Degradation of many short-lived cellular proteins such as the transcription factor MyoD occurs via the ubiquitin-proteasome pathway. MyoD, similar to many rapidly degraded regulatory factors, interacts with several high affinity binding partners, including members of the Id (inhibitors of DNA binding) family. Following transfection to HeLa cells, Id1 is localized to the nucleus and rapidly (t<sub>0.5</sub> h) degraded via the ubiquitin-proteasome system. Mutagenesis of lysine residues within the putative nuclear localization region (amino acids 68–82) directs Id1NLS to the cytoplasm yet confers an increased rate of degradation (t<sub>0.5</sub> > 0.6 h). Addition of a Myc<sub>6</sub> tag to the N terminus of lysine-less Id1 markedly stabilized Id1 (t<sub>0</sub> > 10 h) and suggests degradation via the N terminus-dependent pathway. Co-transfection of MyoD with Id1 or Id1<sup>NLS</sup> increases Id1 or Id1<sup>NLS</sup> within the nucleus and markedly reduces the rate of Id1 degradation. These results thus demonstrate that in vitro MyoD modulates the rate of Id1 degradation and suggest a dynamic interplay of these factors.

 Degradation of many short-lived cellular proteins, such as transcription factors, tumor suppressors, and cell cycle regulators, occurs via the ubiquitin-proteasome pathway (1–3). 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 (4). However, recent studies have shown that the N terminus of a protein substrate may also serve as the site of ubiquitination (5–8), 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 (4). Currently, the relative contribution of each of these two pathways has been described only for MyoD (9).

Among the short-lived proteins degraded by the ubiquitin-proteasome system are several transcription factors including MyoD, a key regulator of muscle differentiation (10). Recent evidence has shown that both of the two (lysine-dependent and N terminus-dependent) pathways are responsible for the rapid degradation of MyoD in vitro and in vivo (5, 11, 12). Degradation of MyoD occurs in both the nucleus and cytoplasm, however, the lysine-dependent and N terminus-dependent pathways play differential roles within these subcellular compartments (9).

A nuclear ubiquitin-proteasome system also appears to be responsible for the degradation of several other transcription factors (13–15) many of which require translocation into the nucleus for degradation. In contrast, others (including some that undergo ubiquitination within the nucleus) (16–22) require nuclear export prior to degradation by the ubiquitin-proteasome system.

Many of the rapidly degraded transcription factors and oncoproteins have regulatory high affinity binding partners. Among these is MyoD, which can form inactive (non-DNA binding) heterodimers with members of the Id family. The four Id (inhibitors of DNA-binding) members are helix-loop-helix proteins that contain no basic region, and thus do not bind DNA. However, they are able to dimerize with one another and with MyoD and its family members, albeit with various affinities (23–25). Langlands et al. (26) reported Id1 as the most active in terms of MyoD binding. Overexpression of Id protein inhibits MyoD-mediated muscle development in vitro (27). Thus, Id proteins are negative regulators of cell differentiation. The turnover of Id proteins appears to be rapid (28, 29). However, the molecular mechanisms and subcellular locus involved in Id degradation is unknown as is its role in MyoD degradation and vice versa. In the present study we show that Id1 is rapidly degraded by the ubiquitin-proteasome system within the nucleus and that interaction with MyoD modulates the degradation of Id1. The importance of these observations lies in understanding the alterations in cellular protein degradation in physiological and pathophysiological states.
Plasmids and Construction of MyoD, MyoD\textsuperscript{NL}, Id1, and Id1 Mutants—Wild type MyoD and MyoD\textsuperscript{NL} both in the pCIneo vector have been previously described (9, 12). Id1 in pcDNA3 was a gift from Burton Wice. Preparation of the NLS and lysine-less mutants of Id1 was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. N-blocked Id1 and N-blocked lysine-less Id1 were prepared by insertion of various tags (HA, sic1p, and myc) into pcDNA3, on the N terminus of Id1 (see below) sic1p in YCplacIII was a gift from David Pellman. Fox1 in pcDNA3 was from Brian Hackett. DNA sequencing using Big Dye version 3.1 (ABI Biosystems) was used to confirm all sequences.

Cell Culture—HeLa cells, which were selected because they do not express MyoD and express Id1 at only minimally detectable levels, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin) (Invitrogen) and maintained in a humidified chamber at 37°C and 5% CO₂. Transient transfections (efficiency ~40–60%) were performed using the FuGENE 6 reagent (Roche Diagnostics) and cells were analyzed 16–24 h later.

Immunofluorescent Localization of Id1 and Id1 Mutants—Subcellular localization of Id1 and its mutants in HeLa cells was determined by direct immunofluorescence using the rabbit polyclonal anti-Id1 antibody (1:300 dilution, Santa Cruz Biotechnology, C-20) followed by incubation with a Alexa Fluor 568 or Alexa Fluor 488 goat anti-rabbit IgG (heavy and light chain) (Molecular Probes). MG132 (20 µM, Peptides International) or leptomycin B (10 nM, Sigma) were added to cells 2 h prior to fixation where indicated. MG132 was prepared as a 10 mM stock solution in DMSO. Leptomycin B was prepared as a 10 µM solution in ethanol. Cells were observed using a Zeiss Axioscope microscope and 10–20 random fields of each culture condition were photographed (magnification ×40) using a Zeiss Axioscan digital camera.

Determination of Degradation of Id1 and Id1 Mutants in Vivo—As previously described (9, 12), 16–24 h after transfection the HeLa cells were incubated with cycloheximide (100 µg/ml, Sigma) to inhibit further protein synthesis. MG132 (20 µM) or leptomycin B (10 mM) were added along with the cycloheximide as noted. The cells were then lysed after 0, 0.5, 1, 2, or 3 h in phosphate-buffered saline containing 5% Igepal, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml leupeptin, and 1 µM pepstatin for at least 30 min after which the cells were sonicated then centrifuged at 14,000 × g 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 an 18% Tris-HCl gel (Bio-Rad) and electroblotted onto nitrocellulose (Osmonics). The blots were probed with polyclonal anti-Id1 antibody (1:300 dilution, Santa Cruz Biotechnology) followed by incubation with a horseradish peroxidase-conjugated antibody and detection by chemiluminescence (Amersham Biosciences). The resulting bands were quantitated using the Kodak EDAS system and the data were graphed using the Excel graphing program (Microsoft). The degradation rate is expressed as half-life (t\textsubscript{1/2}), the time for degradation of 50% of the Id1. Each of the constructs was evaluated by generally three to six independent determinations of t\textsubscript{1/2}. The data are expressed generally as mean ± S.E.

Localization of Id1 and MyoD—HeLa cells were co-transfected with Id1 and MyoD in a molar ratio of 1:1. Subcellular localization of Id1 was determined by indirect immunofluorescence using the rabbit polyclonal anti-Id1 antibody (1:100 dilution, Santa Cruz Biotechnology, C-20) followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (heavy and light chain) (Jackson Immunochemical). MyoD was probed using the mouse monoclonal anti-MyoD (1:100 dilution, Vector Labs, NCL-MyoD1) followed by incubation with a TRITC-conjugated donkey anti-mouse IgG (heavy and light chain) (Jackson Immunochemical). Cells were observed using a Zeiss Axioscope microscope and 10–20 random fields of each culture condition were photographed (magnification ×40) using a Zeiss Axioscan digital camera. Cells were scored and the percent of cells expressing nuclear Id1 or MyoD was determined as previously described (9).

Degradation of Id1/MyoD—HeLa cells were co-transfected with MyoD and Id1 NL\textsuperscript{NL} or MyoD NL\textsuperscript{NL} and Id1 in a 1:1 ratio. 16–24 h after transfection the HeLa cells were incubated with cycloheximide to inhibit further protein synthesis. The half-life experiments on Id1, Id1 NL\textsuperscript{NL}, MyoD, and MyoD NL\textsuperscript{NL} were described previously. The cell lysates were run on a 12% Tris-HCl gel (Bio-Rad), and the blots were probed with polyclonal anti-Id1 or polyclonal anti-MyoD (Santa Cruz Biotechnology, M-318) in dilutions of 1:100 followed by incubation with a secondary horseradish peroxidase-conjugated antibody and detection by chemiluminescence (Amersham Biosciences).

RESULTS

To determine the pathway of Id1 degradation we transfected Id1 cDNA into HeLa cells. Following transfection of Id1 cDNA, Id1 is localized (~72%) to the nucleus (Fig. 1A). Nucleoli are negative. As seen in Fig. 1B, Id1 is rapidly degraded (t\textsubscript{1/2} ~ 1 h). Incubation of cells with MG132, a potent and selective inhibitor of the proteasome, markedly slowed the rate of Id1 degradation (t\textsubscript{1/2} ~ 9 h). These observations suggest that Id1 is degraded via the ubiquitin-proteasome pathway.

Many nuclear proteins appear to be degraded following export from the nucleus, whereas others appear to be degraded within the nucleus. To determine whether Id1 cycles into and out of the nucleus, and ultimately to determine the locus of its degradation, we sought to determine whether Id1 contains a nuclear localization sequence (NLS) that targets it to the nucleus. Evaluation of the Id1 amino acid sequence reveals only one region of high positive charge density (amino acids 68–84: RKLKVTPLQPKNRKVSK). We thus generated a mutant of Id1

FIG. 1. Localization and half-life of wild-type Id1. A, 18 h after transfection with wild type Id1, HeLa cells were fixed and localization of Id1 was visualized by immunofluorescence. B, 18 h after transfection with wild type Id1, HeLa cells were treated with cycloheximide (CHX) or CHX plus MG132. Cells were lysed at 0, 0.5, 1, 2, and 3 h and were evaluated via SDS-PAGE and Western blot for Id1. The pixels for each band were measured and normalized so that the number of pixels at t = 0 was 100%. The log10 of the percent of pixels was plotted versus time for each time point and the t\textsubscript{1/2} calculated from the log of 50%. The t\textsubscript{1/2} for Id1 was 0.9 and 8.9 h when MG132 was added.
Infection with Id1 NLS, HeLa cells were fixed and localization of Id1 was
mediated via this pathway. The presence of LMB does not alter the
localization of Id1. Furthermore, the rate of degradation of Id1NLS (t1/2 = 0.5 h) was unaltered in the presence of LMB (Fig. 2B) (Table I). These results suggest that LMB-sensitive nucleocytoplasmic export is not a significant pathway for Id1.

Because Id1NLS was rapidly degraded, in fact more rapidly than wild type Id1, and because Id1NLS has three of the five lysine (Lys) residues mutated to alanine (Ala), it is possible that one or both of the remaining lysine residues (Lys8 or Lys19) are targets for ubiquitination and proteasomal degradation. To address this, Id1 mutants with modification of each of the lysine residues were generated and the rates of degradation of the resultant proteins were examined. As seen in Table I, Id1K2A was rapidly degraded (t1/2 ~ 0.1 h). Similarly Id1K2R,
Id1NLS,K2A, and Id1NLS,K2A,K19A (also termed lysine-less Id1) are each rapidly degraded (t1/2 ~ 0.6, 0.7, and 0.6 h, respectively) (Table I). Incubation with MG132 markedly slowed the rate of degradation of each of these mutants (Table I). Furthermore, wild type Id1 was localized to the nucleus, whereas Id1NLS and Id1NLS,K2A,K19A (data not shown) were each predominantly localized to the cytoplasm as well as the nucleus. These results demonstrate that Id1 is rapidly degraded via the ubiquitin-proteasome system although this occurs independent of its (five) lysine residues.

To determine whether the free N terminus of Id1 is necessary for its rapid degradation, a series of Id1 mutants were generated in which various inserts were placed at the N terminus of either wild type Id1 or lysine-less Id1. Previous work demonstrated that an N-terminal addition of Myc6 tags (78 amino acids), or alternatively deletion of 11 amino acids from the N terminus of the parent protein, was required to stabilize the degradation of lysine-less MyoD, LMP1, and E7 (5–7). Furthermore, recent studies on sic1p have demonstrated that the lysine-less protein is stable and the N-terminal 31 amino acids do not contain a degradation recognition motif (30). Thus, we generated a mutant of lysine-less Id1 that contained a 78-amino-acid, Myc6 tag at the N terminus. When expressed in HeLa cells, this Myc6-lysine-less Id1 was stable (t1/2 = > 10 h, Fig. 3). No additional stability was conferred by MG132 addition. To determine whether this N-terminal block by the Myc6 tag was the result of a simple bulk addition (78 amino acid residues), we examined the stability of HA7-lysine-less Id1, with 63 amino acids added to the N terminus. HA7-lysine-less Id1 is also rapidly degraded (t1/2 = 0.7 ± 0.1 h). We then generated another lysine-less Id1 mutant with the N-terminal 30 amino acids of sic1p, sic1p-HA7-lysine-less Id1 (an 84 amino acid residue addition), which is also rapidly degraded (t1/2 = 0.7 ± 0.1 h) but stabilized in the presence of MG132 (t1/2 = 4.2 h). Thus, the particular nature of the N-terminal addition, not simply its size, is necessary for abrogation of N-terminal recognition.

To determine whether MyoD interacts with Id1 in vivo and alters the rate of Id1 degradation we transfected HeLa cells with various Id1 and MyoD constructs. Initially we selected MyoD and Id1NLS because interaction of the two might direct Id1NLS to the nucleus via the NLS of MyoD. We evaluated both localization and rate of degradation of Id1NLS and MyoD. As seen in Table II, MyoD expressed alone is localized to the nucleus (99%) and degraded with a t1/2 ~ 0.9 h as we have shown earlier (9). Id1NLS, as seen above, is 28% nuclear and is degraded with a t1/2 ~ 0.5 h (Table II). Transfection of both MyoD and Id1NLS resulted in a marked increase in Id1NLS nuclear localization (28 → 58%) and decrease in the rate of Id1NLS degradation (t1/2 ~ 1.5 h) (Table II). The rate of degr-
Degradation of Id1 Is Modulated by MyoD

Rates of degradation of lysine mutants of Id1

Lysine residues within the Id1 sequence were mutated to alanines (as noted) and the resultant constructs expressed in HeLa cells. The rates of degradation of the expressed proteins were determined in the absence or presence of MG132 or LMB, as described. Each figure represents mean \( \pm \) S.E. (number of determinations).

| Amino acid residue | Rate of degradation |
|--------------------|---------------------|
|                    | MG132               | LMB                  |
| Id1               |                     |                      |
| K                 | 1.0 \( \pm \) 0.1 (33) | 14 \( \pm \) 4.5 (8) |
| Id1<sup>NLS</sup> |                     |                      |
| A                 | 1.1 \( \pm \) 0.5 (2)  | 13 \( \pm \) 1.0 (2) |
| Id1<sup>NLS</sup> |                     |                      |
| K                 | 0.5 \( \pm \) 0.1 (24) | 4.8 \( \pm \) 1.0 (9) |
| Id1<sup>NLS, K213A</sup> |               |                      |
| A                 | 0.6 \( \pm \) 0.1 (8)  | 4.0 \( \pm \) 1.7 (2) |

Table II

Effect of coexpression of Id1 and MyoD on subcellular localization and rates of degradation

HeLa cells were transfected with Id1 (or Id1<sup>NLS</sup>), MyoD (or MyoD<sup>NLS</sup>) or both as indicated. Thereafter the subcellular localization of each expressed protein was determined via immunofluorescence and the rate of degradation of each expressed protein was determined as described. Each figure represents mean \( \pm \) S.E. (three to six determinations).

| Construct | Localization | Rate of degradation |
|-----------|--------------|---------------------|
|           | % nuclear | \( t_{1/2} \) in h |
| A         |           |                     |
| Id1<sup>NLS</sup> | 28       | 0.5 \( \pm \) 0.1  |
| MyoD      | 99        | 0.9 \( \pm \) 0.1  |
| Id1<sup>NLS</sup>/MyoD | 58       | 1.5 \( \pm \) 0.2  |
| B         |           |                     |
| Id1      | 72        | 1.0 \( \pm \) 0.1  |
| MyoD<sup>NLS</sup> | 9        | 1.5 \( \pm \) 0.1  |
| Id1/MyoD<sup>NLS</sup> | 52       | 1.8 \( \pm \) 0.2  |
| C         |           |                     |
| Id1      | 72        | 1.0 \( \pm \) 0.1  |
| MyoD      | 98        | 0.9 \( \pm \) 0.1  |
| Id1/MyoD | 87        | 1.5 \( \pm \) 0.1  |

As seen in Table II, cotransfection with MyoD increased Id1 nuclear localization (72 \( \rightarrow \) 87%) and slowed Id1 degradation (\( t_{1/2} \) \( \rightarrow \) 1.0 to 1.5 h). The nuclear localization of MyoD was essentially unaltered, as was its degradation rate (Table II). Taken together these results demonstrate that cotransfection of MyoD or MyoD<sup>NLS</sup> slows the rate of Id1 degradation.

To determine whether the effect of MyoD on Id1 degradation is specific, cells were cotransfected with Id1 and Foxj1, a nuclear forkhead transcription factor (31). The rate of Id1 degradation (\( t_{1/2} = 0.9 \) h) was not prolonged by Foxj1 expression (data not shown).

DISCUSSION

Despite extensive studies on the regulation of transcription factor synthesis, little attention has been directed at transcription factor degradation and its regulation. MyoD, a key regulator of muscle differentiation, provides an excellent example. While its role in cellular differentiation and regulation of its synthesis is well understood, until recently little was known of the mechanisms involved in its degradation (5, 9, 12). MyoD and its family members (23) are often complexed with one another or with Id1, a small helix-loop-helix protein. To date, there is little or no data regarding the degradation of the active or the inactive complexes. As seen herein (Table II), expression of MyoD results in a decrease in the rate of degradation of Id1. The converse is not seen, expression of Id1 does not result in a decrease in the rate of MyoD degradation.

We show that the degradation rate of MyoD is not diminished while that of Id1 is. This finding is important in its subtlety and suggests a dynamic interplay of these factors. At least two possible mechanisms may underlie these observa-

Fig. 3. Half-life of Myc-lysine-less Id1. 18 h after transfection with Myc-lysine-less Id1, HeLa cells were treated with cycloheximide or cycloheximide plus MG132 and analyzed as described in the legend to Fig. 1. The \( t_{1/2} \) for Myc-lysine-less Id1 was 10 h and 10 h when MG132 was added.
Degradation of Id1 Is Modulated by MyoD

At present it appears that many more proteins are tagged for ubiquitin-dependent degradation on internal lysines than via the free N terminus. Among those degraded via the free N terminus are MyoD (5), the E7 oncoprotein of human papilloma virus (7), LMP1 of Epstein-Barr virus (6), p21 (8), and Id2 (29). Recent studies with MyoD suggest that the lysine-dependent pathway appears to be more active in the cytoplasm than in the nucleus (9). 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. Our results with lysine-less Id1 and N terminus blocked lysine-less Id1 identifies Id1 as another nuclear protein degraded via the N terminus-dependent pathway.

Id1 and MyoD have been shown to interact in vitro in a variety of assays. For example, Langlands et al. (26), using a yeast two-hybrid assay demonstrated Id1 interaction with MyoD and myf-5 but not myogenin nor MRF4/Myf-5. They further showed interaction of Id1 and Id2 with MyoD via co-immunoprecipitation of in vitro translated proteins. In vivo Id1/MyoD interactions have been observed following transient transfections of a modified two-hybrid interaction in HeLa cells (26) or with a split luciferase reporter system (34). Our results (Table II) provide additional insight into Id1/MyoD interactions in vivo. Transfection of Id1-NLS (which by itself is localized 28% to the nucleus) together with MyoD (which by itself is localized 99% to the nucleus) causes substantial redistribution of Id1-NLS to the nucleus (28 → 58% nuclear). This observation suggests that MyoD (via its NLS) is able to chaperon Id1-NLS into the nucleus. Similarly, MyoD is able to increase wild type Id1 within the nucleus. These results thus demonstrate in vivo that Id1 and MyoD interact. Examples of in vitro and in vivo interaction of other members of the helix-loop-helix families have recently been reported (35), including the observation of Imink et al. (36) who applied FRET to examine the in vivo interaction of two basic helix-loop-helix transcription factors and demonstrated both molecular interaction and chaperone-mediated nuclear targeting.

Not only do MyoD and Id1 interact and traffic within subcellular compartments, this interaction influences their degradation. Other reports of degradation of transcription factor pairs are sparse. Zhao et al. (37) showed that Cbf1 and its partner Sma1 are each degraded via the ubiquitin-proteasome system, but their interrelationship was not reported. The dioxin receptor is a ligand-activated transcription factor, which upon ligand binding translocates to the nucleus and complexes with its partner, Arnt, to form a DNA binding heterodimer. Dioxin receptor is constitutively degraded via the E3, C-terminal hsp 70-interacting protein (CHIP), and the ubiquitin-proteasome system via a process termed “protein triage” whereby fate decisions regarding proper folding versus degradation occur (38). XAP2, a molecular chaperone, protects dioxin receptor from degradation during this process (38). Finally, Hattori et al. (39) have shown that the inhibitory/regulatory C/EBP transcription factors are degraded via the ubiquitin-proteasome system and that dimer formation via leucine zipper motifs suppresses this degradation. This suggests a system for the regulation of the cellular complement of non-dimerized binding partners. Our observations with Id1 and MyoD extend this notion in that dimerization alters the rate of degradation of Id1, but not that of MyoD.

The implications of these results are broad and suggest that regulation of the Id1 degradation rate and thus its cellular and tissue levels may significantly impact the biological processes in which it is involved. Recent studies also suggest that transcription factor ubiquitination serves a key role in regulating gene expression by linking transcription factor activity to its...
Degradation of Id1 Is Modulated by MyoD

proteasome-dependent degradation (40–42). Several ubiquitin E3 ligases function as transcriptional co-activators. For example, Kim et al. (43) have shown that the ubiquitin ligase, Skp2, itself an oncoprotein, is a transcriptional co-activator for Myc, and thus connects Myc activity and death. Biochemical and genetic studies have demonstrated that the biological actions of the Id proteins is via a “dominant-negative” effect whereby they sequester the tissue-restricted basic helix-loop-helix proteins (e.g. MyoD) or their ubiquitously expressed partners (e.g. E proteins). Thus, by sequestering these DNA-binding basic helix-loop-helix proteins, Id proteins can control the activity of basic helix-loop-helix proteins in diverse cell lineages (e.g. muscle, lymphocytes, and endothelium). Additional Id protein partners have been recently found, including Rb, ets proteins, and pax family members (44). A variety of mechanisms have been shown to increase Id1 protein expression including signaling via the type 1 insulin-like growth factor receptor and STAT 3 (45). In addition, increased expression of Id1 has been shown to delay cellular senescence in several cell lineages including the endothelium (46). Thus, regulation of Id1 protein level via changes in its rate of degradation is likely to be important in tissue differentiation and developmental programs.

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