

## Phosphorylation of Nuclear MyoD Is Required for Its Rapid Degradation

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**MyoD is a basic helix-loop-helix transcription factor involved in the activation of genes encoding skeletal muscle-specific proteins. Independent of its ability to transactivate muscle-specific genes, MyoD can also act as a cell cycle inhibitor. MyoD activity is regulated by transcriptional and posttranscriptional mechanisms. While MyoD can be found phosphorylated, the functional significance of this posttranslational modification has not been established. MyoD contains several consensus cyclin-dependent kinase (CDK) phosphorylation sites. In these studies, we examined whether a link could be established between MyoD activity and phosphorylation at putative CDK sites. Site-directed mutagenesis of potential CDK phosphorylation sites in MyoD revealed that S200 is required for MyoD hyperphosphorylation as well as the normally short half-life of the MyoD protein. Additionally, we determined that turnover of the MyoD protein requires the proteasome and Cdc34 ubiquitin-conjugating enzyme activity. Results of these studies demonstrate that hyperphosphorylated MyoD is targeted for rapid degradation by the ubiquitin pathway. The targeted degradation of MyoD following CDK phosphorylation identifies a mechanism through which MyoD activity can be regulated coordinately with the cell cycle machinery (CDK2 and CDK4) and/or coordinately with the cellular transcriptional machinery (CDK7, CDK8, and CDK9).**

In animals, skeletal muscle is a critical organ, as it is responsible for carrying out coordinated and directed movements. The developmental events that ultimately give rise to mature skeletal muscle can be divided into two major steps (reviewed in references 27, 47, 48, and 50). The first step is determination of which multipotential mesodermal cells become restricted to the myogenic lineage. The second step is commitment of the myogenically determined cells (myoblasts) to terminal differentiation. Terminal differentiation involves cell cycle arrest, fusion of myoblasts leading to myotube formation, and the production of skeletal muscle-specific structural proteins. Cellular restriction to the myogenic lineage is in large part under the control of determination genes which encode a family of skeletal muscle-specific basic helix-loop-helix transcription factors, including MyoD, Myf-5, myogenin, and MRF4. The first determination gene, *MyoD*, was found by its ability to initiate the conversion of fibroblasts into myoblasts (6), a property subsequently found to be true for all four myogenic determination genes. While mice containing a homozygous deletion of *MyoD* develop normally, mice lacking both *MyoD* and *Myf-5* do not form skeletal muscle (36, 37). These results indicate that *MyoD* and *Myf-5* are critical for the determination step in skeletal muscle development. Once MyoD is activated, it continues to be expressed throughout development, presumably through the ability of the MyoD protein to activate *MyoD* transcription (46).

In addition to activating skeletal muscle-specific genes, *MyoD* expression can also lead to cell cycle arrest, even in the absence of terminal myogenic differentiation (5, 26, 45, 47).

The importance of MyoD as an inhibitor of cell cycle progression can also be seen during wound repair (29). When muscle from mice lacking MyoD is injured, the animals are deficient in the regeneration of muscle tissue. The defect in regeneration appears to be the result of the increased potential of satellite cells for self-renewal rather than fusion and myotube formation. One potential mechanism by which MyoD can arrest cell cycle progression is through the transcriptional activation of the *CIP1* gene, which encodes the cyclin-dependent kinase (CDK)-inhibitory protein p21 (14, 33). Consistent with a role for MyoD in negatively regulating cell cycle progression during G<sub>1</sub>, overexpression of cyclin D1 results in an inhibition of MyoD-dependent transcription and might lead to a decrease in p21 production (35, 42). The cyclin D1-mediated decrease in MyoD transactivation activity correlates with a concomitant increase in the abundance of a phosphorylated form of the MyoD protein, although MyoD has not been shown to be a substrate of cyclin D complexes (35, 42, 46). Alternatively, MyoD has been proposed to inhibit cell cycle progression by binding the Rb protein and interfering with E2F-dependent transcription events, which include the activation of a number of genes required for exit from G<sub>1</sub> or DNA replication (3, 13, 44).

These results suggest that a CDK controls MyoD protein activity, possibly through direct phosphorylation of the MyoD protein. The CDKs have been termed proline-directed protein kinases, as their substrates are phosphorylated on serines or threonines that are preceded by a proline (30). MyoD protein has seven putative CDK phosphorylation sites. We demonstrate here that the MyoD protein is likely to be phosphorylated on a serine residue within one of these CDK consensus sites and that the prevention of phosphorylation at this site leads to the stabilization of the MyoD protein. Furthermore, agents that inhibit the ubiquitin-dependent pathway of protein degradation, a pathway intimately linked to the turnover of several key regulators of cell cycle progression, also cause the

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stabilization of MyoD. Based on these results, we propose that the functional significance of MyoD phosphorylation is to control the level of MyoD protein rather than to alter the transactivating activity of the MyoD protein.

## MATERIALS AND METHODS

**Cell culture.** Cell culture and transient transfections were performed as described previously (15). Nuclear extracts were prepared as described elsewhere (1). Where indicated, extracts were treated with 1 U of calf intestine alkaline phosphatase (Gibco BRL Co.) for 30 min at 37°C prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Plasmids and DNA manipulations.** Plasmid 4TRTK-CAT (kindly provided by S. Konieczny, Purdue University) contains four copies in tandem of the E-box sequence present in the muscle creatine kinase gene enhancer. The E-box sequences are upstream of the thymidine kinase (TK) gene basal promoter that controls the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene.

All DNA manipulations were performed by using standard techniques (38). The *MyoD* gene was liberated from plasmid pVZC11b (6) (kindly provided by A. Lassar) by cleavage with *EcoRI/HindIII* and ligated into the *EcoRI/HindIII* fragment of pALTER-1 (Promega, Madison, Wis.). Site-directed mutagenesis was performed by using the Altered Sites Mutagenesis System II (Promega), with oligonucleotides described in the legend to Fig. 2, according to the manufacturer's instructions. The presence of each mutation was verified by DNA sequencing using Sequenase (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's instructions. An *EcoRI/HindIII* DNA fragment containing *MyoD* was ligated into the *EcoRI/HindIII* fragment of pCB6 (28) (kindly provided by F. Rauscher). Cloning into pCB6 allows for expression under the control of the cytomegalovirus immediate-early gene promoter in mammalian cells (15).

Plasmid pADANS-tx61 (kindly provided by S. Plon), which contains the human *CDC34* gene (9, 34), was used to generate a PCR product consisting of the coding region of *CDC34* with an *EcoRI* site at the 5' end and a *HindIII* site at the 3' end of the DNA fragment, respectively, using *Taq* DNA polymerase with primer 1 (5' GTGGTGAATTCCCCGCGCTGCTCCGACC 3') and primer 2 (5' TTTATTCTGAAGCTTTCAGGACTCTCCGTCGCC 3') according to the manufacturer's instructions (Boehringer Mannheim Corp.). The PCR product was then ligated into the *EcoRI/HindIII* fragment of the mammalian expression vector pCB6 (15) to generate pCB6-CDC34. pCB6-dnCDC34 was generated as follows. Plasmid pADANS-tx61 was used as template for PCR using primers 1 and 4 (5' ACGGGGACGCTGTCTATCTCCATCTCCACCCGCC GGTG 3') as well as primers 2 and 3 (5' CACCGCGGGTGGGAGATGGA GATAGACACGTCCCCCGT 3') in two separate reactions as described above. These two PCR products were then placed together with primers 1 and 2 for several further rounds of amplification. The resulting PCR product was placed into pCB6 as described above.

**Half-life determination.** To determine the MyoD protein half-life, cell cultures were incubated for 16 h following transfection and then harvested by trypsinization. Equal numbers of cells were replated into 60-mm-diameter dishes; 24 h after replating, one set of cultures was harvested and nuclear extracts were prepared. The remaining cultures were treated with cycloheximide (final concentration, 35.5  $\mu$ M). At the indicated time points, one set of cultures was harvested and nuclear extracts were prepared. Equal volumes of nuclear extracts from each sample were separated by SDS-PAGE (10% gel) and analyzed by Western blotting with affinity-purified anti-MyoD. The relative amounts of MyoD protein were quantitated with a Bio-Rad GS-250 Molecular Imager. The half-lives were calculated as described previously (40). Where noted, identical blots were analyzed by Western analysis using anti-Cdc2 antibodies as instructed by the manufacturer (Santa Cruz, Inc.).

**Antibody generation and Western blot analysis.** Plasmid pGEX MyoD (23) (kindly provided by the late H. Weintraub) encodes a glutathione *S*-transferase (GST)-MyoD fusion protein that can be produced in bacteria. Recombinant GST-MyoD was prepared as described previously (23). Purified recombinant GST-MyoD fusion protein was used to prepare rabbit polyclonal antisera (performed by HRP Inc., Denver, Colo.). Antibodies were affinity purified as described previously (9), using the recombinant GST-MyoD fusion protein. SDS-PAGE and Western blotting were performed as described previously (9).

Antibodies against human Cdc34 were generated as follows. PCR was performed on pADANS-TX61 with the primers TCCGCCGCCATATGGCTCG GCCGCTAGTGCC and TTTATTCTGCATATGTTCAGGACTCTCCCGT GCC to generate a DNA fragment containing the human *CDC34* gene with an *NdeI* site at both the 5' and 3' ends. This PCR product was ligated into the *NdeI* site of pET28a (Novagen, Inc.), generating a fusion gene encoding a Cdc34 protein tagged with six histidine residues at the amino terminus. The His-tagged Cdc34 was produced in bacteria as previously described (9), purified by using Ni<sup>2+</sup>-nitrilotriacetic acid agarose (Qiagen Inc.) according to the manufacturer's instructions, and used for the production of antisera (performed by HRP Inc.). Antibodies against human Cdc34 were affinity purified as described previously, using His-tagged Cdc34 (9).

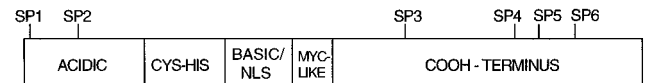


FIG. 1. Schematic diagram of the MyoD protein indicating functional domains and putative CDK phosphorylation sites. NLS, nuclear localization signal.

## RESULTS

**Serine 200 is required for the hyperphosphorylation of MyoD.** Overexpression of cyclin D1 leads to hyperphosphorylation of MyoD protein and inhibition of MyoD-dependent transcription (35, 42). To explore the possibility that MyoD is a CDK substrate, five of the seven potential CDK phosphorylation sites were individually changed to alanines by site-directed mutagenesis. At the sixth and seventh potential CDK sites (GTQSPTPDA), S296 and T298 were changed to alanines simultaneously (Fig. 1). The *MyoD* genes encoding the six mutant proteins or the wild-type protein were subcloned into a mammalian expression vector that places the cDNAs under the control of the cytomegalovirus immediate-early gene promoter and simian virus 40 polyadenylation signals (see Materials and Methods). The expression constructs were transfected into C3H10T1/2 fibroblasts, and nuclear extracts were prepared 36 h later. MyoD proteins were then visualized by Western analysis of the separated nuclear proteins with anti-MyoD antibodies (Fig. 2A). Nuclear extracts were used, as preliminary experiments revealed that the wild-type MyoD protein was difficult to visualize consistently in whole-cell lysates (unpublished observations). Western analysis indicated that cells expressing wild-type *MyoD* or all but one of the serine-to-alanine mutations produced a 45-kDa form of MyoD as well as a slower-migrating form (Fig. 2A, lanes 2 to 4 and 6 to 8). However, cells expressing SP3-*MyoD*, encoding the MyoD S200A protein, lacked the slower-migrating form (Fig. 2A, lane 5). Treatment of nuclear extracts with alkaline phos-

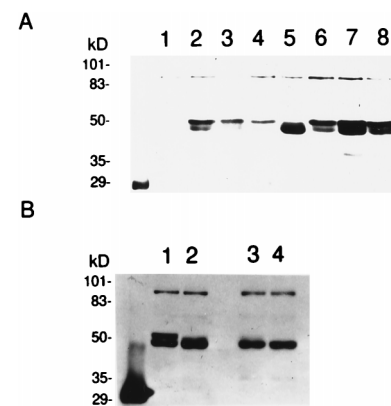


FIG. 2. Hyperphosphorylation of MyoD requires S200. Thirty micrograms of pCB6+ vector or pCB6+ containing wild-type or mutated *MyoD* was transfected into C3H10T1/2 fibroblasts as described in Materials and Methods. At 36 h after transfection, cells were harvested; nuclear extracts were prepared and separated by SDS-PAGE. Separated proteins were subjected to Western analysis using anti-MyoD antibodies. (A) Lanes (oligonucleotide sequences in brackets): 1, vector alone; 2, *MyoD* (wild-type *MyoD*); 3, SP1-*MyoD* (MyoD S5A [ELLSPLLR ELLAPPLR]); 4, SP2-*MyoD* (MyoD S37A [CFDSDPLR CFDAPDLR]); 5, SP3-*MyoD* (MyoD S200A [DASSPRSN DASAPRSN]); 6, SP4-*MyoD* (MyoD S262A [STDSPAAP STDAPAAP]); 7, SP5-*MyoD* (MyoD S277A [PPESPSPGP PPEAPPSPGP]); 8, SP6-*MyoD* (MyoD T296A/S298A [GTQTPSPDA GTQA PAPDA]). (B) Lanes: 1, wild-type MyoD-containing nuclear extracts; 2, wild-type MyoD-containing nuclear extracts pretreated with calf intestine alkaline phosphatase; 3, MyoD S200A-containing nuclear extracts; 4, MyoD S200A nuclear extracts pretreated with calf intestine alkaline phosphatase.

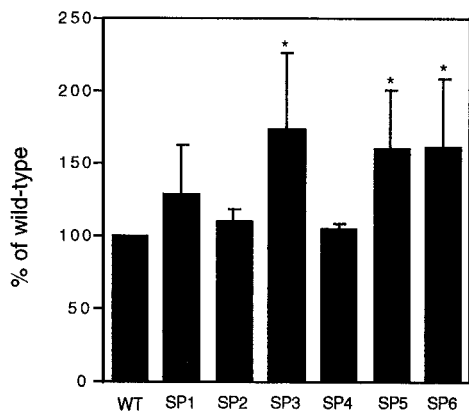


FIG. 3. Increased 4TRTK-CAT activity in C3H10T1/2 fibroblasts expressing MyoD proteins containing mutations of CDK consensus phosphorylation sites. Wild-type (WT) or mutant *MyoD* expression vectors, 4TRTK-CAT, and a TK-luciferase vector were cotransfected into C3H10T1/2 fibroblasts. Cultures were harvested 36 h posttransfection, and luciferase and CAT activities were determined. The results are presented as percentages of the transactivating ability of wild-type MyoD, which was arbitrarily set to 100%. Each experiment was done in duplicate, and each CAT assay contained equivalent amounts of luciferase activity. The results presented are means  $\pm$  standard deviations of three separate experiments.

phatase prior to SDS-PAGE also caused a loss of the slower-migrating form of MyoD expressed from the wild-type gene (Fig. 2B; compare lanes 1 and 2); however, alkaline phosphatase had no effect on the mobility of the MyoD S200A protein (Fig. 2B, lanes 3 and 4). These results are consistent with experiments on endogenous MyoD, demonstrating that phosphorylation of MyoD retards its migration during SDS-PAGE (46). These results indicate that the slower-migrating form of MyoD is phosphorylated and that this phosphorylation event requires a serine residue at position 200 in the MyoD protein. However, from these experiments we cannot determine whether S200 is the site of phosphorylation or whether phosphorylation of S200 is required for phosphorylation at a different position.

**Mutation of serine 200 to alanine increases the ability of MyoD to transactivate a muscle-specific promoter.** Since SP3-MyoD, encoding the MyoD S200A protein, prevented formation of the slower-migrating MyoD species, we set out to determine whether this mutation would also affect MyoD function. One consequence of preventing the formation of the hyperphosphorylated form of MyoD might be an increase or decrease in the ability of the MyoD protein to transactivate muscle-specific genes. To determine the importance of phosphorylation for MyoD transactivation activity, the ability of the mutant proteins to transactivate expression of a reporter construct under the control of the muscle creatine kinase promoter/enhancer E box was examined (see Materials and Methods). In transiently transfected C3H10T1/2 fibroblasts, MyoD S200A exhibited a modest but significant increase in reporter transactivation compared to wild-type MyoD (Fig. 3). All six mutant MyoD proteins were at least as effective as wild-type MyoD at activating transcription from this promoter. These results indicate that the phosphorylation event abolished by the MyoD S200A mutant protein increases MyoD transactivating activity. Interestingly, SP5-MyoD and SP6-MyoD also led to increased 4TRTK-CAT activity, although these mutants had no obvious effect on the abundance of the hyperphosphorylated form of MyoD.

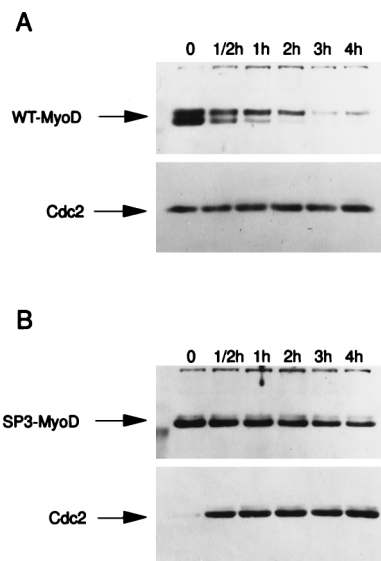


FIG. 4. Increased stability of MyoD S200A. The half-lives of nuclear wild-type MyoD and MyoD S200A were determined as described in Materials and Methods. C3H10T1/2 fibroblasts transiently transfected with either wild-type MyoD (WT-MyoD) (A) or MyoD S200A (B) were treated with cycloheximide for the times indicated; nuclear extracts were prepared and subjected to Western analysis. Identical blots were subjected to Western analysis with anti-Cdc2 antibodies to verify sample loading.

**Mutation of serine 200 to alanine causes an increase in the half-life of MyoD.** CDK-dependent phosphorylation events have been proposed to control protein stability (22, 25). To determine if the SP3-MyoD mutation affected MyoD protein stability, the half-lives of the mutant MyoD proteins were determined as described in Materials and Methods. The half-life of wild-type MyoD in nuclear extracts was found to be about 30 min (Fig. 4 and Table 1), which compares well with the previously determined value of 30 to 60 min (46). The half-life of each of the mutant MyoD proteins described above was also determined (see Materials and Methods). The MyoD S200A mutant was found to have a half-life of about 147 min, approximately fivefold longer than that of the wild-type protein (Fig. 4 and Table 1). These results indicate that one role of MyoD phosphorylation is to destabilize the MyoD protein. Additionally, they suggest that the increased 4TRTK-CAT transactivation in response to SP3-MyoD may be due to increased steady-state levels of the mutant MyoD protein resulting from decreased protein turnover. Interestingly, the SP4-MyoD and SP5-MyoD proteins were also slightly more stable than wild-type MyoD protein (Table 1).

**Requirement for components of the ubiquitin pathway in MyoD degradation.** Short-lived proteins are often degraded by a large, complex protease known as the 26S proteasome (11,

TABLE 1. Half-lives of MyoD proteins

MyoD protein	Mean half-life (min) $\pm$ SD
Wild type	30 $\pm$ 3
S5A	33 $\pm$ 8
S37A	35 $\pm$ 1
S200A	147 $\pm$ 3
S262A	55 $\pm$ 10
S277A	46 $\pm$ 4
T296A/S298A	31 $\pm$ 6

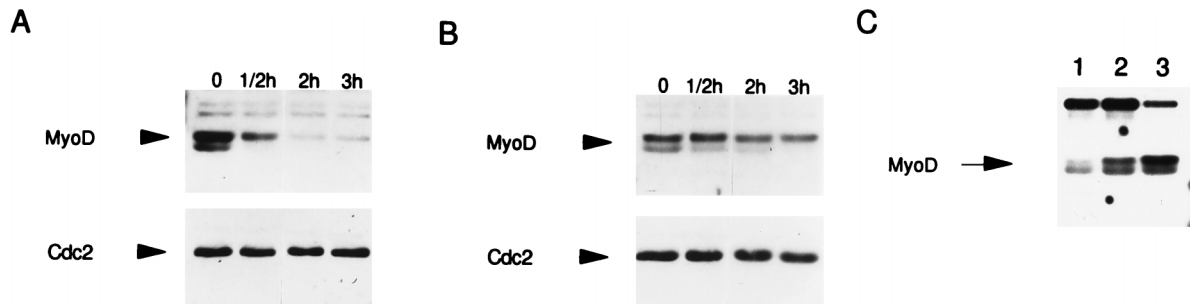


FIG. 5. The proteasome inhibitor LLnL stabilizes the hyperphosphorylated form of MyoD. (A and B) C3H10T1/2 fibroblasts transiently transfected with wild-type MyoD were treated with cycloheximide for the times indicated in the absence (A) or presence (B) of LLnL; nuclear extracts were prepared and subjected to Western analysis. The same blots were subjected to Western analysis with anti-Cdc2 antibodies to verify sample loading. (C) C3H10T1/2 fibroblasts transiently transfected with wild-type MyoD were not treated (lane 1) or treated with LLnL for 1 h (lane 2) or 2 h (lane 3) prior to harvest; nuclear extracts were prepared and subjected to Western analysis.

17). The relatively short half-life of MyoD led us to determine whether the degradation of MyoD might be dependent on 26S proteasome activity. Leucyl-leucyl-norleucinal (LLnL) has been described as a potent inhibitor of the 26S proteasome (39), and therefore the effect of LLnL on the stability of the MyoD protein was determined. In the presence of LLnL, wild-type MyoD protein becomes more stable (compare Fig. 5A and B). This result indicates that the turnover of MyoD is dependent on the function of the 26S proteasome. Further, in the presence of LLnL, there is a preferential accumulation of the phosphorylated form of the MyoD protein under steady-state conditions (Fig. 5C; compare lanes 2 and 3 to lane 1). A preferential accumulation of phosphorylated MyoD would be expected if phosphorylated MyoD were the preferred substrate of the proteasome.

Most proteins that are degraded by the 26S proteasome are first targeted to the proteasome by the attachment of ubiquitin (17, 39). Consistent with this observation, MyoD protein is ubiquitinated *in vitro* following the addition of purified ubiquitin machinery components (12). In yeast, several cell cycle ubiquitination events have been shown to be under the control of the Cdc34p (Ubc3p) ubiquitin-conjugating enzyme (7, 10, 34). Furthermore, Cdc34p has also been shown to control the function of the transcription factors Gcn4p and Rgt1p (21, 24). In mammalian cells, human Cdc34 has been proposed to serve a function analogous to that of its yeast counterpart (32, 34). The prolonged half-life of MyoD in the presence of LLnL as well as the effects of phosphorylation on MyoD stability led us to consider a role for the Cdc34 enzyme in controlling the degradation of MyoD. This possibility was tested directly by examining the effect of expressing wild-type and a dominant-negative allele of human *CDC34* (*dnCDC34*) (2) on the stability of MyoD. C3H10T1/2 fibroblasts were transiently transfected with wild-type *MyoD* and either wild-type *CDC34* or *dnCDC34*. Thirty-six hours later, nuclear extracts were prepared and the steady-state levels of the MyoD protein were compared by Western analysis. The steady-state level of MyoD protein was dramatically elevated by the presence of the *dnCdc34* protein, whereas expression of wild-type *CDC34* had no obvious effect on MyoD levels (Fig. 6A; compare lanes 3 and 4). The effect of *dnCdc34* protein was not due to differential expression of the dominant-negative and wild-type Cdc34 proteins, as both forms of Cdc34 were efficiently produced in the C3H10T1/2 fibroblasts (Fig. 6). Interestingly, the levels of Cdc34 were high in the nuclear extracts (Fig. 6) and low in the cytoplasm (data not shown), indicating that the subcellular location of Cdc34 is also consistent with a role for Cdc34 in MyoD degradation. These results indicate that the

rapid degradation of nuclear MyoD is controlled by Cdc34 ubiquitin-conjugating activity.

## DISCUSSION

We demonstrate here that serine residue 200, located within a CDK consensus phosphorylation site in the MyoD protein, is required for the formation of a specific phosphorylated form of MyoD, often referred to as hyperphosphorylated MyoD. When serine 200 is changed to an alanine, the hyperphosphorylated form of MyoD is absent. Mutations that eliminate other potential CDK phosphorylation sites do not affect the presence of hyperphosphorylated MyoD. While we do not know the nature of the protein kinase responsible for MyoD phosphorylation, we can propose several possible candidates based on the function of MyoD as a transcription factor with roles in differentiation and cell cycle progression. One possibility is that MyoD is a substrate of Cdc2, Cdk2, or Cdk4 complexes that control progression through the cell cycle. Such a phosphorylation event might regulate the cell cycle-inhibitory effects of MyoD. In fact, increasing the levels of cyclin D1 resulted in an eleva-

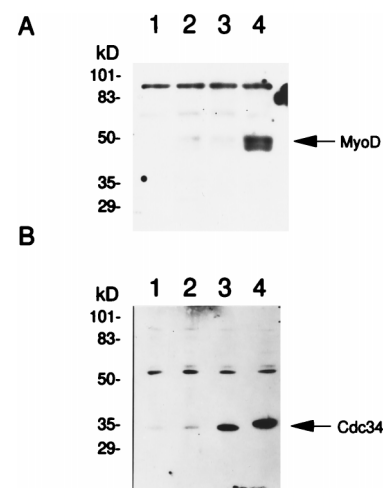


FIG. 6. Expression of a *dnCDC34* stabilizes MyoD. C3H10T1/2 fibroblasts were transiently transfected with *MyoD*, *CDC34*, or both; nuclear extracts were prepared and subjected to Western analysis as described for Fig. 5. (A) Western analysis of nuclear extracts prepared from cells transfected with the indicated plasmids and incubated in the presence of anti-MyoD. Lanes: 1, pCB6+; 2, pCB6+ and wild-type *MyoD*; 3, wild-type *MyoD* and wild-type *CDC34*; 4, wild-type *MyoD* and *dnCDC34*. (B) Western analysis of the samples described for panel A, incubated with anti-Cdc34.

tion of the hyperphosphorylated form of MyoD as well as inhibition of terminal myogenic differentiation (35, 42). However, Cdk4-cyclin D complexes have not been shown to phosphorylate MyoD. An alternative model is that CDKs associated with the transcriptional machinery, Cdk7, Cdk8, and Cdk9, modify MyoD. These kinases and their associated cyclin subunits have been proposed to regulate transcriptional elongation (for a review, see reference 19). In this instance, transcriptional elongation may be regulated by a process that requires the modification and subsequent elimination of transcription factors like MyoD. Under these conditions, MyoD would be required for initiation of transcription, and its targeted degradation would be required for processivity of the transcriptional machinery along the transcribed gene (elongation).

When the half-lives of wild-type and the various mutant MyoD proteins were determined, the S200A change was found to dramatically alter the half-life of MyoD. MyoD S200A has a half-life (147 min) about five times longer than that of wild-type MyoD (30 min). The increased stability of MyoD S200A is likely to be responsible for the increased 4TRTK-CAT activity detected in C3H10T1/2 fibroblasts overexpressing MyoD S200A compared to wild-type MyoD. In contrast, removal of the other potential CDK sites had a much more modest effect on the MyoD half-life. The 30-min half-life was determined for nuclear MyoD. Whether the MyoD present in the cytoplasm has the same half-life is not clear. These results suggest that phosphorylation of MyoD at serine 200 is a prerequisite for MyoD degradation.

Most short-lived proteins in the cell are targeted for degradation by ubiquitin modification (11, 17). Ubiquitination of many proteins leads to their destruction via the 26S proteasome. Indeed, wild-type MyoD protein is a short-lived protein. The steady-state level of wild-type MyoD protein is increased in the presence of the LLnL, a proteasome inhibitor (39), which suggests that inhibition of proteasome activity stabilizes MyoD. Interestingly, in the presence of LLnL, there is a preferential increase in the amount of hyperphosphorylated MyoD, as would be expected if the hyperphosphorylated form of MyoD is the actual target of the proteasome. Recently, Horwitz (18) described a myoblast cell line in which nuclear MyoD had become unstable and the cells had lost the ability to differentiate into myotubes. These studies point to a relationship between MyoD activity and the stability of the MyoD protein.

In this study, we have demonstrated that expression of a dominant-negative allele of human *CDC34* leads to the accumulation of the MyoD protein. While expression of a dnCdc34 protein may indirectly perturb the ubiquitination machinery, we favor the possibility that the Cdc34 protein is directly involved in the destruction of MyoD. The Cdc34 protein was first identified in yeast as a regulator of the G<sub>1</sub>-to-S phase transition (10). It is a member of the family of ubiquitin-conjugating enzymes that can catalyze the addition of ubiquitin to other protein substrates. A characteristic of Cdc34 substrates in yeast is that they must first be phosphorylated by Cdc28-cyclin complexes (8, 16, 22, 43, 51). In mammals, Cdc34 has been invoked to destabilize p27<sup>Kip1</sup> and therefore is a likely regulator of the G<sub>1</sub>-to-S phase transition (32). Furthermore, human Cdc34 is a predominantly nuclear protein (our results and reference 26). MyoD can be ubiquitinated in vitro (12), and its half-life is controlled by phosphorylation through a potential CDK site. Therefore, we propose that phosphorylation of nuclear MyoD leads to its Cdc34-dependent ubiquitination, thus targeting MyoD for destruction via the 26S proteasome.

The potential control of MyoD levels through CDK phos-

phorylation-dependent ubiquitination suggests a mechanism for coordinating transcription events during the G<sub>1</sub>-to-S phase progression in myoblasts. At least two other proteins associated with the transcriptional machinery, p53 and Rb, are substrates of CDKs as well as potential substrates of Cdc34. The stability of the p53 protein also appears to be controlled by CDK-dependent phosphorylation at a site, SSSPQPKKK, very similar to that found in MyoD, ASSPRSN (25). Recently, Connell-Crowley et al. (4) reported that phosphorylation of a similar site in Rb, PSSPLRI, is critical to the inactivation of the Rb protein (see also reference 20). Therefore, depending on cellular growth status, or potentially as a mechanism to enhance transcriptional elongation, the activities of MyoD, Rb, and p53 may be coordinately activated or alternatively eliminated by CDK-dependent phosphorylation. A role for phosphorylation in targeting a regulatory protein for degradation has been postulated in a number of cases, including p27<sup>Kip1</sup> and B-lymphocyte-specific Rag2 (25, 31, 41, 49). In fact, one could speculate that the activities of unrelated cellular events are coordinated by the regulated degradation of several proteins simultaneously.

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A.S. and Q.W. contributed equally to this work.

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