The ubiquitin-proteasome system is responsible for the regulation and turnover of many short-lived proteins both in the cytoplasm and in the nucleus. Degradation occurs via two distinct pathways, an N-terminal pathway and a lysine-dependent pathway. The pathways are characterized by the site of initial ubiquitination of the protein: the N terminus or an internal lysine, respectively. MyoD, a basic helix-loop-helix transcription factor, is a substrate for the ubiquitin-proteasome pathway and is degraded in the nucleus. It is preferentially tagged for degradation on the N terminus and thus is degraded by the N terminus-dependent pathway. Addition of a 6× Myc tag to the N terminus of MyoD can force degradation through the lysine-dependent pathway by preventing ubiquitination at the N-terminal site. Modifications of the nuclear localization signal and nuclear export signal of MyoD restrict ubiquitination and degradation to the cytoplasm or the nucleus. Using these mutants, we determined which degradation pathway is dominant in the cytoplasm and in the nucleus. Our results suggest that the lysine-dependent pathway is the more active pathway within the cytoplasm, whereas in the nucleus the two pathways are both active in protein degradation.

Degradation of many short-lived cellular proteins, such as transcription factors, tumor suppressors, and cell cycle regulators, occurs via the ubiquitin-proteasome pathway (1–4). Through this pathway, proteins are targeted for degradation by the 26 S proteasome via the formation of a polyubiquitin chain. The process begins with activation of ubiquitin by the ubiquitin-activating enzyme (E1), followed by transfer of ubiquitin to E2, a ubiquitin-conjugating enzyme. E2 shuttles the ubiquitin molecule to the substrate-specific ubiquitin ligase (E3), which then delivers the ubiquitin to the substrate to be degraded. Initially, it was thought that ubiquitination occurred only through a lysine-dependent ubiquitination pathway in which ubiquitin is covalently attached to the substrate protein via an amide linkage to the ε-amino group of an internal lysine (5). However, recent studies have shown that the N terminus of a protein substrate may also serve as the site of ubiquitination (6–8), a pathway termed N terminus-dependent ubiquitination. Via either ubiquitination pathway, polyubiquitin chain formation continues by the conjugation of subsequent ubiquitin moieties to the attached ubiquitin, and the substrate-ubiquitin conjugate is then degraded by the 26 S proteasome in an ATP-dependent manner. Isopeptidases cleave the ubiquitin chain, and the single ubiquitin molecules are recycled (5). Currently, the relative contribution of each of these two pathways is unknown.

Among the short-lived proteins degraded by the ubiquitin-proteasome system are several transcription factors, including MyoD. MyoD, a nuclear basic helix-loop-helix transcription factor necessary for skeletal muscle differentiation (9), is rapidly degraded by the ubiquitin-proteasome system both in vitro and in vivo (10). Ubiquitination on the N terminus of MyoD appears to occur in preference to ubiquitination on internal lysines (8), and MyoD appears to be ubiquitinated and degraded in the nucleus by the ubiquitin-proteasome system (11). A nuclear ubiquitin-proteasome system may also be responsible for the degradation of several other transcription factors, including Smad2 and the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor (12, 13), as the activated forms of these proteins require translocation into the nucleus for degradation. In addition, Far1, a protein required for establishing cell polarity of mating yeast and for bringing about cell cycle arrest, is ubiquitinated and degraded in the nucleus (14). In contrast, cyclin D1 (15), p53 (16), p27kip1 (17), IκBα (18), and the aryl hydrocarbon receptor (19) all require nuclear export prior to degradation by the ubiquitin-proteasome system. Lind-gained Smad3, although ubiquitinated in the nucleus, is also exported to the cytoplasm for degradation by the 26 S proteasome (20).

Transport of cellular proteins, including transcription factors such as MyoD, into and out of the nucleus is facilitated by nuclear localization and nuclear export sequences (NLSs and NESs), which facilitate transport of such proteins across the nuclear envelope (21–24). NLSs exist as single sequences (5–12 amino acids) of basic amino acids (lysine or arginine; Ref. 25) or as bipartite regions of basic residues separated by 10–12 nonbasic residues (26). Less is known about NESs, but they are generally comprised of regions of hydrophobic amino acids.

Thus, ubiquitin-mediated protein degradation occurs in both the cytoplasm and the nucleus, and both lysine-dependent and N terminus-dependent ubiquitination pathways exist for several proteins, including MyoD. The aim of the present study was to determine the relationship of these ubiquitin-mediated...
degradation pathways using NLS and NES mutants and inhibitors of nuclear uptake and export. Herein, we report that for MyoD, both the N terminus-dependent and the lysine-dependent pathways function within the nucleus, although the lysine-dependent pathway appears to be more active in the cytoplasm.

The importance of this observation lies in the elucidation of alterations in cellular protein degradation in physiological and pathophysiological states.

EXPERIMENTAL PROCEDURES

Plasmids and Construction of MyoD-NLS and MyoD-NES—Wild-type MyoD and lysine-less MyoD, each of which is in the pcDNA vector, and N-terminal-blocked (6× Myc-tagged) MyoD, which is in the pcPS2+MT vector, have been described previously (8, 11). Preparation of NLS and NES mutants of MyoD was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. DNA sequencing using Big Dye Version 2.0 (Applied Biosystems) was used to confirm all sequences.

Cell Culture—HeLa cells, which were selected because they do not express MyoD, were grown in Dulbecco’s modified Eagle’s medium, which was supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin) (Invitrogen) and was maintained in a humidified chamber at 37 °C and 5% CO2. Transient transfections (efficiency >75%) were accomplished using the FuGENE 6 reagent (Roche Molecular Biochemicals), and cells were analyzed 16–24 h later.

Immunofluorescent Localization of MyoD—Subcellular localization of MyoD and its mutants in HeLa cells was determined by direct immunofluorescence using the mouse monoclonal anti-MyoD antibody (1:100 dilution, NCLMyoD1; NovoCastra) followed by incubation with a TRITC-conjugated donkey anti-mouse IgG (heavy and light chain; Jackson ImmunoResearch) as described previously (11). MG132 (10 μM; Peptides International) or LMB (10 nM; Sigma) were added to cells where indicated 2 h prior to fixation. MG132 was prepared as a 10 mM stock solution in Me2SO, and LMB was prepared as a 10 μM solution in ethanol. Cells were observed using a Zeiss Axioskop microscope, and 10–20 random fields of each culture condition were photographed (magnification ×40) using a Zeiss AxioCam digital camera. Cells were scored, and the percentage of cells expressing nuclear MyoD was determined by methods similar to those of Sachdev et al. (27) and Yagita et al. (28). Specifically, cells were scored according to the cellular localization of MyoD as predominately nuclear, predominately cytoplasmic, or distributed equally between the nucleus and the cytoplasm. For each determination over 100, cells were scored from 3 to 6 independent transfections.

Detection of Degradation of MyoD and MyoD Mutants in Vivo—As described previously (11), 16–24 h after transfection, the HeLa cells were incubated with CHX (100 μg/ml; Sigma) to inhibit further protein synthesis. MG132 (10 μM) or LMB (10 nM) was added along with CHX as necessary. Following incubation for 0, 0.5, 1, 2, and 3 h, the cells were lysed for at least 30 min in phosphate-buffered saline containing 5% Igepal, 1 mM EDTA, 1 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride. Afterward the cells were sonicated and then centrifuged at 14,000 rpm for 10 min at 4 °C in an Eppendorf microcentrifuge to remove cellular debris. The lysates were mixed with an equal amount of 2× Laemmli sample buffer (Bio-Rad), and equal amounts of each sample were run on a 12% Tris-HCl gel (Bio-Rad) and were electroblotted onto nitrocellulose (Osmonics). The blots were probed with monoclonal anti-MyoD antibody (1:100 dilution; NovoCasta) followed by incubation with a secondary horseradish peroxidase-conjugated antibody and detection by chemiluminescence (Amersham Biosciences). The resulting bands were quantitated using the EADS system (Eastman Kodak Co.), and the data were graphed using the Excel graphing program (Microsoft). The degradation rate is expressed as half-life (t1/2), the time for degradation of 50% of the MyoD. Each of the constructs was evaluated by 3–8 independent determinations of t1/2. The data are expressed as ± S.D.

RESULTS

MyoD is localized to the cell nucleus in growing myoblasts (29) and in a variety of cells following transfection (11). To determine the subcellular localization of lysine-less MyoD (Lys → Arg mutations) and N-terminal-blocked (6× Myc-tagged) MyoD, MyoD constructs were transfected into HeLa cells and were visualized 24 h later by immunofluorescence (Fig. 1). Transfected cells were also treated either with MG132 or with LMB 2 h prior to fixation. MG132 inhibits the proteasome, whereas LMB inhibits CRM-1-dependent nuclear export

FIG. 1. Localization of wild-type MyoD, lysine-less MyoD, and N-terminal-blocked MyoD. 24 h following transfection of HeLa cells, cells were treated with MG132 or LMB, and the localization of each construct was visualized by immunofluorescence. Wild-type MyoD, lysine-less MyoD, and N-terminal-blocked MyoD were localized exclusively to the nucleus under all conditions.

FIG. 2. Half-life of wild-type MyoD with MG132. A, 24 h after transfection with wild-type MyoD, HeLa cells were treated with CHX or CHX plus MG132. Cells were lysed at 0, 0.5, 1, 2, or 3 h and were evaluated via SDS-PAGE gel and Western blot for MyoD. B, the pixels for each band were measured and normalized so that the number of pixels at t = 0 was 100%. The log10 of the % pixels was plotted versus time for each time point, and the t1/2 was calculated as the log of 50% (1.7 h). The t1/2 for MyoD was 0.8 h and 11.2 h when MG132 was added. Treatment of the cells with CHX plus LMB increased the t1/2 to 1.3 h (data not shown).
to retain MyoD within the nucleus (30). As seen in Fig. 1, wild-type MyoD, lysine-less MyoD, and N-terminal-blocked MyoD were all localized to the nucleus under basal conditions as well as in the presence of MG132 or LMB. Thus, at steady state, MyoD and its lysine-less and N-terminal-blocked mutants were found exclusively in the cell nucleus.

We next compared the rates of degradation of wild-type, lysine-less, and N-terminal-blocked MyoD in vivo. 16–24 h following transfection, HeLa cells were treated with CHX to inhibit further protein synthesis, and the amount of MyoD at 0, 0.5, 1, 2, and 3 h was determined via Western blot analysis. MG132 or LMB was added together with CHX in replicate samples to determine the degradation rate of these proteins under conditions in which the proteasome or nuclear export was inhibited. As seen in Fig. 2, wild-type MyoD was degraded with a $t_{1/2}$ of 0.8 ± 0.1 h, which was markedly increased to −11 ± 4 h in the presence of MG132. The half-life was slightly increased in the presence of LMB to 1.3 ± 0.2 h (legend to Fig. 2). The half-lives of lysine-less MyoD and N-terminal-blocked MyoD were significantly increased over the half-lives of wild type, with $t_{1/2}$ of −2.7 ± 0.6 h and 2.6 ± 0.6 h, respectively, as seen in Table I. As with wild-type MyoD, the half-lives of lysine-less MyoD and N-terminal-blocked MyoD were markedly increased by incubation with MG132, although they were minimally affected by incubation with LMB (data not shown). Together these data suggest that wild-type MyoD, lysine-less MyoD, and N-terminal-blocked MyoD are each degraded via the ubiquitin-proteasome system within the nucleus.

Nuclear transcription factors, including MyoD, are synthesized in the cytoplasm and are targeted to the nucleus via NLS. To determine whether MyoD or its mutants are degraded within the cytoplasm either prior to nuclear uptake or during nucleocytoplasmic recycling, we sought to identify the NLS within MyoD to generate NLS-deficient mutants.

Based on results from Vandromme et al. (31), we mutated the basic residues within two regions of MyoD (amino acid regions 110–112 and 130–135) to alanine, as Vandromme et al. report that deletion of both of these regions is necessary to prevent nuclear localization of MyoD. However, as seen in Fig. 3, substitution mutagenesis yielded distinctly different results. Mutagenesis of K102A, R103A, K104A, R110A, R111A, and K112A (MyoDNLS1) was sufficient to markedly inhibit nuclear import, whereas mutagenesis of K133A and R134A (MyoDNLS2) did not

Table I

| Localization              | $t_{1/2}$ |
|---------------------------|-----------|
| Wild-type MyoD            | Nucleus   | 0.8 ± 0.1 |
| N terminus-blocked MyoD   | Nucleus   | 2.7 ± 0.6 |
| Lysine-less MyoD          | Nucleus   | 2.6 ± 0.6 |

Fig. 3. Localization and $t_{1/2}$ of MyoD NLS mutants. Basic amino acids of putative NLS sequences in MyoD were mutated to alanines, and the effect on nuclear localization was determined by immunofluorescence. A total of six regions were mutated; MyoD-NLS1 (amino acid region 102–112) and MyoD-NLS2 (amino acid region 117–124) were found to be 83 and 79% in the cytoplasm, respectively. MyoD-NLS1-NLS2 was found to be 87% cytoplasmic. MyoD-NLS2 was 18% cytoplasmic. The rates of degradation of wild-type MyoD and its NLS mutants are described above, with the rates of MyoD-NLS1+2 not determined (ND).
affect subcellular localization. Given these results, the entire amino acid sequence of MyoD was evaluated for additional basic residue-containing regions which may serve as NLS. Mutation of R117A, R119A, R120A, R121A, and K124A (MyoDNLS3) proved to be an additional NLS (Fig. 3). This same region was examined by Vandromme et al. (31) using deletion mutagenesis and was found not to serve as an NLS. Inhibition of nuclear import of MyoDNLS3 (79% cytoplasmic) was comparable with that seen with MyoD NLS1 (83% cytoplasmic). Furthermore, mutation of both NLS1 and NLS3 (MyoDNLS1/3) did not inhibit import substantially more (i.e., ~87% cytoplasmic) than that observed with the single mutation. Therefore it is likely that NLS1 and NLS3 represent subdomains of a single large targeting sequence (amino acid region 102–124) within MyoD. Evaluation of three additional regions in MyoD (R143A, K146A, and R151A; R220A and R221A; R235A, R238A, and K241A) failed to reveal any additional NLS.

The rates of degradation of several of these NLS mutants were examined. The $t_{1/2}$ of wild-type MyoD and MyoDNLS2, both of which localized to the nucleus (96 and 92% nuclear, respectively), were each found to be 0.8 ± 0.1 h. However, the $t_{1/2}$ of MyoD NLS1, MyoD NLS3, and MyoDNLS1+2, each of which localized predominately to the cytoplasm (83, 79, and 79% cytoplasmic, respectively), were found to be 1.3 ± 0.3, 1.2 ± 0.1, and 1.2 ± 0.2 h, respectively. Thus, it appears that the rate of MyoD degradation correlates with its subcellular localization, in that nuclear localization is associated with more rapid degradation.

The NLS1 mutation identified above did not result in complete absence of MyoD from the nucleus. It may be that MyoD undergoes nucleocytoplasmic shuttling and that the mutagenesis of NLS1 causes MyoD to enter the nucleus more slowly than wild-type MyoD, or it may be that the rate at which MyoD exits the nucleus is accelerated when NLS1 is mutated. To test if MyoDNLS1 undergoes nucleocytoplasmic shuttling, 16–24 h after transfection of HeLa cells with MyoDNLS1, LMB was added, and MyoD localization was visualized via immunofluorescence. As seen in Fig. 4, incubation with LMB led to a dramatic accumulation of MyoDNLS1 within the nucleus (17–93% nuclear). This suggests that the MyoDNLS1 mutant cycles between the cytoplasm and the nucleus, although it does not indicate whether MyoDNLS1 is entering the nucleus more slowly or exiting the nucleus more quickly than wild-type MyoD. Localization of this mutant to the nucleus in the presence of LMB was associated with a decrease in the half-life of MyoDNLS1 (1.0 ± 0.1 h) compared with that seen in the absence of LMB (1.3 ± 0.3 h; Fig. 4).

The ability of MyoD to cycle in and out of the nucleus suggests either the presence of an intrinsic NES or of association with a chaperone (32) that supports MyoD nuclear export. Typically, NES are regions high in leucine content. Comparison
of the MyoD sequence to other known NES within various proteins, such as protein kinase A inhibitor (33), cyclin B1 (34), human immunodeficiency virus-1 Rev protein (35), or human Cdc25C (36), suggested a candidate NES within the N terminus region of MyoD. Thus, using the MyoD\(^{\text{NLS1}}\) mutant as a template, Leu-3, Leu-4, Leu-8, Ile-11, and Leu-13 were mutated to alanines (MyoD\(^{\text{NLS1NES}}\)), and the construct was transfected into HeLa cells. A marked increase in nuclear localization of MyoD (17–42% nuclear) was observed (Fig. 5) consistent with the notion that amino acid sequence 3–13 contains a nuclear export signal. Two other regions high in leucine content were mutated on the MyoD\(^{\text{NLS1NES}}\) background (L268A, L269A, and L270A; L248A, L251A, and L254A) to identify other potential NES within MyoD; however, no others were identified.

The NES of wild-type MyoD was then mutated to determine the half-life of MyoD\(^{\text{NES}}\). As expected, MyoD\(^{\text{NES}}\) localized exclusively to the nucleus (100% nuclear) (Fig. 6). The half-life of MyoD\(^{\text{NES}}\) \((t_{1/2} = 1.05 \pm 0.40 \text{ h})\) decreased compared with that seen for MyoD\(^{\text{NLS1}}\) \((t_{1/2} = 1.33 \pm 0.30 \text{ h})\) and slightly increased compared with that seen for wild-type MyoD \((t_{1/2} = 0.81 \pm 0.11 \text{ h})\).

Having identified the major NLS and NES signals in MyoD, we next sought to determine which degradation pathway (N terminus-dependent or lysine-dependent) predominates in the nuclear and cytoplasmic compartments. To localize the various mutants to the cytoplasm or the nucleus, we prepared NLS1 or NES mutants on N-terminal-blocked MyoD and lysine-less MyoD.

The N-terminal-blocked species of MyoD are degraded via the lysine-dependent pathway. The N-terminal-blocked NLS1 mutant (N-terminal-blocked MyoD\(^{\text{NLS1}}\)) was degraded with a shorter \(t_{1/2} (1.6 \pm 0.3 \text{ h})\) compared with the N-terminal-blocked wild-type MyoD \((2.7 \pm 0.6 \text{ h})\), whereas the N-terminal-blocked NES mutant (N-terminal-blocked MyoD\(^{\text{NES}}\)) was degraded with an \(t_{1/2} (2.8 \pm 0.5 \text{ h})\) similar to the N-terminal-blocked wild-type MyoD (Fig. 7A). As expected, N-terminal-blocked wild-type MyoD and N-terminal-blocked MyoD\(^{\text{NES}}\) were nuclear, whereas N-terminal-blocked MyoD\(^{\text{NLS1}}\) was predominately cytoplasmic. The more rapid degradation of the N-terminal-blocked species localized to the cytoplasm (N-terminal-blocked wild-type MyoD) suggests that the lysine-dependent pathway may be more active for MyoD in the cytoplasm than in the nucleus.

The lysine-less species of MyoD are degraded via the N terminus-dependent pathway. Whereas lysine-less wild-type MyoD and lysine-less MyoD with the NES mutation (lysine-less MyoD\(^{\text{NES}}\)) were both nuclear as expected, lysine-less MyoD with the NLS1 mutation (lysine-less MyoD\(^{\text{NLS1}}\)) was only 40% cytoplasmic. This result is somewhat unexpected because the NLS1 mutation on either the wild-type MyoD or N-terminal-blocked MyoD background yielded species with 83 or 75% cytoplasmic localization, respectively (Figs. 3, 4, and 7A). Lysine-less MyoD\(^{\text{NLS1}}\) had a shorter \(t_{1/2} (1.5 \pm 0.4 \text{ h})\) than lysine-less wild-type MyoD \((2.6 \pm 0.6 \text{ h})\). Lysine-less MyoD\(^{\text{NES}}\) was degraded at a substantially slower rate \((t_{1/2} = 5.8 \pm 0.9 \text{ h})\). The slower degradation of the lysine-less species localized to the nucleus (lysine-less MyoD and lysine-less MyoD\(^{\text{NLS1}}\)) suggests that the N terminus-dependent pathway is less active in the nucleus.

### DISCUSSION

The ubiquitin-proteasome system exists in both the cytoplasm and the nucleus and is responsible for the degradation of many short-lived cellular proteins. Ubiquitination of the target protein can occur on an \(\epsilon\)-amino group of an internal lysine or on the N terminus of the protein tagged for destruction. MyoD, a basic helix-loop-helix transcription factor, is ubiquitinated and degraded by the ubiquitin-proteasome system in the nucleus (11). Ubiquitination of the protein occurs preferentially on the N terminus, leading to N terminus-dependent degradation. Blocking of the N terminus with a 6\times Myc tag, via methylation, or through carbamylation of the \(\epsilon\)-amino group leads to preferential degradation by the lysine-dependent pathway (8). Using NLS and NES mutants of N-terminal-blocked and lysine-less MyoD, we determined that the lysine-dependent pathway is more active in the cytoplasm, whereas both degradation pathways have equal activity in the nucleus.

This determination was based on \(t_{1/2}\) calculations of the different N-terminal-blocked and lysine-less mutants, and although the differences in the \(t_{1/2}\) may be small, they are likely to be biologically significant because a 2–4-fold change in the half-life of a protein may underlie a significant change in a protein steady-state level and hence in a biological function. It should also be noted that only the subcellular localization and \(t_{1/2}\) were assayed and that kinetic determinations of MyoD nucleocytoplasmic shuttling were not evaluated. Furthermore, it is certainly possible, if not likely, that these changes have multiple effects on MyoD, affecting such aspects as DNA binding, acetylation, and/or dimerization. Although the results presented herein are specific to MyoD, future studies will focus on
these pathways of cellular localization and degradation and on how the pathways relate to other proteins degraded by the ubiquitin-proteasome system.

To determine the ability of the ubiquitin-proteasome system to degrade MyoD in the cytoplasm, the NLSs of MyoD was mutated in an attempt to inhibit nuclear localization. NLSs are characterized by single or bipartite regions of basic amino acids. In the present study NLS mutants of MyoD were prepared by mutagenesis of clusters of basic amino acids (Lys and Arg) to alanines. Among the six regions evaluated, MyoDNLs1NLs2 and MyoDNLs3NLs2 were found to significantly reduce nuclear localization of MyoD (17 and 21% nuclear, respectively). Mutation of NLS1 and NLS3 together did not significantly inhibit nuclear import to a greater degree than that seen with either single mutation alone. Therefore, it is likely that MyoD possesses one NLS signal (amino acid region 102–124) and that NLS1 and NLS3 function as subdomains of this sequence. These results differ from those previously reported by Vandromme et al. (31) in which they identify two NLS regions (amino acid regions 100–112 and 130–135) that appear to function independently in that deletion of both regions is required to inhibit nuclear localization of MyoD. We find that substitution mutagenesis of NLS1 (amino acid region 102–112) alone is sufficient to inhibit import and that NLS2 (amino acid region 133–134) is not a nuclear targeting sequence. Furthermore, MyoDNLS1NLs2 was no more cytoplasmic than MyoDNLs1 alone. Using MyoDNLs1 as a background, we identified a single NES signal within the N terminus of MyoD (Fig. 5). Although MyoDNLs1NLs3NES was 42% nuclear compared with 17% nuclear for MyoDNLs1, there was still a considerable amount of MyoD in the cytoplasm. Treatment of MyoDNLs1NLs3NES with LMB caused the protein to be greater than 88% nuclear (data not shown), suggesting that mutagenesis of the NES does not fully inhibit nuclear export. One reason for this may be that MyoD associates with cofactors that facilitate nuclear export regardless of the mutated NES, or it may be that a separate NES, consisting of residues other than leucine, exists.

Examination of the rates of degradation of the various MyoD mutants yields a strong correlation between degradation rate and subcellular localization. Constructs that were localized to the nucleus, (96–100%) wild-type MyoD, MyoDNLs2NLs2, and MyoDNLs1NLs2NLs2, were degraded with a t1⁄2 of 0.8–1.0 h, whereas MyoDNLs1, MyoDNLs2NLs1NLs2, or MyoDNLs1NLs2, each of which was predominately cytoplasmic (17–21% nuclear), displayed t1⁄2 of 1.2–1.3 h (Fig. 3). MyoDNES had a slightly longer t1⁄2 than wild-type MyoD (1.05 versus 0.8 h; Fig. 6). One potential reason for this finding may be that we had introduced modifications to the protein at the N terminus, which appears to be its main ubiquitination site (8). Another possibility is that the nuclear degradation machinery is saturated when MyoD is exclusively nuclear. Consistent with this latter possibility, we found that the t1⁄2 for the degradation of wild-type MyoD in the presence of LMB is somewhat slower than that seen without the inhibitor (legend to Fig. 2). To localize N-terminal-blocked MyoD and lysine-less MyoD to the cytoplasm or to the nucleus, we mutated their NLS and NES. The t1⁄2 of N-terminal-blocked MyoD was significantly longer than that of wild-type MyoD (2.7 ± 0.6 h and 0.8 ± 0.1 h, respectively), which is in agreement with previously published data (Fig. 7A; Ref. 8). Cytoplasmically localized N-terminal-blocked MyoD (N-terminal-blocked MyoDNLs1) was degraded with a shorter half-life than either wild-type N-terminal-blocked MyoD or N-terminal-blocked MyoDNLs2, both of which localized to the nucleus (Fig. 7A). This supports the notion that the lysine-dependent pathway is more active in the cytoplasm than in the nucleus.

The locus of activity of the N terminus-dependent pathway is less clear. Mutation of the NLS within lysine-less MyoD did not yield a construct with predominant cytoplasmic localization. Although some cytoplasmic localization was seen, lysine-less MyoDNLs3 was ~60% nuclear (Fig. 7B). The reason or reasons for this finding are unclear. One reason may be that mutagenesis of lysines to arginines may affect DNA binding as lysine 146 has been shown to make contacts with the bound DNA duplex (37). The reduced ability of MyoD to induce myogenesis as a result of mutagenesis of lysines 124 (38) may also contribute to the nuclear localization and shorter half-life of lysine-less MyoDNLs3. Activation and the DNA-binding ability of MyoD are also regulated by acetylation of lysines 99, 102, and 104 (39, 40), which are mutated in the MyoD lysine-less mutant. In addition, lysine-less MyoDNLs1 was degraded more rapidly than wild-type lysine-less MyoD, perhaps consistent with this notion. Lysine-less MyoDNLs2 was nuclear with a half-life considerably longer than that of wild-type lysine-less MyoD (5.8 ± 0.9 h versus 2.6 ± 0.6 h). This finding likely results from the lack of recognition sites for the ubiquitin-proteasome system, as all of the lysines had been mutated to arginines and had been mutated within MyoDNES. The N terminus (amino acid region 3–13) had also been mutagenized, leaving no known site for recognition and ubiquitination, as alterations of amino acids with the protein alter its three-dimensional structure and hence its potential for recognition by the ubiquitin system.

A scheme for the pathways of MyoD degradation is illustrated in Fig. 8. MyoD is synthesized in the cytoplasm and rapidly targeted to the nucleus, where it resides both free and complexed with DNA. An NES sequence allows MyoD to shuttle from nucleus to cytoplasm. In addition, another minor LMB-sensitive (LMB Sens) pathway supports nuclear export. In the nucleus, where the lysine-dependent and N terminus-dependent pathways are equally active, ubiquitin proteasome degradation occurs more rapidly than in the cytoplasm. The lysine-less-dependent pathway was more active than the N terminus-dependent pathway within the cytoplasm.
plasmic MyoD is degraded only through a lysine-dependent pathway, whereas both pathways function equally well in the nucleus. Equivalent activities of the two pathways within the nucleus are also supported by the similar half-lives of wild-type N-terminal-blocked MyoD and wild-type lysine-less MyoD.

At present it appears that many more proteins are tagged for ubiquitin-dependent degradation on internal lysines. In addition, it currently seems that the majority of nuclear proteins require nuclear export prior to degradation. Consistent with these observations, the lysine-dependent degradation pathway appears to be more active within the cytoplasm than in the nucleus. This raises the possibility that nuclear proteins that are degraded in the nucleus undergo degradation via an N-terminus-dependent pathway, as is the case with MyoD.

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