

p57^{Kip2} Stabilizes the MyoD Protein by Inhibiting Cyclin E-Cdk2 Kinase Activity in Growing Myoblasts

EMMANUEL G. REYNAUD, KARINE PELPEL, MARTINE GUILLIER,
MARIE PIERRE LEIBOVITCH, AND SERGE A. LEIBOVITCH*

Laboratoire de Génétique Oncologique UMR 1599 CNRS, Institut Gustave Roussy, 94805 Villejuif, France

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We show that expression of p57^{Kip2}, a potent tight-binding inhibitor of several G₁ cyclin–cyclin-dependent kinase (Cdk) complexes, increases markedly during C2C12 myoblast differentiation. We examined the effect of p57^{Kip2} on the activity of the transcription factor MyoD. In transient transfection assays, transcriptional transactivation of the mouse muscle creatine kinase promoter by MyoD was enhanced by the Cdk inhibitors. In addition, p57^{Kip2}, p21^{Cip1}, and p27^{Kip1} but not p16^{Ink4a} induced an increased level of MyoD protein, and we show that MyoD, an unstable nuclear protein, was stabilized by p57^{Kip2}. Forced expression of p57^{Kip2} correlated with hypophosphorylation of MyoD in C2C12 myoblasts. A dominant-negative Cdk2 mutant arrested cells at the G₁ phase transition and induced hypophosphorylation of MyoD. Furthermore, phosphorylation of MyoD by purified cyclin E-Cdk2 complexes was inhibited by p57^{Kip2}. In addition, the NH2 domain of p57^{Kip2} necessary for inhibition of cyclin E-Cdk2 activity was sufficient to inhibit MyoD phosphorylation and to stabilize it, leading to its accumulation in proliferative myoblasts. Taken together, our data suggest that repression of cyclin E-Cdk2-mediated phosphorylation of MyoD by p57^{Kip2} could play an important role in the accumulation of MyoD at the onset of myoblast differentiation.

Cell cycle progression in eukaryotes is controlled by a series of cyclin-dependent kinases (Cdks) which are in turn modulated by binding to specific cyclins. D-type cyclins (D1, D2, and D3) and cyclin E, termed G₁ cyclins (48), are involved in regulating G₁ progression and S-phase entry. Complexes that control mammalian G₁ progression include cyclin E-Cdk2 and Cdk4/Cdk6 associated with any D-type cyclin and become activated upon phosphorylation of the Cdk subunit by CAK (Cdk-activating kinase), itself a Cdk-related kinase complex (49). These cyclin-Cdk complexes can regulate positively the cell cycle by phosphorylating pRB and thereby inhibit the activity of this cell cycle regulator (48, 57). The discovery of proteins that bind to and inhibit the catalytic activity of cyclin-Cdk complexes has identified kinase inhibition as an intrinsic component of cell cycle control (50). These Cdk inhibitors (Ckis) induce cell cycle arrest in response to antiproliferative signals, including contact inhibition and serum deprivation (42), transforming growth factor β (44), and myogenic (41), myeloid (32), and neuronal (26) differentiation. Ckis can be divided in two families (50, 60). The Ink4 family includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{ARF}. These proteins specifically bind and inhibit Cdk4 and Cdk6 and not other Cdks such as Cdk2 (45). p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, members of the other family of inhibitors, the Cip/Kip family, have the ability to inhibit all G₁/S-phase cyclin-Cdk complexes (19, 49, 56). Although p21^{Cip1} expression during development correlates with terminally differentiating tissues, mice lacking p21^{Cip1} develop normally (9, 39). Similarly, p27^{Kip1}-deficient mice have a grossly normal development and display only phenotypes that seem to be linked to cell proliferation (13, 24, 38). These data suggest the existence of compensatory mechanisms between p21^{Cip1} and p27^{Kip1} during development. p57^{Kip2} is also a tight-binding inhibitor of cyclin A/E-Cdk2 and cyclin

D-Cdk4/Cdk6 complexes and a negative regulator of cell proliferation (25, 33). The expression pattern of p57 mRNA in various adult human tissues indicates that its distribution is more restricted than that of p21^{Cip1} and p27^{Kip1} (25, 33), suggesting that p57^{Kip2} has an important role during development (61, 62).

To undergo differentiation, myogenic cells have to exit the cell cycle through the G₁ checkpoint. Myogenic differentiation is under the control of a family of muscle-specific transcription factors (MRFs) which includes MyoD (7), myogenin (12, 59), Myf5 (4), and MRF4 (45), also known as herculin (34) or Myf6 (5). These proteins share a central basic helix-loop-helix (bHLH) domain that is involved in DNA binding and protein-protein interactions (8). This 70-amino-acid region accounts for their ability to form heterodimers with the E-protein bHLH factors (34, 35), to bind as heterodimers to an E-box DNA consensus sequence (CANNTG) (8), to transactivate muscle genes, and to efficiently convert nonmuscle cells to a myogenic lineage (55, 58). MyoD is expressed in proliferating myoblasts prior to terminal differentiation (55). A number of molecular mechanisms have been proposed to explain the functional inactivation of MyoD in proliferating myoblasts and the coupling of muscle differentiation with the cell cycle arrest (39, 40). These regulatory pathways modulate one or more aspects of myogenic bHLH protein functions such as dimerization with E-protein DNA binding, transactivation, and direct or indirect interaction with cofactors such as MEF-2 (35), pRB (14), p300/CBP (11), or the protein kinase Mos (29). Functional inactivation includes inhibitory phosphorylation of myogenic bHLH proteins (18, 30, 31), inhibition of the myogenic bHLH function via the Id family of dominant-negative HLH factors (2), and either direct or indirect inhibition by the cyclin D-dependent kinases (43, 51). It has been previously shown that overexpression of cyclinD-Cdk complexes inhibited myogenic transcriptional activation mediated by MyoD (15, 16). The role of Cdks in inhibiting muscle differentiation has been substantiated by the observation that forced expression of p21^{Cip1} or p16^{Ink4a} in mitogen-stimulated myoblasts facilitates muscle dif-

* Corresponding author. Mailing address: Laboratoire de Génétique Oncologique UMR 1599 CNRS, Institut Gustave Roussy, 39, rue Camille Desmoulins, 94805 Villejuif, France. Phone: (33) (1) 01 42 11 45 16. Fax: (33) (1) 01 42 11 52 60. E-mail: leibovit@igr.fr.

differentiation in the absence of mitogen deprivation, suggesting that an active cyclin-Cdk complex suppresses MyoD function in proliferating myoblasts (51). It has been recently demonstrated that Cdk phosphorylation of MyoD can target this protein for rapid degradation (52). Indeed, recent data show that direct phosphorylation of MyoD Ser200 by Cdk1 or Cdk2 plays a crucial role in modulating MyoD half-life and myogenic activity (23). Although Ckis appear to be involved in both repression of cyclin-Cdk complexes and activation of MyoD in proliferating myoblasts, no direct relationship between these two events has been described to date.

In the present work, we show that p57^{Kip2} protein expression increases markedly during the early phases of myogenic differentiation. We show that in transient transfection assays, transcriptional transactivation of the mouse creatine kinase (MCK) promoter by MyoD is enhanced by p57^{Kip2}. The Cip/Kip protein family but not p16^{Ink4a} increases the expression level of MyoD. MyoD, an unstable nuclear protein, is stabilized by p57^{Kip2}. In proliferating C2C12 myoblasts, forced expression of p57^{Kip2} represses phosphorylation of MyoD. A dominant-negative form of Cdk2 (Cdk2 DN) which arrests cells at the G₁ phase also induces hypophosphorylation of MyoD. We demonstrate that phosphorylation by cyclin E-Cdk2 complexes of MyoD is inhibited by p57^{Kip2}. In addition, the conserved cyclin and Cdk binding domain of p57^{Kip2} necessary for the inhibition of cyclin E-Cdk2 activity was sufficient to stabilize MyoD, leading to its accumulation in proliferative myoblasts. Taken together, our data suggest that repression of cyclin E-Cdk2-mediated phosphorylation of MyoD by p57^{Kip2} could play an important role in the stability and activity of MyoD during myoblast proliferation.

MATERIALS AND METHODS

Plasmids. pEMSV-MyoD, pGEX-3X-MyoD, and pEMSV-E12 were generous gifts from the H. Weintraub laboratory. Expression vector pCMV-HA-MyoD was generated by cloning three hemagglutinin epitope (HA) tags at the amino terminus of the cDNA insert in pcDNA3 (Invitrogen). The MCK-chloramphenicol acetyltransferase (CAT) reporter plasmid (p1256MCK), generously provided by S. Hauschka, contains the mouse MCK promoter-enhancer region (6). pEX10X-p57^{Kip2} and pBIISK-p27^{Kip1} were kind gifts from J. Massagué. pCEP-WAF1 was a generous gift of B. Vogelstein. CMV-p16^{Ink4a} was a kind gift from B. Heinglein. Cytomegalovirus (CMV)-Cdk2 and CMV-Cdk2 DN were generous gifts from the Ed Harlow laboratory. Cyclin E-Cdk2 was a kind gift from B. Ducummun. To create expression vectors, fragments containing the entire coding sequences were cloned into expression vectors pcDNA3 and/or pEMSV.

pEMSV-p57^{Kip2} was obtained by inserting the *NcoI-HindIII* fragment (filled in with Klenow polymerase) from p57^{Kip2} cDNA into expression plasmid pEMSV at the *EcoRI* site, which was filled in with Klenow polymerase. pEMSV-p57ΔQT was obtained by inserting the *EcoRI-SmaI* fragment from pEMSV-p57^{Kip2} at *EcoRI-SmaI* sites of green fluorescent protein (GFP) expression plasmid pEGFP-C1 (Clontech). The resultant plasmid was used as an intermediate to generate an *EcoRI-MluI* (filled) fragment, inserted into pEMSV at the *EcoRI* site filled in with Klenow polymerase. pEMSV-p57ΔCKI was generated by inserting in frame the *PvuII-HindIII* fragment from p57^{Kip2} at the *PvuII-HindIII* sites of pRSET-C (Invitrogen). This plasmid was further used as an intermediate to generate an *NdeI-HindIII* fragment filled in with Klenow polymerase and inserted into pEMSV at the *EcoRI* site filled in with Klenow polymerase. pEMSV-p57CKI was generated by inserting the *EcoRI-PvuII* fragment from p57^{Kip2} at the *EcoRI-SmaI* sites of pEGFP-C1. This plasmid was used as an intermediate to generate an *EcoRI-MluI* fragment filled in with Klenow polymerase and inserted into pEMSV at the *EcoRI* site filled in with Klenow polymerase. All constructions were tested by *in vitro* translation using the T3 polymerase site of pEMSV.

pGEX-2TKP-p57^{Kip2} was obtained by inserting in frame the *NcoI-HindIII* fragment from pEX10X-p57^{Kip2} into the *NcoI-HindIII* sites of expression plasmid pGEX-2 TKP (a generous gift of L. Kouzarides). pGEX-2T-p57CKI was constructed by inserting in frame the *SmaI-PvuII* fragment from pGEX-2TKP-p57^{Kip2} at the *SmaI* site of expression plasmid pGEX-2T (Pharmacia). pGEX-2TK-p57ΔCKI was constructed by deleting an *NcoI-BglIII* fragment of pGEX-2TKP-p57^{Kip2}. pGEX-2TK-p57ΔQT was generated by inserting in frame the *BspI* (filled)-*HindIII* fragment obtained from pEX10X-p57^{Kip2} at the *SmaI* (filled) site of pGEX-2TK.

Cell cultures, DNA transfections, and CAT assays. The mouse skeletal muscle cell line C2C12 and the fibroblast cell line C3H10T1/2 were maintained in growth medium (GM) supplemented with antibiotics (a mixture of penicillin and streptomycin [Life Technologies, Inc.]) and with 20 and 15% of fetal calf serum (FCS) in Dulbecco's modified Eagle's medium (DMEM), respectively. C2C12 cells were transfected by the calcium phosphate procedure as previously described (28). C3H10T1/2 fibroblasts were transfected by using polyethyleneimine essentially as described previously (3). Briefly, 4 × 10⁴ cells per well were plated onto 24-well plates. On the following day, cells were transfected with various combinations of plasmids as indicated in the figure legends. The total amount of DNA used for each plate was normalized with the relevant empty expression vehicle. CAT activity was determined with aliquots of cell extracts from harvested cells 48 h after transfection in GM as previously described (28). Five hundred nanograms of plasmid pCH110 (Pharmacia) was included in transfections as an internal control for transfection efficiency. All CAT activities were determined with equivalent quantities of proteins in triplicate, and assays were repeated at least twice. A phosphorimager system was used to determine the amount of ¹⁴C-labeled reaction products and substrate from thin-layer chromatographic plates.

Metabolic labeling. Cell cultures were incubated for 1 h in methionine-free medium supplemented with 5% FCS followed by incubation for 30 min in the same medium with 300 μCi of [³⁵S]methionine (Trans³⁵S-label; ICN) per ml. In cold-chase experiments, the [³⁵S]methionine-labeled cultures were rinsed with fresh medium supplemented with 10 mM nonradioactive methionine and 15% FCS and harvested at appropriate times. For immunoprecipitation of HA-MyoD, cells were lysed as described as follows.

Kinase assay. Cyclin E-Cdk2 complexes were mixed with purified glutathione S-transferase (GST) or GST-p57 fusion proteins in 50 mM HEPES (pH 8.0) and incubated for 1 h at 4°C. The kinase reaction was carried out at 30°C for 30 min in a 30-μl reaction mixture containing 50 mM HEPES (pH 8.0), 10 mM MgCl₂, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na₃VO₄, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM ATP, and 150 kBq of [γ-³²P]ATP (5,000 Ci/mmol; Amersham). One microgram of GST-MyoD or MyoD (29) was used as the substrate. The reaction products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and phosphorylated proteins were detected by autoradiography and quantitated with a Fuji BAS-1000 imaging analyzer.

Antibodies, immunoprecipitation, and Western blot analyses. For immunoprecipitation, precleared cell lysates in immunoprecipitation buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol, 0.5% NP-40, 0.5 mM sodium orthovanadate, 50 mM NaF, 80 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM EGTA, 10 μg each of leupeptin, pepstatin, and aprotinin per ml) were incubated with the indicated antibody for 2 to 3 h at 4°C with gentle agitation. Immunocomplexes bound to protein G-Sepharose were collected by centrifugation and washed several times in immunoprecipitation buffer. Immunoprecipitated proteins were resolved by SDS-PAGE (10% gel) followed by fluorography (³⁵S-labeled proteins) and autoradiography.

For immunoblot analyses, total-cell extracts or immunoprecipitates were solubilized in radioimmunoprecipitation assay-EGTA buffer and processed as previously described (27). Analyses were performed on 10% polyacrylamide gels with a 5% polyacrylamide stacking gel. After electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membranes, the membranes were blocked with 50 mM Tris-HCl (pH 7.4)-150 mM NaCl-0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies: polyclonal anti-MyoD antibody C-20 diluted 1/500, polyclonal anti-mouse p57^{Kip2} antibody E-17 diluted 1/250, anti-mouse monoclonal anti-p27^{Kip1} antibody F8 diluted 1/250, monoclonal anti-p21^{Cip1} antibody F5 diluted 1/250, and polyclonal anti-p16 antibody M-156 diluted 1/250 (provided by Santa Cruz Biotechnology, Santa Cruz, Calif.); monoclonal anti-HA antibody 12CA5 (provided by Boehringer Mannheim); and monoclonal anti-troponin T antibody JTL-12 (supplied by Sigma). Membranes were washed and incubated 1 h with a peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1/10,000 with polyclonal antibodies or 1/4,000 with monoclonal antibodies. After several washes, membranes were incubated with an enhanced chemiluminescence (ECL) system (Amersham) according to the manufacturer's instructions. Exposure was done with Agfa Curix RP2 films and intensifying screens.

Phosphatase treatment. Extracts normalized to protein content were immunoprecipitated with the mouse monoclonal anti-MyoD antibody 5.8A (Pharmingen) and protein G-Sepharose beads. After immunoprecipitation, beads were washed twice with ECB buffer without phosphatase inhibitors and resuspended in 50 μl of calf intestine phosphatase (CIP) buffer (10 mM Tris-HCl [pH 8], 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride) for phosphatase treatment with 50 U of CIP (New England Biolabs) for 60 min at 50°C. Proteins were separated by SDS-PAGE, immunoblotted with anti-MyoD polyclonal antibody C-20 (Santa Cruz), and analyzed by ECL.

RESULTS

Increase in p57^{Kip2} protein levels during the differentiation of C2C12 myoblasts to myotubes. Little is known about the role of p57^{Kip2} in myogenic differentiation; we initially charac-

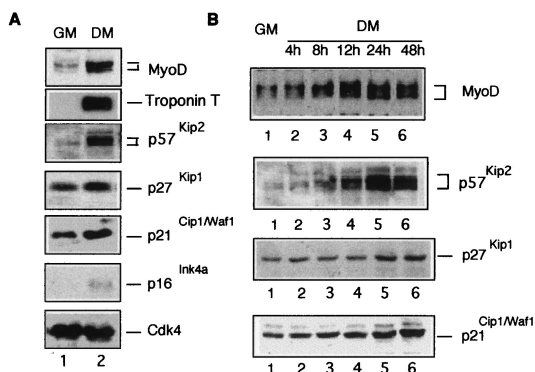


FIG. 1. Protein expression of Ckis during muscle differentiation. C2C12 myoblasts were cultured in high-mitogen medium (20% FCS-containing GM (A, lane 1) or in low-mitogen medium (2% FCS DM) for 48 h (A, lane 2) or for increasing various times as indicated (B, lanes 2 to 6). For each stage, total-cell extracts corresponding to 150 μ g of proteins were resolved by SDS-PAGE on a 10% gel, and MyoD, troponin T, p16^{Ink4a}, p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} were detected by Western blot analysis using specific antibodies as described in Materials and Methods. Exposure times were 5 min for the Cip/Kip protein family, 1 min for MyoD, 1 min for troponin T, and 60 min for p16^{Ink4a}.

terized p57^{Kip2} expression during early myogenic differentiation by Western blot analysis using the C2C12 cell line, a well-defined model for ex vivo differentiation. These cells proliferate as myoblasts in high serum concentrations and can be induced to differentiate by reducing the serum concentration from 20% to 2%. Under our conditions, C2C12 myoblasts fuse into myotubes within 48 to 60 h with an efficiency of 75 to 80%. As shown in Fig. 1A, C2C12 myoblasts express p57^{Kip2} that migrates as a doublet around 57 kDa, as previously observed in SAOS-2 cells (33). The presence of p57^{Kip2} protein in our C2C12 cell line has been confirmed by using four antibodies directed against four different epitopes of p57^{Kip2} protein (data not shown). p57^{Kip2} is detectable in myoblasts as MyoD and accumulates at high levels (about 10-fold) in C2C12 myotubes. In contrast, troponin T, a skeletal muscle marker, is

observed only in differentiated cells, and Cdk4 is equally expressed in both myoblasts and myotubes (51, 63). As Ckis such as p21^{Cip1}, p27^{Kip1}, and p16^{Ink4a} have been implicated in differentiation and cell cycle arrest, we also analyzed their protein expression in C2C12 myogenic differentiation by Western blot analyses (Fig. 1A). p21^{Cip1} expression was stimulated by three-fold in myoblasts cultured in differentiation medium (DM), in agreement with previous observations (17). p27^{Kip1} was induced in C2C12 cells following culture in DM, but this enhancement was only twofold between proliferating myoblasts and differentiated myotubes. In contrast to the Cip/Kip protein family, p16^{Ink4a} protein was not observed in proliferating myoblasts and was barely detectable during myogenic differentiation. As shown in Fig. 1B, p57^{Kip2} expression levels increase as soon as 4 h after cells are placed in DM and peak after 24 h of differentiation. By this time, MyoD is also up-regulated and then slightly decreases during differentiation. Altogether, these results suggest that p57^{Kip2} may play a particular role during early differentiation.

p57^{Kip2} enhances the effect of MyoD transcription on MCK expression. Transient transfection assays were performed to determine the effect of overexpression of p57^{Kip2} on MyoD-mediated transcription of muscle-specific genes. C3H10T1/2 cells were transiently transfected with expression vectors encoding MyoD and/or either p16^{Ink4a}, p21^{Cip1}, p27^{Kip1}, or p57^{Kip2} Ckis along with a skeletal muscle reporter construct containing 1,256 bp from the MCK promoter driving expression of CAT (MCK-CAT) (6). MCK-CAT is not expressed in C3H10T1/2 cells when transfected alone, but it was activated efficiently by cotransfection with a MyoD expression vector (Fig. 2A, lanes 1 and 2, respectively). In a dose-dependent manner, expression of p16^{Ink4a}, p21^{Cip1}, p27^{Kip1}, or p57^{Kip2} increased the transactivation of MCK-CAT by MyoD, with the highest increase (about 3- to 3.5-fold) observed in the presence of p57^{Kip2} (lanes 7 to 9). The vector alone (Fig. 2A, lane 1) or the Ckis alone (lanes 3 to 6) had no effect on MCK promoter activity. In contrast, transactivation of a non-muscle-specific reporter (CMV-CAT) was not significantly altered (reduced by 10 to 15%) when cotransfected with p57^{Kip2} compared to con-

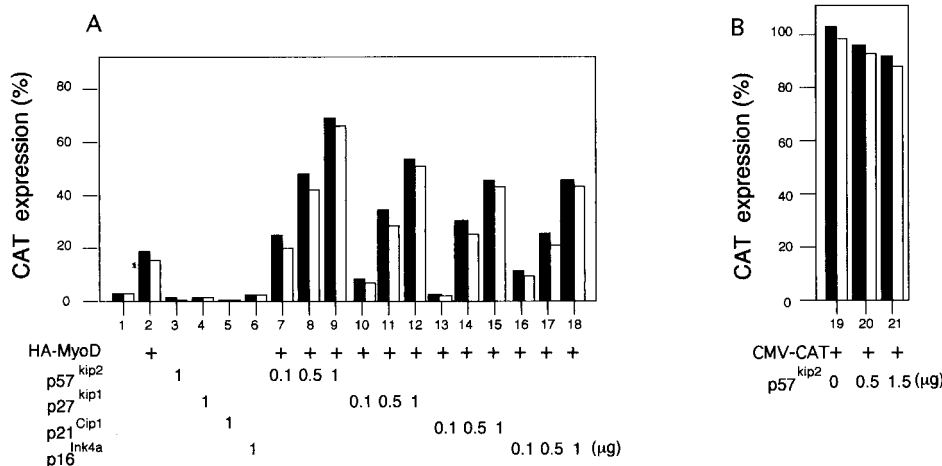


FIG. 2. Effect of ectopically expressed Ckis on MyoD-dependent transcriptional transactivation of the MCK enhancer-promoter. C3H10T1/2 cells were cotransfected with 0.5 μ g of MCK-CAT reporter plasmid (A, lanes 1 to 18) or a non-muscle-specific reporter (CMV-CAT) (B, lanes 19 to 21) together with 1 μ g of an expression vector encoding HA-MyoD (lane 2), pCMV-p57^{Kip2} (lane 3), pCMV-p27^{Kip1} (lane 4), pCMV-p21^{Cip1} (lane 5), or pCMV-p16^{Ink4a} (lane 6) or with 0.5 μ g of pCMV-HA-MyoD (lanes 7 to 18) and increasing amounts of p57^{Kip2} (lanes 7 to 9), p27^{Kip1} (lanes 10 to 12), p21^{Cip1} (lanes 13 to 15), or p16^{Ink4a} (lanes 16 to 18). Expression vector pCMV without insert was included to normalize DNA in all transfections. CAT levels were determined 48 h after transfection in high-serum (20% FCS) medium. Protein concentration was equalized by the Bradford method. Typically, 15 μ g of total-cell extract was used for the reaction. CAT activities in duplicate plates (black and white bars) from a representative experiment are expressed as percentages of acetylated forms of chloramphenicol versus the nonacetylated substrate.

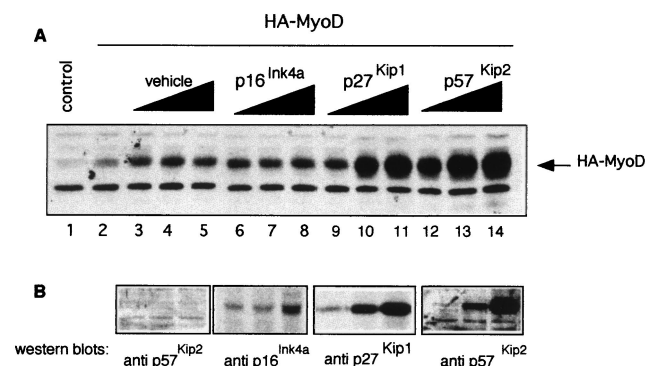


FIG. 3. Differential accumulation of MyoD protein by ectopic expression of Ckis. (A) Immunoblot showing exogenous MyoD and Ckis in transiently transfected C3H10T1/2 cells. As for Fig. 2, C3H10T1/2 cells were transiently transfected with 0.5 μ g of expression vector encoding HA-MyoD (lanes 2 to 14) plus 0.5, 1, or 1.5 μ g of empty expression vector (lanes 3 to 5) or 0.5, 1, or 1.5 μ g of pCMV-p16^{Ink4a} (lanes 6 to 8), pCMV-p27^{Kip1} (lanes 9 to 11), or pCMV-p57^{Kip2} (lanes 12 to 14). Whole-cell lysates (10 μ g) were separated by SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with monoclonal antibody 12CA5 (Boehringer Mannheim) and visualized by ECL (Amersham). (B) Fifty-microgram aliquots of the same lysates from transfected cells were analyzed for expression of exogenous p16^{Ink4a}, p27^{Kip1}, and p57^{Kip2} by Western blotting with an anti-p16^{Ink4a} (M-156; Santa Cruz), anti-p27^{Kip1} (F8; Santa Cruz), or anti-p57^{Kip2} (E-17; Santa Cruz) polyclonal antibody.

control cells, indicating that p57^{Kip2} has no significant effect on the CMV promoter (Fig. 2B, lanes 19 to 21). These results indicate that transactivation of MCK-CAT expression by MyoD is increased by the addition of a Cki expression vector and suggest a functional interaction between Ckis and MyoD during myogenesis.

p57^{Kip2} increases the level of MyoD in cotransfected cells.

We next examined the influence of ectopic expression of p16^{Ink4a} and p57^{Kip2} on the levels of coexpressed MyoD in transiently transfected C3H10T1/2 fibroblasts. Immunoblotting analyses revealed that p57^{Kip2} increased the steady-state level of coexpressed MyoD (Fig. 3) in a dose-dependent manner. This much higher level of MyoD was specific for the Cip/Kip proteins because coexpression of p27^{Kip1} (Fig. 3, lanes 9 to 11) also increased MyoD expression whereas the p16^{Ink4a} expression vector (lanes 6 to 8) or an empty vector (lanes 3 to 5) had no effect.

p57^{Kip2} increases the stability of MyoD. To elucidate the mechanism by which the levels of coexpressed MyoD is enhanced by the Cip/Kip protein family and especially by p57^{Kip2}, we first tested whether p57^{Kip2} affected transcription or translation of the cotransfected MyoD. Northern blot analyses showed that the level of MyoD mRNA did not differ significantly between expression of MyoD alone and coexpression of MyoD and p57^{Kip2} (data not shown). Furthermore, the amount of newly synthesized MyoD was measured by immunoprecipitation analysis with the anti-HA monoclonal antibody in cells pulse-labeled for 30 min with [³⁵S]methionine. The amounts of MyoD did not differ significantly between the single or pairwise transfections of MyoD (Fig. 4A, lanes 2 and 3). The metabolic stability of MyoD was investigated by a pulse-chase experiment. Immunoprecipitation analyses revealed that MyoD has a half-life of 40 to 50 min (Fig. 4B and C), in agreement with previously reported results (1, 23, 52, 55). Surprisingly, when coexpressed with p57^{Kip2}, MyoD showed a half-life extended to 140 min (Fig. 4B and C). This result suggests that the increased level of MyoD observed upon coexpression with p57^{Kip2} is due to the stabilization of the protein by p57^{Kip2}.

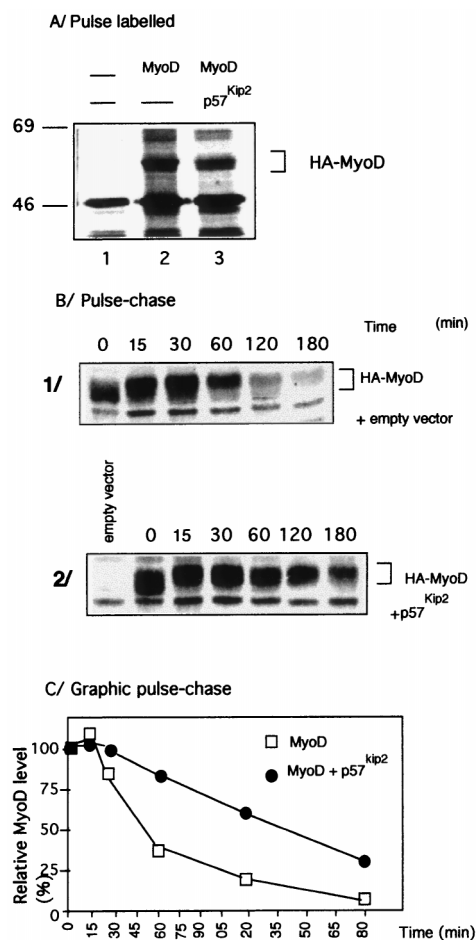


FIG. 4. Stabilization of MyoD protein by p57^{Kip2} coexpression. (A) Pulse-labeling experiment showing the initial translation rate of MyoD with or without p57^{Kip2} coexpression. C3H10T1/2 fibroblasts transfected with either control vector alone (lane 1), HA-MyoD expression vector alone (lane 2), or HA-MyoD plus pCMV-p57^{Kip2} vector (lane 3) were labeled for 30 min with [³⁵S]methionine. Cell lysates having the same radioactive counts were subjected to immunoprecipitation with specific monoclonal antibody 12CA5 and analyzed by SDS-PAGE and autoradiography. (B) Pulse-chase of [³⁵S]-labeled MyoD. C3H10T1/2 fibroblasts were cotransfected by pCMV-HA-MyoD and empty expression vector (1) or cotransfected by pCMV-HA-MyoD and pCMV-p57^{Kip2} expression vectors (2). Cells were cultured in methionine-free medium for 1 h and then pulsed with 300 μ Ci of [³⁵S]methionine per ml for 30 min. Following incubation, cells were washed with medium containing an excess of unlabeled methionine (10 mM) and chased for the indicated times. Cell extracts were immunoprecipitated with specific monoclonal antibody 12CA5. One 60-mm-diameter dish of transfected cells was used for each time point. This experiment was performed three times. (C) Graphic display of intensities of MyoD shown in panel B. Results are representative of three independent experiments. For quantitation, the autoradiograms were scanned with a phosphorimager (Fuji).

MyoD is a substrate for phosphorylation by cyclin E-Cdk2 complexes. Cdk-mediated phosphorylation is known to be often involved in proteolytic degradation; we next examined whether p57^{Kip2} induces an increased stability of MyoD by blocking Cdk2-dependent phosphorylation of MyoD. As shown in Fig. 5A, MyoD is detected as a doublet following transfection of wild-type MyoD (MyoD^{wt}). These two bands contain rapidly and slowly migrating forms of MyoD (Fig. 5A, lane 1). The slowly migrating form is shown to be the hyperphosphorylated species and was resolved into a single fast-migrating species after treatment with the CIP (Fig. 5A, lanes 1 and 2). Because p57^{Kip2} is known as a potent inhibitor of G₁-

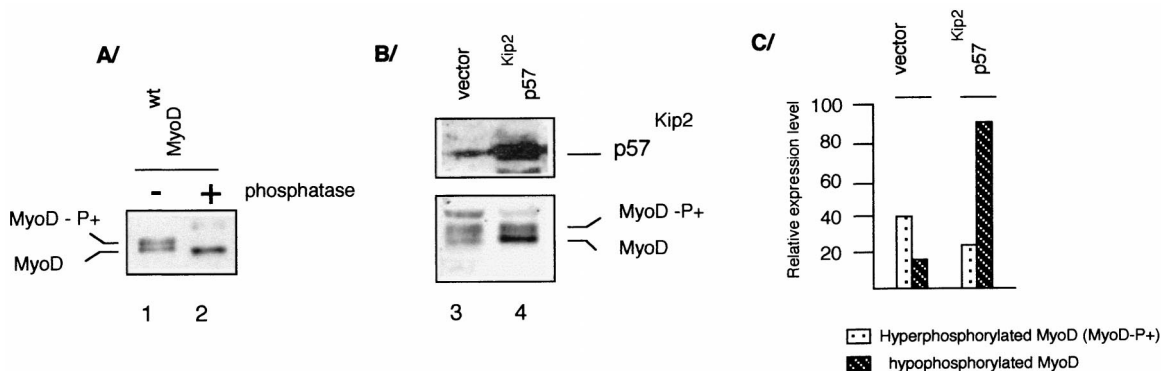


FIG. 5. Forced expression of p57^{Kip2} reduces MyoD phosphorylation in growing myoblasts. (A) Total cellular proteins from C3H10T1/2 fibroblasts transfected by pEMSV-MyoD^{wt} were split in two, immunoprecipitated with a MyoD monoclonal antibody (5.8A; Pharmingen), and resuspended in SDS-containing sample medium (lane 1) or treated with CIP (lane 2) as described in Materials and Methods. The immunoprecipitates were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. MyoD was detected by Western blot analysis using anti-MyoD polyclonal antibody C-20 (Santa Cruz). (B) C2C12 myoblasts were transfected with either the empty vector (lane 3) or pCMV-p57^{Kip2} (lane 4). Transfected cells were grown in GM (DMEM containing 20% FCS) for 48 h. Cells were collected, and 50 μg of whole-cell extract was analyzed by Western blot for MyoD and p57^{Kip2} expression. (C) The signals were quantitated with a Gel Scan (Pharmacia).

and S-phase Cdks (25), we tested its ability to inhibit and/or to reduce hyperphosphorylation of MyoD. For this purpose, a p57^{Kip2} expression vector was transiently transfected into C2C12 myoblasts, transfected cells were grown for 48 h in GM, and MyoD expression was monitored by Western blotting of whole-cell extracts. As shown in Fig. 5B, in C2C12 myoblasts, MyoD is detected as two bands with a major slowly migrating hyperphosphorylated form following transfections with the empty vector. In contrast, the fast-migrating band corresponding to the hypophosphorylated form of MyoD accumulates in p57^{Kip2}-transfected myoblasts compared with control cells transfected with vector alone (Fig. 5B and C). These results show that in vivo, Cdk-dependent phosphorylation of MyoD can be repressed by p57^{Kip2}. Recently Cdk1 and Cdk2 have been shown to phosphorylate MyoD in vitro (23). To verify

that Cdk2 phosphorylates MyoD in vivo, Cdk2 DN clones 1 and 3 were cotransfected with MyoD in C2C12 proliferating myoblasts. As shown in Fig. 6A, expression of Cdk2 DN clones 1 and 3 (lanes 3 and 4) caused a large increase in the G₁ population, whereas wild-type Cdk2 (Cdk2 wt; lane 2) did not affect the cell type distribution. Expression of Cdk2 DN but not Cdk2 wt had noticeable effect on the phosphorylation of MyoD (Fig. 6B, lanes 2 to 4) with respect to MyoD phosphorylation in cells cotransfected with the empty vector (Fig. 6B, lane 1). Expression of Cdk2 DN induces hypophosphorylation of MyoD, as evidenced by the accumulation of the fast-migrating form of MyoD. Altogether, these data strongly suggest that in vivo p57^{Kip2} inhibition of Cdk2 activity plays a role in MyoD-mediated myoblast differentiation.

Phosphorylation of MyoD by cyclin E-Cdk2 is inhibited by p57^{Kip2}. To assess the consequence of inhibition of Cdk2 activity by p57^{Kip2} upon MyoD phosphorylation, MyoD^{wt} was translated in the presence of [³⁵S]methionine in rabbit reticulocyte lysate and subjected to phosphorylation by purified cyclin E-Cdk2. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. As shown in Fig. 7A, phosphorylation of MyoD^{wt} by cyclin E-Cdk2 resulted in a decrease of its electrophoretic mobility. This slower-migrating form corresponded to phosphorylation, as shown by ³²P incorporation in Fig. 7B. We next examined whether p57^{Kip2} was sufficient for the inhibition of cyclin E-Cdk2 activity upon MyoD phosphorylation. We generated GST-fused wild-type

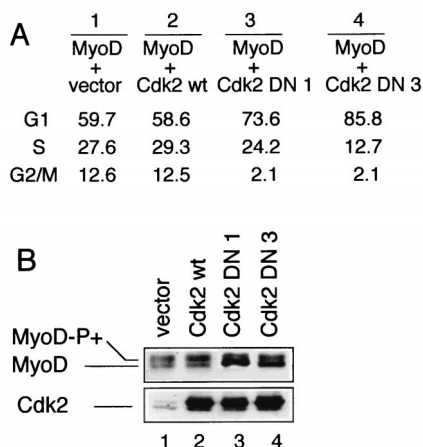


FIG. 6. Cdk2 DN causes the accumulation of C2C12 myoblasts in G₁ and reduces hyperphosphorylation of MyoD. (A) C2C12 proliferating myoblasts were transiently cotransfected with 0.5 μg of CMV-GFP plasmid in combination with 7.5 μg of the CMV vector (lane 1), 7.5 μg of the CMV-Cdk2 wt (lane 2), or 7.5 μg of Cdk2 DN clone 1 (lane 3) or 3 (lane 4). The cells were harvested 48 h after transfection, visualized for GFP fluorescence, stained for DNA content, and analyzed by flow cytometry. (B) Fifty-microgram aliquots of total-cell lysates were used for the expression pattern of MyoD and analyzed by protein immunoblotting with anti-MyoD antibody C-20 (Santa Cruz). The same blot was also analyzed by protein immunoblotting with polyclonal anti-Cdk2 antibody M2 (Santa Cruz).

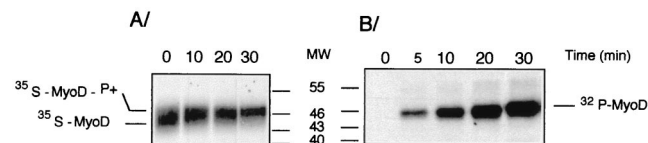


FIG. 7. MyoD is a substrate of cyclin E-Cdk2-dependent phosphorylation. (A) In vitro-translated ³⁵S-labeled MyoD^{wt} was incubated with purified cyclin E-Cdk2 complexes for various periods of time. Phosphorylation shifts were visualized after SDS-PAGE. Shown is the autoradiogram of the SDS-PAGE analysis. (B) Bacterially produced MyoD protein was phosphorylated by purified cyclin E-Cdk2 complex for the time indicated. Shown is the autoradiogram for ³²P phosphorylation reactions. Unphosphorylated MyoD protein migrates as 45-kDa band that progressively shifts to 47 kDa in the course of phosphorylation by cyclin E-Cdk2 kinase reaction. Positions of molecular weight markers (MW) are shown in thousands.

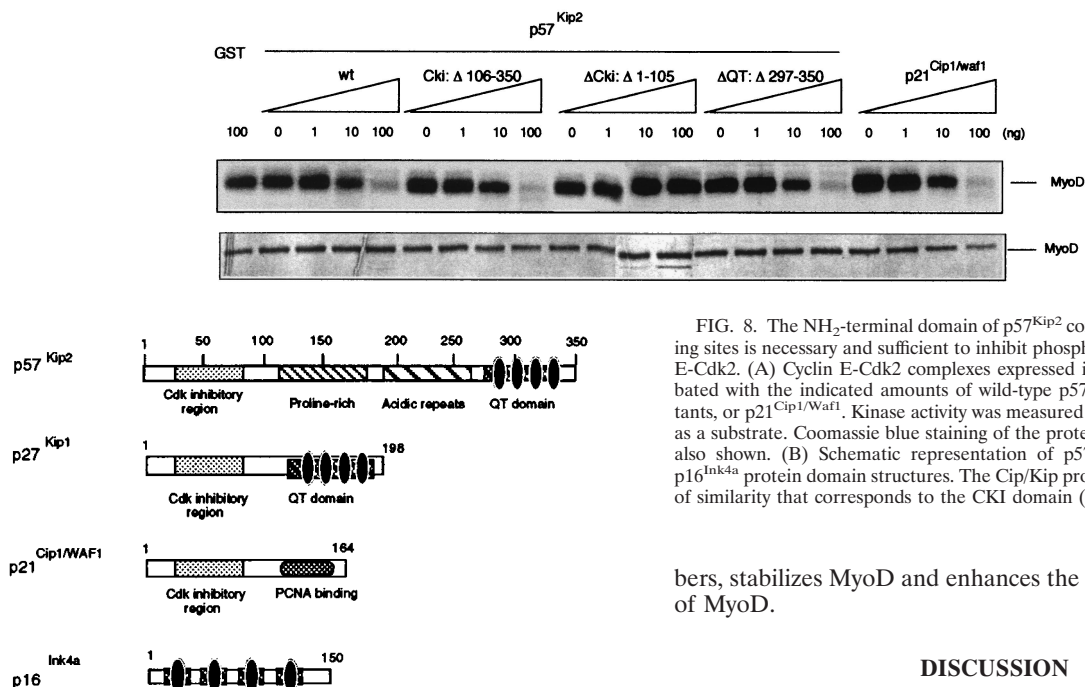


FIG. 8. The NH₂-terminal domain of p57^{Kip2} containing the cyclin-Cdk binding sites is necessary and sufficient to inhibit phosphorylation of MyoD by cyclin E-Cdk2. (A) Cyclin E-Cdk2 complexes expressed in Sf9 insect cells were incubated with the indicated amounts of wild-type p57^{Kip2} (wt), the indicated mutants, or p21^{Cip1/Waf1}. Kinase activity was measured by using GST fused MyoD^{wt} as a substrate. Coomassie blue staining of the protein substrate GST-MyoD^{wt} is also shown. (B) Schematic representation of p57^{Kip2}, p27^{Kip1}, p21^{Cip1}, and p16^{Ink4a} protein domain structures. The Cip/Kip protein family contains a region of similarity that corresponds to the CKI domain (dotted box).

p57^{Kip2} and mutants defective in either cyclin and Cdk binding domains (p57 Δ CKI) or deleted of the COOH-terminal region (p57 Δ QT) or GST-fused CKI domain (p57CKI) and tested their abilities to inhibit phosphorylation of MyoD by the cyclin E-Cdk2 complexes. As shown in Fig. 8A, wild-type p57^{Kip2}, p57CKI, and p57 Δ QT inhibited cyclin E-Cdk2 kinase activity, whereas p57 Δ CKI was defective in kinase inhibitory activities. Thus, both cyclin and Cdk binding domains in the NH₂-terminal region of p57^{Kip2} seems to be required for inhibiting phosphorylation of MyoD by cyclin E-Cdk2. It has recently been reported that CKI domain in the NH₂-terminal region appears to be well conserved among members of the Cip/Kip family (Fig. 8B) (20, 46). We found that GST-fused wild-type p21^{Cip1} was also able to inhibit phosphorylation of MyoD by cyclin E-Cdk2 complexes (Fig. 8A). Based on these findings, it appears that the NH₂ region of p57^{Kip2} is necessary and sufficient to inhibit phosphorylation of MyoD by cyclin E-Cdk2.

MyoD accumulation and transactivation of the MCK promoter by MyoD are enhanced by the NH₂ domain of p57^{Kip2}. To determine whether the NH₂ domain of p57^{Kip2} is also sufficient for the stabilization of MyoD in C2C12 cells, we transfected C2C12 myoblasts with either wild-type or mutant p57 expression vectors. MyoD protein was monitored by Western blot analysis. Figure 9A shows that wild-type p57^{Kip2} or p57 Δ QT induced MyoD to accumulate at higher levels (lanes 2 to 7). In contrast, p57 Δ CKI failed to increase MyoD protein abundance (lanes 8 to 10).

MCK transactivation by MyoD is enhanced by coexpression of p57^{Kip2} (Fig. 2). To investigate if the NH₂ domain of p57^{Kip2} was also sufficient for this activation, C3H10T1/2 cells were transiently transfected with MCK-CAT reporter plasmid in the presence of MyoD and of various mutants of p57^{Kip2}. MCK transcriptional activity was analyzed in GM. As shown in Fig. 9B, in high-mitogen GM, MCK promoter activity was enhanced by ectopic expression of wild-type p57^{Kip2} or p57 Δ QT but not by p57 Δ CKI. Altogether, these data show that the NH₂ region of p57^{Kip2}, common to the other Cip/Kip family mem-

bers, stabilizes MyoD and enhances the transcriptional activity of MyoD.

DISCUSSION

Control of myogenesis is achieved by a network of various factors interacting with each other in positive and negative regulatory mechanisms. MyoD has been implicated as a master regulatory gene in the process of muscle differentiation. Its activity is highly controlled in particular by growth factors, oncogenes, and negative HLH proteins such as Id (2, 39, 40). Phosphorylation of MyoD is one of the crucial mechanisms that control its activity in eukaryotic cells, and recent reports show that phosphorylation of MyoD Ser200 in proliferating myoblasts appears to play a major role in modulating MyoD half-life and myogenic activity (23, 52). The apparent antagonism between proliferation and differentiation implies that signaling pathways driving proliferation must be suppressed to allow induction of differentiation. Indeed, recent works demonstrated the role of G₁ cyclins and their partners (Cdk and Ckis) in the cell cycle arrest and muscular differentiation program (15–17, 51).

In this study, we show that p57^{Kip2} levels are up-regulated during myogenesis (Fig. 1). This raises the possibility that p57^{Kip2} participates in growth arrest of myoblasts through inhibition of Cdks and thereby in the initiation of differentiation. We show that overexpression of Ckis in proliferating myoblasts reverses mitogen-mediated repression of MyoD function. We found that p57^{Kip2} and p27^{Kip1} but not p16^{Ink4a} lead to accumulation of MyoD. We demonstrate that accumulation of MyoD is due to an increased half-life of MyoD protein by coexpressed p57^{Kip2} (Fig. 4C). Such an increased stability of MyoD was also observed recently when Ser200, the major site of Cdk-dependent phosphorylation, was changed to nonphosphorylatable alanine (23, 52). The fact that the resultant protein, MyoD^{Ala200}, was more stable than MyoD^{wt} supports the view that a p57^{Kip2}-induced increase in MyoD half-life occurs via the repression of cyclin-Cdk activities implicated in the phosphorylation of MyoD at Ser200. This specific phosphorylation is a prerequisite for MyoD degradation by the ubiquitin pathway (21). Interestingly, in C2C12 myoblasts, MyoD is expressed as two bands, a major slow-migrating hyperphosphorylated form and a fast-migrating band corresponding to the hypophosphorylated form which accumulates in p57^{Kip2}-transfected myoblasts (Fig. 5). This raises the possibility that MyoD

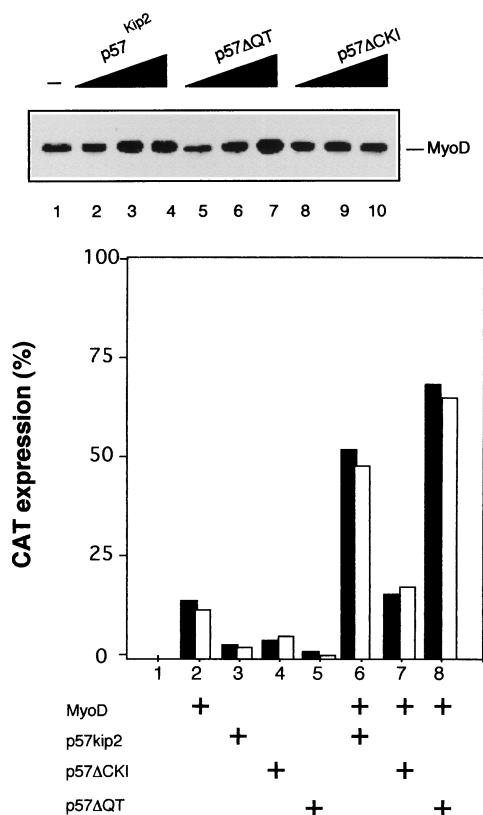


FIG. 9. (A) Differential accumulation of endogenous MyoD protein by ectopically expressed p57^{Kip2} mutants. C2C12 myoblasts (lane 1) were transiently transfected with 0.5, 1, or 1.5 μ g of a pEMSV expression vehicle encoding p57^{Kip2} (lanes 2 to 4), p57 Δ QT (lacking amino acids 297 to 350) (lanes 5 to 7), or p57 Δ CKI (lacking amino acids 1 to 105) (lanes 8 to 10) and were cultured in high-serum (20% FCS) medium. After 48 h, 50 μ g of total-cell extract was analyzed by Western blotting with anti-MyoD antibodies (Santa Cruz). (B) Differential activation of MyoD function by ectopically expressed p57^{Kip2} mutants. Bars represent CAT activities from whole-cell extracts of C3H10T1/2 fibroblasts which were cotransfected with plasmids encoding the MCK-CAT reporter (0.5 μ g) (lanes 1 to 8), MyoD (1 μ g), wild-type p57 (p57^{Kip2}), p57 Δ CKI, and p57 Δ QT (1 μ g). The corresponding empty expression vector was added to normalize DNA content to 3 μ g. CAT levels were determined 48 h after transfection. Protein concentration was equalized by the Bradford method. Typically, 15- μ g aliquots of total cell extracts were used for the reactions. CAT activities in duplicate plates (black and white bars) are from a representative experiment.

is a substrate of Cdc2, Cdk2, or Cdk4 complexes that control progression through the cell cycle and are inhibited by p57^{Kip2}. It has been shown that overexpression of cyclin D1 results in an increase of the hyperphosphorylated form of MyoD and an inhibition of MyoD-mediated MCK-CAT transactivation (41, 51). However, cyclin D1-Cdk4 complexes fail to phosphorylate MyoD (23). Recently, a mechanism has been described to explain this finding. The cyclin-mediated inhibition of myogenesis by cyclin D1 involves nuclear translocation of Cdk4 by cyclin D1 and the subsequent formation of a MyoD-Cdk4 complex that specifically inhibits the transactivation functions of MyoD in the absence of Cdk4 kinase activity (63). This implies that p16^{Ink4a}, which interacts exclusively with Cdk4 and Cdk6, does not increase the stability of MyoD. p16^{Ink4a} binds to and inhibits Cdk4, leading to the sequestration of Cdk4, and promotes the transactivation functions of MyoD (63). In contrast, Cdc2 and Cdk2 have recently been shown to be involved in the direct phosphorylation of MyoD Ser200 in proliferative myoblasts (23). In agreement with these data, we show that ectopic expression of a Cdk2 DN mutant induces hypophos-

phorylation of MyoD (Fig. 6B) and cyclin E-Cdk2 efficiently phosphorylates MyoD in vitro (Fig. 7). Furthermore, when purified as a recombinant protein from bacteria and added to assays of recombinant cyclin E-Cdk2 preparations, p57^{Kip2} inhibits MyoD phosphorylation. p57^{Kip2} is known to inhibit Cdk2, Cdk4, and Cdk6 but is much less effective toward Cdc2-cyclin B and does not associate with CAK (Cdk7-cyclin H) (25). Altogether, these data suggest that p57^{Kip2} preferentially inhibits cyclin A/E-Cdk2 rather than cyclin B-Cdc2 activities in proliferating myoblasts.

Extensive structure-function studies of the Cip/Kip molecules have suggested that binding of both cyclin and Cdk is not only necessary but also sufficient for the inhibition of cyclin-Cdk activities (20, 25). In p57^{Kip2}, the amino- and carboxy-terminal domains are well conserved between species, but the internal region contains proline-alanine repeats in human p57^{Kip2} (33) and a proline-rich region followed by an acidic repeat region in mouse p57^{Kip2} (25). This absence of sequence conservation in the internal region could be attributable to the lack of functional conservation. On the other hand, the carboxy-terminal domain (also termed the QT box) is a structural motif conserved with p27^{Kip1}. The QT box is likely to function in protein-protein interactions. We observed that the NH₂ domain of p57^{Kip2}, which contains the cyclin-Cdk binding sites, is necessary and sufficient for inhibiting phosphorylation of MyoD by cyclin E-Cdk2. This result is supported by the fact that the carboxy-terminal domain of p57^{Kip2} is required neither for cell cycle arrest in SAOS2 cells (33) nor for a positive effect on transactivation of the MCK promoter by MyoD (Fig. 9B). Thus, these data indicate that p57^{Kip2}, independently of its QT box, can function to arrest cell cycle in G₁ and to stabilize MyoD by preventing its phosphorylation by cyclin-Cdk complexes. It is interesting that the cyclin and Cdk binding domains of p57^{Kip2} are highly conserved in the Cip/Kip protein family, suggesting that p21 and p27 could also be involved in vivo as p57^{Kip2}. High-level ectopic expression of MyoD into non-muscle cells is known to stop cell cycle progression and allow cells to undergo myogenic differentiation (53, 58). Controlled degradation of cyclin E-Cdk2-mediated phosphorylation of MyoD by the ubiquitin pathway (21) may be one of the control mechanism necessary to prevent MyoD from reaching a threshold that could interfere with normal cell cycle progression before triggering myogenic differentiation. Furthermore, phosphorylation of MyoD may result in a change in MyoD-associated protein by reducing its association with a partner such as pRB (14), MEF-2 (35), or p300/CBP (11). Cdk-dependent phosphorylation has been shown to modify the interaction between pRB and E2F and to change the interaction specificity of the HLH factor Id3 (9, 19, 22). Finally, degradation of MyoD by the ubiquitin pathway may be regulated by specific DNA binding (21). Altogether, these data indicate that MyoD is subjected to a variety of specific regulatory mechanisms and strongly suggest that MyoD plays a crucial role in a terminal cell cycle withdrawal decision that clearly involves the control of the phosphorylation of MyoD by Cip/Kip inhibitors such as p57^{Kip2}.

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