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Cyclin-Mediated Inhibition of Muscle Gene Expression via a Mechanism That Is Independent of pRB Hyperphosphorylation

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It was recently demonstrated that ectopic expression of cyclin D1 inhibits skeletal muscle differentiation and, conversely, that expression of cyclin-dependent kinase (cdk) inhibitors facilitates activation of this differentiation program (S. S. Rao, C. Chu, and D. S. Kohtz, Mol. Cell. Biol. 14:5259–5267, 1994; S. S. Rao and D. S. Kohtz, J. Biol. Chem. 270:4093–4100, 1995; S. X. Skapek, J. Rhee, D. B. Spicer, and A. B. Lassar, Science 267:1022–1024, 1995). Here we demonstrate that cyclin D1 inhibits muscle gene expression without affecting MyoD DNA binding activity. Ectopic expression of cyclin D1 inhibits muscle gene activation by both MyoD and myogenin, including a mutated form of myogenin in which two potential inhibitory cdk phosphorylation sites are absent. Because the retinoblastoma gene product, pRB, is a known target for cyclin D1-cdk phosphorylation, we determined whether cyclin D1-mediated inhibition of myogenesis was due to hyperphosphorylation of pRB. In pRB-deficient fibroblasts, the ability of MyoD to activate the expression of muscle-specific genes requires coexpression of ectopic pRB (B. G. Novitch, G. J. Mulligan, T. Jacks, and A. B. Lassar, J. Cell Biol., 135:441–456, 1996). In these cells, the expression of cyclins A and E can lead to pRB hyperphosphorylation and can inhibit muscle gene expression. The negative effects of cyclins A or E on muscle gene expression are, however, reversed by the presence of a mutated form of pRB which cannot be hyperphosphorylated. In contrast, cyclin D1 can inhibit muscle gene expression in the presence of the nonhyperphosphorylatable form of pRB. On the basis of these results we propose that G_1 cyclin-cdk activity blocks the initiation of skeletal muscle differentiation by two distinct mechanisms: one that is dependent on pRB hyperphosphorylation and one that is independent of pRB hyperphosphorylation.

Skeletal muscle differentiation is coordinated by the activities of the myogenic basic helix-loop-helix (bHLH) transcription factors (5, 13, 39, 57, 75) and by members of the MEF2 family of transcriptional activators (reviewed in reference 58). Differentiation of skeletal muscle entails transcriptional activation of muscle-specific structural genes coupled with irreversible cell cycle withdrawal (reviewed in reference 40). Ectopic expression of one member of the myogenic bHLH family, MyoD, can initiate both of these processes in a variety of cell types (8, 10, 70). However, because MyoD protein is also expressed in proliferating myoblasts prior to the onset of differentiation (71), it is clear that MyoD itself must be functionally regulated in these committed, proliferating myoblasts.

Studies which address the functional regulation of MyoD have demonstrated that the retinoblastoma gene product (pRB) and the pRB-related proteins p107 and p130 may be critical for normal skeletal muscle differentiation. Viral oncoproteins which sequester and inactivate the pRB family of proteins inhibit muscle gene expression and terminal cell cycle withdrawal in myogenic cell lines (3, 6, 14, 15, 54, 72). Furthermore, pRB itself has been shown to be necessary for MyoD-induced skeletal muscle differentiation in Saos2 osteosarcoma cells (18). Paradoxically, however, mouse embryos in which the *Rb* gene has been deleted by homologous recombination contain differentiated skeletal muscle (7, 28, 42), and myogenic cell lines derived from such mice can be induced to differentiate in vitro (53, 63). However, in vitro analysis of pRB-deficient myocytes revealed that these cells do not permanently withdraw from the cell cycle after differentiating into multinucleate myocytes (53, 63). Furthermore, although pRB-deficient myocytes express normal levels of muscle proteins expressed early in the differentiation program (i.e., myogenin and p21), these cells express only trace levels of late muscle differentiation markers (i.e., myosin heavy chain) (53). Thus, pRB plays a key role in both the terminal cell cycle arrest of skeletal muscle and the transcriptional activation of a subset of muscle-specific genes.

Because G_1 cyclins with their catalytic partners, the cyclin-dependent kinases (cdks), can phosphorylate pRB and thereby inhibit the activity of this cell cycle regulator (reviewed in references 66 and 74), we and others have evaluated the effect of ectopic G_1 cyclin expression on MyoD activity (60, 61, 69). These studies have demonstrated that the expression of cyclin D1 and cyclin D2 (to a lesser extent) inhibits muscle gene expression in fibroblasts expressing MyoD and in C2C12 myoblasts (60, 61, 69). This inhibitory activity of cyclin D1 requires cdk activation and is associated with a shift of MyoD to a more slowly migrating hyperphosphorylated form (69). Cyclin-dependent regulation of muscle gene expression in mitogen-stimulated myoblasts seems to be biologically relevant because forced expression of a combination of cdk inhibitors, $p16^{INK4}$ (64) and $p21^{CIP1/WAF1}$ (12, 19, 25, 52, 77), in nearly confluent, mitogen-stimulated myoblasts facilitates muscle differentiation in the absence of mitogen deprivation (69). Finally, $p21^{CIP1/WAF1}$ is induced early during the skeletal muscle differentiation pro-

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gram (20, 21, 47, 59). In this context, $p21^{CIP1/WAF1}$ may prevent further cyclin-dependent inactivation of MyoD function and preserve the terminal cell cycle arrest of differentiated skeletal muscle.

The present studies were undertaken to further characterize how ectopic expression of G_1 cyclins inhibits skeletal muscle differentiation. Our data indicate that cyclin D1-cdk inhibits muscle gene expression without affecting MyoD DNA binding activity. Because cyclin D-cdk complexes are known to phosphorylate (16, 31, 45) and block pRB function (11, 27), we determined whether G_1 cyclins inhibit myogenesis by hyperphosphorylating pRB. In $Rb^{-/-}$ mouse embryonic fibroblasts reconstituted with wild-type pRB, cyclins A, D1, and E can each inhibit muscle gene expression induced by MyoD. Cyclin A- and cyclin E-mediated inhibition of muscle gene expression can be reversed by expression of a nonhyperphosphorylatable form of pRB, suggesting that these cyclins block muscle differentiation via pRB phosphorylation. In contrast, expression of this mutated form of pRB cannot reverse cyclin D1-mediated inhibition of myogenesis. Thus, G_1 cyclins seem to suppress muscle gene expression by at least two mechanisms: one dependent on pRB hyperphosphorylation and one independent of pRB hyperphosphorylation.

MATERIALS AND METHODS

Cells and cell culture. Mouse 10T1/2 fibroblasts and embryonic fibroblasts derived from $Rb^{-/-}$ mouse embryos (kindly provided by Tyler Jacks) were maintained in growth medium (Dulbecco modified Eagle medium [DMEM] supplemented with 20% fetal calf serum). To induce myogenic differentiation following transfection, cells were cultured in differentiation medium (DMEM supplemented with 2% horse serum and insulin [10 μg/ml]).

Plasmid reagents. Eukaryotic expression plasmids for transient gene expression contained the cytomegalovirus (CMV) enhancer-promoter driving the expression of the following genes: CMV-MyoD (made by the subcloning mouse MyoD cDNA into the *Eco*RI site in the pCSA plasmid [kindly provided by Tom Roberts]); CMV-cyclin A, -cyclin D1(HA), –cyclin D1-gh(HA), and -cyclin E, all cloned into pRC/CMV (kindly provided by Steve Dowdy and Phil Hinds) (27); CMV-E12 (made by subcloning the human E12 cDNA [kindly provided by Robert Davis] into the *Eco*RI and *Eco*RV sites of pCSA); CMV–myogenin-wt and CMV-myogenin 43/170 (S-A) cloned into pCDM-8 (kindly provided by Eric Olson); CMV-human pRB(wt) cloned into pCMV-Neo/Bam (kindly provided by Bill Kaelin); and CMV–mouse pRB-wt (B/X-HA) and -mouse pRB (ΔP34-HA), made by subcloning the respective cDNAs (22) into the pCMV-Neo/Bam plasmid (constructed and kindly provided by Paul Hamel, Bill Sellers, and Bill Kaelin). In addition, an expression plasmid containing the Moloney sarcoma virus long terminal repeat (pEMSV) was used to drive expression of rat myogenin (kindly provided by Woody Wright) employed for immunoblotting.

Reporter plasmids contained the chloramphenicol acetyltransferase (CAT) gene driven by the following: 3,300 bp upstream of the muscle creatine kinase (MCK) gene (MCK-CAT) (kindly provided by Steve Hauschka) (29); four reiterated MEF1 sites driving the thymidine kinase promoter (4XMEF1)-CAT (formerly referred to as 4RtkCAT [76]); reiterated E boxes from the immunoglobulin M heavy chain enhancer [$(E5+E2)_6$-CAT] (kindly provided by Tom Kadesch) (26); and the CMV promoter (made by subcloning the CAT gene from pEMSV-CAT into the *Eco*RI site of pCSA) (CMV-CAT).

Transient transfection and CAT assay. Mouse 10T1/2 fibroblasts and $Rb^{-/-}$ fibroblasts were transfected by using Lipofectamine (Gibco BRL) according to the manufacturer's recommendations as previously described (53, 69). Briefly, 1.5×10^5 cells were plated onto 60-mm-diameter tissue culture dishes. On the following day, cells were transfected with various combinations of plasmids as indicated in the figure legends. For each plate, the appropriate plasmids, each at a stock concentration of 0.1 μg/μl, were aliquoted into a microcentrifuge tube. The total amount of DNA used for each plate (2 to 3 μg, as indicated in the figure legends) was normalized with the respective empty expression plasmids as needed. This DNA mixture was diluted with 100 μl of DMEM; to each diluted DNA mix, 110 μl of diluted Lipofectamine (10% [vol/vol] in DMEM) was added. The DNA-Lipofectamine mix was incubated at room temperature for 45 min. Following this incubation, the mix was diluted with 800 μl of DMEM and then pipetted onto the cells which had just been washed three times and refed with 1 ml of DMEM. Following a 3-h incubation at 37°C in 5% CO_2, the DNA-Lipofectamine mix was washed off the cells and the cells were refed with growth medium without antibiotics. At 36 h following transfection, the cell culture medium was changed to differentiation medium.

After 24 h in differentiation medium, cells were harvested for CAT assay with extracts normalized to protein content, essentially as described previously (17). Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and scraped off the plate into 1 ml of cold PBS. Cells were pelleted by spinning at $2,040 \times g$ and 4°C for 3 to 5 min. The cell pellet was resuspended in 100 μl of 250 mM Tris (pH 8.0) and lysed by sequential freeze-thaw steps. When $Rb^{-/-}$ fibroblasts were used, lysates were incubated at 65°C for 10 min to inactivate deacetylase enzymes. (This step was shown to not be necessary for 10T1/2 cell lysates.) Lysates were clarified by centrifugation at $16,000 \times g$ and 4°C for 5 min. The protein concentration of the supernatant was determined by the Bio-Rad assay. The CAT activity was determined by incubating the same amount of protein from each supernatant (volumes of each sample were equalized to 70 μl with 250 mM Tris [pH 8.0]) with 30 μl of a cocktail containing 20 μl of 8 mM chloramphenicol, 5 μl of 1 mM acetyl coenzyme A, and 5 μl of diluted ^{3}H-acetyl coenzyme A (10% [vol/vol] in sterile H_2O) (NEN catalog number NET-290L) at 37°C for 2 h. Following this, ^{3}H-acetylated chloramphenicol was extracted with 400 μl of cold ethyl acetate followed by mixing by vortex for 30 s and centrifugation at 14,000 rpm for 2 min; 300 μl of the organic phase was used for quantitation by scintillation counting. For each experiment, CAT activity was determined to be within the linear range; when necessary, original samples were diluted and the assay was repeated so that the final quantitation was within the linear range. In the figures, CAT activity is expressed as percent maximal achieved in the absence of cotransfected cyclin (and in the presence of cotransfected pRB for experiments done with $Rb^{-/-}$ fibroblasts). All transfection experiments included duplicate plates, and individual experiments were repeated at least three times with similar results (i.e., approximately 10 to 20% variability between experiments). The data from a representative experiment are shown in the figures.

Electrophoretic mobility shift assay (EMSA). MyoD DNA binding activity in the presence or absence of cotransfected cyclin D1 was determined essentially as described elsewhere (38). Briefly, high-salt extracts from 10T1/2 fibroblasts, which were transfected and cultured as described above, were normalized to 10^7 cells per ml in EMSA lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6], 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pepstatin and leupeptin [10 μg/ml each], aprotinin [1 μg/ml], 10 mM $NaPPO_4$, 1 mM NaF, and 0.1 mM Na_3VO_4). Three microliters of this extract was incubated with 4×10^4 cpm of a MEF1 oligomer (38) (labeled with T4 polynucleotide kinase) in a 30-μl binding reaction mix (containing 20 mM HEPES [pH 7.5], 5 mM EDTA, 50 mM NaCl, and 5% glycerol [final concentrations]). After 15 min at 25°C, samples were separated by electrophoresis using a 4.5% polyacrylamide gel in 0.5× Tris-borate-EDTA and analyzed by autoradiography. For supershift experiments, monoclonal anti-MyoD antibody (5.8A; kindly provided by Peter Houghton) or monoclonal anti-myosin heavy chain antibody (control) (MF20; kindly provided by Don Fischman) was added to the reaction mix after 15 min and the binding reaction was continued for 15 min prior to gel electrophoresis. MyoD protein levels were evaluated in equivalent volumes of whole-cell extract by Western blot (immunoblot) analysis, employing monoclonal anti-MyoD antibody (5.8A) as described below. All gel shift assays and Western analyses were carried out at least three times.

Protein analysis. Steady-state protein levels in the presence or absence of specific cotransfected genes were determined as previously described (69) with 10T1/2 cells or $Rb^{-/-}$ fibroblasts which were transfected and cultured as described above. After 24 hours in differentiation medium, transfected cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 20% glycerol, 1 mM dithiothreitol, and protease and phosphatase inhibitors as described above), sonicated, and clarified by centrifugation. Whole-cell sonicates and high-salt extracts, normalized to equal protein content, were employed for immunoprecipitation and immunoblotting. For detection of MyoD, rabbit anti-MyoD antiserum (71) was used for immunoprecipitation and mouse anti-MyoD monoclonal antibody (5.8A) was used for immunoblotting. For detection of transfected wild-type pRB, rabbit anti-pRB antiserum (0.495; kindly provided by Eva Lee) was used for immunoprecipitation and mouse anti-pRB monoclonal antibodies (11D7 and 245; kindly provided by Eva Lee) were used for immunoblotting. For detection of transfected myogenin, mouse monoclonal antibody to myogenin (F5D; kindly provided by Woody Wright) was employed for immunoprecipitation and for immunoblotting. Immunoblotted proteins were detected by enhanced chemiluminescence (Amersham).

RESULTS

Forced expression of cyclin D1 preferentially inhibits E-box reporter gene expression in 10T1/2 cells cotransfected with myogenic bHLH transcription factors. It has been previously demonstrated that the ability of MyoD to heterodimerize with ubiquitous bHLH transcription factors, E proteins, is critical for MyoD activity (38, 49). MyoD–E-protein heterodimers bind to a consensus sequence (CANNTG) (termed an E box) found in the promoters and enhancers of many muscle-specific genes (38). Whereas MyoD and myogenin are solely expressed in skeletal muscle, E proteins are ubiquitously expressed and

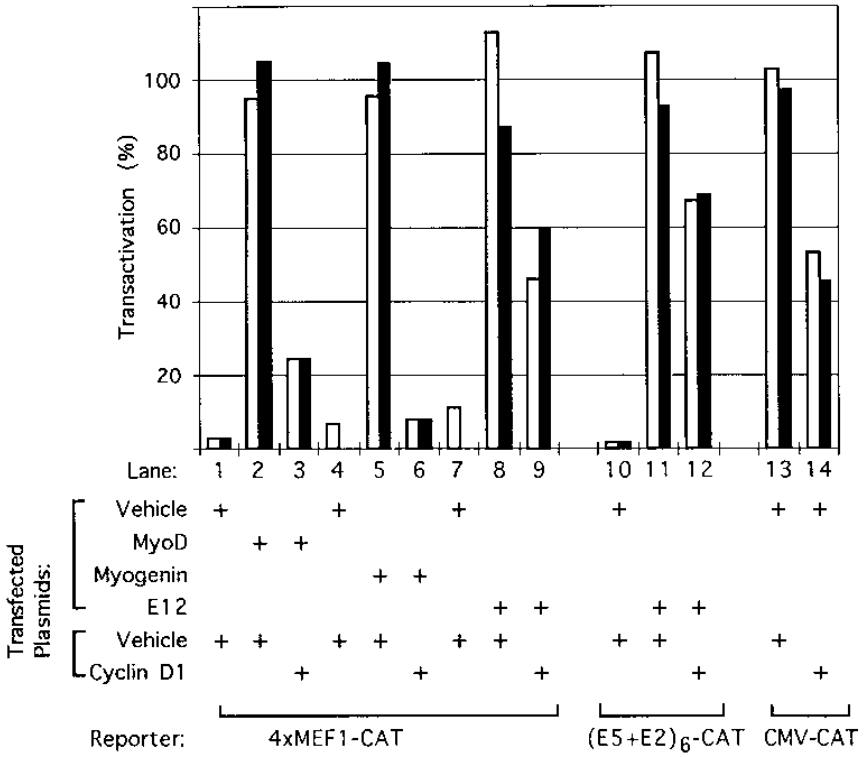

FIG. 1. The ectopic expression of cyclin D1 preferentially inhibits the activity of myogenic bHLH transcription factors versus a non-tissue-restricted bHLH transcription factor. CAT activity from whole-cell extracts of 10T1/2 fibroblasts which were cotransfected with plasmids encoding MyoD (0.5 μg), myogenin (0.5 μg), or E12 (0.5 μg) or the corresponding expression vehicles (0.5 μg); cyclin D1 (0.5 μg) or its empty expression plasmid (0.5 μg); reporter genes (0.5 μg) as indicated; and the corresponding empty expression vehicles (0.5 μg) to normalize DNA content to 2 μg. CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved for each transcription factor in the absence of cyclin D1. The average maximal CAT activity for each condition was as follows: 184,581 cpm (lane 2), 39,750 cpm (lane 5), 34,771 cpm (lane 8), 147,638 cpm (lane 11), and 49,956 cpm (lane 13).

can bind to an E box either as a homodimer in B cells (65) or as a heterodimer with myogenic bHLH transcription factors in myocytes (38). Although E proteins can bind to and transactivate certain E-box-containing regulatory regions (such as the immunoglobulin M heavy chain enhancer [26] or artificial reporters containing reiterated E boxes [9]), they are not myogenic when ectopically expressed in 10T1/2 fibroblasts (9). Because previous studies demonstrated that cyclin D1 could inhibit MyoD-mediated transactivation of a reporter gene containing reiterated E boxes (69), we wanted to determine whether cyclin D1 could similarly inhibit the expression of E-box-dependent reporters in cells cotransfected with either myogenic bHLH proteins or nonmyogenic E proteins.

Cotransfection of cyclin D1 with MyoD decreased the expression of a simplified muscle-specific promoter containing four reiterated E boxes from the MCK enhancer (termed MEF1 sites; 4×MEF1-CAT) (76) to about 20% of that observed in the absence of cyclin D1 (Fig. 1, compare lanes 2 and 3). Similarly, expression of this reporter in the presence of cotransfected myogenin and cyclin D1 was decreased to about 10% of that observed in the absence of cotransfected cyclin D1 (Fig. 1, compare lanes 5 and 6). In marked contrast, expression of cyclin D1 with the nonmyogenic bHLH protein E12 only slightly depressed the expression of either 4×MEF1-CAT (9) or $(E5+E2)_6$-CAT, which contains reiterated E boxes from the immunoglobulin heavy chain enhancer (26), to about 60 to 70% of that observed in the absence of cotransfected cyclin D1 (Fig. 1, lanes 8, 9, 11, and 12). We suspect that this modest suppression of E12-mediated activation of E-box-containing reporters may be due to down-regulation of the CMV promoter employed to express this transcription factor (Fig. 1, lanes 13 and 14). The quantitative differences in cyclin D1-mediated suppression of these reporter genes in the presence of muscle-specific versus non-tissue-restricted bHLH proteins suggest that cyclin D1 preferentially inhibits the expression of genes which may be activated directly or indirectly by myogenic bHLH proteins versus nonmyogenic bHLH proteins.

Constitutive expression of cyclin D1 does not alter MyoD DNA binding activity. As noted above, the activity of myogenic bHLH transcription factors is dependent on their ability to bind to DNA as heterodimers with ubiquitously expressed E proteins. Agents which inhibit myogenic bHLH function have been proposed, in some cases, to block DNA binding by these transcription factors. For example, it has been suggested that fibroblast growth factor administration induces phosphorylation of the basic domain of myogenin which precludes subsequent DNA interaction by this transcription factor (44). Furthermore, proliferating myoblasts express high levels of the dominant-negative HLH protein Id-1, which can sequester E proteins and thereby inhibit MyoD interaction with DNA (1, 30). Because cyclin D1 was shown to inhibit E-box-dependent gene expression in cells cotransfected with myogenic bHLH transcription factors, we addressed whether ectopic expression of cyclin D1 affects MyoD DNA binding activity.

Employing an EMSA, we detected a DNA binding species capable of recognizing an E box in the MCK enhancer (termed a MEF1 site) present in high-salt extracts made from 10T1/2 cells transiently transfected with MyoD (Fig. 2A). This MEF1 site binding complex, which displays an electrophoretic mobility similar to those of MyoD–E-protein complexes present in

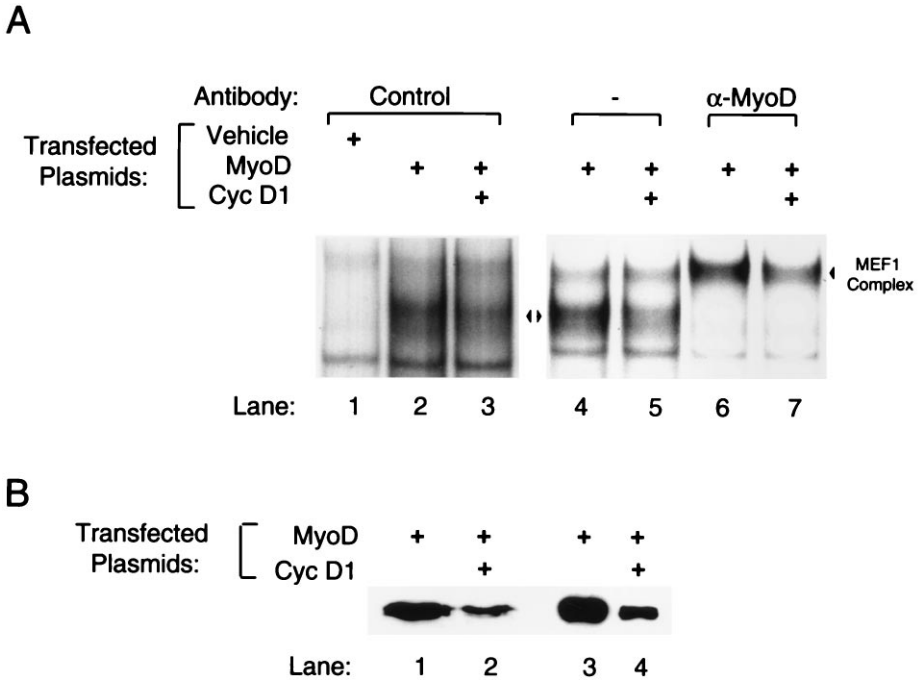

FIG. 2. The ectopic expression of cyclin D1 does not inhibit MyoD DNA binding activity. (A) EMSA showing MEF1 site DNA binding activity in high-salt extracts of 10T1/2 fibroblasts which were transiently transfected with equal amounts (0.5 μg) of plasmids encoding MyoD and either cyclin D1 (Cyc D1) or the corresponding empty expression vehicles. Final DNA content was normalized to 2 μg with the corresponding empty expression vehicles. The shifted MEF1 complex (arrows) contains MyoD which can be supershifted by an anti-MyoD monoclonal antibody (lanes 6 and 7) but is not supershifted by an anti-myosin heavy chain monoclonal antibody (Control) (lanes 2 and 3). (B) Immunoblot for MyoD protein in equal volumes of high-salt extract (used in EMSA) from fibroblasts transfected with equal amounts (0.5 μg) of MyoD and either cyclin D1 (lanes 2 and 4) or the empty expression plasmid (lanes 1 and 3). Final DNA content was normalized to 2 μg with the corresponding empty expression vehicles. Lanes 1 and 2 and lanes 3 and 4 represent two independent experiments used for EMSA experiments shown in Fig. 2A, lanes 1 to 3 and 4 to 7, respectively.

myogenic cell lines (38, 68), was supershifted with an anti-MyoD monoclonal antibody but not with a control monoclonal antibody (Fig. 2A, compare lanes 2, 4, and 6). Cotransfection of 10T1/2 cells with plasmids encoding MyoD and cyclin D1 resulted in a modest loss of MEF1 site DNA binding activity (Fig. 2A, compare lanes 2 and 3, 4 and 5, and 6 and 7). However, this decrease in MEF1 site binding activity corresponded to a parallel decrease in the amount of MyoD protein present in high-salt extracts made from cells that had been cotransfected with cyclin D1 (Fig. 2B). Interestingly, this decrease in MyoD protein in the presence of cyclin D1 was not observed in whole-cell sonicates (Fig. 3A) (69). Although these findings indicate that nuclear retention of MyoD may be affected by cyclin D1, we have not observed significant differences in the salt extractability of nuclear MyoD in either the presence or absence of cotransfected cyclin D1 (68). These findings indicate that interaction of MyoD with a MEF1 binding site was proportional to the amount of MyoD protein in these extracts and unaffected by cotransfection of cyclin D1. This suggests that cyclin D1 has neither induced an inhibitory phosphorylation in the basic domain of MyoD nor disrupted the interaction of MyoD with E proteins.

Cyclin D1-mediated inhibition of muscle gene expression is independent of phosphorylation of serines 43 and 170 in the myogenin transcriptional activation domains. Ectopic cyclin D1 expression in 10T1/2 cells leads to the accumulation of MyoD in a form that displays a slower electrophoretic mobility (Fig. 3A, lanes 1 and 2); this slowed mobility was previously shown to be due to phosphorylation of MyoD (69). Because cyclin D1 inhibits the expression of E-box reporter genes in cells transfected with either myogenin or MyoD, we determined whether there was a similar change in the electrophoretic mobility of myogenin in the presence of inhibitory amounts of cotransfected cyclin D1. Cotransfection of cyclin D1 induced the accumulation of myogenin protein, including a more slowly migrating form (Fig. 3A, lanes 3 and 4). The decreased electrophoretic mobility of the more slowly migrating form of myogenin has previously been shown to be due to phosphorylation (78). When exposures of equivalent intensity were compared, the relative ratios of hyper- and hypophosphorylated myogenin appeared to be similar in both the presence and absence of cyclin D1 (Fig. 3A, lanes 3, 4, 5, and 6). Therefore, because there is no relative change in the ratio of hypo- versus hyperphosphorylated myogenin in the presence of cyclin D1, the importance of the absolute increase in hyperphosphorylated myogenin in the presence of cyclin D1 is unclear.

The electrophoretic mobility of the slowly migrating form of myogenin is due to dimerization-dependent phosphorylation of a serine residue (serine 43) which is one of two serine residues whose phosphorylation appears to inhibit myogenin activity (78). These two serine residues lie within the transcriptional activation domains of myogenin (outside of the bHLH motif) and are similar to consensus cdk phosphorylation sites (51). Therefore, we tested whether a form of myogenin in which both of these serine residues have been mutated to alanine (myogenin 43/170 S-A) could be inhibited by cotransfected cyclin D1. Ectopic expression of cyclin D1 inhibited expression of both a complex muscle-specific reporter, MCK-CAT, and a simplified muscle reporter, 4xMEF1-CAT, in cells cotransfected with either wild-type myogenin or myogenin 43/170 S-A (Fig. 3B). This result indicates that cyclin D1 can

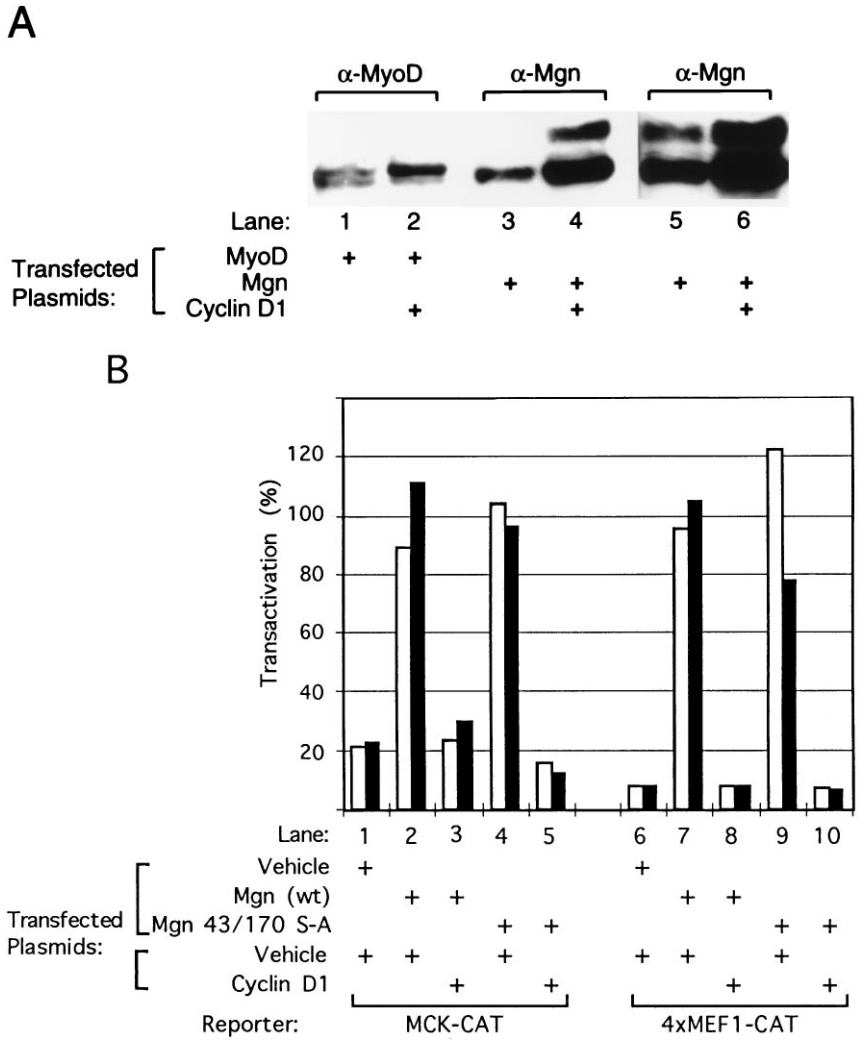

FIG. 3. Cyclin D1 can inhibit myogenin transactivation without phosphorylating specific serine residues. (A) Immunoblot of MyoD and myogenin (Mgn) from whole-cell sonicates of 10T1/2 fibroblasts transfected with expression plasmids encoding MyoD, myogenin, and cyclin D1 as indicated. Lanes 5 and 6 represent a longer exposure of lanes 3 and 4. (B) CAT activity from fibroblasts which were cotransfected with plasmids encoding wild-type myogenin [Mgn (wt)] (0.5 μg), mutated myogenin (Mgn 43/170 S-A) (0.5 μg), or the corresponding empty expression vehicle (0.5 μg); cyclin D1 (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); reporter genes (0.5 μg) as indicated; and the corresponding empty expression vehicles (0.5 μg) to normalize DNA content to 2 μg. CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved for each transcription factor in the absence of cyclin D1. The average maximal CAT activity for each condition is as follows: 15,811 cpm (lane 2), 27,959 cpm (lane 4), 39,750 cpm (lane 7), and 72,056 cpm (lane 9).

inhibit the activity of myogenin independent of inducing phosphorylation at either serine 43 or serine 170 of this protein.

Cyclin D1 can inhibit muscle-specific gene expression in the absence of pRB association or phosphorylation. Because expression of ectopic cyclin D1 leads to pRB phosphorylation (16, 31, 45) and relieves pRB-induced cell cycle arrest (11, 27), we investigated whether cyclin D1-mediated inhibition of muscle gene expression could be due to inactivation of pRB function. To evaluate this, we assayed whether cotransfection of cells with a nonhyperphosphorylatable form of pRB would render myogenesis in these cells resistant to the negative effects of ectopic cyclin D1. In this and subsequent experiments, we used a form of pRB in which eight putative cdk phosphorylation sites have been mutated (22, 23). This form of pRB, pRB-ΔP34-HA, resists cyclin-cdk-mediated phosphorylation (23, 68) and shows enhanced activity in vivo (4). Transfection of pRB-ΔP34-HA into 10T1/2 fibroblasts cotransfected with MyoD augmented the expression of MCK-CAT approximately twofold (Fig. 4A, compare lanes 2 and 5). Expression of cyclin D1 decreased MCK-CAT expression by about 50% in either the absence or presence of pRB-ΔP34-HA (Fig. 4A, compare lanes 2 and 3 and lanes 5 and 6), which suggests that the positive effects of pRB and the negative effects of cyclin D1 are independent and separable. We noted that although cyclin D1 slightly decreased MyoD protein levels in high-salt extracts isolated from cotransfected cells (Fig. 4B, compare lanes 1 and 2), addition of pRB-ΔP34-HA to these cells significantly boosted MyoD function (Fig. 4A, compare lanes 3 and 6) yet did not increase MyoD protein levels (Fig. 4B, rightmost lane). Thus, the efficiency of MyoD-mediated transactivation of reporter genes does not correlate with the absolute levels of MyoD protein in high-salt extracts isolated from cells cotransfected with cyclin D1 in the absence or presence of pRB-ΔP34-HA.

The physical interaction between the "pocket" of pRB and the D-type cyclins, via a conserved L-X-C-X-E motif in the

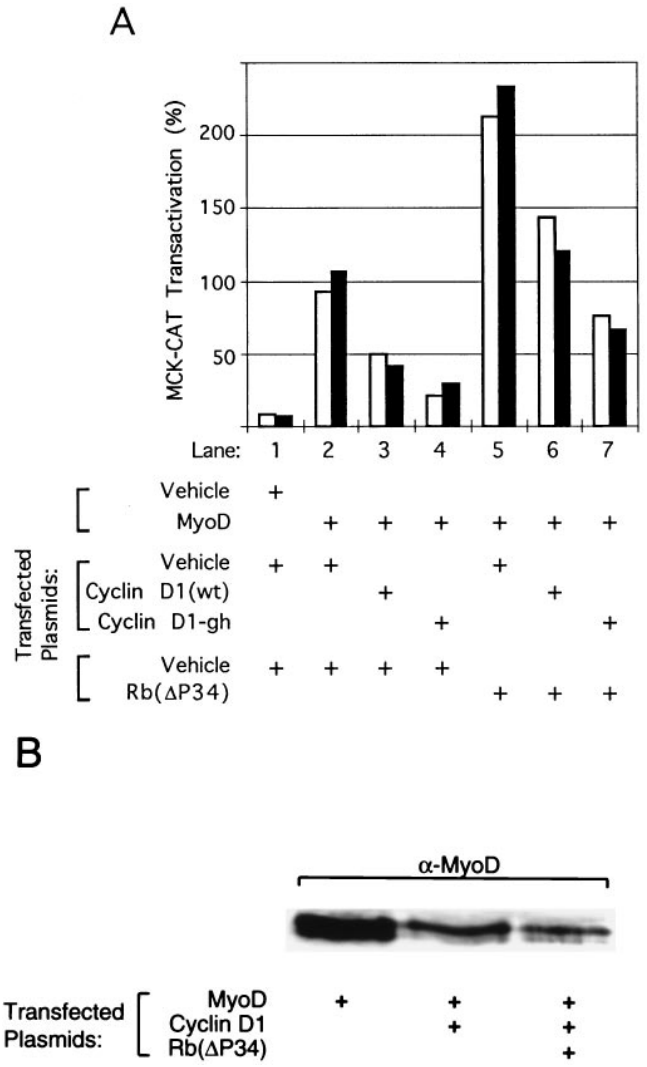

FIG. 4. The expression of cyclin D1 and pRB has opposing effects on MyoD-mediated transactivation of a muscle-specific promoter. (A) CAT activity from whole-cell extracts of 10T1/2 fibroblasts which were cotransfected with plasmids encoding the MCK-CAT reporter (0.5 μg) (all lanes); MyoD (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); wild-type (wt) cyclin D1 (0.5 μg), mutated cyclin D1-gh (0.5 μg), or the corresponding empty expression vehicle (0.5 μg); and nonhyperphosphorylatable pRB(ΔP34) (1.0 μg) or the corresponding empty expression vehicle (1.0 μg). CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved in the absence of cyclin D1 (lane 2). The average maximal CAT activity for MyoD activation of MCK-CAT was 29,574 cpm (lane 2). (B) Immunoblot of MyoD immunoprecipitated from high-salt extracts of 10T1/2 fibroblasts transfected with expression plasmids encoding MyoD, cyclin D1, and pRB(ΔP34) as indicated.

amino terminus of the D-type cyclins (11), may have important functional implications. There is evidence that this interaction with pRB is necessary for cyclin D2 to negatively regulate pRB (16). In addition, there is evidence that direct pRB binding of D-type cyclins may negatively regulate the activity of these cyclins (11; reviewed in reference 51). We therefore wanted to investigate whether interaction of cyclin D1 with pRB-ΔP34-HA was important for cyclin D1-mediated inhibition of myogenesis. For this analysis, we employed a mutated form of cyclin D1 (cyclin D1-gh) (11) in which the pRB-binding motif has been disrupted. Cyclin D1-gh is expressed at the same level as wild-type cyclin D1 in 10T1/2 cells (62) and when cotransfected with MyoD reduced the activity of MCK-CAT by about 75% in either the absence or presence of pRB-ΔP34-HA (Fig. 4A, compare lanes 2 and 4 and lanes 5 and 7). Rao and colleagues have similarly found that this mutated form of cyclin D1 inhibited myogenesis in 10T1/2 fibroblasts (60). Thus, a form of cyclin D1 which has low affinity for pRB (11) and cannot induce hyperphosphorylation of mutated pRB-ΔP34-HA (23, 68) is fully competent to inhibit the expression of muscle-specific reporter genes.

Cyclin D1 inhibits muscle gene expression in pRB-deficient cells reconstituted with a nonhyperphosphorylatable form of pRB. The experiments described above suggest that cyclin D1 can inhibit muscle gene expression in the presence of a nonhyperphosphorylatable form of pRB; however, 10T1/2 cells also contain wild-type pRB, which can be inactivated by ectopic cyclin D1. Therefore, the inhibition of myogenesis in these cells may be due to inhibition of endogenous pRB function. To address this possibility, we monitored the effects of ectopic cyclin D1 expression on myogenesis in pRB-deficient mouse embryo fibroblasts (MEFs) that had been transiently reconstituted with pRB-ΔP34-HA. In these MEFs, the expression of exogenous muscle reporter genes in cells cotransfected with MyoD is dependent upon the activity of exogenously supplied pRB (53). Indeed, the ability of MyoD to enhance the expression of MCK-CAT in these cells was completely dependent upon the addition of pRB-ΔP34-HA (Fig. 5, compare lanes 2 and 3). The pRB-dependent increase in MyoD activity in these cells occurred in the absence of any alteration in MyoD protein levels (Fig. 6B). When these cells were supplemented with the nonhyperphosphorylatable form of pRB (pRB-ΔP34-HA), cotransfection with MyoD and either wild-type cyclin D1 or cyclin D1-gh efficiently inhibited the expression of MCK-CAT (Fig. 5, lanes 3 to 5).

Transfection of this mutated form of pRB also significantly augmented the expression of the nonmuscle reporter CMV-CAT in these cells (Fig. 5, lanes 6 and 7). Importantly, however, this augmented expression was not attenuated by cotransfection with either wild-type cyclin D1 or cyclin D1-gh (Fig. 5, lanes 8 and 9), indicating that transcriptional inhibition induced by ectopic cyclin D1 was specific to muscle reporter genes and was not a global effect on transcription. Thus, cyclin D1 can inhibit muscle gene expression under conditions in which muscle gene expression is completely dependent on the presence of an exogenous nonhyperphosphorylatable form of pRB. These findings confirm that cyclin D1 can inhibit the expression of genes which are downstream of the MyoD gene in the absence of pRB hyperphosphorylation.

Unlike cyclin D1, cyclins A and E can suppress muscle gene expression only in the presence of a hyperphosphorylatable form of pRB. The experiments described above indicated that cyclin D1 can inhibit the expression of MCK-CAT independent of pRB hyperphosphorylation. However, because pRB function is necessary for the expression of muscle gene reporters in pRB-deficient MEFs cotransfected with MyoD, we determined whether other G_1 cyclins could inhibit myogenesis in these cells when reconstituted either with wild-type pRB or with pRB-ΔP34-HA. pRB-deficient MEFs were transiently cotransfected with MyoD and either wild-type pRB (Fig. 6A, lanes 1 to 6) or pRB-ΔP34-HA (Fig. 6A, lanes 7 to 11); MCK-CAT expression was monitored in the presence of either cyclin D1-gh, A, or E. When cotransfected with wild-type pRB, cyclins D1-gh, A, and E each depressed the expression of MCK-CAT in cells transfected with MyoD. Cyclin D1-gh depressed MCK-CAT expression to levels observed in the absence of cotransfected MyoD, whereas cyclins A and E diminished MCK-CAT expression to a lesser degree (Fig. 6A, lanes 4 to 6).

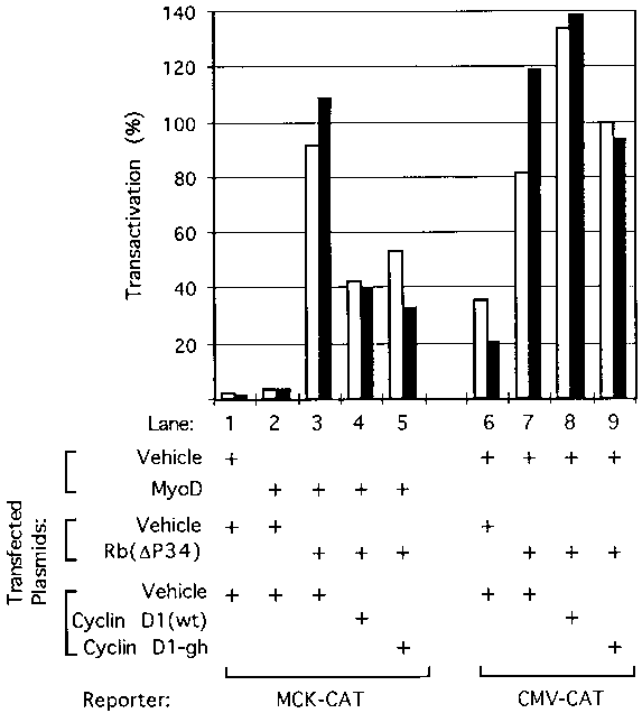

FIG. 5. The ectopic expression of cyclin D1 inhibits MyoD-mediated transactivation of a muscle-specific promoter independent of pRB phosphorylation in $Rb^{-/-}$ fibroblasts. CAT activity from whole-cell extracts of $Rb^{-/-}$ fibroblasts which were cotransfected with plasmids encoding MyoD (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); nonhyperphosphorylatable pRB(ΔP34) (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); wild-type (wt) cyclin D1 (1.5 μg), mutated cyclin D1-gh (1.5 μg), or the corresponding empty expression vehicle (1.5 μg); and either a muscle-specific reporter (MCK-CAT) (0.5 μg) or a non-muscle-specific reporter (CMV-CAT) (0.5 μg). CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved in the absence of cyclin D1 and in the presence of pRB (lanes 3 and 7). The average maximal CAT activity in the presence of ectopic pRB for MCK-CAT was 31,2095 cpm (lane 3) and for CMV-CAT was 57,664 cpm (lane 7).

Expression of pRB-ΔP34-HA in $Rb^{-/-}$ MEFs suppressed the inhibitory effects of both cyclins A and E on MCK-CAT activity (Fig. 6A, compare lanes 3, 5, and 6 with lanes 8, 10, and 11). In contrast, cyclin D1-mediated inhibition of MCK-CAT expression was not reversed by pRB-ΔP34-HA (Fig. 6A, compare lanes 4 and 9). These findings indicate that the inhibitory effects of cyclins A and E on muscle gene expression may depend on the ability of these cyclins (in a complex with cdk2) to hyperphosphorylate pRB, whereas cyclin D1 may inhibit muscle gene expression independent of pRB hyperphosphorylation.

In these cells, there was no significant change in MyoD protein levels following cyclin transfection (Fig. 6B), suggesting that inhibition of MyoD function by the different G_1 cyclins in these cells was due neither to apoptosis nor to loss of MyoD protein. In contrast to 10T1/2 fibroblasts, in $Rb^{-/-}$ fibroblasts, the electrophoretic mobility of MyoD in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was not significantly altered by cotransfection with these G_1 cyclins (Fig. 6B). The transfected cyclins, however, did induce the accumulation of a hyperphosphorylated, slowly migrating form of pRB to various degrees (Fig. 6B, lanes 2 to 5); whereas cyclin D1-gh induced little or no hyperphosphorylated pRB, cyclins A and E clearly promoted pRB hyperphosphorylation. Therefore, although the relative ability of these G_1 cyclins to phosphorylate pRB did not correlate with their ability to inhibit muscle gene expression, these data do indicate that expression of G_1 cyclins which induce various degrees of pRB hyperphosphorylation can suppress muscle gene expression.

Because the catalytic partners for cyclin D1 and cyclins A and E are different (i.e., cdk4/6 and cdk2, respectively), it was conceivable that the relative differences in MCK-CAT inhibition observed with these cyclins were due to differences in the availability of the cognate cdks in these cells. Therefore, we assayed MCK-CAT expression in $Rb^{-/-}$ MEFs ectopically expressing MyoD and each cyclin with its cognate cdk. In the presence of MyoD and pRB-ΔP34-HA, cyclin D1-gh plus cdk4 completely extinguished the expression of MCK-CAT (Fig. 6C, compare lanes 2 and 3) while combinations of either cyclin E plus cdk2 or cyclin A plus cdk2 had relatively little effect on this reporter (Fig. 6C, lanes 4 and 5). Thus, whereas the inhibitory activities of cyclin A-cdk2 and cyclin E-cdk2 on muscle gene expression are reversed by the inclusion of a nonhyperphosphorylated form of pRB, the negative effects of cyclin D1-cdk4 are not ameliorated by a nonhyperphosphorylatable form of pRB.

DISCUSSION

In this study, we have further explored the molecular basis for cyclin D1-mediated inhibition of muscle gene expression in cells expressing MyoD. Our data indicate that (i) cyclin D1 preferentially inhibits the expression of muscle genes in cells transfected with myogenic transcription factors in comparison to the non-tissue-restricted bHLH transcription factor, E12; (ii) cyclin D1 does not affect MyoD DNA binding; (iii) cyclin A-cdk2 and cyclin E-cdk2 can attenuate muscle gene expression by inducing hyperphosphorylation of pRB; and (iv) cyclin D1 can also attenuate muscle gene expression through a mechanism that is independent of pRB hyperphosphorylation. On the basis of these data, we propose a model in which there is a balance between positively acting pRB and negatively acting cyclin D1 (and other G_1 cyclins) in the regulation of muscle gene expression (Fig. 7); changes in this balance may be critical for regulating the functional activity of muscle differentiation factors during the transition from proliferating myoblasts to differentiating myocytes.

Cyclin D1 inhibits muscle gene expression in cells expressing MyoD without compromising MyoD-DNA interaction. Because MyoD protein is expressed in proliferating myoblasts prior to terminal differentiation (71), a number of molecular mechanisms have been proposed to explain the functional inactivation of MyoD in proliferating myoblasts and the coupling of cell cycle arrest with myogenic differentiation (reviewed in references 40 and 56). These include negative regulation of myogenic bHLH factors by peptide growth factors like fibroblast growth factor (24, 44); intracellular signal transducers like notch (35), ras (34, 41, 55), and jun (2, 43); or transcription factors that are associated with cell cycle progression, like myc (46) and E2F (73). It is conceivable that these regulatory pathways modulate one or more aspects of myogenic bHLH protein function such as dimerization with E proteins, DNA binding, transactivation, or direct or indirect interaction of these bHLH factors with cofactors such as MEF2 (48) or pRB (18, 53, 63).

Regulation of the DNA binding activity of myogenic bHLH transcription factors is known to occur by at least two mechanisms. Id-1, an HLH-containing protein, can inhibit MyoD DNA binding by sequestering E proteins and thereby regulate MyoD–E-protein heterodimerization (1). Id-1 is highly expressed in myoblasts, decreases upon mitogen deprivation and

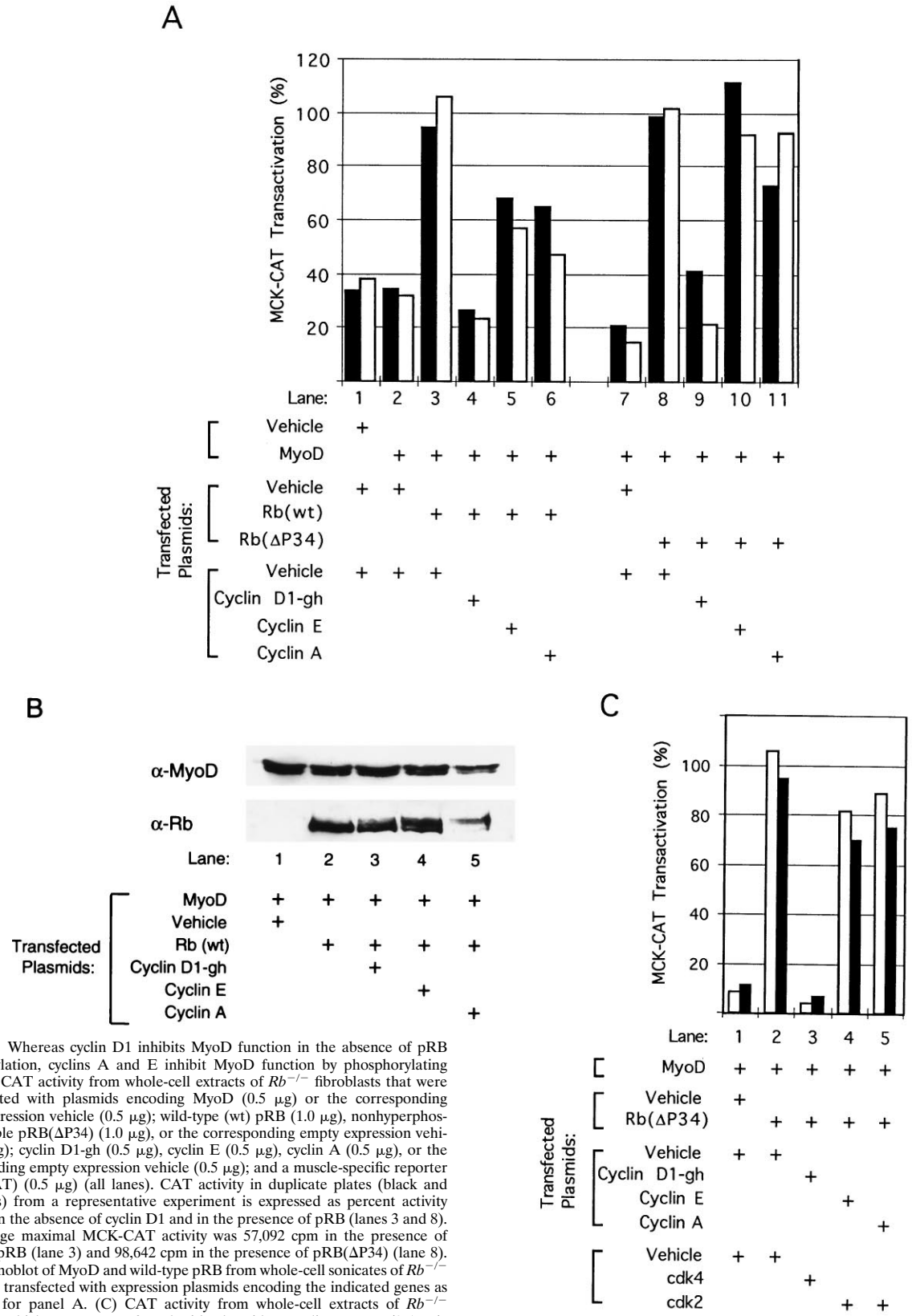

FIG. 6. Whereas cyclin D1 inhibits MyoD function in the absence of pRB phosphorylation, cyclins A and E inhibit MyoD function by phosphorylating pRB. (A) CAT activity from whole-cell extracts of $Rb^{-/-}$ fibroblasts that were cotransfected with plasmids encoding MyoD (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); wild-type (wt) pRB (1.0 μg), nonhyperphosphorylatable pRB(ΔP34) (1.0 μg), or the corresponding empty expression vehicle (1.0 μg); cyclin D1-gh (0.5 μg), cyclin E (0.5 μg), cyclin A (0.5 μg), or the corresponding empty expression vehicle (0.5 μg); and a muscle-specific reporter (MCK-CAT) (0.5 μg) (all lanes). CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved in the absence of cyclin D1 and in the presence of pRB (lanes 3 and 8). The average maximal MCK-CAT activity was 57,092 cpm in the presence of wild-type pRB (lane 3) and 98,642 cpm in the presence of pRB(ΔP34) (lane 8). (B) Immunoblot of MyoD and wild-type pRB from whole-cell sonicates of $Rb^{-/-}$ fibroblasts transfected with expression plasmids encoding the indicated genes as described for panel A. (C) CAT activity from whole-cell extracts of $Rb^{-/-}$ fibroblasts which were cotransfected with plasmids encoding MyoD (0.5 μg); nonhyperphosphorylatable pRB(ΔP34) (0.5 μg) or the corresponding empty expression vehicle (0.5 μg); cyclin D1-gh and cdk4 (0.75 μg each) (lane 3), cyclin E and cdk2 (0.75 μg each) (lane 4), cyclin A and cdk2 (0.75 μg each) (lane 5), or the corresponding empty expression vehicles (0.75 μg each); and a muscle-specific reporter (MCK-CAT) (0.5 μg) (all lanes). CAT activity in duplicate plates (black and white bars) from a representative experiment is expressed as percent activity achieved in the absence of cyclin D1 and in the presence of pRB (lane 2). The average maximal MCK-CAT activity was 87,792 cpm in the presence of Rb(ΔP34) (lane 2).

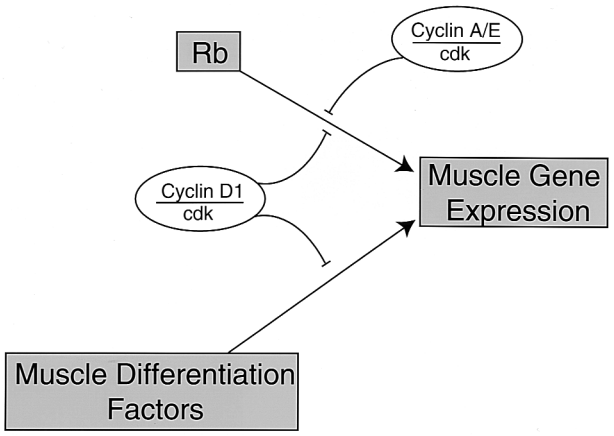

FIG. 7. Schematic model for pRB-dependent and pRB-independent regulation of skeletal muscle differentiation by cyclins. Myogenic differentiation factors, such as MyoD, myogenin, and MEF2, cooperate with hypophosphorylated pRB to combinatorially activate skeletal muscle-specific genes. G_1 cyclins which lead to pRB hyperphosphorylation can limit pRB-mediated enhancement of muscle gene expression. In addition, cyclin D1 can inhibit muscle differentiation via a mechanism that is independent of pRB hyperphosphorylation.

myogenic differentiation, and delays skeletal muscle differentiation when constitutively expressed (30). A second mechanism demonstrated to regulate DNA binding of myogenic regulatory proteins is phosphorylation of residues in the basic region of these proteins. For example, fibroblast growth factor signaling has been shown to induce protein kinase C-mediated phosphorylation of myogenin at a threonine residue which is conserved in the basic domain of all myogenic bHLH factors; phosphorylation at this residue inhibits DNA binding by myogenin (44). These data are consistent with a model in which mitogenic stimuli maintain the functional inactivation of some myogenic differentiation factors by inhibiting DNA binding. However, recent data suggest that other mechanisms of myogenic bHLH regulation exist, because (i) nuclear extracts isolated from proliferating myoblasts have been found to contain MyoD-E complexes that are fully competent to bind DNA (37, 67), (ii) fibroblast growth factor-dependent regulation of MRF4 is independent of threonine phosphorylation in the DNA-binding basic region (24), and (iii) a fusion protein containing MyoD and E47 tethered together as a forced heterodimer can overcome Id-mediated inhibition but cannot fully restore MyoD activity in proliferating cells (50).

In our experiments, cyclin D1 expression did not significantly affect the DNA binding activity of MyoD (Fig. 2A). This observation is consistent with the findings of Rao and colleagues, who demonstrated that a mutated version of myogenin which lacks the protein kinase C phosphorylation site in the basic domain was still susceptible to cyclin D1-mediated inhibition (60). Recently, it was demonstrated that activated ras inhibits myogenic differentiation without decreasing DNA binding by myogenic bHLH factors (33), raising the possibility that activated ras and ectopic cyclin D1 inhibit myogenesis by a similar mechanism. However, it should be noted that cyclin D1 inhibits MyoD-mediated transactivation of both complex and simplified muscle-specific reporters in 10T1/2 cells whereas activated ras did not appear to inhibit the expression of a simplified muscle-specific reporter (containing reiterated MEF1 sites) (33).

Phosphorylation of pRB by G_1 cyclin-cdk complexes inhibits muscle gene expression in cells expressing MyoD. pRB is critical for both high-level expression of late muscle differentiation markers (53) and irreversible cell cycle arrest of differentiated myocytes (53, 63). Consistent with a positive role for pRB in activating muscle gene expression, we have found that ectopic expression of cyclin A or E, which induces pRB hyperphosphorylation, can attenuate muscle gene expression in pRB-deficient MEFs reconstituted with wild-type pRB and MyoD. In contrast, when these cells are reconstituted with a form of pRB that is not hyperphosphorylatable, the negative effects of cyclins A and E on muscle gene expression are nullified. This finding strongly supports the notion that ectopic expression of cyclins A and E inhibits muscle gene expression via pRB hyperphosphorylation. Although previous studies have failed to uncover significant modulation of MyoD activity by either cyclin A or E in 10T1/2 cells (60, 69), when 10T1/2 cells were cotransfected with both cyclin A and cdk2, muscle-specific gene expression was inhibited (68). Therefore, we think that the previously reported absence of an effect of cyclin A or E on muscle differentiation may be due to decreased levels of cdk2 in 10T1/2 cells incubated under differentiation conditions (20, 32, 54). At present it is unclear how pRB functions to augment muscle gene expression; nonetheless, our data suggest that cdk-mediated hyperphosphorylation of this cell cycle regulator can abrogate its positive effects on muscle gene expression.

Cyclin D1 can inhibit muscle gene expression by a mechanism that is independent of pRB hyperphosphorylation. In contrast to cyclins A and E, cyclin D1 was able to extinguish muscle gene expression in $Rb^{-/-}$ MEFs that were reconstituted with a form of pRB that was immune to cdk phosphorylation. These findings indicate that cyclin D1 plus cdk4/6 may be able to inhibit MyoD function by a mechanism that is independent of pRB hyperphosphorylation. It should be noted, however, that our data do not rule out the possibility that cyclin D1-cdk activity blocks myogenesis by phosphorylation of pRB at sites other than those mutated in pRB-ΔP34-HA. The functional overlap provided by two cyclin-dependent pathways to inhibit the expression of muscle differentiation genes (outlined in Fig. 7) may ensure that the onset of terminal differentiation is restricted to the G_0 phase of the cell cycle.

It is not clear how cyclin D1 inhibits muscle differentiation independent of pRB hyperphosphorylation. However, it seems unlikely that inhibition of MyoD function by cotransfected cyclin D1 is due to apoptosis (36), as evidenced by the nearly identical levels of transfected MyoD in cells cotransfected with either cyclin D1, A, or E (Fig. 6B). Furthermore we have found that cotransfection of cells with the anti-apoptotic adenovirus 21-kDa E1B protein fails to reverse a cyclin D1-mediated inhibition of muscle differentiation (68).

Previously, we had noted that ectopic cyclin D1 could induce hyperphosphorylation of MyoD in 10T1/2 cells (69) (Fig. 3A and 4B). In contrast, in $Rb^{-/-}$ MEFs reconstituted with exogenous wild-type pRB, cotransfection of cyclin D1 efficiently blocked MyoD function but did not significantly alter MyoD phosphorylation (Fig. 6B). In addition, we have found that in 10T1/2 cells, cyclin D1 can inhibit myogenin activity without apparently changing the relative ratio of hyper- to hypophosphorylated myogenin (Fig. 3A) and that cyclin D1 can efficiently inhibit the activity of a mutated form of myogenin which lacks two putative cdk phosphorylation sites which are known to regulate its transactivation potential (78). Furthermore, we have found that baculovirus-produced cyclin D1-cdk4 failed to increase phosphate incorporation into glutathione *S*-transferase–MyoD under conditions in which glutathione *S*-transferase–pRB was highly phosphorylated (68). Although we cannot exclude the possibility that cyclin D1-cdk inhibits MyoD function directly, these results suggest that cyclin D1 inhibits the expression of muscle genes through mechanisms

that are independent of pRB hyperphosphorylation and also independent of direct cdk-mediated phosphorylation of the myogenic bHLH transcription factors.

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