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Direct inhibition of G_1 cdk kinase activity by MyoD promotes myoblast cell cycle withdrawal and terminal differentiation

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MyoD has been proposed to facilitate terminal myoblast differentiation by binding to and inhibiting phosphorylation of the retinoblastoma protein (pRb). Here we show that MyoD can interact with cyclin-dependent kinase 4 (cdk4) through a conserved 15 amino acid (aa) domain in the C-terminus of MyoD. MyoD, its C-terminus lacking the basic helix–loop–helix (bHLH) domain, or the 15 aa cdk4-binding domain all inhibit the cdk4-dependent phosphorylation of pRb *in vitro*. Cellular expression of full-length MyoD or fusion proteins containing either the C-terminus or just the 15 aa cdk4-binding domain of MyoD inhibit cell growth and pRb phosphorylation *in vivo*. The minimal cdk4-binding domain of MyoD fused to GFP can also induce differentiation of C2C12 muscle cells in growth medium. The defective myogenic phenotype in MyoD-negative BC3H1 cells can be rescued completely only when MyoD contains the cdk4-binding domain. We propose that a regulatory checkpoint in the terminal cell cycle arrest of the myoblast during differentiation involves the modulation of the cyclin D cdk-dependent phosphorylation of pRb through the opposing effects of cyclin D1 and MyoD.

Keywords: cell cycle/G_1 cdks/MyoD/myogenesis

Introduction

Following the discovery of the MyoD family of muscle gene regulatory factors and their myogenic and growth suppressive effects, these proteins were shown to play a role in the maintenance of the differentiated state and the regulation of the myoblast cell cycle (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990; Buckingham, 1992; Olson, 1992; Emerson, 1993; Weintraub, 1993; Lassar and Munsterberg, 1994; Lassar *et al*., 1994). The retinoblastoma protein (pRb) was proposed to participate in this regulation since there was a direct correlation between the phosphorylation status of pRb and the cycling state of the cell (Chen *et al*., 1989; Furukawa *et al*., 1990). Forced expression of MyoD in a variety of different cell backgrounds, including normal, transformed and tumor cell lines, was shown to induce growth arrest and this was correlated with the presence of the MyoD basic helix–loop–helix (bHLH) domain (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990). Furthermore, growth arrest required functional pRb (Schneider *et al*., 1994; Novitch *et al*., 1996). *In vitro* protein binding and immunoprecipitation studies indicated that both MyoD and myogenin bound to underphosphorylated pRb directly through the bHLH domain (Gu *et al*., 1993). A general model emerged suggesting that pRb mediates terminal cell cycle withdrawal during muscle cell differentiation by interacting directly with the myogenic factors to block pRb phosphorylation and cell growth.

Evidence for another linkage between MyoD and cell cycle regulatory proteins came from the demonstration that the ectopic expression of cyclin D1 inhibited MyoD activation of a muscle-specific reporter (Rao *et al*., 1994; Skapek *et al*., 1995) independently of pRb phosphorylation (Skapek *et al*., 1996). We have recently found that cyclin D1 inhibits myogenesis by promoting the nuclear translocation of cyclin-dependent kinase 4 (cdk4). Nuclear cdk4 disrupts the DNA-binding and transactivation functions of MyoD through the formation of a MyoD–cdk4 complex that does not require cdk4 kinase activity (J.-M.Zhang *et al*., 1999). These observations suggested that MyoD may be acting in two ways, one to form a transcriptionally inactive complex with cdk4 and the second to inhibit pRb function through an interaction with cdk4.

Experiments presented here demonstrate that the MyoD–cdk4 interaction, in addition to inhibiting the transactivation functions of MyoD, directly inhibits the cdk4 kinase activity. We can find no evidence for a direct MyoD or myogenin–pRb interaction using the two-hybrid system. The MyoD–cdk4 interaction does not involve the highly conserved bHLH domain but requires a conserved 15 amino acid (aa) domain in the C-terminus of MyoD. This interaction can inhibit cell growth and the cdk4-dependent phosphorylation of pRb *in vitro* as well as in 10T1/2 mouse fibroblasts. Evidence for the *in vivo* importance of this domain during myogenesis is supported by the observation that rescue of the defective myogenic phenotype of BC3H1 cells depends upon the minimal cdk4-binding domain in MyoD.

Results

Efforts to establish a propagating muscle cell line using mouse 10T1/2 cells transfected with the chicken MyoD homolog, CMD1, under the transcriptional control of the Rous sarcoma virus long terminal repeat (RSV LTR), were unsuccessful (Lin *et al*., 1989). In every instance only a few post-mitotic, myosin-positive single cells were observed, even in the mitogen-rich growth medium used to promote the expansion of any myogenically converted cells. In contrast, a stable, propagating muscle cell line could be established in growth medium if the SV40 promoter was used to express CMD1, a promoter that is ~5-fold weaker than the RSV LTR (Gorman *et al*., 1982).

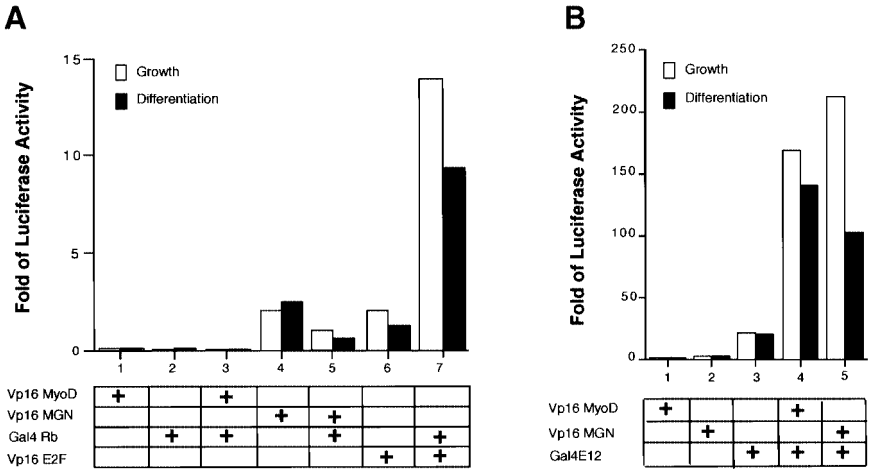


Fig. 1. In the mammalian two-hybrid system MyoD(CMD1) and myogenin (ch-myogenin) do not demonstrate significant interaction with pRb in C2C12 under growth or differentiation conditions. (**A**) gal4-pRb and vp16-E2F interact to activate luciferase expression 10- to 15-fold routinely (lane 7), whereas vp16-MyoD and vp16-myogenin show no significant activation above background with gal4-pRb (lanes 3 and 5). (**B**) Both vp16-MyoD and vp16-myogenin interact strongly with gal4-E12 (lanes 4 and 5).

This result suggested that only a limited concentration of MyoD within the cell was compatible with cell division and the absence of differentiation under standard growth conditions, and was consistent with the idea that high levels of MyoD in the cell resulted in sequestration of pRb and the subsequent inhibition of cell growth as previously proposed (Gu *et al*., 1993).

In order to determine whether either MyoD or myogenin was interacting directly with pRb in differentiating muscle, the mammalian two-hybrid system was employed in C2C12 muscle cells. Full-length human pRb and chicken E12 were each cloned into the gal4 DNA-binding domain vector and human E2F1, MyoD(CMD1) and myogenin [chicken (ch)-myogenin] were each inserted into the vp16 activation domain plasmid. Both gal4-pRb and vp16-E2F, even in the presence of the endogenous levels of these proteins, exhibited significant interaction (10- to 15-fold activation of the reporter) in this type of assay (Figure 1A, lane 7). In contrast, there was no evidence for any interaction between gal4-pRb and either vp16-MyoD or vp16-myogenin (ch-myogenin) in growing or differentiating muscle cells (Figure 1A, lanes 3 and 5). Both the vp16-MyoD(CMD1) and vp16-myogenin(ch-myogenin) constructs used in the two-hybrid assay interacted strongly (Figure 1B, lanes 4 and 5) with gal4-E12 (ch-E12), a known partner for these myogenic factors (Lassar *et al*., 1991; Shirakata *et al*., 1993). As a further control, both the MyoD and myogenin vp16 constructs could efficiently convert 10T1/2 mouse fibroblasts to muscle cells (data not shown).

The failure to find any evidence for a pRb interaction with the myogenic factors during C2C12 differentiation suggested that MyoD was impacting pRb function indirectly, possibly by modulating the phosphorylation status of pRb through the G_1 cdks. To investigate this possibility further, we decided to test for a direct interaction between cdk4 and any of the myogenic factors. Results obtained both *in vivo* and *in vitro* demonstrated that, of the four vertebrate myogenic factors, only MyoD bound specifically to cdk4, either as a cdk4–cyclin D1 complex or with cdk4 alone (J.-M.Zhang *et al*., 1999). In order to map the MyoD cdk4-binding domain, various portions of MyoD(CMD1) fused to glutathione *S*-transferase (GST) (Figure 2A) were bound to glutathione agarose and used in binding experiments (Kaelin *et al*., 1991) with cdk4 produced in the baculovirus system (Kato *et al*., 1993). In this assay, cdk4 bound tightly to the C-terminus of MyoD(CMD1) in the absence of the bHLH domain (Figure 2B, lane 4). Further deletion mapping of the MyoD(CMD1) C-terminus located the cdk4-binding region to a maximum of 15 aa located between residues 189 and 203 (Figure 2B, lanes 4 and 5). To confirm that these 15 aa in MyoD were responsible for the binding of cdk4, the peptide was joined to GST and tested for its ability to bind the baculovirus-produced cdk4 present in Sf9 cell extracts. The MyoD C-terminal 15 aa domain bound cdk4 as efficiently as full-size MyoD(CMD1) (Figure 2D, lanes 2 and 3).

We have previously shown that cdk4 can bind to MyoD to block DNA binding in the absence of cdk4 kinase activity (J.-M.Zhang *et al*., 1999). We now wanted to test the possibility that MyoD could also inhibit the ability of cdk4 to phosphorylate pRb. Using an *in vitro* cdk4-dependent pRb kinase assay, both MyoD(CMD1) and myogenin (ch-myogenin), the latter used as a control since it does not bind cdk4 (J.-M.Zhang *et al*., 1999), were tested for their ability to inhibit the phosphorylation of pRb at serine 780 using aa 769–928 of pRb joined to GST, a standard target for cdk4 phosphorylation studies *in vitro* (Kato *et al*., 1993). Extracts produced from Sf9 cells co-infected with virus stocks encoding cyclin D1 and cdk4 were used as a source of active kinase (Kato *et al*., 1993). Either direct phosphorylation with γ-ATP or antibody binding to phosphoserine 780 were used to measure the phosphorylation status of the pRb target (Kitagawa *et al*., 1996; Knudsen and Wang, 1997). Using extracts from Sf9 cells infected singly with either wild-type virus, cyclin D1- or cdk4-encoding virus stocks produced background levels of GST–pRb phosphorylation (Figure 3A, lanes 1–3). The co-infected extracts phosphorylated GST–pRb specifically since phosphorylation was inhibited by >90% with the addition of p16 to the kinase reaction (Serrano *et al*., 1993; Okamoto *et al*., 1994) (Figure 3A, lanes 4 and 5). Full-size as well as the

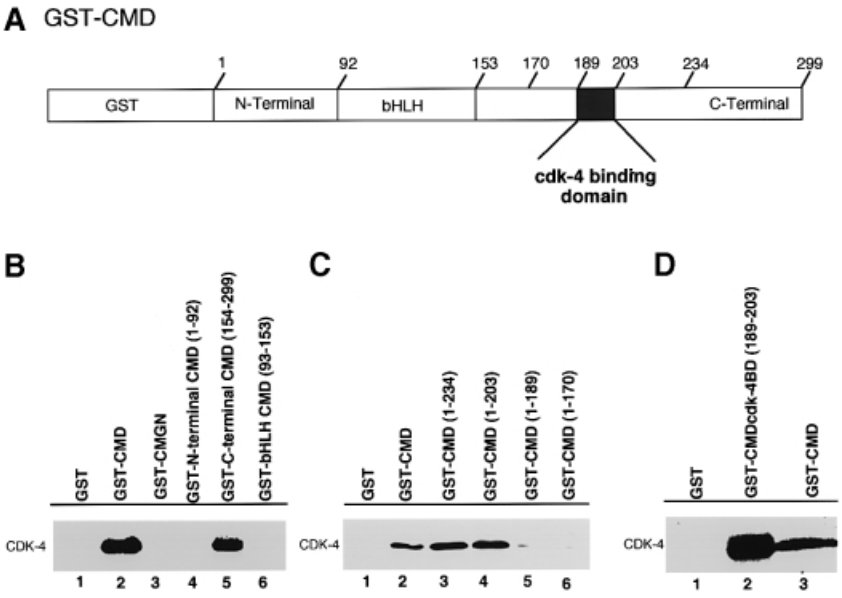


Fig. 2. Characterization of the cdk4-binding domain in MyoD(CMD1). (**A**) Map of GST–MyoD(CMD1) defining the protein regions used to study MyoD–cdk4 interactions: N-terminus (aa 1–92), bHLH (aa 93–152), C-terminus (aa 153–299). (**B**) cdk4 produced in Sf9 cells binds to the C-terminus of MyoD (lane 4) but does not bind to the other vertebrate myogenic factors (J.-M.Zhang *et al*., 1999). (**C**) Further deletion analysis defines a 15 aa domain between 189 and 203 that is required for cdk4 binding (lanes 4 and 5). (**D**) The 15 aa cdk4-binding domain in MyoD (aa 189–203) binds cdk4 as efficiently as full-size MyoD when joined to GST (lanes 2 and 3).

C-terminal domain of MyoD(CMD1), both expressed and purified from *Escherichia coli* as His_6-tagged proteins (Shirakata *et al*., 1993; Kato *et al*., 1994), were titrated into the cdk4-dependent phosphorylation reaction to test their ability to inhibit kinase activity. His-tagged myogenin, which does not bind to cdk4, was again used as the control. Both the full-size and the C-terminus of MyoD(CMD1) inhibited the phosphorylation of pRb in a concentration-dependent manner (Figure 3B, second and third rows, respectively), whereas myogenin (ch-myogenin) had no inhibitory effect (Figure 3B, top row).

The 15 aa cdk4-binding domain from MyoD(CMD1) joined in-frame to GST was also able to inhibit specifically the phosphorylation of the pRb target (Figure 3B, bottom row) as efficiently as full-size MyoD(CMD1), whereas neither the bHLH domain nor GST protein alone were inhibitory in the reaction and behaved similarly to myogenin (data not shown). Therefore, the MyoD–cdk4 interaction *in vitro* resulted in the specific inhibition of pRb phosphorylation on serine 780, a known target for cdk4 phosphorylation (Kitagawa *et al*., 1996; Knudsen and Wang, 1997). Similar results were obtained with antibodies to serine 795, another cdk4 phosphorylation site (data not shown). These results lead us to predict that the forced expression of the MyoD cdk4-binding domain should inhibit cdk4 in cells to block the phosphorylation of pRb and to arrest cell growth.

To test whether or not expression of either the C-terminus or the minimal 15 aa cdk4-binding domain of MyoD(CMD1) could induce growth arrest in cultured cells, the previously described growth assay measuring BrdU incorporation was employed (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990). 10T1/2 cells in growth medium were cotransfected with plasmids expressing full-size MyoD(CMD1) or the indicated domains, either as GST fusions in GST–pCEFL or as subclones in pcDNA3, along with the lacZ expression construct pCH110 to mark transfected cells. A typical result used in the scoring assay to measure the percentage of BrdU-labeled cells expressing lacZ is shown (Figure 4A). Because active cdk4 and MyoD are both nuclear proteins, all the MyoD constructs used in these experiments were targeted to the nuclei of transfected cells, as illustrated here for the 15 aa cdk4-binding domain fused to GST (Figure 4B). Full MyoD(CMD1) demonstrated growth inhibitory activity, in agreement with previous reports (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990) (Figure 4C), whereas the MyoD(CMD1) N-terminus (aa 1–92) was ineffective in growth inhibition. The bHLH domain (aa 93–153) and, to a greater extent, the C-terminus (aa 153–298) were both effective at inhibiting cell growth (Figure 4C, upper section). The empty vectors (pcDNA3 or GST–pCEFL) did not inhibit cell growth (Figure 4C). Consistent with the binding experiments and the *in vitro* phosphorylation studies, the 15 aa cdk4-binding domain from the C-terminus (aa 189–203) was nearly as potent as full-size MyoD(CMD1) in growth inhibitory activity (Figure 4C, lower section).

To determine whether growth arrest by MyoD was correlated with a change in the phosphorylation status of pRb, 10T1/2 cells were cotransfected with expression plasmids for a pRb target (aa 767–928 of pRb joined to GST targeted to the nucleus) and MyoD(CMD1) or the various subdomains of MyoD indicated in Figure 4. Whole cell extracts were prepared 48 h after transfection and passed over glutathione agarose to capture the GST–pRb target for Western blot analysis with phosphoserine-specific antibodies to the cdk4 target sites, serines 780 and 795. Gel loadings for phosphoserine analysis were adjusted for equal amounts of GST–pRb target by preliminary Western analysis (Figure 5A, middle row). The full-size MyoD(CMD1) (Figure 5B, top row), the C-terminal domain (Figure 5B, third row down) and the 15 aa cdk4-binding domain (Figure 5B, fourth row down), reduced

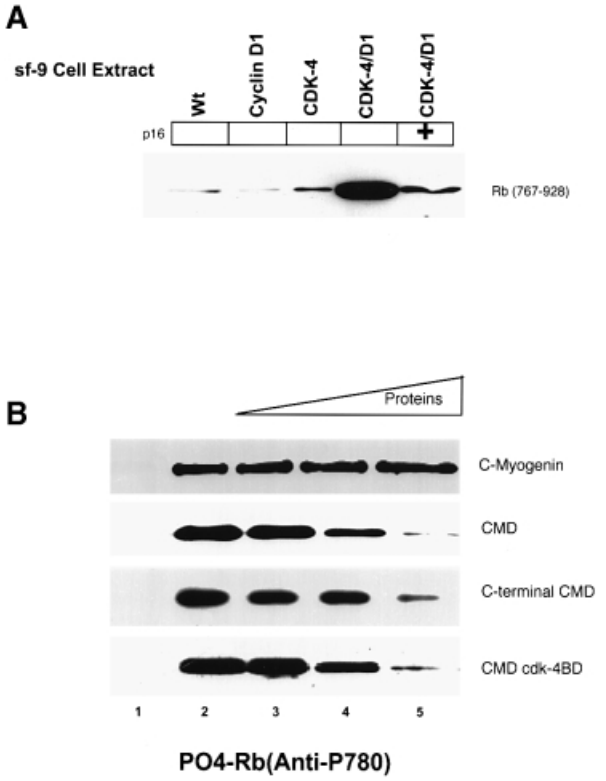


Fig. 3. Either full-size MyoD(CMD1), the C-terminus containing the 15 aa cdk4-binding region, or GST fused with the 15 aa cdk4-binding site all inhibit the cdk4-dependent *in vitro* phosphorylation of pRb in a dose-dependent fashion. (**A**) Extracts from Sf9 cells co-infected with wild-type virus (lane 1), a cyclin D1-encoding virus (lane 2) or a cdk4-encoding virus (lane 3) give background levels of kinase activity on serine 780 (antibody to phosphoserine 780). Co-infections with baculovirus stocks encoding cdk4 and cyclin D1 produce active cdk4 (lane 4) that specifically phosphorylates pRb (GST–pRb; aa 767–928) on serine 780 and this phosphorylation is inhibited to background levels by the cdk4-specific kinase inhibitor, p16 (lane 5, added as GST–p16). (**B**) Full-size MyoD(CMD1) (second row), the C-terminus of MyoD(CMD1) (third row) and the 15 aa cdk4-binding domain fused to GST (fourth row) inhibit the cdk4-dependent phosphorylation of pRb (GST–pRb) *in vitro*, whereas myogenin (first row) has no inhibitory affect. Proteins were added to the kinase reaction in 2-fold increments from 75 to 300 ng. Proteins encoding the N-terminus and the bHLH domains of MyoD or GST alone were also not inhibitory in this assay and behaved like myogenin (data not shown).

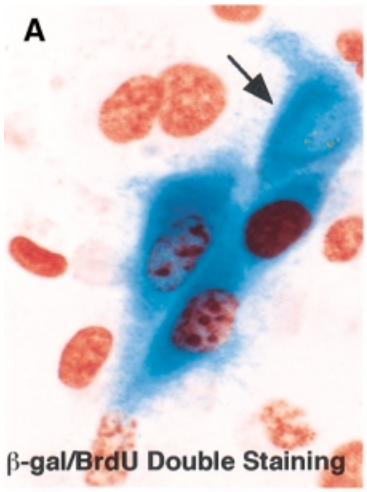
the phosphorylation of serines 780 and 795 on the cotransfected pRb target protein. In agreement with the growth inhibition studies, the N-terminus of MyoD (Figure 5B, second row) had no inhibitory effect on the phosphorylation status of the pRb target *in vivo*. Although the bHLH domain does not bind to (Figure 3C) or inhibit cdk4 kinase activity directly *in vitro* (data not shown), expression of the bHLH domain in cells resulted in the decreased phosphorylation of the pRb target, consistent with the growth inhibitory effects of this domain (Figure 5B, bottom row).

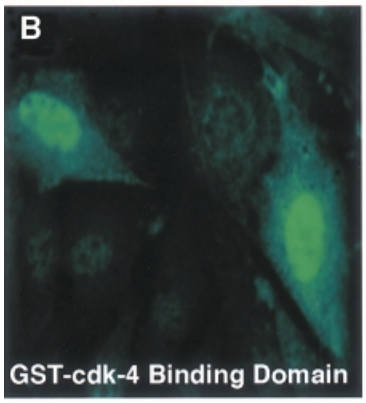
The growth arrest and pRb phosphorylation studies with the 15 aa cdk4-binding domain predict that this domain should facilitate cell cycle withdrawal and terminal differentiation of myoblasts even in moderate levels of serum. To test this directly, the 15 aa cdk4-binding domain with a nuclear localization signal (NLS) was fused to the C-terminus of GFP and transfected into C2C12 myoblasts maintained in 10% fetal calf serum (FCS; growth medium). GFP without the domain was expressed in both the nuclear and cytoplasmic compartments of undifferentiated myoblasts (Figure 6A). GFP containing the 15 aa MyoD cdk4-binding domain was expressed in the nuclei of well formed myotubes maintained in growth medium (Figure 6B). We conclude that the MyoD cdk4-binding domain itself is sufficient to promote cell cycle exit and terminal differentiation in C2C12 myoblast in growth medium, consistent with its inhibitory effects on cdk4 function and the reduced phosphorylation of pRb.

We have shown that the MyoD cdk4-binding domain can regulate cell growth and enhance terminal differentiation through its regulatory interaction with cdk4. However, in order to determine whether this type of interaction plays a similar role during myogenesis *in vivo*, we looked at the myogenic conversion of BC3H1 cells, which are negative for MyoD expression (Brennan *et al*., 1990), with our different MyoD constructs. Although BC3H1 cells express some skeletal muscle markers in differentiation medium, the cells are defective in their myogenic phenotype, do not fuse or express fast myosin heavy or light chains and do not commit to terminal differentiation. Ectopic expression of MyoD in BC3H1 cells will restore fusion and the expression of myosin light chain and will commit the cells to terminal differentiation, similar to that seen is skeletal muscle (Brennan *et al*., 1990). Myogenic conversion of BC3H1 cells with a MyoD lacking a portion of the C-terminus that includes the cdk4-binding domain (aa 1–189; see Figure 2D) resulted in the formation of a few predominantly mononuclear myocytes expressing fast skeletal muscle myosin, a marker for fast myosin light chain (Figure 6C). These converted BC3H1 cells looked similar to primary MyoD$^{-/-}$ muscle cells cultured from the knockout mouse (Sabourin *et al*., 1999). However, myogenic conversion with the same C-terminal truncated MyoD containing just the additional 15 aa of the cdk4-binding domain (aa 1–203; see Figure 2D) resulted in the formation of fast skeletal muscle MHC positive multinucleated myotubes indistinguishable from cells converted with full-size MyoD (Figure 6C). Thus, the MyoD cdk4-binding domain appears necessary in order for BC3H1 myogenic cells to commit to terminal differentiation even though BC3H1 cells express high levels of myogenin (Brennan *et al*., 1990). This rescue is not simply due to the inhibition of cdk4 since transfection of BC3H1 cells with the GFP cdk4-binding domain fusion that triggered C2C12 differentiation in growth medium (Figure 6B) did not rescue fusion or fast myosin heavy chain expression. The cdk4-binding domain must function in the context of MyoD to effect rescue.

Discussion

The two-hybrid system has been used successfully to determine the *in vivo* interactions of a variety of proteins involved in transcription control, signal transduction, cell cycle regulation and early development (Fields and Sternglanz, 1994). Our attempts to detect interaction between MyoD or myogenin and pRb in the two-hybrid assay were unsuccessful. In contrast to the suggestion from *in vitro* immunoprecipitation studies, neither MyoD nor myogenin were found to interact with pRb in C2C12 muscle cells under growth or differentiation conditions (Gu *et al*.,





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CDK-4 Binding		CMD aa	No. BrdU+ Cells 500 β-gal+ Cells (n=6)	%Inhibition
		Vector	88.4 ± 6.2	0 %
+	N-CMD bHLH C-CMD	1-299	**37.1 ± 4.2**	**58 %**
−	N-CMD	1-92	86.8 ± 4.8	2 %
−	bHLH	93-153	58.4 ± 3.9	34 %
+	C-CMD	153-299	**46.9 ± 6.4**	**47 %**
−	GST N-CMD	1-92	96.7 ± 5.9	0 %
+	GST	189-203	**57.4 ± 4.3**	**41 %**

Nucleal Localization Signal (NLS) CMD cdk-4 Binding Domain

Fig. 4. Inhibition of cell growth by MyoD(CMD1) and its subdomains as measured by BrdU incorporation. (**A**) Cells transfected with MyoD(CMD1) or its subdomains are marked with a lacZ (blue) expression vector and are analyzed for BrdU incorporation by antibody staining (brown). Note the blue cell in the upper right corner (marked by the arrow) that has no brown nuclear staining. (**B**) The MyoD(CMD1) domains lacking NLSs were constructed with an NLS to target nuclear expression. GST fused to the 15 aa cdk4-binding domain with an NLS is localized to nuclei. (**C**) Growth inhibitory effects of the various MyoD(CMD1) subdomains in either the pCDNA3 or GST–pCEFL vector. The number of BrdU-positive cells per 300 lacZ-positive cells, normalized to vector and averaged for six independent assays is shown. In the top panel constructs were expressed with the pCDNA3 vector. In the lower panel constructs were fused to eukaryotic expression vector, GST–pCEFL, and contained an NLS.

1993). However, all the control interactions between pRb-E2F and MyoD/myogenin/E12 were clearly seen, even with endogenous levels of these proteins competing in the two-hybrid assay in some instances. Thus, if there is any direct interaction between pRb and the myogenic factors in C2C12 cells it appears to be weak. In support of our observations, early targets of MyoD transcription are fully induced in cells lacking pRb, making the significance of the *in vitro* MyoD–pRb interaction questionable (Novitch *et al*., 1996).

Observations that do not mandate a direct interaction between MyoD and pRb during terminal differentiation have also been reported. For example, it has been shown in transfection studies that MyoD can upregulate the promoter for the cdk inhibitor protein p21, implying similar regulation during myogenesis (Halevy *et al*., 1995). However, mice lacking both the MyoD and myogenin genes have normal expression patterns of p21 (Parker *et al*., 1995), and mice lacking the p21 gene develop normally, suggesting that the role of p21 in myogenesis is either redundant or minor (Deng *et al*., 1995). Interestingly, mice lacking both p21 and p57 fail to form myotubes, and myoblasts show increased proliferation and apoptosis (P.Zhang *et al*., 1999), suggesting that p21 and p57 redundantly control skeletal muscle differentiation. p57 is predominantly expressed in differentiated tissues, and the 7 kb mRNA for this inhibitor is only detectable in skeletal muscle and heart, so its importance in cdk regulation in muscle may be dominant compared with p21 (Lee *et al*., 1995; Yan *et al*., 1997). It has also been proposed that active cdk4 in dividing myoblasts may phosphorylate MyoD to inhibit MyoD function (Skapek *et al*., 1995). Data presented here, however, demonstrate that cdk4 is unlikely to phosphorylate MyoD since MyoD can inhibit cdk4 kinase activity. Phosphopeptide analysis of cdk4 kinase reactions carried out *in vitro* with MyoD as substrate, in the presence and absence of the cdk4 inhibitor p16, give no evidence for cdk4-dependent phosphorylation of MyoD (data not shown).

We have demonstrated that growth inhibition by MyoD not only involves the highly conserved bHLH region, previously implicated in the MyoD interaction with pRb

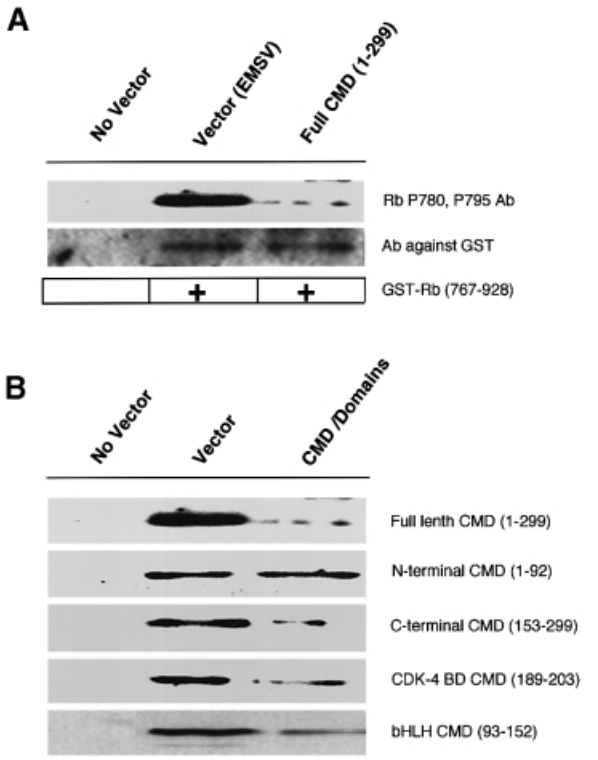

Fig. 5. Inhibition of pRb phosphorylation in C2C12 myoblasts by various domains of MyoD(CMD1). (**A**) Myoblasts were cotransfected with a GST–pCEFL pRb expression plasmid (pRb aa 767–928) containing an NLS along with an expression plasmid for either MyoD [EMSV MyoD(CMD1)] or the empty vector. GST–pRb was recovered from whole cells, adjusted for equal levels of GST protein in each sample (middle row) and loaded on SDS–PAGE for Western blot analysis by pRb phosphoserine 780 and 795-specific antibodies (top row). Expression of MyoD inhibits the phosphorylation of serines 780 and 795 in pRb. (**B**) The N-terminus of MyoD does not inhibit pRb phosphorylation *in vivo*, whereas the C-terminus, the 15 aa cdk4-binding domain and the bHLH region of MyoD all inhibit phosphorylation on pRb serines 780 and 795.

and growth arrest (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990; Gu *et al*., 1993), but is also dependent upon a 15 aa cdk4-binding domain in the C-terminus that blocks cdk4 kinase activity. Previous studies on the growth arrest properties of MyoD took advantage of a series of mutations and deletions developed for the initial characterization of MyoD function (Tapscott *et al*., 1988). One mutation, B2ProB3, which contains a proline residue between the second and third basic cluster in the DNA-binding domain, did not bind DNA or induce differentiation, yet still exhibited a relatively high level of growth arrest (Crescenzi *et al*., 1990), suggesting that DNA binding and transcriptional activation were not prerequisites for growth inhibition. This observation is consistent with our growth arrest studies using the 15 aa cdk4-binding domain in the C-terminus of MyoD. The mechanism underlying the growth inhibitory activity of the bHLH domain remains to be determined but it has been reported that myogenin, MRF4 and the E2A (E12/E47) proteins all inhibit the growth of NIH 3T3 fibroblasts and this inhibition depends upon the dimerization domain (Peverali *et al*., 1994). In the cases involving growth arrest by E47 and MyoD, the HLH protein Id reversed the growth-suppressive effects suggesting that DNA binding and transcriptional activity of both E47 and MyoD were involved in growth regulation.

Although the MyoD$^{-/-}$ mouse develops normally, recent studies have demonstrated that MyoD expression is required in order for satellite cells to become differentiation-competent myogenic precursor cells during muscle regeneration (Megeney *et al*., 1996). Furthermore, primary MyoD$^{-/-}$ myogenic cells grown *in vitro* continue to proliferate to yield predominantly mononuclear myocytes under conditions that normally induce differentiation, even though they express 4-fold higher levels of myf-5 mRNA and 20-fold more protein. This defect can be rescued by the ectopic expression of MyoD (Sabourin *et al*., 1999). A similar 'knockout' phenotype was observed in our studies with BC3H1 cells stably transfected with MyoD truncated on the C-terminus to remove the cdk4-binding domain. The normal conversion phenotype seen with full-size MyoD, i.e. cell fusion, expression of fast myosin light chain and commitment to terminal differentiation, was restored by just adding the 15 aa cdk4-binding domain to the truncated MyoD. This suggests that MyoD plays an additional role during terminal differentiation of the myoblast, possibly by suppressing any residual nuclear cyclin D cdk activity along with the other cyclin kinase inhibitors (Lassar and Munsterberg, 1994). Our results do not rule out the possibility that the 15 aa domain interacts with additional protein(s) other than the cdks to rescue the differentiation defect in BC3H1 cells. This notion is supported by the observation that transfection of the domain itself or p16, the cdk4 inhibitor, does not rescue myogenesis (data not shown), so suppression of cdk4/6 activity is not the sole mechanism of action of this domain in the context of MyoD.

The cdk4-binding domain itself has been relatively well conserved in the MyoD proteins of mammals, but the amino acid sequence homology is reduced in the MyoD-related proteins from the lower vertebrates and is only partially conserved in the invertebrate MyoD family members characterized to date (Table I). Preliminary experiments indicate that the MyoD homologs from *Drosophila* (*nautilus*) (Paterson *et al*., 1991) and *Caenorhabditis elegans* (*hlh-1*) (Chen *et al*., 1994) can specifically bind vertebrate cdk4 and can inhibit cell growth in the BrdU incorporation assay. The cdk4-binding domains have not yet been characterized in either of these proteins but the conservation of the bHLH domain within the MyoD family suggests that binding does not involve this region since only MyoD bound cdk4.

The concentration-dependent MyoD inhibition of cdk4 activity *in vitro* coupled with the MyoD-dependent repression of pRb phosphorylation in cells provides an explanation for our inability to establish a muscle cell line with RSV MyoD(CMD1) (Lin *et al*., 1989). Moderate levels of MyoD expression driven by the SV40 promoter are compatible with cell division and suggest that there is enough active cdk4 not bound to MyoD to drive cell cycle functions. Higher levels of nuclear MyoD resulting from the higher expression with the RSV LTR promoter titrate all the active cdk4 and trigger exit from the cell cycle and the differentiation of single myoblasts, even in high serum growth conditions.

Preliminary results on the interaction between cdk6, the other G_1 cyclin D cdk, and MyoD indicate similar binding to the 15 aa cdk4-binding domain (B.M.Paterson, unpublished observations). This is not suprising since cdk4 and

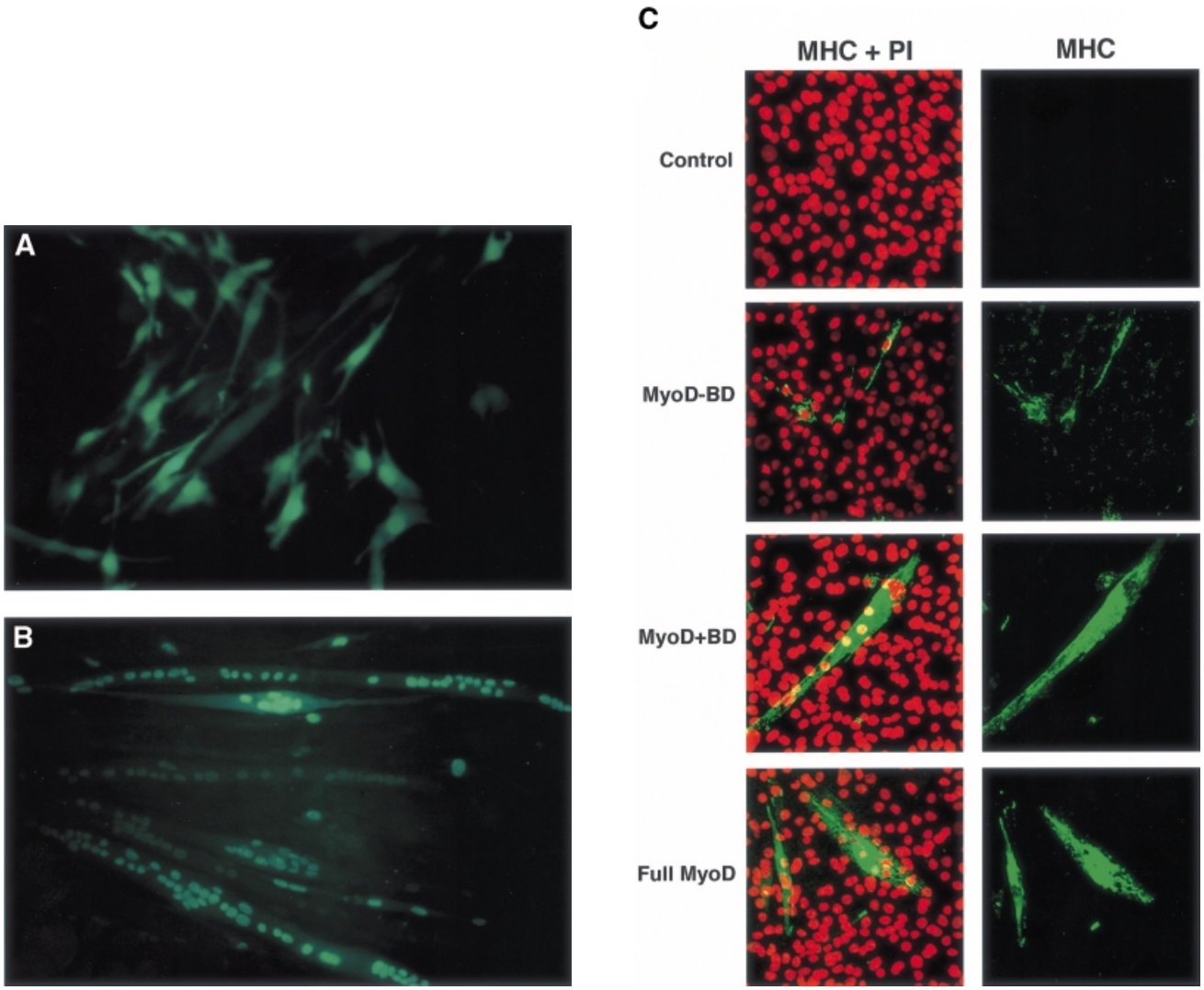

Fig. 6. The 15 aa cdk4-binding domain of MyoD(CMD1) can promote terminal differentiation of C2C12 myoblasts in growth medium and restore terminal differentiation in BC3H1 cells transformed by MyoD lacking the domain. (**A**) Myoblasts transfected with GFP vector alone are not fused and express GFP throughout in cells maintained in growth medium. (**B**) Myoblasts transfected with a GFP expression construct containing the 15 aa cdk4-binding domain with an NLS show nuclear GFP in fully differentiated myotubes maintained in growth medium. (**C**) BC3H1 cells stably transfected either with GFP–MyoD or GFP–MyoD deletions lacking or containing the 15 aa cdk4-binding domain. Control: untransfected cells stained for fast skeletal muscle MHC, a marker for fast myosin light chain. MyoD–BD: cells expressing C-terminal truncated MyoD missing the 15 aa cdk4-binding domain (–BD) and stained for fast skeletal muscle MHC. Note the MHC-positive single cells. MyoD+BD: cells expressing C-terminal truncated MyoD with the additional 15 aa cdk4-binding domain (+BD) and stained for fast skeletal muscle MHC. Note the terminally differentiated multinucleated fibers expressing MHC. Full MyoD: cells expressing the full-size MyoD and stained for fast skeletal muscle MHC.

Table I. Comparison of the cdk4-binding domains in vertebrate MyoDs

Species	Peptide sequence
Chicken	YSGPPCSSRRRNSYDS
Human	YSGPPSGARRRNCYEG
Rat	YSGPPSGPRRQNGYDA
Murine	YSGPPSGPRRQNGYDT
Pig	YSGPPSGARRRNCYDG
Consensus	YSGPPC/SS/G-RRR/QN-YD/E
Xenopus-A	YNSPPCGSRRRNSYD
Xenopus-B	YNSPPCSSRRRNSYD
Zebra fish	FMGPTCQTRRRNSYD

cdk6 are 71% similar at the amino acid level (Meyerson *et al*., 1992; Meyerson and Harlow, 1994). Western blot analysis indicates that the levels of cdk4 and cdk6 are similar in C2C12 cells, that both are translocated to the cytoplasm in differentiated muscle and that both can be relocated to the nuclei of myotubes with the ectopic expression of a stable cyclin D1 (Q.Wei and B.M.Paterson, unpublished data; J.-M.Zhang *et al*., 1999). Unlike the cdk4 binding studies with various domains of MyoD (Figure 2), our effort to map the MyoD-binding domain in cdk4 has not given a clear answer. Both MyoD and the 15 aa cdk4-binding domain seem to bind throughout cdk4. This result may reflect the fact that cdk4, like cdk2, consists of an N-terminal lobe of predominantly β-sheet (aa 1–85 in cdk2) joined to a larger, mostly α-helical, C-terminal lobe (Jeffrey *et al*., 1995). The cleft between these two lobes contains the ATP binding site and the catalytic residues conserved among all eukaryotic kinases (Lys33, Glu51 and Asp145 in cdk2), and is the presumed site for substrate binding and catalysis. Our results suggest

that MyoD binding is dependent upon contact points located throughout cdk4 on either face of the cleft between the N-terminal β-sheets and the α-helices in the C-terminus. Similar results were reported for cdk4 residues involved in cyclin D1 and p16 binding, although many of the mutations that reduced binding of these molecules were located in the N-terminal half of cdk4 (Coleman *et al*., 1997). Our initial results with the two-hybrid system suggest that MyoD binds nearer to the N-terminus of cdk4, since gal4-cdk4 fails to show interaction with vp16-MyoD *in vivo*, whereas gal4-MyoD and vp16-cdk4 show interaction. This could indicate steric hindrance with the formation of the gal4 dimer blocking access to the cdk4 N-terminus at the gal4-cdk4 junction. Structural studies will be likely to be the only way to resolve the nature of the MyoD–cdk4 interaction.

Virtually all cyclin D1-dependent kinase activity in proliferating mouse fibroblasts can be attributed to cdk4 (Miller, 1990; Matsushime *et al*., 1994) and cyclin D1 is the only ectopically expressed cyclin that will inhibit myogenesis, consistent with a unique role for the MyoD–cdk4 interaction (Rao *et al*., 1994; Skapek *et al*., 1995). Our results, however, do not rule out a similar interaction with cdk6 since we observe binding between the 15 aa MyoD cdk4-binding site and cdk6 (unpublished observations).

It would be of interest to know whether the presence of MyoD and pRb in cyclin D1–cdk4 complexes is mutually exclusive, as this could have implications with regard to the threshold levels for MyoD inhibition of cdk. However, attempts to identify complexes between pRb and the D-type cyclins in intact cells have been unsuccessful, and only overexpression in insect Sf9 cells has shown stable complex formation between pRb and cyclins D2 or D3, but not D1 (Kato *et al*., 1993). These complexes are rapidly destabilized by co-expression of cdk4; thus pRb–cyclin D complex formation is inhibited by cdk4-mediated phosphorylation of pRb (Ewen *et al*., 1993; Kato *et al*., 1993). This suggests that only MyoD–cyclin D1 cdk4 complexes would be formed as the pRb–D1 cdk4 complex is unstable, and inactive kinase would be cytoplasmic in location (J.-M.Zhang *et al*., 1999).

The dynamics of the MyoD–cdk4 interaction in the myoblast can be represented by the following model. The forced overexpression of MyoD inhibits the cdk4-dependent phosphorylation of pRb to trigger growth arrest and the exit from the cell cycle (likely to include cdk6 as well). Dephosphorylated pRb is thought to be required to maintain cell cycle arrest by inhibiting the growth promoting actions of E2F/DP family members, the induction of apoptosis, and DNA replication in myotubes (Novitch *et al*., 1996; Wang *et al*., 1996; Dyson, 1998). As we have shown, excess cyclin D1 activates more cdk4 and the complex is translocated to the nucleus where it inhibits MyoD and the activation of the myogenic program (J.-M.Zhang *et al*., 1999). Excess expression of nuclear cdk4 triggers phosphorylation of pRb allowing dissociation of E2F/DP and cell growth. On the other hand, excess ectopic expression of MyoD depletes active cdk4, prevents the phosphorylation of pRb and induces growth arrest while activating target genes to drive myogenesis. Thus, excess cyclin D1 and cdk4 induce growth and block differentiation, whereas excess MyoD induces exit from the cell cycle and differentiation. D-type cyclins act as growth factor sensors (Sherr and Roberts, 1999) and levels of cyclin D1 appear to be rate limiting in the formation of active cdk4, based upon the half-life of each protein (Diehl and Sherr, 1997). The ectopic expression of cyclin D1 can increase nuclear levels of cdk4 to shorten the G_1 phase of the cell cycle (Quelle *et al*., 1993). Thus, the relative nuclear ratios of MyoD and cyclin D cdks in the cell would appear to be key determinants in the cell cycle decisions of the myoblast during terminal differentiation, and this ratio is determined by growth factor modulation of cyclin D1 expression levels and the cyclin D1-dependent translocation of active cdk4 to the nucleus (J.-M.Zhang *et al*., 1999), as illustrated in the scheme in Figure 7.

The interaction between MyoD and the cyclin D cdks provides an explanation for the lack of DNA binding and transcriptional activation by MyoD in dividing myoblasts (Tapscott *et al*., 1988; Mueller and Wold, 1989). Furthermore, the mutual inhibitory interaction between MyoD and the cyclin D cdks described here can impact the cell cycle without requiring a direct interaction between MyoD and the pRb (Gu *et al*., 1993). In support of our observations, which fail to show interaction between MyoD and the pRb in C2C12 cells, early targets of MyoD transcription are fully induced in the absence of pRb (Novitch *et al*., 1996). Although p21 can be activated by MyoD during muscle differentiation (Halevy *et al*., 1995), more recent studies have shown that the cip/kip inhibitors p21 and p27 are essential activators of the cyclin D cdks and play a role in their assembly and nuclear import (LaBaer *et al*., 1997; Cheng *et al*., 1999; Sherr and Roberts, 1999). In support of this observation, cyclin D cdk pRb kinase activity is found in complexes containing the Cip/Kip proteins and cyclin D1 cdk4–p21/p27 complexes (LaBaer *et al*., 1997; Sherr and Roberts, 1999). *In vitro* binding studies show that one molecule of p21 bound to a cdk complex stimulates kinase activity, whereas a second bound molecule is inhibitory (Zhang *et al*., 1994). Thus, the induction of p21 by MyoD is not necessarily an immediate inhibitor of the cyclin D cdks, and MyoD may act as a more potent inhibitor of these kinases during terminal differentiation, although this will have to await a more detailed analysis.

Myf5 activity is also silenced in myoblasts and this apparently involves a different mechanism than the cdk4/6 regulation of MyoD (Zhang *et al*., 1999). Although an increase in the level of MyoD expression can trigger the rapid terminal differentiation of single myoblasts (Lin *et al*., 1989), in the $MyoD^{-/-}$ mouse myf5 mRNA is upregulated 4-fold with a concomitant 20-fold increase in myf5 protein expression, yet the myoblasts fail to make the normal transition from proliferation to differentiation and remain in a compartment of prolonged self renewal (Megeney *et al*., 1996; Sabourin *et al*., 1999; Yablonka-Reuveni *et al*., 1999). This may reflect the fact that Myf5 is absent from mitotic cells and is degraded during each cell cycle via a phosphorylation-dependent ubiquitylation pathway, whereas MyoD appears to be present throughout the cell cycle (Lindon *et al*., 1998). This result also suggests that once mitogens are reduced during terminal differentiation, myf5 phosphorylation would decrease allowing myf5 to reach the levels necessary to promote the activation of myogenin and the onset of myogenesis.

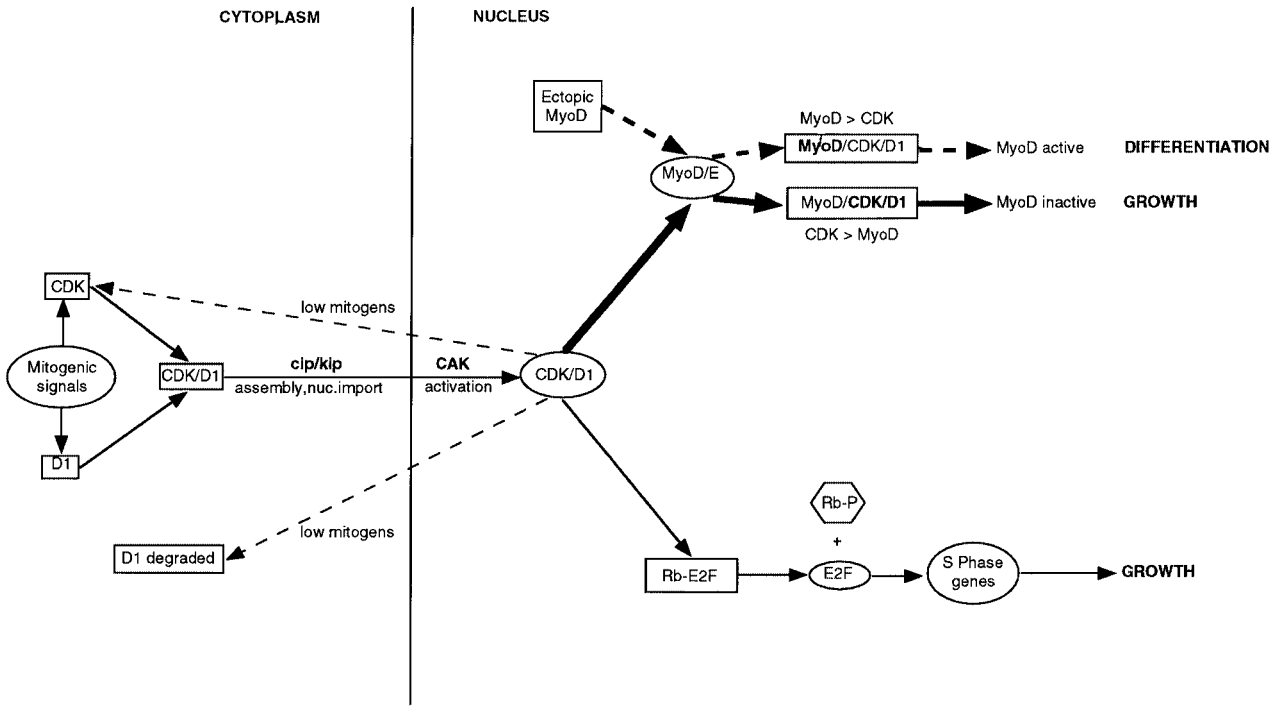

Fig. 7. The dynamic interaction between MyoD and nuclear cyclin D cdks regulates transcriptional activation by MyoD and the cdk-dependent phosphorylation of pRb. The net outcome of this interaction determines whether or not myoblasts will continue to divide (when active cyclin D cdks are in excess of MyoD, CDK > MyoD) or differentiate (when MyoD is in excess of nuclear cyclin D cdks, MyoD > CDK). In dividing myoblasts the path defined by the heavy black arrow predominates to keep MyoD inactive and E2F active (lower path marked by thin arrows). Ectopic MyoD, indicated by the heavy broken arrow, can drive differentiation by sequestering cyclin D cdks in an inactive complex, even in the presence of mitogenic signals. Normally, when mitogenic signals are reduced ('low mitogens'), cyclin D is degraded (thin broken arrow), cdks leave the nucleus to the cytoplasmic pool (thin broken arrow) and MyoD is able to activate target genes to drive differentiation. In this scheme, cdk4 and cdk6 are assumed to behave identically since cdk6 can also bind MyoD and is relocalized to myotube nuclei with the ectopic expression of stable cyclin D1, as reported previously for cdk4 (J.-M.Zhang *et al*., 1999) (B.M.Paterson, unpublished observations). Active molecules are outlined by an oval, whereas inactive molecules are outlined by a rectangle. The status of phosphorylated pRb is not clear.

Regardless of the presence or absence of MyoD, dividing myoblasts respond to reduced levels of mitogens to regulate the cyclin D cdks and eventually exit the cell cycle, as shown by the normal phenotype of the MyoD$^{-/-}$ mouse (Rudnicki and Jaenisch, 1995).

The fact that cdk4-deficient mice can live and develop relatively normal muscle although the mice are 50% smaller than wild-type litter mates, suggests that cdk6 is redundant in this regulatory pathway and may fill the role as the major cyclin D cdk when cdk4 is absent (Rane *et al*., 1999). Our recent studies with cdk6 suggest that it will behave like cdk4 with regard to MyoD binding and inhibition of kinase activity (B.M.Paterson, unpublished) so both the cyclin D cdks are potentially affected by MyoD during terminal muscle differentiation.

The differential regulation of the cyclin D cdks by MyoD and cyclin D1 places these cdks at a key switch point in myogenesis. Our observations raise the possibility that other terminally differentiating systems may use a similar regulatory mechanism involving the interaction of tissue-specific transcription factors with the cdks during exit from the cell cycle and the onset of differentiation.

Materials and methods

Plasmid constructs

RSV-CMD and pSV2-CMD constructs were described previously (Lin *et al*., 1989). For the mammalian two-hybrid assays, chicken MyoD(CMD1) and myogenin were PCR-amplified from pRSET clones (Shirakata *et al*., 1993) using Pfu polymerase (Stratagene), a 5′ primer containing a *Bam*HI site and a 3′ primer with a *Hin*dIII site, and cloned into the corresponding sites in the vp16 vector pVP16 (Clontech). E2F was cut from CMV-E2F (from E.Harlow; Helin *et al*., 1993) with *Bam*HI, flush ended with T4 DNA polymerase and cloned into pVP16 cut with *Mlu*I and flush ended as before. Human pRb (Kaye *et al*., 1990) was cloned as a flush *Bam*HI–Asp718 fragment into the flush *Mlu*I site in the gal4 vector pM (Clontech). Chicken E12 (Shirakata *et al*., 1993) was amplified by PCR as before with a 5′ primer containing a *Bgl*II site and a 3′ primer containing a *Hin*dIII site, and was cloned into the *Bam*HI–*Hin*dIII sites of pM. Various domains of MyoD(CMD1) shown in Figure 3 were amplified by PCR with Pfu, with 5′ primers containing a *Bam*HI site and 3′ primers with an *Eco*RI site, and were cloned into the same sites either in pCDNA3 (Invitrogen) or GST–pCEFL (a eukaryotic GST expression vector from S.Gutkind, NIDCR, NIH). An NLS (MCPKKRKV) was incorporated into the 5′ primer when the fragment being amplified did not have an NLS. MyoD deletions for expression in stably transformed BC3H1 cells contained MyoD aa 1–189 (–BD; no cdk4-binding domain), aa 1–203 (+BD; with the 15 aa cdk4-binding domain) and full-size MyoD fused in-frame to pEGFP-C1 (Clontech) using PCR-amplified DNA inserts made with specific primers to generate the necessary restriction sites. The GFP tag was used to look at BC3H1 cell myogenic conversion in transient assays but to no avail. The inserts were removed from the GFP plasmid as *Nhe*I (flush)–*Bam*HI inserts and cloned into pCEMSV, a modified pCDNA vector with the EMSV promoter (B.M.Paterson laboratory). For expression in bacteria, proteins were cloned either into the PGEX vectors (Pharmacia) or into the pRSET vectors (Invitrogen) cut with *Bam*HI–*Eco*RI for production of GST or His_6-tagged fusion proteins, respectively. Proteins were expressed in the *E.coli* strain BL21(DE3) and purified as before (Shirakata *et al*., 1993).

Cell culture and transfections

10T1/2 fibroblasts or C2C12 myoblasts (3×10^5 cells per 60 mm dish or multiwell plate) grown in Dulbecco's modified Eagle's medium

(DMEM) with either 10 or 20% FCS, respectively, were transfected with the various plasmid DNAs using Fugene-6 (Boehringer Mannheim) according to the manufacturer's directions. After 24–36 h, if required, cells were placed in 2% horse serum plus insulin (10 μg/ml) to induce differentiation. After an additional 24–48 h, cells were either harvested for reporter assays (luciferase) or Western blot analysis, or fixed in 10% formalin solution (Sigma HT50-1-2) for immunostaining and lacZ expression. Luciferase assays were performed with a Promega kit (catalogue No. E4030) and analyzed on a Victor 1420 multi-label counter. For the two-hybrid assays, 1 μg of gal4 luciferase reporter, 2 μg each of the gal4 and vp16 constructs, and 300 ng of a β-galactosidase expression plasmid, pCH110 (Pharmacia), were used for a total of 5.3 μg of DNA for each transfection. For the conversion assay, 1 μg of either RSV-CMD or pSV2-CMD was used. For growth inhibition studies, 2 μg of the various constructs along with 0.3 μg of pCH110 (lacZ) were transfected into 10T1/2 cells. Cultures were maintained in 20% FCS. Seventy-two hours after transfection cultures were refed with the same medium and 12 h later they were given a 1 h pulse with BrdU (10 μM) prior to fixation, as previously reported for growth inhibition studies with MyoD (Crescenzi *et al*., 1990; Sorrentino *et al*., 1990). Cells were then reacted with rabbit anti-BrdU antibody (Becton Dickinson) and Xgal. Stable transformants of BC3H1 cells expressing the various MyoD deletions joined to the C-terminus of the the pEGFP-C1 plasmid (Clontech) were grown in 20% FCS and selected in G418. Clones were pooled for a representative stock of stably transfected cells. Differentiation studies were performed as described previously (Brennan *et al*., 1990) with the following changes: cells were reacted with primary monoclonal antibody (MY32) to fast skeletal muscle myosin (Sigma, catalogue No. M4276), a marker for fast myosin light chain, and then with Alexa 488 green fluorophore secondary antibody (Molecular Probes). Nuclei were stained with propidium iodide and the images for myosin expression and nuclear localization were taken with a Bio-Rad confocal.

cdk4 kinase assays

Active cdk4 was produced in Sf9 cells co-infected with baculovirus stocks encoding cdk4 and cyclin D1 (from C.J.Sherr) and assayed as described (Kato *et al*., 1993). The cdk4-specific kinase inhibitor p16 (from D.Beach; Serrano *et al*., 1993) was used as a GST–p16 fusion added to the kinase reaction. The GST–pRb fusion (767–928) was used as a kinase target as previously described (Kato *et al*., 1993). To test for kinase inhibition, the various proteins and protein domains prepared as before (Shirakata *et al*., 1993; Kato *et al*., 1994) were added to the reaction in 2-fold increments from 75 to 300 ng. Total protein concentration was kept constant in the kinase reaction by adding either the His_6-tagged N-terminus of MyoD(CMD1) (aa 1–92), which does not bind cdk4, or GST protein, for the pCDNA3 constructs and the GST constructs, respectively. After the assay the total reaction was run on SDS–PAGE and Western blotted as described (see below). Specific phosphorylation of pRb was measured with rabbit polyclonal antibodies to pRb phospho-serines 780 or 795 (New England Biolabs).

Immunostainings and Western blots

For immunostaining cultures, the first antibodies for myosin or BrdU were used at 1:200–500 dilution (rabbit anti-myosin, Sigma; rabbit anti-BrdU, Jackson Laboratories; rabbit anti-GST, B.M.Paterson laboratory). The second antibody detection used the Vectastain Kit according to the manufacturer. Cultures were reacted with Xgal (Parker *et al*., 1995) to mark transfected cells in the growth inhibition assay. For Western blots on HyBond ECL (Amersham), primary antibody was diluted 1:2000–5000 (rabbit anti-cdk4, Santa Cruz; rabbit anti-GST, B.M.Paterson laboratory; rabbit anti-phosphoserine 780 and 795, New England Biolabs) and the second antibody, HRP anti-rabbit, 1:10 000 (Pierce, catalogue No. 31460). Signal was detected using SuperSignal Ultra Reagent (Pierce, catalogue No. 34075).

Protein function assays

cdk4-binding assays with the various glutathione agarose-bound GST fusion proteins were carried out essentially as described (Kaelin *et al*., 1991) using Sf9 cell extracts from cells infected with wild-type virus, cyclin D1-encoding virus, cdk4-encoding virus or extracts co-infected with cyclin D1- and cdk4-encoding viral stocks (Kato *et al*., 1993). Bacterial cdk4 bound to MyoD(CMD1) just as specifically as cdk4 produced in Sf9 cells (Shirakata *et al*., 1993; Kato *et al*., 1994). Washed beads were loaded directly onto SDS–PAGE for Western blot analysis with anti-cdk4 antibody as described above.

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References

Brennan,T.J., Edmondson,D.G. and Olson,E.N. (1990) Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of BC3H1 cells (published erratum appears in *J. Cell Biol.*, 1990, **110**, 2231). *J. Cell Biol.*, **110**, 929–937.

Buckingham,M. (1992) Making muscle in mammals. *Trends Genet*., **8**, 144–148.

Chen,L., Krause,M., Sepanski,M. and Fire,A. (1994) The *Caenorhabditis elegans* MYOD homologue HLH-1 is essential for proper muscle function and complete morphogenesis. *Development*, **120**, 1631–1641.

Chen,P.L., Scully,P., Shew,J.Y., Wang,J.Y. and Lee,W.H. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell*, **58**, 1193–1198.

Cheng,M., Olivier,P., Diehl,J.A., Fero,M., Roussel,M.F., Roberts,J.M. and Sherr,C.J. (1999) The $p21^{Cip1}$ and $p27^{Kip1}$ CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.*, **18**, 1571–1583.

Coleman,K.G., Wautlet,B.S., Morrissey,D., Mulheron,J., Sedman,S.A., Brinkley,P., Price,S. and Webster,K.R. (1997) Identification of CDK4 sequences involved in cyclin D1 and p16 binding. *J. Biol. Chem.*, **272**, 18869–18874.

Crescenzi,M., Fleming,T.P., Lassar,A.B., Weintraub,H. and Aaronson,S.A. (1990) MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl Acad. Sci. USA*, **87**, 8442–8446.

Deng,C., Zhang,P., Harper,J.W., Elledge,S.J. and Leder,P. (1995) Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G_1 checkpoint control. *Cell*, **82**, 675–684.

Diehl,J.A. and Sherr,C.J. (1997) A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin-dependent kinase 4 (CDK4) and its phosphorylation by CDK-activating kinase. *Mol. Cell. Biol*., **17**, 7362–7374.

Dyson,N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.*, **12**, 2245–2262.

Emerson,C.P.,Jr (1993) Skeletal myogenesis: genetics and embryology to the fore. *Curr. Opin. Genet. Dev.*, **3**, 265–274.

Ewen,M.E., Sluss,H.K., Sherr,C.J., Matsushime,H., Kato,J. and Livingston,D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*, **73**, 487–497.

Fields,S. and Sternglanz,R. (1994) The two-hybrid system: an assay for protein–protein interactions. *Trends Genet*., **10**, 286–292.

Furukawa,Y., DeCaprio,J.A., Freedman,A., Kanakura,Y., Nakamura,M., Ernst,T.J., Livingston,D.M. and Griffin,J.D. (1990) Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **87**, 2770–2774.

Gorman,C.M., Merlino,G.T., Willingham,M.C., Pastan,I. and Howard,B.H. (1982) The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl Acad. Sci. USA*, **79**, 6777–6781.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavi,V. and Nadal-Ginard,B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, **72**, 309–324.

Halevy,O., Novitch,B.G., Spicer,D.B., Skapek,S.X., Rhee,J., Hannon,G.J., Beach,D. and Lassar,A.B. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science*, **267**, 1018–1021.

Helin,K., Harlow,E. and Fattaey,A. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol*., **13**, 6501–6508.

Jeffrey,P.D., Russo,A.A., Polyak,K., Gibbs,E., Hurwitz,J., Massague,J. and Pavletich,N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA–CDK2 complex. *Nature*, **376**, 313–320.

Kaelin,W.G.,Jr, Pallas,D.C., DeCaprio,J.A., Kaye,F.J. and Livingston,D.M. (1991) Identification of cellular proteins that can

interact specifically with the T/E1A-binding region of the retinoblastoma gene product. *Cell*, **64**, 521–532.
Kato,J., Matsushime,H., Hiebert,S.W., Ewen,M.E. and Sherr,C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.*, **7**, 331–342.
Kato,J.Y., Matsuoka,M., Strom,D.K. and Sherr,C.J. (1994) Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol. Cell. Biol.*, **14**, 2713–2721.
Kaye,F.J., Kratzke,R.A., Gerster,J.L. and Horowitz,J.M. (1990) A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. *Proc. Natl Acad. Sci. USA*, **87**, 6922–6926.
Kitagawa,M. *et al*. (1996) The consensus motif for phosphorylation by cyclin D1–Cdk4 is different from that for phosphorylation by cyclin A/E–Cdk2. *EMBO J.*, **15**, 7060–7069.
Knudsen,E.S. and Wang,J.Y. (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol. Cell. Biol.*, **17**, 5771–5783.
LaBaer,J., Garrett,M.D., Stevenson,L.F., Slingerland,J.M., Sandhu,C., Chou,H.S., Fattaey,A. and Harlow,E. (1997) New functional activities for the p21 family of CDK inhibitors. *Genes Dev.*, **11**, 847–862.
Lassar,A. and Munsterberg,A. (1994) Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. *Curr. Opin. Cell Biol.*, **6**, 432–442.
Lassar,A.B., Davis,R.L., Wright,W.E., Kadesch,T., Murre,C., Voronova,A., Baltimore,D. and Weintraub,H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo. Cell*, **66**, 305–315.
Lassar,A.B., Skapek,S.X. and Novitch,B. (1994) Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr. Opin. Cell Biol.*, **6**, 788–794.
Lee,M.H., Reynisdottir,I. and Massague,J. (1995) Cloning of $p57^{KIP2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev.*, **9**, 639–649.
Lin,Z.Y., Dechesne,C.A., Eldridge,J. and Paterson,B.M. (1989) An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. *Genes Dev.*, **3**, 986–996.
Lindon,C., Montarras,D. and Pinset,C. (1998) Cell cycle-regulated expression of the muscle determination factor Myf5 in proliferating myoblasts. *J. Cell Biol.*, **140**, 111–118.
Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J. and Kato,J.Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.
Megeney,L.A., Kablar,B., Garrett,K., Anderson,J.E. and Rudnicki,M.A. (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.*, **10**, 1173–1183.
Meyerson,M. and Harlow,E. (1994) Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.*, **14**, 2077–2086.
Meyerson,M., Enders,G.H., Wu,C.L., Su,L.K., Gorka,C., Nelson,C., Harlow,E. and Tsai,L.H. (1992) A family of human cdc2-related protein kinases. *EMBO J.*, **11**, 2909–2917.
Miller,J.B. (1990) Myogenic programs of mouse muscle cell lines: expression of myosin heavy chain isoforms, MyoD1 and myogenin. *J. Cell Biol.*, **111**, 1149–1159.
Mueller,P.R. and Wold,B. (1989) *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR (published erratum appears in *Science*, 1990, **248**, 802). *Science*, **246**, 780–786.
Novitch,B.G., Mulligan,G.J., Jacks,T. and Lassar,A.B. (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G_2 phases of the cell cycle. *J. Cell Biol.*, **135**, 441–456.
Okamoto,A. *et al.* (1994) Mutations and altered expression of p16INK4 in human cancer. *Proc. Natl Acad. Sci. USA*, **91**, 11045–11049.
Olson,E.N. (1992) Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.*, **154**, 261–272.
Parker,S.B., Eichele,G., Zhang,P., Rawls,A., Sands,A.T., Bradley,A., Olson,E.N., Harper,J.W. and Elledge,S.J. (1995) p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. *Science*, **267**, 1024–1027.
Paterson,B.M., Walldorf,U., Eldridge,J., Dubendorfer,A., Frasch,M. and Gehring,W.J. (1991) The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl Acad. Sci. USA*, **88**, 3782–3786.
Peverali,F.A., Ramqvist,T., Saffrich,R., Pepperkok,R., Barone,M.V. and Philipson,L. (1994) Regulation of G_1 progression by E2A and Id helix–loop–helix proteins. *EMBO J.*, **13**, 4291–4301.
Quelle,D.E., Ashmun,R.A., Shurtleff,S.A., Kato,J.Y., Bar-Sagi,D., Roussel,M.F. and Sherr,C.J. (1993) Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. *Genes Dev.*, **7**, 1559–1571.
Rane,S.G., Dubus,P., Mettus,R.V., Galbreath,E.J., Boden,G., Reddy,E.P. and Barbacid,M. (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. *Nature Genet.*, **22**, 44–52.
Rao,S.S., Chu,C. and Kohtz,D.S. (1994) Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix–loop–helix regulators. *Mol. Cell. Biol.*, **14**, 5259–5267.
Rudnicki,M.A. and Jaenisch,R. (1995) The MyoD family of transcription factors and skeletal myogenesis. *BioEssays*, **17**, 203–209.
Sabourin,L.A., Girgis-Gabardo,A., Seale,P., Asakura,A. and Rudnicki,M.A. (1999) Reduced differentiation potential of primary $MyoD^{-/-}$ myogenic cells derived from adult skeletal muscle. *J. Cell Biol.*, **144**, 631–643.
Schneider,J.W., Gu,W., Zhu,L., Mahdavi,V. and Nadal-Ginard,B. (1994) Reversal of terminal differentiation mediated by p107 in $Rb^{-/-}$ muscle cells. *Science*, **264**, 1467–1471.
Serrano,M., Hannon,G.J. and Beach,D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704–707.
Sherr,C.J. and Roberts,J.M. (1999) CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev.*, **13**, 1501–1512.
Shirakata,M., Friedman,F.K., Wei,Q. and Paterson,B.M. (1993) Dimerization specificity of myogenic helix–loop–helix DNA-binding factors directed by nonconserved hydrophilic residues. *Genes Dev.*, **7**, 2456–2470.
Skapek,S.X., Rhee,J., Spicer,D.B. and Lassar,A.B. (1995) Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science*, **267**, 1022–1024.
Skapek,S.X., Rhee,J., Kim,P.S., Novitch,B.G. and Lassar,A.B. (1996) Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol. Cell. Biol.*, **16**, 7043–7053.
Sorrentino,V., Pepperkok,R., Davis,R.L., Ansorge,W. and Philipson,L. (1990) Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature*, **345**, 813–815.
Tapscott,S.J., Davis,R.L., Thayer,M.J., Cheng,P.F., Weintraub,H. and Lassar,A.B. (1988) MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science*, **242**, 405–411.
Wang,J., Huang,Q., Tang,W. and Nadal-Ginard,B. (1996) E2F1 inhibition of transcription activation by myogenic basic helix–loop–helix regulators. *J. Cell Biochem.*, **62**, 405–410.
Weintraub,H. (1993) The MyoD family and myogenesis: redundancy, networks and thresholds. *Cell*, **75**, 1241–1244.
Yablonka-Reuveni,Z., Rudnicki,M.A., Rivera,A.J., Primig,M., Anderson,J.E. and Natanson,P. (1999) The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Dev. Biol.*, **210**, 440–455.
Yan,Y., Frisen,J., Lee,M.H., Massague,J. and Barbacid,M. (1997) Ablation of the CDK inhibitor $p57^{Kip2}$ results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.*, **11**, 973–983.
Zhang,H., Hannon,G.J. and Beach,D. (1994) p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.*, **8**, 1750–1758.
Zhang,J.-M., Wei,Q., Zhao,X. and Paterson,B.M. (1999) Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4. *EMBO J.*, **18**, 926–933.
Zhang,P., Wong,C., Liu,D., Finegold,M., Harper,J.W. and Elledge,S.J. (1999) $p21^{CIP1}$ and $p57^{KIP2}$ control muscle differentiation at the myogenin step. *Genes Dev.*, **13**, 213–224.

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