Critical Role for Lysine 133 in the Nuclear Ubiquitin-mediated Degradation of MyoD*

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The ubiquitin-proteasome system is responsible for the regulation and turnover of the nuclear transcription factor MyoD. The degradation of MyoD can occur via an NH2 terminus-dependent pathway or a lysine-dependent pathway, suggesting that MyoD ubiquitination may be driven by different mechanisms. To understand this process, deletion analysis was used to identify the region of MyoD that is required for rapid proteolysis in the lysine-dependent pathway. Here we report that the basic helix-loop-helix domain is required for ubiquitination and lysine-dependent degradation of MyoD in the nucleus. Site-directed mutagenesis in MyoD revealed that lysine 133 is the major internal lysine of ubiquitination. The half-life of the MyoD K133R mutant protein was longer than that of wild type MyoD, substantiating the implication of lysine 133 in the turnover of MyoD in myoblasts. In addition, the MyoD K133R mutant displayed activity 2-3-fold higher than the wild type in transactivation muscle-specific gene and myogenic conversion of 10T1/2 cells. Taken together, our data demonstrate that lysine 133 is targeted for ubiquitination and rapid degradation of MyoD in the lysine-dependent pathway and plays an integral role in compromising MyoD activity in the nucleus.

Ubiquitination and degradation of many proteins are essential for cell cycle progression, signal transduction, and development (1). However, it is now clear that the ubiquitin-proteasome pathway can also carry out various non-proteolytic functions, controlling activities as diverse as transcription, nucleotide excision repair, receptor internalization, and ribosome function (see review, Ref. 2). The ubiquitination of the target protein is mediated by a cascade of ubiquitin transfer reactions that require a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) that acts in the last step of the cascade (3). Ubiquitin protein ligases regulate the timing and substrate specificity in protein degradation. MyoD is a basic helix-loop-helix (b-HLH) transcription factor that controls proliferation and differentiation (4). The mechanism by which MyoD induces myogenesis involves both the withdrawal from the cell cycle and the activation of muscle-specific genes expression (5, 6). The HLH domain is required for dimerization with the ubiquitously expressed E-proteins, and the basic domain is responsible for DNA binding. Heterodimers bind to the consensus E-box (CANNTG) DNA sequence motif found in the promoters of many muscle-specific genes (7). Structurally, MyoD contains several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, and nuclear localization (8, 9). Recent data demonstrate a direct link between MyoD levels and cell cycle regulation (10, 11), development (12), and regeneration (13). In this last process, MyoD is required for myogenic stem cell function in adult skeletal muscle (14). The interplay between G1 cyclins and Cdk inhibitors on one hand and MyoD and its cofactors on the other hand plays a critical role in myoblast cell cycle withdrawal. Accurate synchronization of dividing myoblasts revealed that MyoD is subject to specific cell cycle-dependent regulation (see review, Ref. 15). Specific phosphorylation of MyoD at serine 200 implies that Cdk2-cyclin E and up-regulation of its inhibitors (p57Kip2 and p21Cip1) control MyoD levels and subsequent myoblast cell cycle progression or exit into differentiation (11, 16, 17). The signal-inducing phosphorylation of MyoD leads to its degradation by the ubiquitin-proteasome pathway that includes CDC34 ubiquitin-conjugating enzyme activity (E2) (18). On the other hand, recent data have shown that MyoD could be regulated independently of its phosphorylation state, implicating other(s) recognition signal(s), mechanism(s), or multiprotein complexes. For example, up-regulation of the cyclin-Cdk inhibitor p57Kip2 stabilizes MyoD by direct interaction with MyoD (19), whereas in a cell-free system specific DNA binding stabilizes MyoD (20) and in vitro MyoD is degraded via the ubiquitin-proteasome pathway in HeLa nucleoplasm (21). It has been shown that MyoD could be degraded by the ubiquitin-proteasome system via two distinct pathways, an NH2-terminus-dependent pathway (22) and a lysine-dependent pathway (23). MyoD appears to be ubiquitinated and degraded in the nucleus (23), but the molecular determinants and the factors necessary for ubiquitination and targeting of MyoD to the proteasome are not well known.

In the present work we have studied the regions of MyoD that target it for destruction by the ubiquitin-proteasome pathway. We show that the NH2-terminal residue is not essential for conjugation and degradation of nuclear MyoD. Ubiquitination of MyoD implies lysine 133. Preventing ubiquitination at this site by mutation (K133R) leads to stabilization of the MyoD protein. We demonstrate that the MyoD mutant protein K133R shows both a higher activity in transactivating muscle-specific gene expression and a greater ability to convert 10T1/2 cells to muscle cells. Taken together, these data suggest that the ubiquitination of MyoD on lysine 133 may serve two functions in signaling protein degradation by the proteasome and in regulation of the transcriptional activity.

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The abbreviations used are: b-HLH, basic helix-loop-helix; HA, hemagglutinin; MCK, muscle creatine kinase; WT, wild type.
Lysine 133 Is Implicated in Nuclear MyoD Degradation

**MATERIALS AND METHODS**

**Reagents**—MG132 (N-carbobenoxyl-Leu-Leu-leucinal), chloroquine, E-64 (trans-epoxysucinyl-L- leucylamid (4-guanidino)butane) were purchased from Sigma. Clasto-lactacystin β-lactone was purchased from Calbiochem. 35S-translabeled was obtained from ICN.

**Plasmid Constructs**—pEMS-V-MyoD was a generous gift from the Weintraub laboratory. Expression vector pCDNA3-HA was generated by cloning three hemagglutinin epitope tags (3 HA) at the amino terminus of the MyoD cDNA insert in pCDNA3 (Invitrogen). The reporter plasmid MCK-Luc (p1256 MCK), generously provided by S. Hauscka, contains the promoter-enhancer region from the mouse muscle creatine kinase.

Expression vectors pCDNA3-HA-MyoD 2–114 and pCDNA3-HA-MyoD 173–318 were obtained by inserting in-frame an EcoRI-XhoI fragment generated by polymerase chain reaction using pEMSV-MyoD as template into the EcoRI-XhoI site of the pCDNA3-HA expression plasmid. Expression vector pCDNA3-HA-b-HLH was obtained by inserting in-frame the MyoD insert BasHII-NarI filled by the Klenow polymerase fragment into the EcoRI-filled site of the pCMV-HA expression plasmid. All lysine-mutated species of MyoD were generated in the laboratory of K. Breitschopf and were obtained from Dr. A. Harel-Bellan. Inserts were subcloned in pCDNA3-HA expression vector.

**Site-specific Mutagenesis of MyoD Protein**—MyoD mutants were obtained by oligonucleotide-directed mutagenesis using a QuikChange PCR-based site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The mutations were confirmed by sequencing.

**Cell Cultures, DNA Transfections, and Luciferase Assays**—The fibroblastic cell line 10T1/2 was maintained in growth medium supplemented with antibiotics (a mixture of penicillin and streptomycin, in vitrogen) and with 15% fetal calf serum. 10T1/2 cells were transfected by using jetPEI (QiBogene). Briefly, 3 × 10^4 cells/well were plated onto 24-well plates. On the following day, cells were transfected with various combinations of plasmids as indicated in the legends of the figures. The total amount of DNA used for each plate was normalized with the respective empty expression vehicle. Forty-eight hours after transfection, luciferase activity was determined using 10-μl aliquots of cell extracts from harvested cells in 1× reporter lysis buffer (Promega) with equivalent quantities of proteins in triplicate. The experiments were repeated at least twice.

**Metabolic Labeling**—Cell cultures were incubated for one hour in methionine-free medium supplemented with 5% fetal calf serum folowed by incubation for 30 min in the same medium with 300 μCi/ml 35S-methionine (Trans-35S label; ICN). In cold-chase experiments, the 35S-methionine-labeled cultures were rinsed with fresh medium supplemented with 10 μM non-radioactive methionine and 15% fetal calf serum and harvested at appropriate times. For immunoprecipitation of HA-MyoD, cells were lysed as described above.

**Antibodies, Immunoprecipitation, and Western Blot Analyses**—For immunoprecipitation, precleared cell lysates in IP buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM Na-orthovanadate, 10 mM NaF, 3 mM Na-pyrophosphate, 20 mM sodium phosphate, 5 mM dithiothreitol, 0.5 mM EDTA, and 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml apronin) were incubated with the indicated antibody overnight at 4°C with gentle agitation. Immunocomplexes bound to protein G-Sepharose were collected by centrifugation and washed several times in IP buffer. Immunoprecipitated proteins were resolved by SDS-PAGE followed by fluorography (35S-labeled proteins) and autoradiography.

For ubiquitination, cells were lysed in a lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% NaOxovanadate, 10 mM NaF, 3 mM Na-pyrophosphate, 20 mM sodium phosphate, 5 mM dithiothreitol, 0.5 mM EDTA, and 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml apronin. Then cell lysates were incubated with the indicated antibodies.

For immunoblot analyses, total cell extracts or immunoprecipitates were solubilized in radiolmmunoprecipitation assay (RIPA) buffer and analyzed on 10% polyacrylamide gels. Following electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose membranes, they were blocked with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies: polyclonal anti-MyoD (C20) diluted 1:500, monoclonal anti-Ub (P4D1) diluted 1:10000 (Santa Cruz Biotechnology), monoclonal anti-α-tubulin (DM1A) diluted 1:5000, and monoclonal anti-Troponin T (JL7-12) diluted 1:5000 (Sigma). Nitrocellulose membranes were then washed and incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1:1,000,000 for blots with polyclonal primary antibodies or 1/4,000 with blots using monoclonal primary antibodies. After several washes, membranes were incubated with an enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer’s instructions. Blots were exposed with Agfa Curix RP2 films.

**Cycloheximide Treatment**—10T1/2 cells were transfected with expression vectors in 6-well plates as described above. 24 h after transfection, cells were trypsinized, pooled, and redistributed in the number of necessary wells for the experiment in order to homogenize the transfection for the half-life determination. 48 h after transfection, cells were treated with cycloheximide (Sigma) at 50 μg/ml for the indicated times and harvested for Western blot analyses. HA-MyoD-wt and the derivatives were detected using anti-HA antibodies (12CA5). For each experiment, α-tubulin was used as an internal control. Western blots were scanned and quantified using Gel Scan (Amersham Pharma Biotech).

**In Vitro Ubiquitination Assay**—An in vitro ubiquitination assay was performed in a reconstituted cell-free system as described previously (11). In vitro translated 35S-labeled MyoD-wt and/or the various mutants were incubated with 10T1/2 cell extracts (5 mg/ml) in the presence of an ATP-regenerating system (50 mM Tris, pH 7.5, 5 mM MgCl2, 10 mM creatine phosphate, 5 units/ml phosphoketrate kinase, 5 mM ATP) together with 1 μM ubiquitin aldehyde, 1 mg/ml ubiquitin, 1 mM dithiothreitol, and a proteasome inhibitor mixture (1 mM σ-lysine acid, 25 μM MG132, and 25 μM clasto-lactacystin β-lactone). The reactions (10 μl) were carried out at 37°C for 0, 30, and 60 min and were stopped with Laemmli sample buffer containing β-mercaptoethanol. The products were subjected to SDS-PAGE (8%) and fluorography.

**Immunofluorescence Staining**—Cells were cultured on coverslips and fixed with paraformaldehyde (2%) for 30 min and then permeabilized with 0.5% Triton X-100 for 30 min at room temperature. Cells were immunostained with anti-HA, anti-C20, or anti-Troponin T antibodies. The Texas red-conjugated Fab′, fragment of goat anti-mouse IgG or the fluorescein isothiocyanate-conjugated Fab′, fragment of goat anti-rabbit IgG were used to visualize the mouse monoclonal or polyclonal antibodies. Cells were rinsed in phosphate-buffered saline containing DAPI (Sigma), mounted in citifluor, and viewed and photographed with a Zeiss axiophot fluorescent microscope.

**RESULTS**

**Proteolysis of MyoD by the Ubiquitin-Proteasome Pathway: Deletion of the NH2-terminal Residue Is Not Essential for Inhibition of MyoD Degradation**—The MyoD protein has been shown to be a short-lived protein that is degraded in the nucleus by the ubiquitin-proteasome pathway (20), and the NH2-terminal residue has been implicated in conjugation and degradation of the protein in vitro and in COS cells (22). On the other hand, inhibition of the ubiquitin-dependent proteasome pathway by MG132 resulted in accumulation of ubiquitylated MyoD protein in which three HA tags replaced the NH2-terminal Met residue in 10T1/2 cells (11). Recently it has been shown that the NH2 terminus-blocked (6× Myctagged) species of MyoD are degraded via ubiquitination of internal lysines (23). Taken together these data suggest that two distinct pathways mediate ubiquitination and degradation of MyoD: an NH2 terminus-dependent pathway and a lysine-dependent ubiquitination pathway. First we investigated whether three HA tags could modify MyoD turnover in vivo. We transiently expressed untagged MyoD (MyoD-wt) and 3HA-tagged MyoD (HA-MyoD) in 10T1/2 cells. The amount of newly synthesized MyoD was measured by immunoprecipitation analysis with the anti-MyoD polyclonal antibody (C20) in [35S]methionine pulse-labeled cells. The localization and the amounts of MyoD did not differ significantly between untagged MyoD-wt and HA-MyoD. (Fig. 1, A and B). We then compared the rates of degradation of the MyoD proteins. 10T1/2 cells were treated with cycloheximide, and the amount of MyoD at various times was determined by Western blot analysis. When analyzed by pulse-chase experiments and/or cycloheximide treatment (Fig. 1C), the half-life of MyoD-wt was ~50 min (24, 25) and that of HA-MyoD was roughly identical (45 and 50 min, respectively), indicating that the replacement of the NH2-terminal Met residue by the
activity in the nuclear degradation of MyoD.

The b-HLH Domain Is Implicated for Ubiquitination and Degradation of MyoD—To determine which region is required for the proteolysis of nuclear MyoD, we constructed a panel of MyoD deletion mutants. The expression constructs were transiently expressed in 10T1/2 cells, and the localization and the stability of MyoD proteins were determined 48 h later. As shown in Fig. 2, the NH2 (MyoD 2–114, which contains only a part of nuclear localization sequence 1; Ref. 23) and the COOH domains of MyoD (MyoD 173–318) were found exclusively in the cytoplasm. They showed a half-life of ~75 and 130 min, respectively, and were stable with regard to MyoD-wt. In contrast, the central domain of MyoD (amino acids 83–172, termed b-HLH-MyoD) was found exclusively in the nucleus, and the half-life was reduced to ~10 min. These data suggest that, independent of the serine 200 previously shown to be implicated in the modulation of the half-life and the myogenic activity of MyoD (16), MyoD stability seems to be governed by the action of various elements: the NH2 and COOH domains that promote MyoD stabilization and the b-HLH domain that promotes MyoD destruction.

We next investigated whether b-HLH-MyoD was subject to specific lysine-dependent degradation via the ubiquitin-proteasome pathway. We first compared the localization and the half-lives of b-HLH-MyoD and the lysine-less b-HLH MyoD in which Lys-99, -102, -104, -112, -124, -133, and -146 are mutated to arginine (Fig. 3A). These b-HLH MyoD constructs were transfected in 10T1/2 cells and 48 h later were analyzed by immunofluorescence. Wild type b-HLH-MyoD and the lysine-less b-HLH MyoD were both localized in the nucleus (Fig. 3B). The rates of degradation of MyoD proteins were compared in cycloheximide-treated 10T1/2 cells as described in Fig. 1. As previously shown in Fig. 2, b-HLH-MyoD was degraded with a half-life of about 10 min, which was found to be markedly increased to 60 min in the lysine-less b-HLH MyoD (Fig. 3B).

Finally, we examined the effect of different protease inhibitors on the amount of b-HLH-MyoD in transiently transfected cells. Addition of MG132 or lactacystin, two specific inhibitors of the 26 S proteasome (3), induced an accumulation of b-HLH-MyoD protein, whereas the cysteine protease inhibitor E64 or chloroquine, an inhibitor of lysosomal proteolysis, was ineffective (Fig. 3C). Addition of MG132 or lactacystin induced the accumulation of polyubiquitinated forms of b-HLH-MyoD. In contrast, mutation of all the lysine residues rendered ineffective the addition of MG132 or lactacystin and blocked ubiquitination of lysine-less b-HLH-MyoD (Fig. 3, D and E). These data strongly suggest that the b-HLH domain contains the lysine(s) implicated in MyoD ubiquitination and destabilization.

Implication of Lysine 133 for Ubiquitination of MyoD—MyoD contains nine lysine residues at positions 58, 99, 102, 104, 112, 124, 133, 146, and 241. From the data presented above, our results suggest that the lysine-dependent degradation pathway of MyoD implicates lysine residues located between amino acids 83 and 172 in the b-HLH domain. Lysines at position 99, 102, and 104 of MyoD, the major acetylation determinants essential for muscle differentiation (26), are probably not implicated in ubiquitination because although acetylation of MyoD increased during differentiation, the turnover of MyoD is not modified in differentiated myotubes (25). In the context of the full-length MyoD molecule and to map more precisely the lysine residue(s) implicated in the ubiquitination of MyoD, point mutants in which lysines are progressively replaced by arginines starting at the NH2 terminus were used in vitro and in vivo experiments (Fig. 4A). As suggested by the results shown in Figs. 2 and 3, the replacement of the five lysines (at positions 58, 99, 102, 104, and 112) did not affect either the

Fig. 1. Cellular localization and stability of MyoD are not modified by NH2-terminal extension. A, 10T1/2 cells transiently transfected with either control vector alone (lane 1), an expression vector encoding MyoD-wt (pEMSV-MyoD, lane 2), or a MyoD-wt deleted to its NH2-terminal residue with [35S]methionine. Cell lysates having the same radioactive counts were subjected to immunoprecipitation with specific anti-MyoD antibodies. MyoD was analyzed by SDS-PAGE and following autoradiography. B, 10T1/2 cells were transiently transfected with expression plasmids encoding MyoD-wt and/or HA-MyoD. Thirty-six hours later, transfected cells were fixed and stained for MyoD expression and DNA. C, pulse-chase of [35S]-labeled MyoD. 10T1/2 cells were transiently transfected with pcDNA3-HA-MyoD (HA-MyoD) or with pEMSV-MyoD (MyoD-wt) expression vectors. Cells were cultured in methionine-free media for 1 h and then pulsed with 300 μCi/ml [35S]methionine for 30 min. Following this incubation, cells were washed with media containing an excess of cold methionine (10 mM) and chased for the indicated times. Cell extracts were immunoprecipitated with specific anti-MyoD antibodies. One dish (60 mm) of transfected cells was used for each time point. This experiment was performed three times. On the other hand, 10T1/2 cells were transiently transfected with pcDNA3-HA-MyoD expression vector and grown for 48 h in proliferative medium before addition of cycloheximide (50 μg/ml) to the medium for various times. MyoD and α-tubulin protein levels were determined by immunoblot analysis at the indicated times after cycloheximide addition using anti-HA and anti-α-tubulin antibodies, respectively. Immunoblots were quantified by densitometric scanning. MyoD protein levels (corrected with respect to tubulin expression) were expressed relative to that observed before cycloheximide treatment, set as 100%.

3× HA tag does not noticeably affect the stability of MyoD in vivo. These data suggest that the NH2 terminus-dependent pathway and the lysine-dependent pathway have the same
half-life or the ubiquitination of MyoD in vivo and in vitro (Fig. 4, A–C). Additional mutation of lysine 124 did not significantly modify the detection of MyoD conjugation to ubiquitin in the form of high molecular weight polyubiquitin conjugates nor the half-life (~50 min). In contrast, the level of polyubiquitin conjugation was markedly affected by replacement of lysine 133 to arginine 133 and the half-life of mutant 3 was significantly increased (up to 180 min). Additional mutation of lysine 146 in mutant 4 did not noticeably modify these findings. Because mutant lysines are located within the nuclear localization signals (9), we looked at the cellular distribution of the MyoD mutants. Immunofluorescent experiments confirmed that all the mutants are properly shuttled and retained in the nucleus (Fig. 4A and data not shown). However, as previously shown (26) the mutant forms of MyoD (mut 1–mut 4) that are mutated on lysines 99, 102, and 104 cannot be acetylated and are impaired in their transcriptional ability (Fig. 4D).

Following these results, a sequence comparison revealed that lysine 133 and the surrounding residues are conserved in MyoD homologs from species as distant as Drosophila and human and that Myf-5, myogenin, and muscle regulatory factor 4 (MRF4) fully conserve this motif (Fig. 4, E and F). To assess the consequence of lysine 133 ubiquitination on MyoD stability, we first mutated Lys-133 to Arg-133 (K133R) and compared the half-life of MyoD-wt and MyoD K133R. As shown in Fig. 5A, substitution mutagenesis was sufficient to markedly stabilize MyoD by increasing its half-life (~150 min) and to significantly reduce its ubiquitination in vivo and in vitro (Fig. 5, A–C). Because the mutation of lysine 133 to arginine prevented degradation of MyoD, we set out to determine whether this mutation would also affect MyoD function by comparing the abilities of MyoD-wt and MyoD K133R to transactivate muscle-specific gene expression. Plasmids expressing either MyoD-wt, MyoD K133R, or the lysine-less MyoD were cotransfected with a luciferase reporter gene containing the muscle creatine kinase promoter (MCK-Luc) in 10T1/2 cells. Transfected cells were incubated in proliferative medium for 48 h, and transactivation of the reporter gene was estimated by luciferase assay. As expected, the low basal activity of the MCK-Luc reporter gene was highly enhanced by MyoD-wt. Interestingly, MyoD K133R further increased the level of Luc reporter activity 2.5-fold over that obtained with MyoD-wt (Fig. 5D). Such an increase was not observed with a lysine-less MyoD (26). We next compared the abilities of MyoD-wt and MyoD K133R proteins to trigger myogenic conversion. 10T1/2 cells were transfected with expression vectors encoding MyoD-wt, MyoD K133R, or the lysine-less MyoD. Transfected cells were incubated in differentiation medium for 4 days and then analyzed by Western blotting for expression of MyoD and Troponin T as a differentiation marker. After 48 h in growing medium (Fig. 5E, GM, lanes 2–4), various levels of MyoD-wt, MyoD K133R, and the lysine-less MyoD were observed, probably as a result of differences in protein half-life (see below). Only a very small amount of Troponin T expression is found with MyoD K133R and is not observed with MyoD-wt or the lysine-less MyoD. After 96 h in differentiation medium, Troponin T was expressed at levels 4-fold higher in MyoD K133R than MyoD wt and was not detectable in the lysine-less MyoD-overexpressing cells. As observed in normal growth medium, it is worth noting that under differentiation culture conditions MyoD-wt protein level appears to be about 2-fold lower than MyoD K133R and 6-fold lower than the lysine-less MyoD, reflecting differences in protein stability. The efficiency of myogenic conversion was also estimated by immunofluorescence on cells expressing MyoD and Troponin T. As shown in Fig. 5F, MyoD K133R was significantly more efficient than MyoD-wt in converting 10T1/2 cells into myotubes. Perhaps more importantly, MyoD K133R-expressing cells formed large interconnected myotubes, which were never observed with the MyoD-wt protein. Indeed, after 4 days in differentiation medium, nearly all MyoD K133R-expressing cells had differentiated into Troponin T-positive myotubes, whereas 40% of MyoD-wt-expressing cells remained negative for Troponin T and the lysine-less MyoD did not allow the myogenic conversion of 10T1/2 cells. Altogether, these data show that ubiquitination on lysine 133 in MyoD appears to restrict MyoD activity, because mutation of lysine 133 to arginine significantly increased the half-life of MyoD and enhanced its transcriptional activity as well as its ability to induce myogenic conversion of 10T1/2 cell.

**DISCUSSION**

The muscle-specific transcription factor MyoD is a short-lived nuclear protein that is degraded by the ubiquitin-proteasome pathway. Previous studies have shown that after transfection in COS cells and/or HeLa cells, ubiquitination of MyoD occurs at the NH$_2$ terminus, leading to NH$_2$ terminus-dependent degradation. On the other hand, blocking the NH$_2$ terminus with a 6× Myc tag or via methylation or carbamylation of the α-amino group led to preferential degradation by the lysine-dependent pathway (22, 23). Here we show that blocking the NH$_2$ terminus of MyoD with a 3× HA tag did not modify the half-life of HA-MyoD when compared with untagged MyoD when expressed in 10T1/2 cells. Using two different experimental approaches, our data indicate that the lysine-dependent pathway is as efficient as the NH$_2$ terminus degradation pathway in 10T1/2 cells (Fig. 1). In addition, an expression vector encoding
an NH₂ terminus-blocked MyoD with a FLAG tag showed an identical half-life of 50 min when transfected in 10T1/2 cells and/or in C2C12 myoblasts (data not shown). These findings suggest that in MyoD-expressing cells, the lysine- and the NH₂ terminus-dependent pathways are both active in the ubiquitination and degradation of nuclear MyoD.

Using deletion mutants of the MyoD protein, we show that the b-HLH domain, which is highly conserved in all the MyoD protein family, is the target for rapid turnover of the MyoD protein. We demonstrate that the b-HLH domain contains sufficient sequence information to be degraded by the ubiquitin-proteasome system via the lysine-dependent pathway. Because the b-HLH domain contains 7 of the 9 lysine residues found in MyoD, identification of the ubiquitination site(s) involved in the lysine-dependent pathway was undertaken by progressively replacing the lysine residues to arginine in the MyoD protein. The half-lives of MyoD-wt and the various MyoD mutant proteins were determined; the K133R change was found to
dramatically alter the half-life of MyoD. MyoD K133R has a half-life (~150 min) about three times longer than MyoD-wt (45 min) and is significantly less ubiquitinated than MyoD-wt. The increased stability of MyoD K133R is likely to be responsible for the increased MCK-Luc activity detected in 10T1/2 cells overexpressing MyoD K133R compared with MyoD-wt. On the other hand, MyoD mutants 1 and 2 that cannot be acetylated (lysines 99, 102, and 104 are mutated) are impaired in their abilities to promote transcriptional and myogenic potency (26), although their half-lives and ubiquitination are similar to

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**Fig. 4. Ubiquitination of MyoD requires Lys-133.** A. MyoD protein exhibits 9 lysines on its amino acid sequence. Point mutations in which the lysines were replaced to arginines were introduced sequentially as indicated (e.g., in mutant 1, lysines 58, 99, 102, and 104 are replaced to arginines). The half-lives of the MyoD wt and the MyoD mutants were carried out as described in Fig 1C. B, 10T1/2 cells were transiently transfected with expression vectors encoding HA-MyoD and/or the MyoD mutants 1–4 together with pCMV-HA-ubiquitin and grown for 48 h in proliferative medium before treatment with 30 μM MG132. Cell lysates were prepared 5 h later, and 50 μg of total proteins were subjected to Western blot with anti-HA antibodies (upper panel). 500 μg of total cell lysates were first immunoprecipitated with polyclonal anti-MyoD antibodies (C20; Santa Cruz Biotechnology). Ubiquitn-MyoD conjugates were identified in immunoprecipitates by Western blot analysis using anti-HA antibodies to detect both MyoD and the conjugation to ubiquitin in the form of high molecular weight polyubiquitin conjugates. C, in vitro ubiquitination. MyoD-wt and the MyoD mutants were in vitro translated in the presence of [35S]methionine. The labeled MyoD proteins were then incubated in 10T1/2 extracts at 37 °C as described under "Materials and Methods." Aliquots of the reactions were then quenched at the indicated times in SDS sample buffer followed by SDS-PAGE and fluorography. * indicates nonspecific bands present in most samples. D, the MCK-Luc reporter construct was cotransfected in 10T1/2 cells with expression vector alone or encoding HA-MyoD-wt or the MyoD mutants. Transfected cells were grown for 48 h in proliferating medium, and luciferase activity was measured and corrected with respect to MyoD protein levels (upper panel). Values shown are means ± S.D. of three separate readings. The experiment was repeated at least twice. E, conservation of the ubiquitinated lysine 133 (arrow) from *Drosophila* to human MyoD. F, lysine 133 (arrow) ubiquitinated in MyoD is conserved in every member of the murine myogenic b-HLH protein family.
that of MyoD-wt. However, the lysine-less MyoD has a half-life longer than that of MyoD K133R. This non-acetylable MyoD mutant, which is stable, is also impaired in its ability to promote transcription. Altogether, these differences between MyoD K133R and the MyoD mutants may affect either a minor ubiquitination site or, more probably, an interaction with other proteins that mediate transcriptional activity as observed for the transcription factor GATA-1, which cannot be acetylated and is impaired in its ability to promote transcription (27, 28) and to induce erythroid differentiation (28). Furthermore, a conservative change of lysine to arginine does not affect nuclear localization sequence 2 function (9), because a nuclear
localization was observed for MyoD K133R as well as for mutants 3 and 4 and the lysine-less MyoD. Thus, the increasing transcriptional activity of MyoD K133R protein cannot be explained by differences in cellular localization.

Our data show that the degradation of MyoD depends on the ubiquitination of Lys-133 and, moreover, the mutation of lysine to arginine results in an increased transcriptional activity of MyoD. Although ubiquitination of proteins plays a major role in protein degradation, the ubiquitin-proteasome system may also control transcriptional activity (2). The ubiquitin ligase SCAF/Met30 has been shown to oligoubiquitinate the transcription factor Met-4 and to block its transcriptional activity by hindering the recruitment of a transcriptional partner protein (29). Therefore, these data suggest that ubiquitination can influence transcription factors by regulating the association of transcription factors with partner proteins that are required for activation of specific genes. Nevertheless, it is likely that additional mechanisms, perhaps affecting protein-protein interactions, may contribute to the overall effect of K133R mutation on MyoD function.

Among the nine lysines found in the MyoD protein, six residues (Lys-99, -102, -104, -124, -133, and -146) are well conserved in the other muscle regulatory factors. Lysines at position 99, 102, and 104 of MyoD are the major acetylation determinants necessary for the execution of the muscle program (26). Lysine at position 124 is implicated in myogenic transcription, and lysine at position 146 contacts position C5 and A6 in the E-box motif (CANTNG) (30). These data strongly suggest that the high conservation of these lysines is an important regulatory mechanism for the activity of myogenic b-HLH proteins. It is important to note that the residues immediately surrounding and including Lys-133 are highly conserved in MyoD from many different species but they are also conserved in the other members of the myogenic b-HLH family, possibly with the same functional consequence. Future studies should establish whether mutation of these residues in the other muscle regulatory factors affects their myogenic activity.

Phosphorylation of MyoD is one of the crucial mechanisms that control its level and transcriptional activity in the cell cycle. Phosphorylation of MyoD at serine 200 plays a crucial role in modulating its half-life and transcriptional activity during myoblast proliferation (16, 18). In addition, MyoD phosphorylated on serine 200 has been shown to be degraded by the proteasome and CDC34 ubiquitin-conjugating enzyme activity (E2) (18). These data suggest that in proliferating myoblasts phosphorylation of serine 200 by cyclin-Cdk complexes represents the destruction signal for MyoD. Interestingly, although MyoD phosphorylation changes during the course of differentiation, being hyperphosphorylated in myoblasts compared with myotubes (16), the half-life of MyoD remains unchanged (25). However, the MyoD mutants spanning amino acids 83–172, which display a short half-life (10 min), are degraded by the ubiquitin-proteasome pathway. Thus, these data suggest that lysine 133 could be dependent on ubiquitination and not on the phosphorylation of serine 200. Accordingly, signal-induced phosphorylation of MyoD does not seem to be a prerequisite for ubiquitination of MyoD in vitro and in differentiated myotubes, suggesting that MyoD ubiquitination could be mediated by different E3 ubiquitin ligases. Further studies should help to determine candidate molecular mediators of MyoD ubiquitination. Mainly, the ubiquitin protein ligase(s) (E3) responsible for the signal-induced ubiquitination of MyoD remain to be determined.

In conclusion, it appears that the ubiquitin-proteasome pathway via phosphorylation-dependent and -independent mechanisms regulates the turnover of MyoD, suggesting that MyoD ubiquitination on the NH2 terminus or on lysine 133 could be mediated by different ubiquitin ligases during myogenesis. Attachment of ubiquitin implies different recognition signal(s) and/or biological mechanism(s) characteristic of ligases that could be specific to control MyoD levels in the course of muscle differentiation. It will be interesting to study the signals that govern the targeting of MyoD to the NH2 terminus or to the internal lysine 133 that expose the determinants for the ubiquitin ligases during cellular differentiation and regeneration.

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