Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4

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Proliferating myoblasts express the muscle determination factor, MyoD, throughout the cell cycle in the absence of differentiation. Here we show that a mitogen-sensitive mechanism, involving the direct interaction between MyoD and cdk4, restricts myoblast differentiation to cells that have entered into the G_0 phase of the cell cycle under mitogen withdrawal. Interaction between MyoD and cdk4 disrupts MyoD DNA-binding, muscle-specific gene activation and myogenic conversion of 10T1/2 cells independently of cyclin D1 and the CAK activation of cdk4. Forced induction of cyclin D1 in myotubes results in the cytoplasmic to nuclear translocation of cdk4. The specific MyoD–cdk4 interaction in dividing myoblasts, coupled with the cyclin D1-dependent nuclear targeting of cdk4, suggests a mitogen-sensitive mechanism whereby cyclin D1 can regulate MyoD function and the onset of myogenesis by controlling the cellular location of cdk4 rather than the phosphorylation status of MyoD.
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Introduction

Terminal differentiation of skeletal muscle myoblasts is modulated by mitogens and requires that the cell exit from the cell cycle in G_1 and enter into the quiescent post-mitotic state known as G_0 (Olson, 1992). This process is thought to involve mitogen-responsive interactions between the MyoD family of muscle gene regulatory proteins and the proteins that control cell cycle progression (Gu *et al*., 1993; Lassar and Munsterberg, 1994; Schneider *et al*., 1994; Deng *et al*., 1995; Halevy *et al*., 1995; Parker *et al*., 1995; Skapek *et al*., 1995; Novitch *et al*., 1996). The most striking finding so far is that ectopic expression of cyclin D1 inhibits a MyoD-activated reporter (Rao *et al*., 1994; Skapek *et al*., 1995), independently of pRb phosphorylation (Skapek *et al*., 1996), and triggers the apparent hyperphosphorylation of MyoD. This was interpreted to suggest that an active cyclin D1–cyclin-dependent kinase (cdk) complex phosphorylated MyoD to suppress MyoD function in proliferating cells (Skapek *et al*., 1995). However, evidence for the direct cdk-dependent phosphorylation of MyoD or that MyoD interacts specifically with the cdks has not been reported to support this hypothesis further. Here we describe a

mechanism to explain the cyclin-mediated inhibition of myogenesis that involves the cyclin D1-dependent nuclear translocation of cdk4 and the subsequent formation of a MyoD–cdk4 complex that specifically inhibits the trans-activation functions of MyoD in the absence cdk4 kinase activity.

Results

Cyclin D1 has been shown to partner specifically with cdk4 in mammalian cells (Matsushime *et al*., 1994). In order to examine further the possibility of a direct MyoD–cdk4 interaction in the regulation of myogenic differentiation, we investigated the potential binding between MyoD and cdk4 both *in vitro* and *in vivo*. Sf9 cell extracts produced from cells co-infected with baculoviral stocks encoding cdk4 and cyclin D1 were used as a source of active cdk4 (Kato *et al*., 1993). The extract was incubated with glutathione–agarose bound with either glutathione S-transferase (GST) alone, or GST fusion proteins encoding one of the four different vertebrate myogenic factors from chicken: CMD1 (MyoD), Ch-myogenin, Ch-myf5 and Ch-mrf4 (Shirakata *et al*., 1993). Only the GST–MyoD was found to bind cdk4 specifically (Figure 1A, lane 2). Binding assays performed either with extracts from Sf9 cells infected with cdk4 viral stocks or with bacterially produced cdk4 gave identical results (data not shown). The ubiquitously expressed basic helix–loop–helix (bHLH) factors E12 and E47, proteins that form active heterodimers with MyoD (Shirakata *et al*., 1993), did not bind cdk4 when used as GST fusion proteins in this type of assay (data not shown). These data allow us to conclude that active kinase is not required in order for cdk4 to bind specifically to MyoD *in vitro* and that the interaction is not mediated by unknown proteins in the Sf9 cell extracts.

In order to determine whether or not the observed MyoD–cdk4 interaction also occurred *in vivo*, three different approaches were taken. First, co-immunoprecipitation reactions (Lassar *et al*., 1991) were performed using cell extracts from Sf9 cells producing either MyoD and cdk4 or MyoD and cyclin D1 protein combinations (Kato *et al*., 1993). MyoD bound cdk4 but not cyclin D1 in this assay (Figure 1B, lanes 3 and 5). However, when MyoD, cdk4 and cyclin D1 proteins were all co-expressed in Sf9 cells, MyoD formed immune complexes containing both cdk4 and cyclin D1 (Figure 1B, lanes 9 and 11). Therefore, MyoD bound to the cyclin D1–cdk4 complex does not dissociate the cyclin from the kinase. Co-immunoprecipitation reactions substituting either virally expressed myf5 or myogenin for MyoD failed to produce immune complexes with cdk4, in agreement with the *in vitro* GST fusion-binding results (data not shown). MyoD is assumed to bind to cdk4 as a monomer in the immunoprecipitation
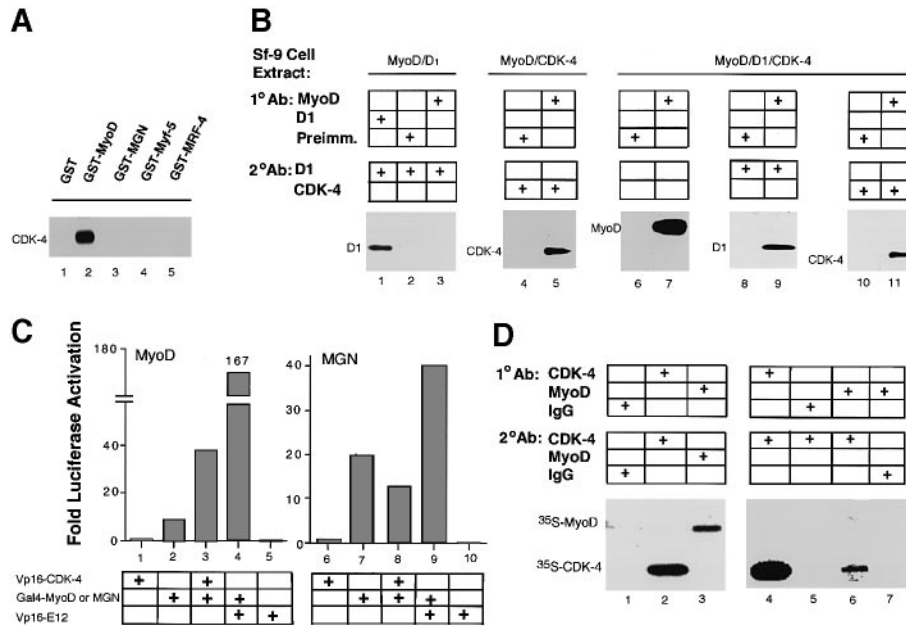
Fig. 1. Specific interaction between MyoD and cdk4 *in vitro* and *in vivo*. (**A**) Cdk4 binds specifically to MyoD. Lysates of Sf9 cells co-infected with baculoviral stocks encoding cyclin D1 and cdk4 (Kato *et al.*, 1993) were incubated either with bacterial GST or purified GST fusion proteins encoding the chicken myogenic factors, MyoD (CMD1), Ch-myf5, Ch-mgn and Ch-mrf4 (Shirakata *et al.*, 1993). GST complexes were recovered on glutathione–Sepharose beads (Kaelin *et al.*, 1991) and analyzed by immunoblot with anti-cdk4 affinity-purified rabbit polyclonal antibody. (**B**) MyoD binding to cdk4 does not depend upon cyclin D1. Lysates of Sf9 cells co-infected with baculovirus encoding MyoD and cyclin D1 (lanes 1–3), MyoD and cdk4 (lanes 4 and 5), and MyoD, cdk4 and cyclin D1 (lanes 6–11) protein combinations, containing equal amounts of protein, were co-immunoprecipitated sequentially with the indicated antibodies (Lassar *et al.*, 1991). Proteins were detected by immunoblot with the indicated antibody combinations. (**C**) MyoD and cdk4 interact *in vivo* in the mammalian two-hybrid system. 10T1/2 mouse fibroblasts were co-transfected with the indicated combinations of gal4 and vp16 expression constructs along with a gal4–luciferase and a pSV2-β-galactosidase reporter, the latter used to normalize transfection efficiencies. MyoD interacts with cdk4 (lanes 1–5) but myogenin does not demonstrate measurable interaction (lanes 6–10). Gal4MyoD–vp16E12 and gal4MGN–vp16E12 interactions, previously established heterodimer partner combinations (Lassar *et al.*, 1991; Shirakata *et al.*, 1993), were used as positive controls (lanes 4 and 9, respectively). (**D**) MyoD and cdk4 interact in dividing C2C12 myoblasts. Nuclear extracts prepared from ^{35}S-labeled cultures (90 min labeling) of C2C12 myoblasts maintained in growth medium, containing equal counts, were co-immunoprecipitated with the indicated primary and secondary antibody combinations, as described (Lassar *et al.*, 1991).

reactions from Sf9 cell extracts since MyoD expressed in Sf9 cells is phosphorylated and does not readily form homodimers *in vitro* unless it is dephosphorylated (Mitsui *et al.*, 1993).

To investigate further specific *in vivo* interactions between MyoD and cdk4, the mammalian version of the yeast two-hybrid system was used as a second approach (Finkel *et al.*, 1993). MyoD and myogenin (used as a control since it does not bind cdk4 *in vitro*) were each cloned into the gal4 DNA-binding domain vector. Cdk4 was inserted into the vp16 activation domain plasmid. Either gal4–MyoD or gal4–myogenin were co-transfected along with vp16–cdk4 and a luciferase reporter into 10T1/2 mouse fibroblasts. Despite the high cellular levels of endogenous cdk4 competing in the two-hybrid reaction (Skapek *et al.*, 1995), a 4-fold activation of vp16 cdk4-dependent luciferase reporter activity was detected routinely with the use of the gal4–MyoD construct (Figure 1C, lanes 2 and 3), whereas no activation was observed with gal4–myogenin (Figure 1C, lanes 7 and 8). This 4-fold activation was observed even above the levels of the expected transcriptinal activation activity of gal4 MyoD since gal4 fusions of any of the MyoD family members will activate transcription in the absence of a vp16 partner (Braun *et al.*, 1990; Schwarz *et al.*, 1992; Finkel *et al.*, 1993).

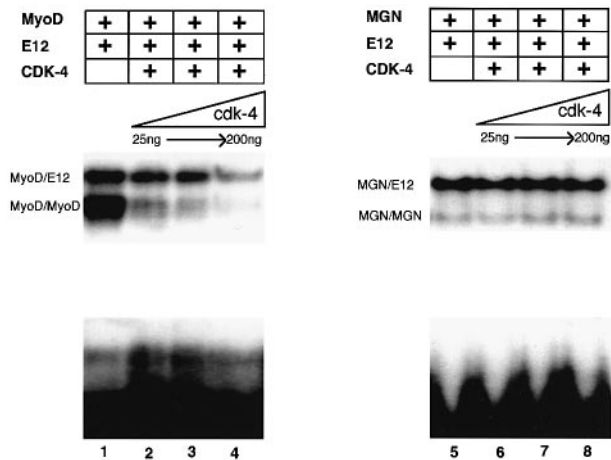In the third and most important test for a MyoD–cdk4 interaction *in vivo*, C2C12 mouse myoblasts were cultured

Fig. 2. Cdk4 specifically inhibits MyoD–E12 DNA binding in the absence of cyclin D1. Band shift assays were performed using the bacterially expressed and histidine-tagged purified proteins (Shirakata *et al.*, 1993). The MyoD (10 ng), myogenin (10 ng) and E12 (30 ng) proteins from chicken were used with increasing 2-fold increments (50–200 ng) of mouse cdk4 protein in the band shift reaction. Cdk4 protein inhibited DNA binding by both the MyoD–E12 heterodimer and the MyoD homodimer (lanes 1–4). DNA binding by the myogenin–E12 heterodimer and the myogenin homodimer (lanes 5–8) were unaffected at the equivalent concentrations of cdk4 protein in the binding reaction. Similar results were obtained with Sf9 cell extracts expressing cdk4 (data not shown).

in mitogen-rich growth medium (GM) and labeled with [^{35}S]methionine, the nuclei were isolated and nuclear extracts were subjected to co-immunoprecipitation reactions with the indicated antibodies to look for endogenously formed MyoD–cdk4 immune complexes (Lassar *et al.*, 1991). Specific MyoD–cdk4 immune complexes were identified only in the nuclei of dividing myoblasts (Figure 1D, lane 6). No such complexes were detected in the nuclear extracts from differentiated muscle cultures (data not shown). Although we can immunodeplete MyoD quantitatively from cell extracts, we are unable to do so with cdk4 since it is in such excess compared with MyoD. Judging from the levels of MyoD in the extract compared with the levels of cdk4 (Figure 1D, lanes 2 and 3) it would appear that at least 50% of the total MyoD is in a complex with cdk4 and this is likely to be an underestimate since the stability of the MyoD–cdk4 complex is somewhat lower in the high salt nuclear extraction buffer (data not 3shown). The combined results from these three different approaches demonstrate that MyoD and cdk4 interact specifically *in vitro* and *in vivo*, and that this interaction is not directly dependent upon cyclin D1 association with cdk4.

To ascertain whether or not cdk4 can interfere directly with MyoD transactivation functions, initially the DNA-binding activity of both MyoD homodimers as well as MyoD–E12 heterodimers was measured in band shift assays (Shirakata *et al.*, 1993) in the presence of increasing amounts of bacterially produced cdk4 protein. Bacterially expressed cdk4 and cyclin D2 can be renatured then incubated with CAK activity to produce active kinase (Kato *et al.*, 1994). Excess amounts of MyoD protein were used in the band shift reactions in order to observe any cdk4 effects on both the MyoD homodimer and the MyoD–E12 heterodimer. DNA binding by the myogenin–E12 heterodimer was used as a control since myogenin does not bind cdk4 *in vitro*. As shown for the bacterial cdk4, a concentration-dependent inhibition of DNA binding was observed with the MyoD homo- and heterodimer complexes (Figure 2, lanes 1–4). Similar results were obtained with Sf9 cell extracts containing cdk4 (data not shown), but use of the purified bacterially expressed proteins ruled out any additional effects due to unidentified components in the cell extracts. The MyoD homodimer was more sensitive to inhibition than the heterodimer, as expected, since DNA binding by the homodimer is significantly weaker than that by the MyoD–E12 heterodimer (Shirakata *et al.*, 1993). No cdk4 inhibition of DNA binding was observed with the myogenin–E12 complex using the same concentration range (50–200 ng) of cdk4 protein in the band shift reaction (Figure 2, lanes 5–8). The actual amount of properly renatured cdk4 protein in the binding reaction is unknown, so the effective inhibitory ratio of cdk4 to the MyoD–E12 complex is likely to be significantly less than 5-fold as suggested here. We conclude that cdk4 dissociates the MyoD–E12 DNA-binding complexes, since no evidence for a supershift was noted with the addition of either cdk4 protein or cdk4 antibody to the shift reactions (data not shown). Most importantly, in agreement with the earlier protein-binding studies, cdk4 inhibition of DNA binding by the MyoD–E12 heterodimer did not depend upon cyclin D1 and, therefore, does not require the active kinase.

Since cdk4 is expressed at similar levels in both myoblasts and myotubes (Skapek *et al.*, 1995), the cdk4 inhibition of MyoD DNA binding suggested that the nuclear localization of cdk4 in differentiated myotubes would be incompatible with MyoD-dependent gene activation. To determine whether or not cdk4 was localized differentially in nuclei during myogenesis, dividing as well as differentiated cultures of C2C12 mouse myoblasts were reacted with cdk4 antibody and examined by immunofluorescence. Although cdk4 protein is located predominately in the nuclei of dividing C2C12 myoblasts kept in GM (Figure 3a), cdk4 is dramatically reduced or absent in myotube nuclei (Figure 3b and c), as seen by comparing the Hoechst staining pattern with the cdk4 pattern, and is essentially translocated to the cytoplasmic compartment in well-formed myotubes. Some single myoblasts in differentiation medium (DM) continue to show weaker nuclear and cytoplasmic cdk4 staining, but the exact status of these cells has not been determined (Figure 3b).

Previous reports have shown that cyclin D1 mRNA and protein decline rapidly during myogenesis (Rao *et al.*, 1994; Skapek *et al.*, 1995). Furthermore, the half-life of cyclin D1 ($t_{1/2}$ = 30 min) is much shorter than that of cdk4 ($t_{1/2}$ = 4 h), enabling the cyclin to act as the rate-limiting partner in the formation of the active kinase (Diehl *et al.*, 1997). In addition, virtually all cyclin D1-dependent kinase activity in rodent fibroblasts can be attributed to cdk4 (Matsushime *et al.*, 1994) and this is likely to be the case for both mouse C2C12 cells and for muscle cell lines derived by myogenic conversion of rodent fibroblasts with MyoD. As expected, there is essentially no detectable cyclin D1 expression in either myotubes or single cells kept in mitogen-depleted DM (Figure 3e and f), whereas the nuclei of rapidly dividing myoblasts maintained in mitogen-rich GM have high levels of nuclear cyclin D1 (Figure 3d). Thus, in the absence of cyclin D1, inactive cdk4 is translocated into the cytoplasmic compartment of newly formed myotubes.

If the absence of cyclin D1 results in the cytoplasmic compartmentalization of cdk4 in differentiated muscle cells, as suggested with the *in situ* studies, then the ectopic expression of cyclin D1 might relocalize and maintain cdk4 in myotube nuclei and, by inference, in myoblast nuclei as well, inhibiting myogenesis. An ecdysone-inducible cyclin D1 construct was transiently transfected into C2C12 myoblasts along with an ecdysone-inducible β-galactosidase reporter plasmid to mark transfected cells. Cyclin D1 is degraded rapidly in the absence of growth factors, conditions used to differentiate muscle (Rao *et al.*, 1994; Diehl *et al.*, 1997). Therefore, a cyclin D1 protein containing a threonine to alanine substitution at position 286 that renders it resistant to degradation through the phosphorylation-dependent ubiquitination pathway (Diehl *et al.*, 1997) was used in place of the wild-type cyclin D1 for the expression induction studies in myotubes. Upon the induction of cyclin D1(T286A) in myotubes with the addition of the ecdysone derivative muristerone (1 μM), cyclin D1 nuclear staining was now observed (Figure 3g and h) and cdk4 was translocated rapidly to the nuclear compartment (Figure 3i and j). This result is consistent with the interpretation that the nuclear localization of cdk4 in C2C12 myoblasts can be regulated by the cellular level
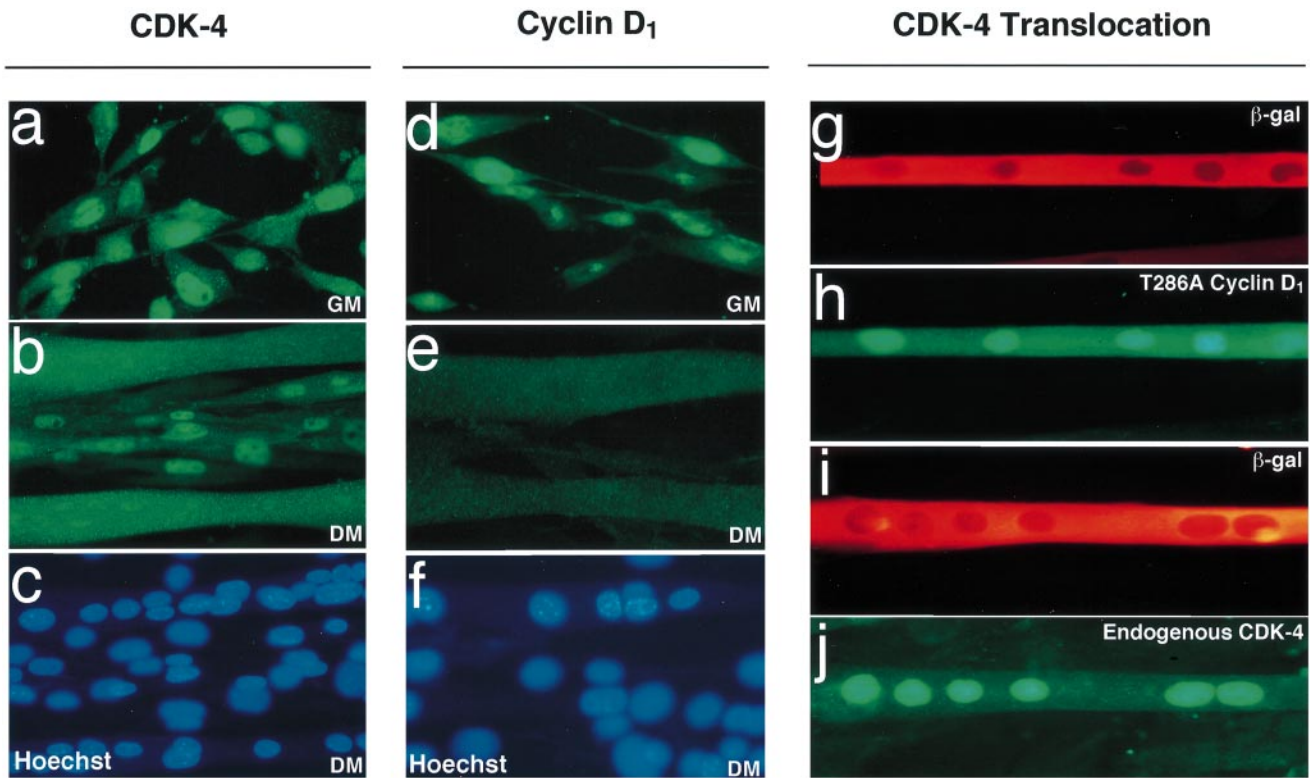
Fig. 3. The nuclear localization of cdk4 in differentiating C2C12 muscle cells and its regulation by cyclin D1. Dividing myoblasts in growth medium (GM) and well differentiated cultures of C2C12 muscle cells in differentiation medium (DM) were reacted with antibodies to cdk4 and cyclin D1. Cdk4 is located in the nuclei of dividing myoblasts (**a**) but translocates to the cytoplasm in differentiated myotubes (**b**), as seen when compared with the nuclear Hoechst staining in the same myotube (**c**). Cyclin D1 is expressed in the nuclei of dividing cells (**d**) and is completely absent in the nuclei of well differentiated myotubes (**e**) when compared with the Hoechst staining in the same myotube (**f**). Induction of both β-galactosidase (**g** and **i**) and stable cyclin D1 (T286A) (**h**) by muristerone (1 μM) in well-formed co-transfected myotubes results in the cytoplasmic to nuclear translocalization of endogenous cdk4 (**j**). Secondary antibodies were tagged with either Alexa 594 (red) or Alexa 488 (green) (Molecular Probes).

of cyclin D1 which, in turn, is dependent upon the levels of mitogens in the growth medium. It should be noted that neither the addition of GM to control myotube cultures nor the muristerone induction of a wild-type cyclin D1 construct resulted in the nuclear translocation of detectable cdk4 (data not shown). In the latter instance, we assume that the kinase triggering the ubiquitination and degradation of wild-type cyclin D1 is active in myotubes, regardless of the growth conditions. In addition, the induction of nuclear cdk4 in myotube nuclei did not result in DNA synthesis in either low or high serum, as measured by bromodeoxyuridine (BrdU) incorporation (data not shown).

Based upon the DNA binding results and the *in situ* studies, we hypothesized that the inhibition of myogenic differentiation by cyclin D1 is a consequence of the cyclin D1-dependent nuclear translocation of cdk4 during myogenesis and not a direct effect of cdk4 kinase activity. A further critical prediction from the nuclear translocation experiment is that either wild-type or kinase-negative cdk4(–CAK, T172A) (Kato *et al.*, 1994) targeted to the myoblast nucleus in the absence of cyclin D1 should also block muscle-specific reporter activation and the myogenic conversion of fibroblasts. To determine whether or not ectopically expressed cdk4 can interfere with the trans-activation functions of MyoD to block muscle differentiation, three types of experiments were performed. First, 10T1/2 mouse fibroblasts in GM, conditions which promote the nuclear localization of cdk4, were co-transfected either with a MyoD or myogenin expression plasmid, the MCK-CAT muscle-specific reporter plasmid (Skapek *et al.*, 1995) and either the wild-type or kinase-negative cdk4(–CAK) expression constructs. Vector alone was used as the control. The increased ectopic expression of wild-type cdk4 was able significantly to suppress the MyoD activation of the MCK-CAT reporter (Figure 4A, upper panel, lanes 3 and 4), whereas little suppression was observed on the myogenin-dependent activation of MCK-CAT (Figure 4A, middle panel, lanes 3 and 4). Secondly, to ascertain whether or not cdk4 in the absence of cyclin D1 could inhibit reporter activation directly, the same experiments were performed in low serum DM, conditions that result in the degradation and loss of cyclin D1 and the cytoplasmic translocation of inactive cdk4 (see Figure 3). The ectopic expression of wild-type cdk4 did not significantly inhibit reporter activation either by MyoD (Figure 4A, upper panel, lanes 7 and 8) or by myogenin (Figure 4A, middle panel, lanes 7 and 8) in cells maintained in DM. Thirdly, wild-type and kinase-negative cdk4 expression constructs with a nuclear localization signal (NLS) on the N-terminus and a hemagglutinin tag (the HA tag is used in later experiments) on the C-terminus were now used in the same reporter assay on cells maintaned in low serum DM conditions where cyclin D1 is degraded and absent from the cells (see Figure 3). In this instance, MyoD activation of MCK-CAT was again inhibited by both wild-type (Figure 4A, upper panel, lanes 11 and 12) and kinase-negative cdk4 (Figure 4A, lower

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MyoD (CMD)

A

GM

DM

DM

Myogenin (CMGN)

GM

DM

DM

MyoD(CMD)

Myogenin(CMGN)

DM

DM

B

C
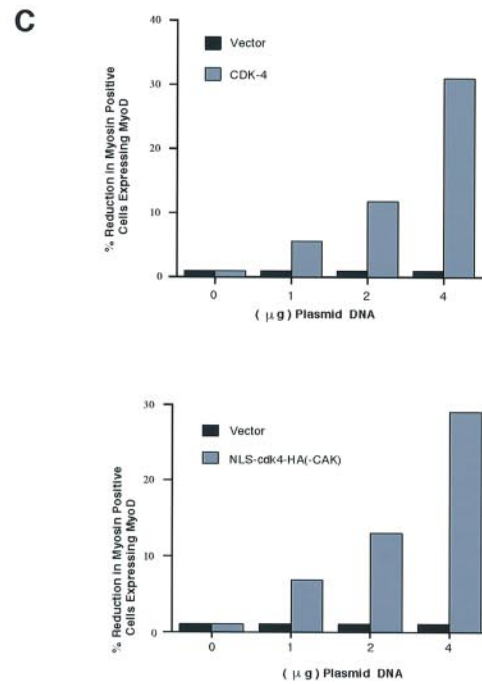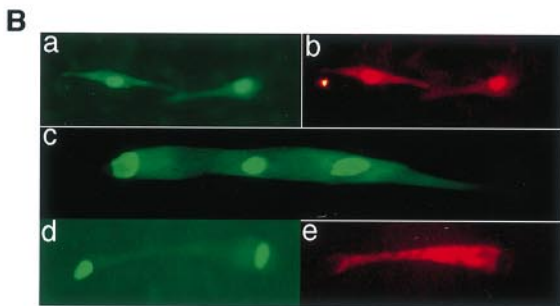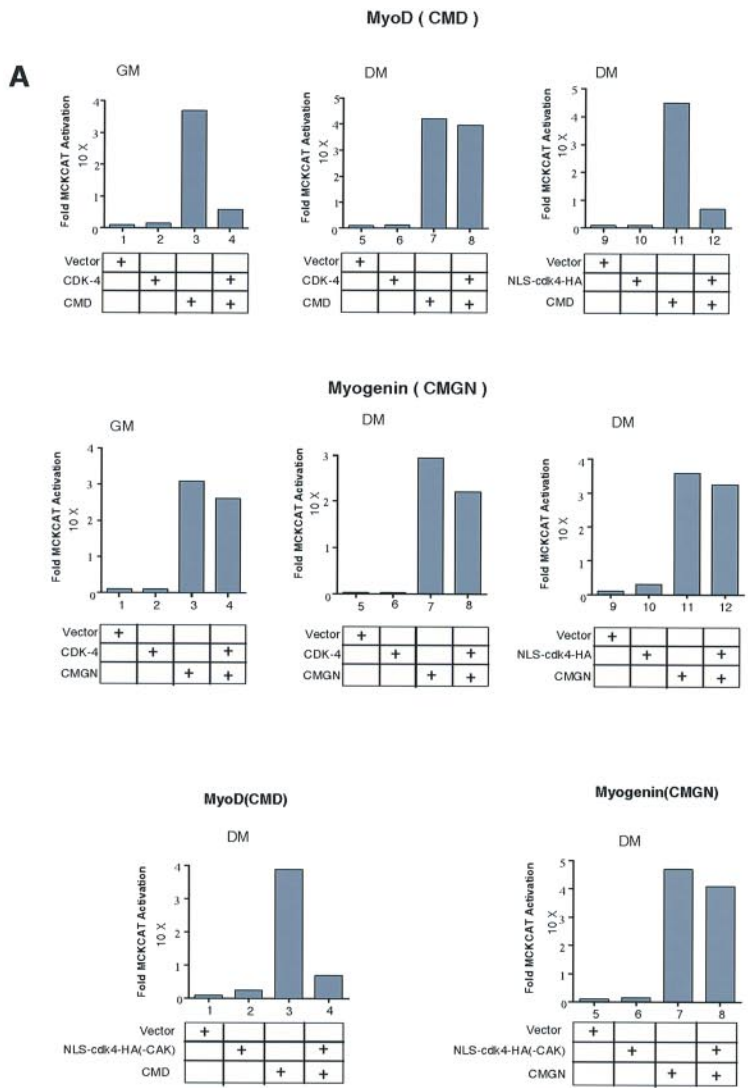
panel, lanes 3 and 4), similarly to cells maintained in GM, whereas myogenin activation of the same reporter was essentially unaffected by either wild-type (Figure 4A, middle panel, lanes 11 and 12) or kinase-negative cdk4 (Figure 4A, lower panel, lanes 7 and 8). Thus, the nuclear localization of cdk4, either through cyclin D1 or an NLS in the absence of cyclin D1, preferentially inhibits MyoD transactivation functions without comparably affecting the myogenin activation of the same reporter, confirming the specificity of the MyoD–cdk4 interaction *in vivo*. Furthermore, although cyclin D1 expression results in the nuclear translocation of cdk4, active kinase is not required to inhibit MyoD function since the forced nuclear localization of either wild-type or kinase-negative cdk4 also specifically inhibited MyoD activation functions.

A hallmark of MyoD activity is its ability to convert 10T1/2 mouse fibroblasts into muscle cells capable of differentiating into multinucleated myotubes that express myosin heavy chain (Buckingham, 1992; Olson, 1992; Emerson, 1993; Weintraub, 1993). A prediction from the reporter activation studies suggests that ectopic expression of the nuclear targeted wild-type or kinase-negative cdk4 should also block myogenic conversion of mouse embryonic fibroblasts. 10T1/2 cells were co-transfected with increasing amounts of both the wild-type and kinase-negative cdk4 expression constructs along with a fixed amount of MyoD expression plasmid. Myosin expression in MyoD-positive cells was then determined as a function of cdk4 plasmid input in the co-transfection. Cultures were maintained in low mitogen DM to destabilize and eliminate cyclin D1 (see Figure 3) and to induce differentiation of the converted cells, as measured by myosin synthesis and cell fusion. The forced nuclear localization of wild-type cdk4 (Figure 4B, panel b, stained for HA) resulted in an increased number of MyoD-positive single cells (Figure 4B, panel a) that were negative for myosin expression when compared with the cells treated with vector alone (Figure 4B, panels c–e). This inhibition of myosin expression in up to 30–35% of the transfected cells ($p = 0.02$) was proportional to the increased expression of nuclear wild-type cdk4 in the transfected cells (Figure 4C, upper panel). Identical results were obtained for the expression of the kinase-negative cdk4 in 10T1/2 cells (Figure 4C, lower panel). Therefore, either the expression of wild-type cdk4 targeted to the nucleus in the absence of cyclin D1 or the nuclear expression of kinase-negative cdk4 can significantly inhibit the myogenic conversion activity of MyoD in a concentration-dependent manner, as measured by the reduction in the number of myosin-positive/MyoD-positive 10T1/2 mouse fibroblasts. Taken together with the reporter transactivation studies, we conclude that cdk4 kinase activity is not required to inhibit myogenesis since cyclin D1 is not expressed in cells maintained in DM, and the kinase-negative cdk4(–CAK) inhibits myogenesis identically to wild-type cdk4 when targeted to the nucleus.

Discussion

It has been proposed previously that cdk4 phosphorylates MyoD directly to inhibit MyoD transactivation functions (Skapek *et al.*, 1995). However, later studies with myogenin demonstrated that putative cdk-dependent phosphorylated sites were not required for cyclin D1-dependent inhibition of myogenin reporter activation (Skapek *et al.*, 1996), suggesting that cdk phosphorylation was not involved. Our data provide an alternative hypothesis that does not require active cdk4 kinase to inhibit MyoD function. We recently have carried out additional experiments that demonstrate that a 15 amino acid region in the C-terminus of MyoD outside the bHLH domain binds to cdk4 to block cell growth and inhibits the cdk4-dependent phosphorylation of a pRb target both *in vitro* and *in vivo* (J.-M.Zhang, X.Zhao, Q.Wei and B.M.Paterson, in preparation). This interaction does not result in the cdk4-dependent phosphorylation of MyoD (J.-M.Zhang, X.Zhao, Q.Wei and B.M.Paterson, in preparation).

Even though cyclin D1 can induce the nuclear localization of cdk4, the increased expression of cyclin D3 in differentiated muscle does not result in the nuclear import of cdk4 and, by implication, the formation of active kinase (Rao *et al.*, 1994; Skapek *et al.*, 1995). Neither protein has an obvious NLS (Diehl and Sherr, 1997), and it has been shown recently that a dominant-negative mutant of cyclin D1 that cannot be phosphorylated on Thr156 will still associate with cdk4 while preventing its nuclear localization (Diehl and Sherr, 1997). This was interpreted to imply that other cellular factors are required for the nuclear import of cdk4 and cyclin D1. It is interesting to note that differentiated muscle either maintains or can regulate these additional factor(s) since ectopic induction of cyclin D1 in myotubes promotes the nuclear import of cdk4. Since cyclin D1 can regulate the myogenic pathway through a cdk4 nuclear import mechanism, alterations in the expression of cyclin D1 may also affect the differentiation and growth properties of a variety of normal and transformed cell types by modulating the cellular location of cdk4 and its potential interaction with other tissue-specific transcription factors. Different D-type cyclins negatively regulate gene expression in a cell type-specific manner since cyclins D2 or D3 but not D1 inhibit

Fig. 4. Nuclear targeting of either wild-type or kinase-negative cdk4 selectively inhibits MyoD transactivation functions and the myogenic conversion of 10T1/2 cells. (**A**) Nuclear targeting of wild-type cdk4, either through cyclin D1 (upper panel, cells in GM, lanes 1–4) or through an NLS tag (NLS-cdk4-HA) in the absence of cyclin D1 (upper panel, cells in DM, lanes 9–12) inhibits MyoD (compare lanes 3 and 4 with lanes 11 and 12) but not myogenin (middle panel, compare lanes 3 and 4 with lanes 11 and 12) activation of the MCK-CAT reporter plasmid in 10T1/2 cells. Kinase-negative cdk4 [NLS-cdk4-HA(–CAK)] behaves like wild-type cdk4 in the specific inhibition of MyoD reporter activation (lower panel, compare lanes 3 and 4) with no comparable effect on myogenin reporter activity (lower panel, compare lanes 7 and 8). (**B**) Forced nuclear expression of cdk4 (NLS-cdk4-HA) inhibits myogenic conversion of 10T1/2 cells in the absence of cyclin D1 expression (cells in DM): a, MyoD (green); b, NLS-cdk4-HA (red, HA staining) in the same cell. MyoD-converted cells in the absence of NLS-cdk4-HA fuse, express MyoD (green) (c and d) and synthesize myosin heavy chain (red) (e, myosin staining in the same myotube as in d). Identical results were obtained with the kinase-negative cdk4 [NLS-cdk4-HA(–CAK), data not shown]. (**C**) Increased expression levels of either wild-type or kinase-negative NLS-cdk4-HA inhibit myogenic conversion of 10T1/2 cells in DM in the absence of cyclin D1. The percentage of myosin-positive cells per MyoD-positive cell was scored for a minimum of 300 MyoD-positive cells for each plasmid concentration in four independent experiments ($p = 0.02$).

granulocyte differentiation of 32D myleoid cells (Kato and Sherr, 1993).

A prediction from the cdk4 nuclear import model suggests that overexpression of MyoD would titrate cellular levels of cdk4 to inhibit cell growth. In fact, this was observed when we were unable to establish a MyoD-derived muscle cell line in 10T1/2 cells using a Rous sarcoma virus (RSV)-MyoD expression construct; however, we were able to do so using pSV2-MyoD with the 5-fold weaker SV40 promoter (Gorman *et al.*, 1982; Lin *et al.*, 1989). Thus, a model emerges that is consistent with an indirect interaction between MyoD and pRb that depends upon a balance between the nuclear levels of MyoD and cdk4, the latter controlled by cyclin D1, to regulate the terminal cell cycle decisions of the myoblast independently of cdk4 kinase activity on MyoD. Clearly, active kinase is not required for the cdk4 inhibitory effect, and this result suggests that cyclin D1 overexpression blocks myogenesis in a manner that is qualitatively different from cdk4 overexpression. Cyclin D1 is the limiting factor in the formation of active cdk4 (Kato and Sherr, 1993; Diehl and Sherr, 1997; Diehl *et al.*, 1997) and G_1 progression (Quelle *et al.*, 1993). Overexpression of cyclin D1 would be likely to increase the levels of active cdk4 and phosphorylated Rb to promote the activation of growth-related genes such as Id (Benezra *et al.*, 1990) to affect reporter activation by both MyoD and myogenin (Rao *et al.*, 1994; Skapek *et al.*, 1996). Overexpression of p16 and p21 blocks cdk4 activation in this process (Skapek *et al.*, 1995). However, overexpression of either cdk4 or kinase-negative cdk4 would not be predicted to affect Rb phosphorylation or growth-related genes but would allow cdk4 to interact with MyoD specifically to block its function without affecting myogenin, as demonstrated here.

Gene targeting experiments have provided a great deal of insight into the role of the myogenic regulatory factors in the determination and differentiation of myogenic precursors in skeletal muscle formation (Weintraub, 1993; Rudnicki and Jaenisch, 1995). In the MyoD$^{-/-}$ mouse, activated satellite cells, the stem cell of adult muscle, increase in number and fail to progress through the myogenic differentition program (Megeney *et al.*, 1996). The defect appears to be a satellite cell failure to exit the proliferative compartment during muscle regeneration. Further analyses of primary myoblasts from the MyoD$^{-/-}$ mouse indicate that even though the cells express 4-fold higher levels of myf-5, the myoblasts continue to proliferate under conditions that normally induce differentiation to yield reduced numbers of predominantly mononuclear myocytes. Transfection of a MyoD expression cassette rescued the differentiation defect (M.Rudnicki, personal communication). These data are interpretated to suggest that, in the absence of MyoD, myogenic stem cells remain at an intermediate proliferative stage unable to become full myogenic precursor cells. Thus, MyoD plays a critical role in terminal cell cycle decisions during myogenic differentiation and this probably involves the MyoD–cdk4 interaction described here.

Materials and methods

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% fetal calf serum, respectively, were transfected with the various plasmid DNAs (see below) 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 and destabilize cyclin D1. After a further 24–48 h, cells were either harvested for reporter assays (CAT, β-galactosidase or luciferase) or Western blot analysis, or fixed in 10% formalin solution (Sigma HT50-1-2) for immunostaining with the listed antibodies. For the two-hybrid assays, 1 μg of gal4 luciferase reporter, 2 μg each of the gal4 (pM vector, Clonetech) and vp16 (pvp16 vector, Clonetech) constructs, and 300 ng of the β-galactosidase reporter pCH110 (Pharmacia), for a total of 5.3 μg DNA, were used in each transfection. For the CAT assays, 2 μg of EMSV MyoD or myogenin and 2 μg of EMSV cdk4 or empty vector, for a total of 4 μg of DNA, as well as 0.3 μg of pCH110 were used. For the NLS-cdk4-HA experiments, 10T1/2 cells were transfected with 1 μg of pCDNA3 MyoD, 1 μg of MCK-CAT and 1–4 μg of pCDNA3-NLS-cdk4-HA. Total transfected DNA was adjusted to 6 μg with pCDNA vector DNA.

Reporter assays
Reporter assays were performed according to the manufacturer's directions: CAT (Promega, cat. No. E1000, liquid scintillation counting method), β-galactosidase (Tropix, cat. No. E4030), luciferase (Promega, cat. No. E4030). The luciferase and β-galactosidase luminescent assays were measured in a Victor 1420 Multilabel Counter. All reporter assays were repeated at least three times with similar results. Both the CAT and luciferase reporter assays were normalized for transfection efficiency with a co-transfected β-galactosidase reporter and by total protein in the cell extract with similar results.

Plamid constructs
To construct the gal4 luciferase reporter, the CAT cassette from pG5 (Clonetech) was exchanged for the luciferase cassette in pGL3 (Promega). Chicken MyoD, myogenin and E12 were cloned by standard PCR methods into pM and pVP16 vectors (Clonetech). Mouse cdk4 was amplified by PCR from baculovirus stocks (from C.J.Sherr) (Kato *et al.*, 1993) and cloned into the indicated vectors. For the ecdysone-inducible construct, cyclin D1 was cloned into the pIND vector (Invitrogen). Thr286 was changed to alanine directly in the pIND vector using the QuickChange Kit (Stratagene, cat. No. 200518). For the transfections with NLS-cdk4-HA, containing a C-terminal HA tag (YPYDVPDYA) and an N-terminal NLS (MCPKKRKV), cdk4 was first PCR amplified from pVP16-Cdk4 with primers containing an NLS 5′ and HA tag 3′ then cloned into pCDNA3 (Invitrogen). Kinase-negative NLS-cdk4-HA was prepared by changing Thr172 to alanine in the parent clone using the QuickChange mutagenesis kit (Stratagene). GST–Rb (amino acids 767–928) was prepared by PCR and cloned into pGEX2 (Pharmacia). All constructed clones were checked by sequence analysis on a Perkin-Elmer 310 Genetic Analyzer.

The ecdysone induction system
C2C12 cells were transfected with 2 μg each of pIND/cyclin D1(T286A) and pIND/lacZ (Invitrogen). After 24 h, cells were switched to DM (2% horse serum in DMEM with 10 μg/ml insulin). After a further 48 h, after well-formed myotubes appeared in the cultures, muristerone was added to a final concentration of 1 μM (Invitrogen). Cells were fixed 24 h post-induction and immunostained for cdk4, cyclin D1 and lacZ (see below).

Immunoassays
For immunostaining cultures, the first antibodies were used at 1:200–1:500 dilution (rabbit or mouse anti-cdk4 and anti-cyclin D1 and rabbit anti-MyoD, Santa Cruz; mouse monoclonal anti-lacZ, Gibco-BRL; mouse monoclonal anti-HA tag, BabCo; rabbit anti-chicken MyoD, Paterson laboratory). Secondly antibody dilutions were also 1:200–1:500 with either Alexa 488 (green) goat anti-rabbit or mouse, or Alexa 594 (red) goat anti-rabbit or mouse (Molecular Probes) for the immunofluorescent reactions. For Western blots on HyBond ECL (Amersham), the indicated first antibody was diluted 1:2000–1:5000 and the second antibody, horseradish peroxidase (HRP)–anti-rabbit, 1:10 000 (Pierce cat. No. 31460). Signal was detected using SuperSignal Ultra Reagent (Pierce cat. No. 34075). Co-immunoprecipitations were performed sequentially, either from Sf9 cell extracts (adjusted for equal protein concentrations in each reaction) (Lassar *et al.*, 1991; Kato *et al.*, 1993) or 10T1/2 cell nuclear extracts labeled with [^{35}S]methionine for 90 min (adjusted for equal counts in each reaction), under sequential low (no SDS) and

high (with SDS) stringency conditions with the indicated antibody combinations using antibody dilutions of 1:200 (Lassar *et al*., 1991).

Protein function assays

For the GST binding experiments, Sf9 cell extracts were prepared as described (Kato *et al*., 1993) and the binding and wash conditions were as previously defined (Kaelin *et al*., 1991). In the band shift assays, his-tagged cdk4 in pRSETC was expressed in the *Escherichia coli* strain BL21 and renatured through sequential dialysis from urea (Kato *et al*., 1994). His-tagged MyoD, myogenin and E12 from chicken were prepared and used in the band shift assays as described earlier (Shirakata *et al*., 1993).

Acknowledgements

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