Inhibition of Myogenic Differentiation in Proliferating Myoblasts by Cyclin D1–Dependent Kinase

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Although the myogenic regulator MyoD is expressed in proliferating myoblasts, differentiation of these cells is limited to the G_0 phase of the cell cycle. Forced expression of cyclin D1, but not cyclins A, B, or E, inhibited the ability of MyoD to transactivate muscle-specific genes and correlated with phosphorylation of MyoD. Transfection of myoblasts with cyclin-dependent kinase (Cdk) inhibitors p21 and p16 augmented muscle-specific gene expression in cells maintained in high concentrations of serum, suggesting that an active cyclin-Cdk complex suppresses MyoD function in proliferating cells.

The process of terminal skeletal muscle differentiation, regulated by the MyoD family of basic helix-loop-helix (bHLH) transcription factors (*1*), is intimately coupled to cell cycle arrest in G_0 (*2*). Although several models address the coupling of cell cycle arrest and differentiation (*3*), recent evidence directly links cell cycle regulatory proteins to the regulation of skeletal muscle differentiation (*4, 5*). Terminal cell cycle withdrawal of differentiated myotubes and MyoD transactivation function in SAOS2 osteosarcoma cells requires the function of the retinoblastoma gene product (Rb) (*5*), whose activity is controlled by a family of regulatory proteins, G_1-cyclins, and their catalytic partners, Cdks (*6–10*). In addition, the functional activity of MyoD seems to be inhibited by constitutive expression of cyclin D1 (*11*). Here we address the mechanism of cyclin-mediated inhibition of MyoD function and explore whether endogenous Cdk activity inhibits myogenic differentiation in proliferating cells.

10T1/2 fibroblasts were transfected with a plasmid encoding MyoD under the control of the cytomegalovirus (CMV) promoter (CMV-MyoD), a reporter plasmid consisting of the muscle creatine kinase promoter-enhancer driving the chloramphenicol acetyltransferase (CAT) gene (MCK-CAT), and plasmids containing the CMV promoter driving expression of different cyclins. Expression of cyclin D1 inhibited transactivation of MCK-CAT, whereas expression of other G_1 and mitotic cyclins had less or no effect (Fig. 1A). Cyclins A and E, which can phosphorylate and inactivate Rb (6), only weakly inhibited MyoD-mediated MCK-CAT transactivation, suggesting that Rb inactivation may not be sufficient to inhibit MyoD function in this assay. Accordingly, a mutant form of cyclin D1 (cyclin D1-gh) that reverses Rb-induced cell cycle arrest more effectively than wild-type cyclin D1 (*8*) was slightly less effective at inhibiting MyoD function than wild-type cyclin D1 (*11, 12*). Although it is possible that cyclin D1 specifically inactivates other Rb-related proteins, our findings indicate that the relative abilities of various G_1 cyclins to inhibit MyoD function do not correlate with the ability of such cyclins to inhibit Rb activity. Transcriptional activation of a reiterated MyoD binding site (MEF1 site) upstream of the thymidine kinase (tk) promoter (4Rtk-CAT) was similarly inhibited by expression of ectopic cyclin D1 (Fig. 1A), suggesting that the target of this cyclin is the MyoD-E protein heterodimer. In contrast to transactivation of muscle-specific reporters, which was inhibited by ectopic cyclin D1 to 10% of that in control cells, transactivation of a nonmuscle-specific reporter (CMV-CAT) was reduced to only 60% of that in control cells (Fig. 2A).

Although cyclin D1 expression did not alter the amount of MyoD protein in transfected cells, the electrophoretic mobility of MyoD was affected (Fig. 1B). When isolated from differentiated myotubes or transfected 10T1/2 cells, MyoD migrated in SDS-polyacrylamide gels as a doublet containing equivalent amounts of fast and slowly migrating forms (Fig. 1B) (*13*). Because the slowly migrating form was resolved into a single, fast migrating species upon treatment with calf intestinal phosphatase (CIP), this slowed mobility is apparently due to phosphorylation (Fig. 1B) (*13*). MyoD isolated from cells transfected with cyclin D1 consisted primarily of the slowly migrating, hyperphosphorylated species (Fig. 1B). Ectopic expression of either cyclin A or E, which did not markedly affect transcriptional activation by MyoD (Fig. 1A), did not alter the phosphorylation state of MyoD (Fig. 1B). Thus, the ability of cyclin D1 to inhibit MyoD-mediated transactivation of muscle genes correlates with phosphorylation of MyoD protein.

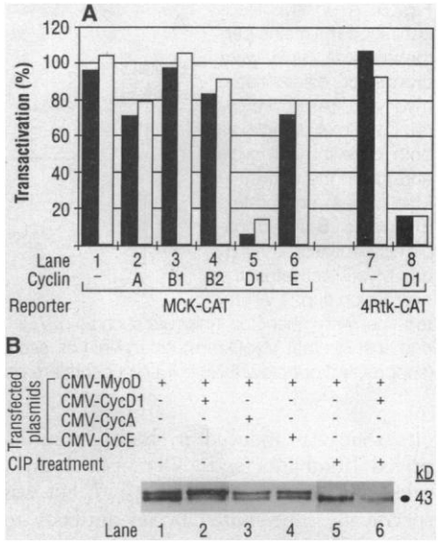

Fig. 1. Inhibition of muscle-specific gene expression by ectopic cyclin D1 correlates with phosphorylation of MyoD protein. (**A**) 10T1/2 fibroblasts were transfected (*20*) with equal amounts of CMV-MyoD and either MCK-CAT (lanes 1 to 6) or 4Rtk-CAT (lanes 7 and 8) and a CMV expression vehicle either without an insert (lanes 1 and 7) or with a cyclin gene (CMV-cyclin) (*20*) (lanes 2 to 6 and lane 8). Open and closed bars represent duplicate plates from a representative experiment. (**B**) 10T1/2 cells were transfected with equal amounts of CMV-MyoD and the indicated CMV-cyclin construct and cultured as described (*20*). After 84 hours, MyoD was isolated by immunoprecipitation and either directly resuspended in SDS loading buffer (lanes 1 to 4) or treated with CIP before resuspension (lanes 5 and 6) (*21*). The CIP-dependent alteration in MyoD electrophoretic mobility was not observed in the presence of phosphatase inhibitors (*12*). After SDS-PAGE, MyoD was detected by protein immunoblot (*21*). Molecular size (in kilodaltons) is indicated on the right.

In transient transfection assays, expression of both cyclins D1 and D2 inhibited MyoD-mediated activation of MCK-CAT (Fig. 2A); the degree of inhibition was proportional to the expression of each construct (Fig. 2B). In contrast, expression of cyclin D3, which was similar to that of cyclin D2, did not specifically inhibit muscle gene expression (Fig. 2, A and B). In proliferating C2C12 myoblasts, cyclin D1 levels are high and decrease upon differentiation (Fig. 2C). In contrast, cyclin D3 levels are low in proliferating myoblasts and are induced during muscle differentiation (Fig. 2C). Cyclin D2 RNA was barely detectable in myoblasts or myotubes (*12, 14*). The catalytic partner for cyclin D1, Cdk4 (*10, 15*), is expressed in both myoblasts and myotubes (Fig. 2C). Thus, cyclin D1 is the only D-type cyclin

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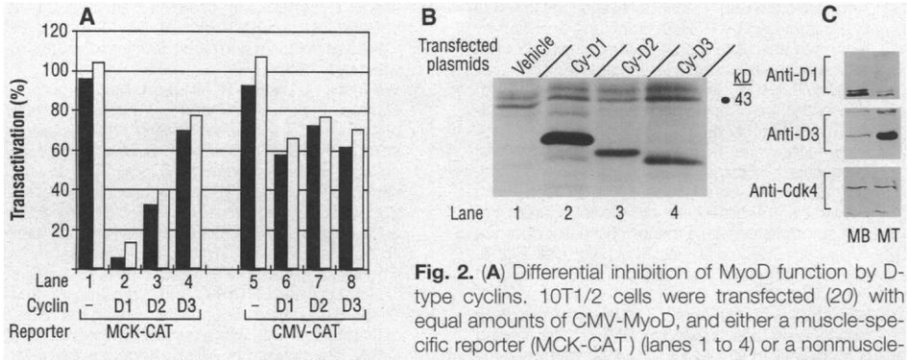

Fig. 2. (**A**) Differential inhibition of MyoD function by D-type cyclins. 10T1/2 cells were transfected (*20*) with equal amounts of CMV-MyoD, and either a muscle-specific reporter (MCK-CAT) (lanes 1 to 4) or a nonmuscle-specific reporter (CMV-CAT) (lanes 5 to 8), plus a CMV expression vehicle without an insert (lanes 1 and 5) or the indicated CMV-cyclin (*20*) (lanes 2 to 4 and lanes 6 to 8). (**B**) Expression of ectopic D-type cyclins. 10T1/2 cells were transfected with equal amounts of CMV-MyoD (lanes 1 to 4) and with a CMV expression vehicle either without an insert (lane 1) or encoding a D-type cyclin that was tagged with the hemagglutinin epitope (lanes 2 to 4). After 84 hours, cells were incubated with ^{35}S-methionine and cyclins were isolated by immunoprecipitation and SDS-PAGE (*21*). Molecular size (in kilodaltons) is indicated on the right. (**C**) Differential expression of endogenous cyclins D1 and D3 during muscle differentiation. Proteins from whole-cell extracts of proliferating murine C2C12 myoblasts (MB) or differentiated myotubes (MT) were resolved by SDS-PAGE. Cyclins D1 and D3 and Cdk4 were detected by protein immunoblot (*22*).

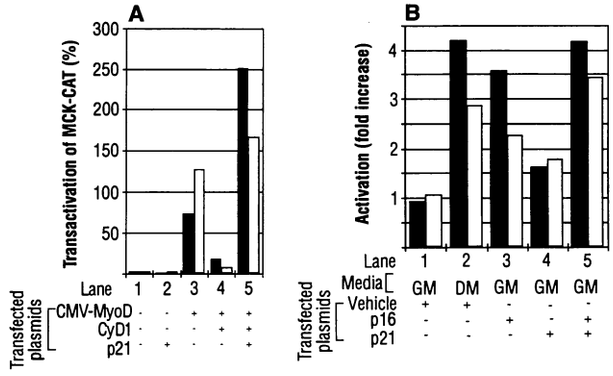

Fig. 3. (**A**) Reversal of cyclin D1–mediated inhibition of MyoD by ectopic p21. 10T1/2 cells were transfected (*20*) with MCK-CAT (*20*) (lanes 1 to 5) plus CMV-MyoD (lanes 3 to 5), CMV–cyclin D1 (lanes 4 and 5), and CMV-p21 (*17*) (lanes 2 and 5). CMV expression vehicles without an insert were included to normalize DNA in all transfections. (**B**) Effect of ectopically expressed Cdk inhibitors on muscle-specific gene expression in mitogen-stimulated myoblasts. C2C12 cells (30% confluent in 60-mm dishes) were transfected with 0.5 μg of MCK-CAT and 1.5 μg of a plasmid in which a CMV promoter drives expression of p21 or p16, or 0.75 μg of each plasmid (*20*). CMV expression vehicles without an insert were included to normalize DNA in all transfections. After 12 hours, cells were trypsinized; one-half of these cells were plated onto 100-mm dishes and refed with GM every 12 hours. To monitor gene expression under differentiation conditions, we cultured vehicle-transfected cells in DM (lane 2). After 72 hours (at which time cells were approaching confluence), cells were collected for CAT assay (*20*). CAT activity is expressed as fold increase over vehicle-transfected cells cultured in GM (lane 1).

that is highly expressed in proliferating myoblasts and inhibits MyoD function in transient assays.

Cyclin D1 can interact with and potentiate Cdk4 or Cdk6 activity (*10*, *15*, *16*). To determine whether Cdk activity is required for cyclin D1–mediated repression of MyoD, we examined the effect of coexpressing p21 (CIP1, WAF1, sdi, CAP20, Pic1), a potent inhibitor of several Cdks (*17*). Cotransfection of 10T1/2 cells with p21 and cyclin D1 reversed cyclin D1–mediated inhibition of MyoD function and led to superactivation of the MCK-CAT reporter (Fig. 3A). Expression of ectopic p21 did not alter the amount of ectopic cyclin D1 (*12*) and did not activate the MCK-CAT reporter in the absence of MyoD (Fig. 3A). The reversal of cyclin D1–mediated repression of MyoD by ectopic p21 indicates that cyclin D1 inhibits MyoD function by activating a Cdk.

To determine whether endogenous cyclin-Cdk complexes similarly inhibit MyoD function in proliferating myoblasts, we transfected C2C12 myoblasts with MCK-CAT and p21 or the Cdk4 inhibitor p16 (*18*) and then split the cells into either growth medium (GM) or differentiation medium (DM). Cells were harvested for CAT activity 60 hours after replating, when cells in GM had just become confluent. Under these conditions, culture in DM activated MCK-CAT expression to levels three to four times that in cells incubated in GM (Fig. 3B). Ectopic expression of either p21, which induced cell cycle withdrawal of these cells (*12*), or p16 partially reversed the inhibitory effect of GM on MCK-CAT expression in mitogen-stimulated myoblasts (Fig. 3B). Coexpression of both p21 and p16 resulted in MCK-CAT expression equal to that achieved in serum-starved cells (Fig. 3B). When expression of endogenous muscle gene (myosin heavy chain, MHC) was monitored by immunofluorescence, we consistently observed a two- to threefold increase in MHC expression in mitogen-stimulated muscle cells transfected with a CMV–β-Gal reporter plus the combination of p16 and p21 versus similarly cultured cells transfected with the CMV–β-Gal reporter plus empty expression vehicles (*12*).

The above findings indicate that ectopic p21 and p16 expression augments the expression of muscle-specific genes in confluent cultures of mitogen-stimulated myoblasts. However, when myoblasts, transfected and cultured as described above, were harvested at earlier time points (and were thus less confluent), there was less differential effect of GM versus DM and no consistent enhancement of MCK-CAT expression by forced expression of p21 or p16 (or both) (*12*). These findings suggest that other factors in addition to Cdks negatively regulate the activity of MyoD in subconfluent myoblasts (*3*). However, in confluent myoblasts maintained in high-mitogen media, the forced expression of a Cdk inhibitor reverses this serum-dependent effect and augments expression of both exogenous and endogenous muscle genes.

Our results demonstrate that cyclin D1 inhibits MyoD function by a Cdk-dependent mechanism and that Cdk activity prevents muscle differentiation in mitogen-stimulated myoblasts. Cyclin D1 is a good candidate as a physiologic regulator of MyoD function because its Cdk-associated activity is present throughout the cell cycle (*15*) and cyclin D1 RNA and protein levels decline in mature skeletal myotubes (Fig. 2C) (*14*). This role for cyclin D1 is supported by the observation that p16, a specific inhibitor of cyclin D–dependent kinases (*18*), augments expression of MCK-CAT in mitogen-stimulated myoblasts (Fig. 3B). Although cyclins D1, A, and E similarly cause phosphorylation of Rb and overcome Rb-induced cell cycle arrest (*6*, *9*), only the expression of cyclin D1 resulted in phosphorylation of MyoD and inhibition of MyoD function, suggesting that cyclin D1–Cdk activity inhibits MyoD function by a mechanism other than Rb phosphorylation. These data suggest that a nodal point in the coordination of cell cycle withdrawal and muscle differentiation may be the down-regulation of cyclin D1–associated Cdk activity and are consistent with our recent observation that the Cdk inhibitor p21 is induced during skeletal muscle differentiation (*19*).

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20. 10T1/2 fibroblasts or C2C12 myoblasts grown in GM (20% fetal calf serum) were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's directions. After 36 hours, cells were fed DM [2% horse serum plus insulin (12 μg/ml)]; 48 hours later, cells were harvested for CAT assay, normalized to protein content, essentially as described [C. M. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)]. All experiments for CAT expression were repeated at least three times with similar results. Except where indicated, CAT activity is expressed as percent of CAT expression observed in the absence of ectopic cyclins. Plasmids for transfection contained the CMV promoter driving expression of the following: MyoD (CMV-MyoD) (provided by B. Novitch); cyclins A, B1, B2, D1, D2, D3, and E (provided by S. Dowdy and P. Hinds) (6); $p21^{CIP1}$ (provided by S. Elledge); nuclear β-galactosidase (CMV–β-GAL) (pCS2+nβ-GAL) (provided by D. Turner, R. Rupp, and H. Weintraub); and $p16^{INK4}$, which was made by subcloning p16 (provided by D. Beach) into the Not I and Apa I sites in pRC/CMV (Invitrogen). Reporter plasmids contained the CAT gene driven by 3300 base pairs of the muscle creatine kinase promoter-enhancer (MCK-CAT) [J. Jaynes *et al.*, *Mol. Cell. Biol.* **8**, 62 (1988)] (provided by S. Hauschka), four reiterated MEF1 sites driving the tk promoter (4Rtk-CAT) [H. Weintraub *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5623 (1990)], or the CMV-promoter (CMV-CAT) from the pCSA plasmid (provided by T. Roberts).
21. For immunoprecipitations, 10T1/2 cells were transfected and cultured as described (*20*). For MyoD immunoprecipitation, cells were lysed in NP-40 lysis buffer [50 mM tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, and 20% glycerol with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin (10 μg/ml each), aprotinin (100 μg/ml), and phosphatase inhibitors (10 mM $NaPPO_4$, 1 mM NaF, 0.1 mM Na_3VO_4)]. Cell extracts were sonicated and precleared with Zysorbin (Zymed Laboratories) and centrifugation. Extracts, normalized to DNA content by fluorometry (Hoefer Scientific), were immunoprecipitated with a rabbit polyclonal antiserum to the COOH-terminus of MyoD (*13*) and protein A–Sepharose beads. After immunoprecipitation, beads were washed with NP-40 buffer without phosphatase inhibitors, split, and resuspended in SDS gel loading buffer or in CIP buffer [10 mM tris (pH 8.0), 1 mM $MgCl_2$, 1 mM PMSF, aprotinin (1 μg/ml)] for phosphatase treatment with 50 U of CIP (New England Biolabs) at 50°C for 60 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), immunoblotted with a monoclonal antibody to MyoD (5.8A) (provided by P. Houghton), and analyzed by enhanced chemiluminescence (Amersham). For detection of hemagglutinin (HA)-tagged D-cyclins (provided by P. Hinds), cells were labeled for 3 hours with ^{35}S-methionine (TransLabel, ICN), lysed in RIPA buffer [10 mM tris (pH 7.4), 200 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors as described (*21*)], sonicated, and precleared as described (*21*). Extracts, with equivalent ^{35}S-methionine incorporation, were immunoprecipitated with a monoclonal antibody to the HA epitope (12CA5) [J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988)] and analyzed by SDS-PAGE and fluorography.
22. Whole-cell extracts from C2C12 myoblasts (MB) (subconfluent and growing in GM) or differentiated myotubes (MT) (in DM for 72 hours) were made in NP-40 lysis buffer as described (*21*). Proteins were separated by SDS-PAGE and analyzed by immunoblotting and enhanced chemiluminescence (Amersham). Cyclin D1 was detected with mAb D1-72-13G, D3 was detected with mAb D3-18B6-10B, and Cdk4 was detected with rabbit antiserum Rz (*15*) (provided by C. Sherr).

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p53-Independent Expression of $p21^{Cip1}$ in Muscle and Other Terminally Differentiating Cells

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Terminal differentiation is coupled to withdrawal from the cell cycle. The cyclin-dependent kinase inhibitor (CKI) $p21^{Cip1}$ is transcriptionally regulated by p53 and can induce growth arrest. CKIs are therefore potential mediators of developmental control of cell proliferation. The expression pattern of mouse p21 correlated with terminal differentiation of multiple cell lineages including skeletal muscle, cartilage, skin, and nasal epithelium in a p53-independent manner. Although the muscle-specific transcription factor MyoD is sufficient to activate p21 expression in 10T1/2 cells, p21 was expressed in myogenic cells of mice lacking the genes encoding MyoD and myogenin, demonstrating that p21 expression does not require these transcription factors. The p21 protein may function during development as an inducible growth inhibitor that contributes to cell cycle exit and differentiation.

Proper development of a multicellular organism is complex and requires precise spatial and temporal control of cell proliferation. A large network of regulatory genes has evolved to specify when and where in the embryo cells divide. This control is superimposed upon the basic cell cycle regulatory machinery.

Cell proliferation requires the action of cyclins, which serve as activators of their cognate cyclin-dependent kinases (Cdks). (*1*) D- and E-type cyclins have been implicated in controling passage through the "restriction" point (*2*), after which cells become committed to a round of cell division (*3*). G_1 cyclin accumulation is required for cell cycle entry and members of this family, particularly D-cyclins, have been identified as targets of growth factors (*1*).

Equally important in the execution of developmental programs is the arrest of growth once the program is complete. Whereas the control of terminal differentiation may be mediated by multiple, possibly redundant, mechanisms, cell cycle arrest through inactivation of Cdks is likely to be a central feature. Possible mediators of such negative control are two classes of Cdk inhibitory (CKI) proteins typified by $p21^{CIP1}$ (*4*) and $p16^{INK4/MTS1}$ (*5*). The p21 protein inhibits G_1 cyclin complexes containing Cdk2, Cdk3, Cdk4, and Cdk6 and is transcriptionally induced by overexpression of the tumor suppressor protein p53 (*6*) or by activation of p53 after DNA damage, consistent with a role for p21 in the p53-dependent G_1 checkpoint (*7*).

The ability of p21 to function as an inducible cell cycle inhibitor suggests that it

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