RG2), which can bind Rb and p21 but not p300 (Stein et al., 1990; Chattopadhyay, D., and M.L. Harter, unpublished data), as judged by the incorporation of the thymidine analogue BrdU (Fig. 6, d and g) and the stain-

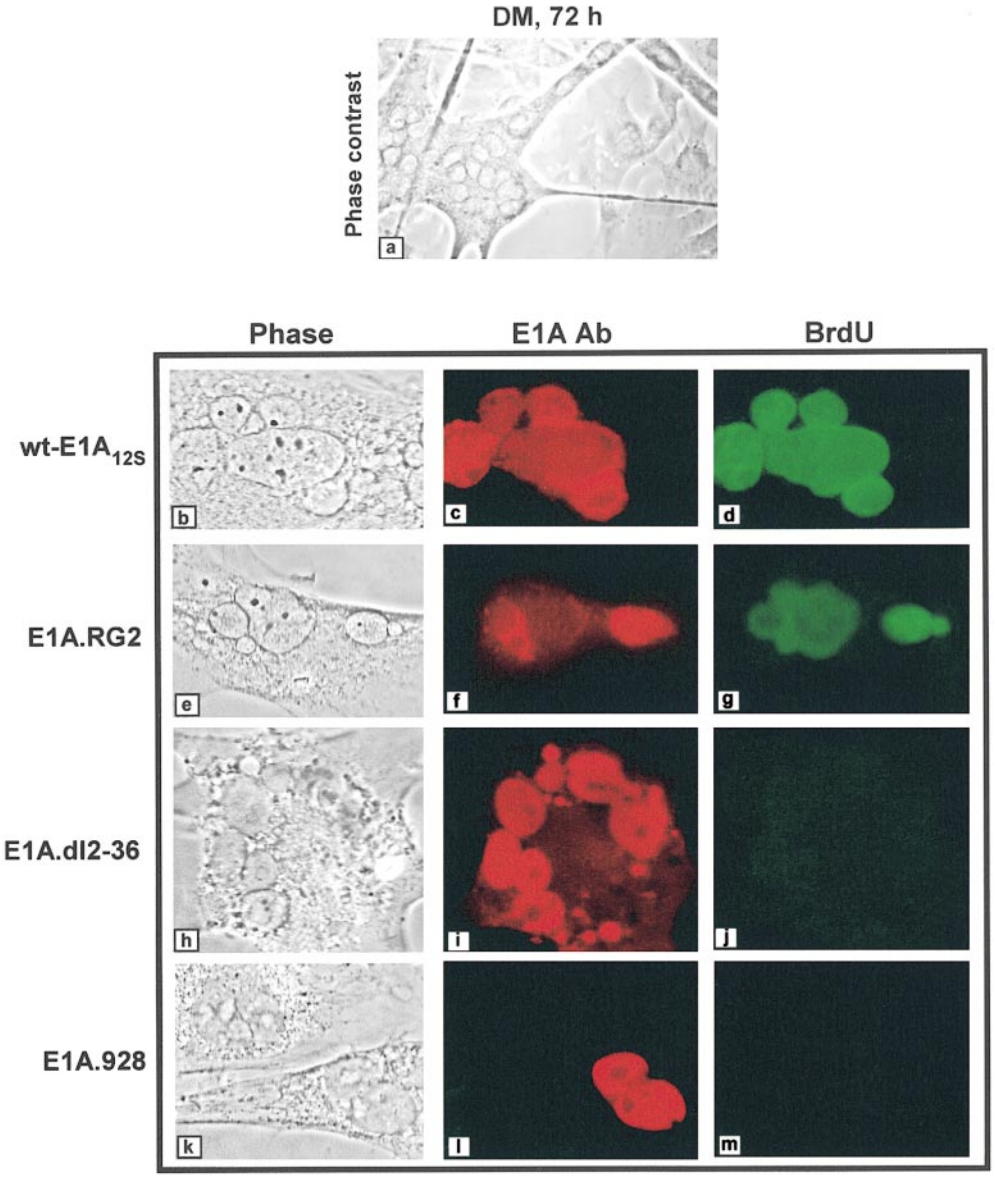

Figure 6. E1A mutants failing to bind p21 or Rb cannot induce DNA synthesis in differentiated muscle cells. C2 cells grown on glass coverslips and cultured in DM for 72 h were transfected in parallel with pCMV-E1A$_{12S}$, pCMV-E1A.RG2, pCMV-E1A.dl2-36, or pCMV-E1A.928. 24 h later, BrdU was added to the media and after 12 h, the cultures were fixed (see Materials and Methods) and sequentially immunostained for E1A and incorporated BrdU. Phase-contrast photomicroscopy of fixed C2 cells cultured in DM for 72 h (a) or after transfection with pCMV-E1A$_{12S}$ (b), pCMV-E1A.RG2 (e), pCMV-E1A.dl2-36 (h), or pCMV-E1A.928 (k) is shown. Myotubes in c and d and f and g, which correspond to the same field shown in b and e, respectively, display E1A staining (red) and BrdU incorporation (green). Myotubes in i and l, which correspond to the same fields shown in h and k, respectively, show E1A staining but are negative for BrdU (j and m).

ing of E1A (Fig. 6, c and f). More importantly, however, the E1A.dl2-36 mutant, which fails to bind p21 and restore kinase activity (Figs. 4 and 5), was incapable of inducing DNA synthesis in these cells (Fig. 6, h–j). Although the E1A.928 mutant, which fails to bind Rb, can induce DNA synthesis in quiescent cells (Flint and Shenk, 1997) albeit with a much lower efficiency, it was completely ineffective in inducing DNA replication in myotubes (Fig. 6, k–m), despite its ability to restore kinase activity to cyclin–cdk2 complexes in these cells (Fig. 5). Nevertheless, this result is consistent with what has been previously shown with the use of an E1A.928 adenovirus mutant (Tiainen et al., 1996b). To be noted is that myotubes transfected with control plasmid were constantly BrdU-negative, and in parallel experiments, the muscle differentiation marker MHC was easily detectable (data not shown). Also to be noted is that some of the nuclei of the BrdU-labeled myotubes that were transfected with wild-type E1A or the RG2 mutant appeared to show a doubling in DNA content (Fig. 6, b–g). Since mitosis in multinucleated myotubes as a result of adenovirus infection induces the coalescence of formerly individual nuclei (Crescenzi et al., 1995), this observation is consistent. A summary of these results and the binding properties of mutant E1As used in this study are shown in Table I.

Taken together, these experiments suggests that both p21 and Rb many be necessary for preventing the reoccurrence of DNA replication in differentiated C2 cells.

Restoration of Kinase Activity in Differentiated Muscle Cells Leads to the Phosphorylation of Rb

As in other mammalian systems, hypophosphorylated or active Rb in proliferating C2 myoblasts becomes phosphorylated when cells enter S phase. Once these cells undergo differentiation, however, the property of the Rb molecule appears to become permanently confined to a hypophosphorylated state (Lassar et al., 1994). Although the E1A.928 mutant, which fails to bind Rb, cannot induce DNA replication in differentiated C2 myotubes (Fig. 6,

Table I. Binding Properties of E1A Mutants and Their Ability to Induce DNA Synthesis in Differentiated Muscle Cells

	Binding			BrdU-positive cells
Mutant	p300	Rb	p21	C2C12 myotubes
wt E1A	+*	+*	+‡	+
E1A.928	+*	−*	+‡	−
E1A.RG2	−*	+*	+‡	+
E1A.dl2-36	−*	+	−‡	−

+, positive for binding to a cellular protein; −, negative for binding to a cellular protein.
*Data obtained from the work of Stein et al. (1990) and Wang et al. (1993).
‡Work described herein, and unpublished results (Chattopadhyay, D., and M.L. Harter).

h–j), it can restore kinase activity to cyclin E–cdk2 complexes in these cells (Figs. 3 and 5). Given this paradox, and the importance of phosphorylated Rb in allowing cells to enter S phase, we examined the phosphorylation state of Rb in E1A transfected C2 myotubes. As shown previously (Gu et al., 1993; Thorburn et al., 1993), hyperphosphorylated forms of Rb were present in proliferating C2 myoblasts, but only the hypophosphorylated form of Rb was detectable in the myotubes (Fig. 7, lanes 1 and 2, respectively). However, after these cells were transfected with wild-type E1A or the E1A.928 mutant, a small amount of hyperphosphorylated Rb became apparent (Fig. 7, lanes 3 and 4, respectively), but not in cells that were transfected with the E1A.dl2-36 mutant (lane 5). The percentage of hyperphosphorylated Rb found in this experiment was roughly equal to that of the transfection efficiency (15–20%), and the expression of each of the transfected E1As in myotubes was equivalent (Fig. 7, lower panel), as judged by Western analysis. Thus, although the expression of wild-type E1A or the E1A.928 mutant in differentiated C2 cells are both equally capable of inducing the phosphorylation of Rb, this in itself is not sufficient for inducing DNA replication in these cells (Fig. 6).

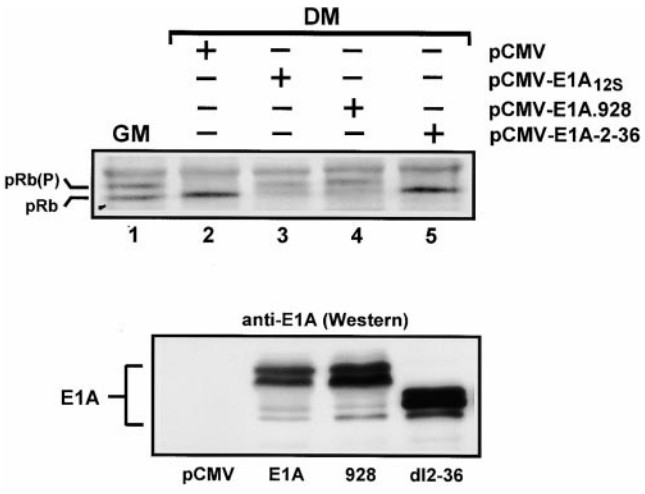

Figure 7. Restoration of kinase activity in differentiated C2 cells leads to the phosphorylation of Rb. Transfection of C2 myotubes (DM) and the preparation of whole cell extract is described for Fig. 5. These extracts were electrophoresed alongside whole cell extracts of C2 myoblasts (GM), transferred to polyvinylidene difluoride membrane, and probed with an Rb-specific polyclonal antibody followed by enhanced chemiluminescence detection. In the lower panel, the same extracts were subject to immunoblot analysis and enhanced chemiluminescence, using anti-E1A as probe.

Discussion

The regulatory pathways, which are important to maintaining the silencing of DNA replication in terminally differentiated C2 cells, are unique. This is exemplified by the fact that the exogenous expression of the transcription factor E2F1, a protein critical for S phase function, cannot promote these cells, unlike quiescent fibroblasts, into S phase (Tiainen et al., 1996a). In proliferating C2 myoblasts as well as in other cell types, it has often been suggested that E2F1, which activates a number of S phase genes, is negatively regulated by Rb, and that this is one way in which Rb inhibits the progression of cells into S phase. Upon muscle differentiation, the expression of E2F1 dramatically decreases (Wang et al., 1995; Puri et al., 1998), whereas the expression of active or hypophosphorylated Rb increases (Gu et al., 1993; Thorburn et al., 1993). In this context, the function of Rb may be other than that of controlling transcription factors that regulate genes responsible for the transition into S phase. What this function might be is still unclear, but studies indicate that Rb may be playing a critical role in preventing aberrant DNA synthesis in terminally differentiated muscle cells (Schneider et al., 1994; Novitch et al., 1996). It is worth noting, however, that the cdk inhibitor p21, which is induced after myogenesis, may also be important in maintaining the absence of DNA synthesis in these cells (Andres and Walsh, 1996). This work reveals that both Rb and p21 are necessary for this activity, and demonstrates with the use of the viral oncoprotein E1A that they cooperate in maintaining the postmitotic state.

The mechanistic details of what prevents DNA synthesis from occurring in differentiated muscle cells are poorly understood. The data available thus far (Fig. 2 A) suggest that after C2 cells differentiate in culture, they continue to express low amounts of cyclin E, and this is consistent with what others have observed in other types of differentiated cells (Gao and Zelenka, 1997). A majority, if not all, of the cyclin E molecules were found to be associated with the cdk2 kinase (Fig. 2 B), but despite this interaction, the cyclin E–cdk2 complexes were enzymatically inactive (Fig. 1). The underlying basis for this inactivity most likely originates from the cdk inhibitor p21, which had accumulated in these cells and was shown to be contained in cyclin E immune complexes as apposed to its closely related form p27 (Fig. 2, B and C). Although we were unable to measure the physiological levels of p21 in the differentiated muscle cells, we were able to demonstrate a correspondence between the time p21 is maximally expressed and the time cdk2 activity becomes inhibited (Fig. 1), an occurrence that came rather late during the differentiation process.

What role might p21 play in differentiated muscle cells? Since a single p21 monomer is sufficient to inhibit a cyclin–cdk2 complex (Hengst et al., 1998), we propose that the activity of cyclin E–cdk2 complexes in differentiated C2 cells is purposely held in check by the binding of p21. If true, then perhaps one function of p21 in these cells is to indirectly prevent the phosphorylation of key regulatory

proteins (see below) that are temporally important to the initiation of DNA synthesis. This contention is supported by the fact that when p21 is inactivated in differentiated C2 cells, a key member of the mini chromosome maintenance (MCM) family of proteins becomes phosphorylated (Mal, A., and M.L. Harter, manuscript in preparation). The MCM proteins are known components of prereplication complexes (pre-RCs), and current models suggest that they make nuclei competent for DNA replication (Dutta and Bell, 1997).

The striking restoration of cdk2 activity in extracts of differentiated C2 cells after the addition of purified E1A (Fig. 3 A), and the fact that a wild-type E1A or an E1A mutant failing to bind Rb can efficiently release p21 from cyclin E–cdk2 complexes (Fig. 3, B and C), supports the notion that p21 is taking an active role in arresting the activity of this kinase in vivo. The removal of p21 from these complexes most likely correlates with E1A's ability to bind directly to p21 (Fig. 4 C). This finding is consistent with E1A's capacity to directly associate with the cdk inhibitor p27, a family member of p21 (Mal et al., 1996; Nomura et al., 1998; Orend et al., 1998). The fact that NH_2-terminal residues, specifically amino acids 2–36, appear to be critical to the binding of p21 (Fig. 4 C) is not too surprising, since most of the cellular proteins (e.g., p300 and Rb) known to interact with E1A bind to subregions within the NH_2 terminus of this protein (Stein et al., 1990).

We have also demonstrated the sequences required for p21 and E1A binding, and find that the E1A binding site of p21 does not appear to include either of the two cyclin or cdk domains or the proliferating cell nuclear antigen (PCNA) domain (Fig. 4, A and B). Instead, the sequences that appear to be responsible for binding E1A are located between amino acids 80–120. It is interesting to note, however, that this particular region of p21 is distinct from that which binds the human papillomavirus E7 oncoprotein (Funk et al., 1997), which has been recently shown to associate with p21 and abrogate its ability to inhibit cyclin E–cdk2 complexes during keratinocyte differentiation (Funk et al., 1997; Jones et al., 1997). Indeed, the E7 binding site of p21 seems to be displaced toward the COOH-terminal end and overlaps with the PCNA binding domain. The reason the sequences in p21 that bind E1A or E7 are distinctly different is not presently understood. However, it should be noted that apart from preventing p21 from inhibiting cyclin E–cdk2 complexes, E7 also has the ability to neutralize p21's activity in inhibiting PCNA-dependent DNA replication (Funk et al., 1997). Furthermore, unlike the p21 binding site of E1A, which maps upstream (between amino acids 2–36) of the Rb binding site (Fig. 4 C), the p21 binding site of E7 appears to include sequences that are responsible for the binding of Rb (Jones et al., 1997). Thus, although both E1A and E7 can abrogate the inhibitory activities of p21, it appears that they may have evolved different ways of doing so.

The discovery that the viral oncoproteins E1A or simian virus 40 large T could overcome the proliferative block of terminally differentiated C2 cells by reactivating authentic DNA synthesis (Crescenzi et al., 1995; Endo and Nadal-Ginard, 1989) was significant, since it suggested that these cells had retained the potential to undergo a complete and well-orchestrated S phase. As such, the unlocking of pathways to DNA replication in differentiated muscle cells cannot be achieved by mitogenic signaling (Lassar et al., 1994) but can by the simple expression of E1A or simian virus 40 large T. Indeed, that these two proteins alone can induce DNA synthesis in differentiated C2 cells suggests, at the very least, that components (ORC, Cdc6, MCMs) of pre-RCs are still being expressed in these cells. If true, then maintaining their assembly at origins of DNA replication and inactivity in DNA synthesis would require an absence of cdk activity, and possibly the functioning of other proteins as well (Dutta and Bell, 1997). Normally, pre-RCs require the activation of cdk2 activity to induce DNA synthesis in mammalian systems (Dutta and Bell, 1997). Thus, if E1A were to switch on cyclin E–cdk2 activity in differentiated muscle cells by interfering with p21's activity, then this could be one way in which E1A is able force these cells into S phase. In support of this model, our data suggest that E1A or E1A mutants failing to bind Rb or p300 (data not shown) can efficiently restore activity to cdk2 complexes in differentiated C2 cells, whereas the E1A mutant (dl2-36) failing to bind p21 cannot (Fig. 5 A). Moreover, with the exception of E1A.dl2-36, wild-type E1A and the other E1A mutants are equally efficient in reducing the amount of p21 in association with cdk2 complexes, both in vitro (Fig. 3) and in vivo (Mal, A., and M.L. Harter, manuscript in preparation), and we suspect that this correlates with their ability to interact with p21 when expressed in myotubes (Fig. 5 B). Finally, although we believe, but as yet cannot prove, that components, or at least parts thereof, of pre-RCs are being constitutively produced in differentiated C2 cells, we cannot rule out the possibility that some of these factors may in fact be induced once E1A is expressed in these cells.

Regulatory cascades control the initiation of muscle cell differentiation, and the players considered to be most important to this process are MyoD, p21, and Rb. For example, in response to differentiation signals, the transcriptional factor MyoD, which is primarily responsible for initiating the myogenic program, upregulates p21, which in turn inhibits cdk activity, causing cells to irreversibly arrest in G1 and maintain Rb in an active or hypophosphorylated state (Lassar et al., 1994). In vitro studies have shown that Rb can bind directly to MyoD, and this has led others to postulate that Rb is totally required for the withdrawal of muscle cells from the cell cycle (Gu et al., 1993). This notion, however, is challenged by the fact that Rb-deficient fibroblasts can still exit the cell cycle and undergo differentiation after MyoD is ectopically expressed in these cells (Novitch et al., 1996). Moreover, ectopic expression of cyclin D1 can inhibit muscle cell differentiation independently of Rb hyperphosphorylation (Skapek et al., 1995). Thus, although Rb's role in the early stages of myogenesis appears to be comparatively obscure, its activity in regulating or maintaining the postmitotic state of differentiated muscle cells is, perhaps, a little more definitive. For example, despite the fact that Rb-deficient myocytes can successfully differentiate into myotubes, they do not appear permanently withdrawn from the cell cycle, since DNA replication can occur in these cells upon mitogenic stimulation (Schneider et al., 1994; Novitch et al., 1996; Wang and Walsh, 1996). Furthermore, myotubes in the absence of Rb also display a higher frequency of apoptosis

(Wang et al., 1997), perhaps because of endoreduplication, since nuclei in the back muscles of $Rb^{-/-}$ fetuses show a DNA content of >2 N (Zacksenhaus et al., 1996). Such results forces one to reinterpret Rb's role in differentiated muscle cells, and instead of binding to or repressing E2F as it does when restraining the G1–S transition in proliferating myoblasts, Rb may be targeting or affecting proteins that are mainly involved in the temporal aspect of DNA replication. Indeed, it is interesting that an active Rb mutant devoid of phosphorylation sites can interfere with DNA synthesis in S phase–committed cells (Knudsen et al., 1998) and that pRb can bind directly to the DNA licensing factor MCM7 (Sterner et al., 1998).

Our observations further substantiate Rb's importance in assuring that DNA synthesis does not occur in differentiated C2 cells; however, critical issues remain. Although we can conclude that wild-type E1A and an E1A mutant (RG2) failing to bind p300 are both very efficient in inducing DNA synthesis in differentiated C2 cells, the E1A.dl2-36 mutant, which fails to bind p21, cannot, despite the fact that it can still bind Rb (Fig. 6). A second issue is that the E1A.928 mutant, which can no longer bind to Rb, is also defective in inducing DNA synthesis in these cells (Fig. 6), even though it can bind to p21 and restore kinase activity to cdk2 (Figs. 3, 4, and 5). In addition, this mutant can also induce the phosphorylation of Rb, whereas the dl2-36 mutant cannot (Fig. 7). Although there are 16 bona fide sites on Rb that can be potentially phosphorylated by cdks (Dyson, 1998), the phosphorylated form of Rb that is generated in this case is inadequate in allowing the reinitiation of DNA synthesis in differentiated C2 cells. This result is consistent with previous findings, which suggest that Rb's activities may be controlled in part by the site(s) at which Rb is phosphorylated (Knudsen and Wang, 1996). Given this set of circumstances, we speculate that the activation of cdk activity and the loss of Rb function are both necessary requirements to reinitiate DNA replication in differentiated muscle cells. We also postulate that Rb is functioning downstream of p21, and possibly other cdk inhibitors as well, to maintain the absence of DNA synthesis in these cells.

Finally, it would appear that one of the purposes for p21 in differentiated muscle cells is to prevent the escape of cdk activity so as to neither activate nor indirectly induce the components of pre-RCs, which might lead to inappropriate DNA synthesis in these cells. However, the purpose of a functionally active Rb protein in differentiated muscle cells has yet to be defined. Although Rb is best known for its role in repressing E2F1-mediated transcription, these cells have very little if any E2F1 expression. Although this in itself does not rule out the possibility that Rb may still be acting as a negative regulator of transcription, it does leave us with the prospect that Rb may be directly or indirectly targeting factors, which could be networking between DNA replication and transcriptional processes.

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