UBIQUITIN-PROTEASOME-MEDIATED DEGRADATION, INTRACELLULAR LOCALIZATION, AND PROTEIN SYNTHESIS OF MYOD AND ID1 DURING MUSCLE DIFFERENTIATION

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Running Title: MyoD and Id1 degradation in muscle differentiation

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Mammalian skeletal myogenesis results in the differentiation of myoblasts to mature syncytial myotubes, a process regulated by an intrinsic genetic network of at least three protein families, muscle regulatory factors (MRFs), E proteins and Id proteins. MyoD, a key MRF, and its negative regulator Id1 have both been shown to be degraded by the ubiquitin-proteasome system. Using C2C12 cells and confocal fluorescence microscopy, we show that MyoD and Id1 co-localize within the nucleus in proliferating myoblasts. In mature myotubes, in contrast, they reside in distinctive subcellular compartments, with MyoD within the nucleus and Id1 exclusively in the cytoplasm. Cellular abundance of Id1 is markedly diminished from the very onset of muscle differentiation, while MyoD abundance is reduced to a much lesser extent and only at the later stages of differentiation. These reductions in MyoD and Id1 protein levels appear to result from a change in the rate of protein synthesis rather than degradation. In vivo protein stability studies reveal that the rates of ubiquitin-proteasome-mediated MyoD and Id1 degradation are independent of myogenic differentiation state. Id1 and MyoD are both rapidly degraded each with a t1/2 ~ 1h in myoblasts and in myotubes. Furthermore, relative protein synthesis rates for MyoD and Id1 are significantly diminished during myoblast to myotube differentiation. These results provide insight as to the interaction between MyoD and Id1 in the process of muscle differentiation, and have implications for the involvement of the ubiquitin-proteasome-mediated protein degradation and protein synthesis in muscle differentiation and metabolism under abnormal and pathological conditions.

Skeletal muscle differentiation is characterized by the myoblast’s terminal withdrawal from the cell cycle, activation of muscle-specific gene expression and cell fusion into multinucleated myotubes. These events are coordinated by a family of four muscle-specific basic helix-loop-helix (bHLH) transcription factors: MyoD, Myf5, myogenin, and Mrf4, termed the muscle regulatory factors (MRFs) (1-4). Mice lacking myogenin appropriately specify the skeletal muscle lineage, but fail to terminally differentiate. Mrf4 is required for the maintenance of the differentiated myotubes. While the specification of the myogenic lineage requires MyoD and Myf5, as double knockout of both genes yields mice with no skeletal muscle (5), MyoD is also required for healthy self-renewing proliferation of the adult skeletal muscle satellite cells (6-8). MRFs form heterodimers with ubiquitous E proteins, and activate myogenic differentiation, through their subsequent binding to specific sequences, termed E boxes, in the promoter-regulatory regions of muscle-restricted target genes (4). The transcriptional activities of MRFs are negatively regulated by a family of inhibitors of DNA-binding (Id) proteins. The 4 Id proteins (Id1, Id2, Id3 and Id4) are helix-loop-helix (HLH) proteins, which contain no basic region, and thus do not bind DNA. However, they
are able to dimerize with one another and with MyoD or E proteins, albeit with different affinities (9-11). Id1 is most active in terms of MyoD binding. The binding affinity of Id1 for the E proteins is considerably higher than its affinity for MyoD. Sequestering ubiquitous E proteins allows Id1 to control the transcriptional activity of muscle-specific MyoD. In cultured myoblasts, Id1 overexpression via a “dominant-negative” effect inhibits the transactivation by MyoD, thereby inhibiting the synthesis of proteins participating in muscle differentiation, and consequently the fusion of myoblasts to myotubes (12).

MyoD and Id1 have both been shown to be degraded by the ubiquitin-proteasome system(13-19). This pathway involves the activation of ubiquitin by the ubiquitin-activating enzyme E1, followed by transfer of ubiquitin to E2, an ubiquitin-conjugating enzyme. E2 shuttles the ubiquitin to the substrate-specific ubiquitin ligase E3, which then delivers the ubiquitin to the protein substrate to be degraded. The ubiquitin-proteasome proteolytic system is recognized as a versatile and efficient mechanism for the control of gene expression. The level of expression of MyoD and Id1 are vitally important during muscle differentiation. Both mRNA and protein levels of Id1 are down-regulated upon initiation of differentiation (20). MyoD mRNA levels change only slightly during differentiation (21), yet the level of MyoD protein is decreased in reserve cells during muscle differentiation (22). Reducing the level of MyoD protein by its destabilization has been shown to be associated with inhibition of myogenic differentiation under abnormal or pathophysiological conditions. For example, accelerated MyoD degradation resulting from hypoxia blocked the accumulation of early myogenic differentiation markers such as myogenin, p21 and pRb, and prevented both permanent cell cycle withdrawal and terminal differentiation (23). In addition, tumor necrosis factor α (TNFα) inhibits myogenic differentiation through destabilizing MyoD protein in a NF-κB-dependent manner, interferes with skeletal muscle regeneration and may contribute to muscle wasting (24-26). Studies in a cell culture model, which has a phenotype similar to that observed in myoblast cultures derived from Myotonic dystrophy 1 patient muscle, suggest that C2C12 myogenic differentiation was disrupted by mutant DMPK 3'-UTR transcripts via posttranscriptional reduction of MyoD protein levels (27). Previous studies in vitro or in non-muscle cells also shown that MyoD degradation is regulated by phosphorylation, DNA binding and protein-protein interactions (19,28). Phosphorylation of MyoD is required for its rapid degradation. The specific DNA sequence to which MyoD binds can inhibit MyoD degradation. Id1, which inhibits the binding of MyoD complexes to DNA, abrogates the effect of DNA on stabilization of MyoD. Furthermore, protein degradation studies following co-transfection of MyoD and Id1 to Hela cells have shown that MyoD is able to modulate both the localization and degradation of Id1 (15). It remains unclear, however, how the morphological and biological changes involved in myogenic differentiation affect the ubiquitin-proteasome mediated degradation of MyoD and Id1 in muscle cells, and how their degradation and interaction contribute to their cellular abundance and thus regulate differentiation.

Mouse C2C12 myoblast cells are a well characterized myogenic cell line. In the presence of mitogen-rich serum, they proliferate as an undifferentiated population expressing MyoD and Myf5. Terminal differentiation of C2C12 cells may be induced by serum deprivation, which initiates a series of chronologically ordered events with expression of myogenin as the earliest detected. Thereafter, cells permanently withdraw from the cell cycle, contractile proteins such as myosin begin to accumulate, and cell fusion then takes place resulting in the formation of myotubes. Using C2C12 cells as our model, we show here that MyoD and Id1 co-localize within the nucleus in proliferating myoblasts. In mature myotubes, in contrast, they reside in distinctive subcellular compartments, with MyoD within the nucleus and Id1 exclusively in the cytoplasm. Both MyoD and Id1 are rapidly degraded by the ubiquitin-proteasome pathway during the differentiation of myoblasts to myotubes. Further, the rate of their degradation appears to be unaffected by the differentiation state. While a reduction of MyoD and Id1 synthesis rate was observed during myogenic differentiation.

**MATERIALS AND METHODS**

**Plasmids and construction of Id1-HA**

Wild type MyoD in pClneo and Id1 in
pcDNA3 have been described previously (15). Id1 with a 1×HA tag at the C-terminus was constructed by insertion of the HA tag into pcDNA3 harboring wild type Id1 encoding DNA. PCR Primers were purchased from IDT. DNA sequencing using Big Dye Version 3.1 (ABI Biosystems) was used to confirm all sequences.

**Cell culture**

The C2C12 mouse myoblast cell line was obtained from the American Type Culture collection. The cells were routinely propagated in growth medium (GM), Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen), and maintained in a humidified chamber at 37 °C with 5% CO₂. Myogenic differentiation was induced by changing the growth medium to differentiation medium (DM), Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum (HyClone), 100 units/ml penicillin G and 100 µg/ml streptomycin, when cells reach confluency. Cells were then maintained in differentiation medium for 6 days with medium being changed every 24 hours. Transient transfections of C2C12 myoblasts were performed using the Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instruction.

**Immunofluorescence**

C2C12 cells on glass cover slips were first washed with PBSc, a phosphate-buffered saline solution (PBSa, Fisher Biotech) supplemented with 100 mM CaCl₂ and 50 mM MgCl₂, fixed in 4% paraformaldehyde, quenched in 0.1M ethanolamine (pH 8.0) and permeabilized in 1% Triton X-100 (Sigma). Subcellular localization of MyoD, Id1, myogenin and myosin heavy chain in C2C12 myoblasts or myotubes was then determined by indirect immunofluorescence using the rabbit polyclonal antibodies (anti-MyoD (C-20), anti-Id1 (C-20), anti-myogenin (M-225) and anti-myosinHC (H-300), Santa Cruz Biotechnology) followed by incubation with Alexa Fluor 568 goat anti-rabbit IgG (heavy and light chains) (Molecular Probes) after blocking the cells in PBSc containing 1% BSA and 0.01% TW-80. Cells were observed using a Zeiss Axioskop microscope, and images were taken using a Zeiss AxioCam digital camera. Blocking control experiments for the detection of MyoD and Id1 were performed by pre-incubating the probing primary antibody with the corresponding peptides (MyoD (C-20)P and Id1 (C-20)P, Santa Cruz Biotechnology) which were initially used to generate the antibodies. For double-immunofluorescence of MyoD and Id1, a mouse monoclonal anti-MyoD (Novocastra) coupled with tetramethyl rhodamine isocyanate (TRITC)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) and the rabbit polyclonal anti-Id1 (C-20) coupled with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) are used as probes. Cells were viewed with a confocal laser scanning microscope (Fluoview 500, Olympus) using a 60x objective oil immersion lens.

**Protein expression level during myogenic differentiation**

C2C12 cells were washed with PBSa twice on ice and harvested at different stages of myogenic differentiation (D-1: 70-80% confluent C2C12 myoblasts in GM, D0: 100% confluent C2C12 cells and the time point for switching the medium to DM, D1 to D6: C2C12 cells cultured in DM for 1 to 6 days). All cells were lysed for at least 30 minutes in PBSa containing 5% Igepal, 1 mM dithiothreitol, 1 µM pepstatin, 2.5 µg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride. The collected cells were then been sonicated briefly and centrifuged at 14,000 rpm for 10 minutes at 4°C to remove cellular debris. Protein concentration of each freshly prepared cell lysate was determined by Bradford assay (Bio-Rad). After mixing with an equal volume of 2× Laemmli sample buffer (Bio-Rad), cell lysates containing equal amounts of total protein were loaded in each lane, resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Osmonics). Membranes were probed with rabbit polyclonal antibodies (anti MyoD (C-20), anti Id1 (Z-8), anti myogenin (M-225) and anti myosin heavy chain (H-300), Santa Cruz Biotechnology) followed by incubation with a secondary horseradish peroxidase-conjugated antibody. Protein bands were detected by chemiluminescence (ECL, Amersham Biosciences). Blocking control experiments for MyoD western blot were performed by pre-incubating the probing primary antibody with peptide (MyoD (C-20)P, Santa Cruz Biotechnology) before applying to the membrane.
### Determination of protein degradation half life

C2C12 cells were incubated with CHX (cycloheximide, 100 μg/ml, Sigma) to inhibit further protein synthesis. The proteasome inhibitor MG132 (N-benzyloxycarbonyl-leu-leu-leucinal, 20 μM, International Peptides) was added along with CHX when necessary. Following incubation for 0, 0.5, 1, 2 and 3 hours, cells were harvested, lysed, and cell lysates were collected as described above. After treatment with Laemmli sample buffer (Bio-Rad), equal volumes of each sample were loaded in each lane for gel electrophoresis. Western blotting was performed in the same fashion as described above. A rabbit polyclonal anti HA antibody (Upstate) was used for detection of Id1-HA protein. Desired protein bands from the westerns were quantitated using the EADS system (Eastman Kodak Co.), and the data were graphed using the Excel graphing program (Microsoft). Protein degradation rate is expressed as half-life (t1/2), the time for degradation of 50% of the protein. Each of the half-life data reported was evaluated by 3-6 independent determinations and is expressed as mean ± S.D.

### Comparison of protein synthesis rate

C2C12 cells were incubated with MG132 (20 μM). Following incubation for 0, 1, 2, 4 hours, cells were harvested, lysed, and cell lysates were collected as described above. After treatment with Laemmli sample buffer (Bio-Rad), equal volumes of each sample were loaded in each lane for gel electrophoresis. Western blotting was performed in the same fashion as described above. The pixels for each band were measured and normalized so that the number of pixels at t=0 was 1. The pixels of each band were plotted versus time. Protein synthesis rates were compared based on the initial slope from plots of data from 0-4 hours.

### RNA isolation and RT-PCR

Total RNA samples from C2C12 myoblasts and differentiated myotubes were obtained using RNAZol reagents (Tel-test, Inc.) following the manufacturer’s instructions. RT-PCR was performed for Id1, MyoD and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) using ProSTAR ultra HF RT-PCR system (Stratagene). The Id1 primers are as follows: TGGACGAGCAGCAGTGAACG, and GCACCGCAGTAGAGAAGTG (with a product of 243 base pairs). The MyoD primers are: GACAGGACAGGACAGGGAGG and GCACCGCAGTAGAGAAGTG (with a product of 358 base pairs).

### RESULTS

To study MyoD and Id1 degradation during muscle differentiation, we chose the murine C2C12 myoblasts cells as our model (12,29). By shifting confluent C2C12 cells from mitogen-rich growth medium to 2% horse serum containing differentiation medium, we were able to observe the expected morphological changes of C2C12 myogenic differentiation, namely cell alignment and fusion into multinucleated myotubes (Figure 1A). By day 1 in DM, C2C12 cells were compact and aligned, but there is no obvious cell fusion. By day 2 in DM, about 5% of cells were fused to form scattered, small multinucleated cells. During the next two days, the cell fusion rate was rapid. By day 4 in DM, about 50% of cells completed fusion, and a large number of tubular syncytial cells were observed with various numbers of nuclei mostly arranged in linear arrays. These myotubes further expanded by incorporating additional neighboring mononucleated cells and fusion with nearby myotubes and increases in size. By day 6 in DM, fused cells account for up to 70% of the cells, while a subpopulation of cells remain undifferentiated as reserve cells (22). The observed morphology transition from myoblasts to myotubes was also confirmed by immunofluorescent staining of myosin heavy chain, a major muscle contractile protein, coupled with DAPI nuclear staining. As a marker for mature muscle cells, myosin heavy chain was only detected in myotubes with multi-DAPI-positive nuclei inside single cells (Figure 1A).

Previous studies have shown that MyoD subcellular localization markedly influences its degradation rate, and that the degradation of Id1 can be modulated by MyoD (14,15). In order to determine the locus of MyoD and Id1 degradation during differentiation and the possible interaction between this pair, we first set out to determine the localization of MyoD and Id1 in C2C12 myoblasts and myotubes (Figure 1B). By indirect immunofluorescent examination, MyoD is localized to the cell nucleus in mononucleated myoblasts as well as in multinucleated myotubes. In myoblasts, Id1 is observed to be predominantly co-localized to the nucleus with MyoD, with low
level cytoplasmic staining, suggesting they interact in vivo. In contrast, MyoD and Id1 appear to reside in distinctive subcellular compartments in myotubes, with MyoD within the nucleus and Id1 exclusively in the cytoplasm. Pre-incubation of the probing antibody with its corresponding peptide completely abolished the observed immunofluorescent signals for MyoD and Id1 in our blocking control experiments (data not shown), demonstrating that the localization of both proteins in myoblasts and myotubes are not the result of non-specific binding.

We then determined MyoD and Id1 protein abundance during the time course of differentiation via immunoblot. Cellular abundance of Id1 is markedly diminished from the very onset of muscle differentiation, while MyoD abundance is reduced to a much lesser extent and only at the later stages of differentiation (Figure 2). After only 2 days in differentiation media, the Id1 protein level has already decreased to less than 20% that found in proliferating myoblasts. During this same period of differentiation, the MyoD protein level remains about the same, if not somewhat increased. By day 6 in differentiation media, Id1 protein is reduced 10 fold, while MyoD protein is reduced less than 3 fold. Overall, the relative ratio between MyoD and Id1 increased as the cells committed to differentiation. The abundance of the myogenic regulatory factor myogenin and the contractile protein myosin heavy chain were also determined in the same cell lysates (Figure 2). Myogenin, which promotes terminal differentiation, became detectable the first day in differentiation media after cells reached confluency. We also observed progressive accumulation of myosin heavy chain from day1 onward. These observations verified that our model exhibited the biological characteristics of muscle differentiation in a chronologically appropriate manner.

To determine how ubiquitin-proteasome-mediated degradation contributes to the regulation of MyoD and Id1 abundance during muscle differentiation, we first compared their protein degradation half lives in C2C12 myoblasts and myotubes. As shown in Figure 3, MyoD was rapidly degraded in both cases with the same half life (t1/2 ~ 0.9h). Incubation of cells with MG132, a potent and selective inhibitor of the proteasome, markedly slowed the rate of MyoD degradation (t1/2 ≥ 10h). For Id1, rapid degradation was also seen both in myoblasts and myotubes (t1/2 ~ 0.8h). The presence of MG132 greatly stabilized Id1 (t1/2 > 10h). All these results suggested that both MyoD and Id1 were degraded by the ubiquitin-proteasome pathway during muscle differentiation, and that the degradation rate is unchanged in myoblasts and in myotubes regardless of the morphological and biological changes. Herein, of note is that only the hyperphosphorylated MyoD species (i.e. the upper, slower migrating band) in Figure 3A was used to calculate the half life. This practice is somewhat different from that used in previous reports where both MyoD bands, although not well separated in many cases, are used for the calculation of MyoD degradation. We believe this method best serves our purpose since, MyoD degradation requires its prior phosphorylation (28), and since the hypophosphorylated band appears to be much more stable.

Although no change was observed in the degradation rate of MyoD or Id1 in myoblasts (D-1) and myotubes (D6), the possibility remains that MyoD or Id1 could be degraded at a different rate at a specific stage between the onset of muscle differentiation and its completion. We thus determined MyoD and Id1 degradation rates at various time points throughout C2C12 differentiation. As seen in Figure 4, MyoD, as well as Id1 was degraded at about the same rate during differentiation from proliferating, mononucleated myoblasts, to mature syncytial muscle cells. These results suggest that ubiquitin-proteasome-mediated MyoD and Id1 degradation is independent of muscle differentiation state.

Studies in Hela cells following co-transfection of MyoD and Id1 have suggested that MyoD can modulate the rate of Id1 degradation (15). We have shown that MyoD and Id1 co-localize in the nucleus of myoblasts (Figure 1B), suggesting there is possible interaction between the pair. To determine if the relative abundance of MyoD or Id1 influences degradation rate, we over-expressed MyoD or Id1 in myoblasts by transient transfection. Following the transient transfection of MyoD or Id1 to myoblasts, each was rapidly degraded (t1/2 ~ 0.9h for MyoD and t1/2 ~ 0.8h for Id1) at the same rate as was observed for endogenous MyoD or Id1 in C2C12 myoblasts (Figure 5). Furthermore, incubation with MG132...
greatly stabilized the protein’s degradation in each case. An HA tag was attached to the C-terminus of the Id1 protein to allow discrimination from the endogenous protein. Western blots using both anti-Id1 and anti-HA antibodies yielded identical half lives.

As no obvious alteration in protein stability was seen for MyoD or Id1 during myogenic differentiation, we compared their protein synthesis rates in myoblasts and myotubes. We took advantage of MG132, the proteasome inhibitor, and determined the rate of MyoD and Id1 accumulation. Under the experimental conditions in which MyoD and Id1 protein degradation is abolished, the relative rate of MyoD and Id1 protein accumulation correlates with the relative rates of protein synthesis in vivo. As seen in Figure 6, within 2hrs of incubation in MG132, the rate of accumulation of Id1 and MyoD are both substantially slower in myotubes than in myoblasts, suggesting that the rate of Id1 (~4 fold) and MyoD (~3.5 fold) protein synthesis is more rapid in myoblasts than in myotubes. To determine if the down-regulation of the protein synthesis rates is general phenomenon of myogenic differentiation, similar studies were also performed on a variety of other proteins. For several, we observed no decrease in protein synthesis rate in myotubes versus myoblasts. For example, the relative synthesis rate of cdk4 was identical in myoblasts and myotubes (Figure 6C). Cdk4 is degraded via the ubiquitin-proteasome system, as incubation of C2C12 cells with MG132 markedly stabilized cdk4 (data not shown).

To gain further insights into the mechanism responsible for the observed down-regulation of protein synthesis rate of MyoD and Id1, we also compared their mRNA levels before and after differentiation (Figure 6D). Semi-quantitative RT-PCR results revealed a marked down-regulation of Id1 mRNA level (~3.5 fold) from myoblasts to myotubes, while no significant reduction of MyoD mRNA level was detected. Therefore, for Id1, the down-regulation of its transcript level from myoblasts to myotubes correlates well with the reduction in relative protein synthesis rate, suggesting that there is transcriptional regulation for Id1 expression during myogenic differentiation. In the case of MyoD, the difference in the relative protein synthesis rate in myoblasts and myotubes does not appear to be due to a difference in transcript level.

**DISCUSSION**

Muscle differentiation is a process involving profound morphological and biological changes. Previous studies have shown that ubiquitin-proteasome-mediated protein degradation can regulate protein abundance by affecting protein stability during muscle differentiation. For example, during myogenic differentiation of C2C12 cells, the stability of extracellular signal-regulated kinase 3 (ERK3) increases with time, resulting in a marked up-regulation of the protein (30). Enhanced degradation of 6-phosphofructo-2-kinase is responsible for its down-regulation during myogenic differentiation (31). MyoD, a key regulator of muscle differentiation, and its binding partner Id1 both decrease dramatically during muscle differentiation, although the time courses are substantially different (Figure 2). In addition, both MyoD and Id1 are degraded by the ubiquitin-proteasome system. We show here that the rates of ubiquitin-proteasome-mediated MyoD and Id1 degradation appear to be independent of myogenic differentiation state. Thus, the reduction in MyoD and Id1 protein levels during myogenic differentiation more likely results from a change in the rate of protein synthesis rather than in protein degradation.

We show that the rate of ubiquitin-proteasome-mediated degradation of MyoD is not affected by the differentiation state in vivo. As seen in Figures 3 and 4, endogenous MyoD from both myoblasts and myotubes exists in both hyperphosphorylated and hypophosphorylated forms (both bands were confirmed by control blocking experiments using the peptides that were initially used for the generation of the MyoD antibody, data not shown). As mentioned earlier, only the hyperphosphorylated upper band was used for calculation of the MyoD half life reported here. The reasons for this determination were two-fold. First, previous studies have shown that phosphorylation is required for MyoD degradation (28), consistent with our results herein showing that while the top hyperphosphorylated MyoD is degraded over time, the bottom hypophosphorylated MyoD band is more stable.
regardless of experimental conditions (t1/2 ≥ 10h, plot not shown). Second, we found that different MyoD antibodies have different affinities towards the two MyoD forms depending on their phosphorylation state. Thus the two MyoD bands differ greatly in intensity depending upon the details of the Western procedure (e.g. primary antibody incubation time). For example, one antibody predominantly recognized the hypophosphorylated MyoD species and thus resulted in an apparent longer half life for MyoD, thus overestimating the true degradation rate which results from the degradation of the hyperphosphorylated MyoD form. This issue is of importance in comparison of half lives during muscle differentiation, should the relative abundance of the two species change during muscle differentiation. An analysis of the relative distribution of the two MyoD species with all lysates from the various differentiation states were obtained at the same time and analyzed on the same Western (Figure 2 and data not shown). Indeed, we find that the fraction of hypophosphorylated MyoD increases from D-1 to D1, reaches its peak between D1 and D2 (i.e. the early stage of the differentiation), then returns to its initial value (i.e. D-1), at which it remains thereafter.

Although this half life calculation does not include the apparently stabilized hypophosphorylated MyoD form, it does imply that this species of MyoD is important in MyoD biology. Previous studies of MyoD degradation following transfection into Hela cells have shown that hypophosphorylated MyoD is sequestered to be phosphorylated and degraded over time (14). Largely for this reason, previous determinations of MyoD half life used both species in the calculation. Since MyoD engages in a network of transcriptional activities in C2C12 cells as well as in other cells of the muscle lineage, one potential explanation for the inaccessibility of the hypophosphorylated MyoD species to phosphorylation and subsequent ubiquitin-proteasome-mediated degradation is that it is actively engaged in the transcription process, possibly associated with DNA or other binding partners. Consistent with this notion, it has been shown that formation of MyoD-ubiquitin conjugates is inhibited by the specific DNA sequence to which MyoD binds; conjugation and degradation of a MyoD mutant protein which lacks the DNA-binding domain are not inhibited (19). Moreover, substitution of serine 200 in MyoD to a non-phosphorylatable alanine abolished the slower-migrating hyperphosphorylated form of MyoD; this mutant also significantly enhances both the muscle gene-specific transcriptional activity of MyoD and the ability of MyoD to induce myogenic conversion of non-muscle cells (32). These results suggest that hypophosphorylated MyoD is more likely to be the transcriptionally active species. Taken together, our results with endogenous MyoD degradation in C2C12 cells suggest that although MyoD, following phosphorylation, is degraded at the same rate during myogenic differentiation, the ubiquitin-proteasome system contributes to the regulation of MyoD cellular abundance via interactions with phosphorylation, DNA-binding and possibly other MyoD post-translational modifications. Our results also imply that the ubiquitin-proteasome-mediated MyoD degradation, and its independence of the muscle differentiation state, is important for muscle differentiation. As such, MyoD may be a selective target molecule for altering the differentiation under abnormal or pathological conditions (23,24,26,27).

We further show that Id1 co-localizes with MyoD to the nucleus in myoblasts, whereas in myotubes, Id1 exclusively localizes to the cytoplasm. Id1, as a 14 kDa monomer, is small enough to diffuse through nuclear pores freely (33). Its co-localization within the nucleus with MyoD strongly suggests they interact with one another in vivo, consistent with MyoD’s ability to chaperon Id1 to the nucleus (15). This result is also similar to the observation that Id3 translocates from the cytoplasm to the nucleus following co-transfection with E47 in COS cells (34). Therein the E protein regulates the available pool of its own inhibitory partner. A similar mechanism is likely responsible for Id1’s localization to the cytoplasm in myotubes, wherein, Id1 likely associates with other higher affinity binding partners; thus its retention in the cytoplasm of myotubes, and sequestration from MyoD or other MRFs which drive muscle differentiation. For example, Id1 may interact with E proteins, which have a higher affinity than MyoD for Id1. Other possibilities certainly exist. These results thus
suggest that, in addition to the regulation of its cellular abundance, Id1 activity during muscle differentiation may also be regulated by nuclear translocation of Id1 via binding partner-mediated chaperone mechanisms. The implications of this mechanism are broad, as ubiquitously expressed Id proteins have important roles in regulation of tissue-specific bHLH transcription factors in differentiation of multiple cell lineages and developmental programs.

The possible involvement of Id1 with other binding partners makes the interpretation of Id1 half life more complex, since dimerization can modulate Id protein half life, as demonstrated previously following co-transfection of Id1 and MyoD to Hela cells, and co-transfection of Id3 and E47 to COS cells (34). In both cases, Id protein is stabilized by its binding partner. As Id1 is one species of the multi-protein regulatory network in muscle differentiation, it is exceedingly difficult to determine all of the factors which could contribute to its half life observed here. Further, by modulating Id1 stability, it is possible that ubiquitin-proteasome-mediated degradation also regulates Id1 protein level during muscle differentiation.

Regulation of MyoD and Id1 protein abundance is essential for muscle differentiation, as down-regulation of Id1 protein, together with its intracellular translocation, provides the driving force for the initiation of MyoD transcriptional activity. We show that during differentiation from myoblast to myotube the down-regulation of Id1 protein, and that of MyoD protein as well, is due predominantly to a reduction of their protein synthesis rates rather than a change of their protein degradation rates. Reduction in the protein synthesis rate is not a general feature of all myoblast proteins during differentiation, as no difference was observed, for example, for cdk4 protein synthesis rate. For Id1, its protein synthesis rate reduction from myoblast to myotube correlates well with the reduction of its mRNA abundance, suggesting that Id1 is regulated transcriptionally during muscle differentiation. For MyoD, however, the lack of significant change in its mRNA abundance suggests that mechanisms other than transcriptional regulation govern the changes observed. Overall, our results suggest that protein abundance is highly regulated by the combination of protein synthesis and degradation during muscle differentiation, with different mechanisms or combinations thereof governing the regulation of specific protein species.

During muscle differentiation, MyoD and Id1 expression is tightly coupled to that of the E proteins (E12 and E47) (11,12,35,36), which are also degraded by the ubiquitin-proteasome system (37,38). In contrast to the well-documented reduction of MyoD and Id1 protein levels during differentiation, the regulation of E protein levels is unclear. Future studies should determine whether E12 and E47 are able to regulate the rates of degradation of MyoD, Id1, or one another. Thus, ubiquitin-proteasome-mediated E protein degradation may play a role in muscle differentiation.

REFERENCES


FIGURE LEGENDS
Figure 1. C2C12 cell differentiation and localization of MyoD and Id1 in myoblasts and myotubes. A, Phase contrast microscopic images of the C2C12 cells: (a) proliferating myoblasts, (b) myoblasts grown to confluency, and (c) differentiated to myotubes by day 6 in differentiation media; and double staining of C2C12 cells with anti-myosin heavy chain and DAPI: (d) myoblasts, and (e) myotubes. B, C2C12 myoblasts (f, MyoD; g, Id1; h, overlay) and myotubes (i, MyoD; j, Id1; k, overlay) were fixed and localization of endogenous MyoD and Id1 were determined with immunofluorescent staining and confocal laser scanning microscopy.

Figure 2. Cellular abundance of MyoD, Id1, myogenin and myosin heavy chain during C2C12 myogenic differentiation. Proliferating C2C12 myoblasts at 70-80% confluency (D-1) in GM were grown to reach confluency, switched to DM (D0), and then maintained in DM for up to 6 days (D1 to D6) with fresh media changed every 24 hours. Cells were harvested, lysed and were evaluated via SDS-PAGE and Western blot for MyoD, Id1, myogenin, and myosin heavy chain. Equal amounts of total protein were loaded for each lysate. The pixels for each band were measured and normalized so that the number of pixels at D0 was 100. The number of pixels for each band were plotted versus time to show the change of MyoD and Id1 protein abundance during the course of C2C12 myogenic differentiation.

Figure 3. Half life of endogenous MyoD and Id1 in C2C12 myoblasts and myotubes. C2C12 myoblasts or myotubes (D6) were treated with cycloheximide (CHX), or CHX plus MG132. Cells were harvested, lysed at 0, 0.5, 1, 2, and 3h and were evaluated via SDS-PAGE and Western blot for MyoD and Id1. An equal volume of each lysate was loaded onto the gel. 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 and the t1/2 was calculated from the log10 of 50%.

Figure 4. Half life of endogenous MyoD and Id1 at D-1, D0, D2, D4, and D6 during C2C12 myogenic differentiation. C2C12 cells at D-1, D0, D2, D4 and D6 were treated with CHX. Protein half life analysis was performed as described in legend to figure 3.

Figure 5. Half life of exogenous MyoD and Id1 in C2C12 myoblasts. 18h after transfection with wild-type MyoD and Id1-HA, C2C12 myoblasts were treated with CHX, or CHX plus MG132. Protein half life analysis was performed as described in legend to figure 3.

Figure 6. Relative MyoD, Id1 and cdk4 synthesis rate and semi-quantitative RT-PCR of MyoD and Id1 in myoblasts and myotubes. For protein synthesis, C2C12 myoblasts (♦) and myotubes (D6) (■) were treated with MG132. Cells were lysed at 0, 1, 2, 3, and 4h and were evaluated via SDS-PAGE and Western blot for MyoD, Id1 or cdk4. An equal volume of each lysate was loaded onto the gel. The pixels for each band were measured and normalized so that the number of pixels at t=0 was 1. The pixels of each band were plotted versus time. For RT-PCR, total RNA samples were extracted from C2C12 myoblasts and myotubes. The pixels for each band were measured and plotted for comparison of MyoD and Id1 mRNA levels in myoblasts and myotubes. RT-PCR for GAPDH was also performed with the same samples as a control for the amount of reverse-transcribed cDNA present in the samples.
Figure 1A

myoblasts

myotubes

Myosin HC
DAPI
Figure 1B

C2C12 myoblasts

C2C12 myotubes

MyoD

Id1

overlay
Figure 2

myoblasts → myotubes

MyoD

Id1

myogenin

myosinHC

D-1  D0  D1  D2  D3  D4  D5  D6

time course (days in differentiation media)

relative protein abundance

MyoD

Id1

D-1  D0  D1  D2  D3  D4  D5  D6

time course (days in differentiation media)
Figure 3

A: MyoD

myoblasts

CHX+
MG132

CHX

T0 T0.5 T1 T2 T3

myotubes

CHX+
MG132

CHX

T0 T0.5 T1 T2 T3

log % initial

CHX+
MG132

t1/2 16 h

CHX

t1/2 0.9 h

log % initial

CHX+
MG132

t1/2 11 h

CHX

t1/2 0.8 h

B: Id1

myoblasts

CHX+
MG132

CHX

T0 T0.5 T1 T2 T3

myotubes

CHX+
MG132

CHX

T0 T0.5 T1 T2 T3

log % initial

CHX+
MG132

t1/2 15 h

CHX

t1/2 0.8h

log % initial

CHX+
MG132

t1/2 11 h

CHX

t1/2 0.8h
### Figure 4

#### A: MyoD

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<td>![image]</td>
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<tr>
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#### B: Id1

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#### Time Course

![Graph for MyoD](image)

![Graph for Id1](image)
Figure 5

A: MyoD

B: Id1
Figure 6

A: Id1

B: MyoD

C: cdk4

D: RT-PCR
Ubiquitin-proteasome-mediated degradation, intracellular localization, and protein synthesis of MyoD and Id1 during muscle differentiation
Liping Sun, Julie S. Trausch-Azar, Aaron Ciechanover and Alan L. Schwartz

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