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A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein

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The ubiquitin proteolytic pathway is a major system for selective protein degradation in eukaryotic cells. One of the first steps in the degradation of a protein via this pathway involves selective modification of ε-NH_2 groups of internal lysine residues by ubiquitination. To date, this amino group has been the only known target for ubiquitination. Here we report that the N-terminal residue of MyoD is sufficient and necessary for promotion of conjugation and subsequent degradation of the protein. Substitution of all lysine residues in the protein did not affect significantly its conjugation and degradation either *in vivo* or *in vitro*. In cells, degradation of the lysine-less protein is inhibited by the proteasome inhibitors MG132 and lactacystin. Inhibition is accompanied by accumulation of high molecular mass ubiquitinated forms of the modified MyoD. In striking contrast, wild-type MyoD, in which all the internal Lys residues have been retained but the N-terminus has been extended by fusion of a short peptide, is stable both *in vivo* and *in vitro*. In a cell-free system, ATP and multiple ubiquitination are essential for degradation of the lysine-less protein. Specific chemical modifications have yielded similar results. Selective blocking of the α-NH_2 group of wild-type protein renders it stable, while modification of the internal Lys residues with preservation of the free N-terminal group left the protein susceptible to degradation. Our data suggest that conjugation of MyoD occurs via a novel modification involving attachment of ubiquitin to the N-terminal residue. The polyubiquitin chain is then synthesized on an internal Lys residue of the linearly attached first ubiquitin moiety.

Keywords: MyoD/N-terminus/proteolysis/ubiquitin

Introduction

Ubiquitination of many proteins plays important roles in basic cellular processes. In most cases, the modification signals proteins for degradation by the 26S proteasome. Formation of ubiquitin conjugates of a specific protein requires the sequential action of three enzymes: the ubiquitin-activating enzyme, E1, one of several ubiquitin-carrier proteins (or ubiquitin-conjugating enzymes), E2s (or UBCs), and a member of the ubiquitin-protein ligase family, E3. E3s play an essential role in specific substrate recognition (Coux *et al.*, 1996; Hochstrasser, 1996; Hershko and Ciechanover, 1998). The ubiquitin pathway is involved in processing and proteolysis of many cellular proteins including, for example, mitotic and G_1 cyclins and their regulators, oncoproteins and tumor suppressors, transcriptional activators, cell surface receptors and ER membrane proteins, and MHC class I-restricted antigens. It also removes in a selective manner abnormal and mutated proteins.

Proteins destined for degradation by the 26S proteasome are commonly modified by a multi-ubiquitin chain anchored to an internal ε-NH_2 group of one or more lysine residues. As for specific recognition, the N-terminal domain may play a role in the targeting of a few proteins: in certain rare cases, the stability of a protein is a function of its N-terminal residue, which serves as a binding site for the E3 ('N-end-rule'; Bachmair *et al.*, 1986; reviewed in Varshavsky, 1996; Hershko and Ciechanover, 1998). For the Mos protein, it was found that its stability is governed primarily by the penultimate Pro residue and by a phosphorylation/dephosphorylation cycle of Ser^3 ('second codon rule'; Nishizawa *et al.*, 1993). One interesting case involves the artificial fusion protein ubiquitin–Pro-β-galactosidase. In this chimera, the ubiquitin moiety has been fused to the N-terminal Pro residue of the protein. Unlike other ubiquitin–X-β-galactosidase species (where X is any of the remaining 19 amino acid residues), here ubiquitin is not removed by isopeptidases and serves as a degradation signal following generation of a polyubiquitin chain that is anchored to Lys^{48} of the fused ubiquitin moiety (Johnson *et al.*, 1992). However, in this case the attachment of the ubiquitin moiety to the N-terminal residue is not the result of a natural, cellular enzyme-catalyzed, modification.

There is no consensus as to the specificity of the internal Lys residues that are targeted by ubiquitin. In some cases distinct lysines are required, while in others there is little or no specificity. Signal-induced degradation of IκBα involves two particular Lys residues, 21 and 22 (Scherer *et al.*, 1995). In the case of Gcn4, lysine residues in the vicinity of a specific PEST degradation signal serve as ubiquitin attachment sites (Kornitzer *et al.*, 1994). Mapping of ubiquitination sites of the yeast iso-2-cytochrome *c* has revealed that the polyubiquitin chain is synthesized almost exclusively on a single lysine (Sokolik and Cohen, 1991). In two other examples, the proto-oncogene product Mos (Nishizawa *et al.*, 1993) and the 'N-end rule' substrate X-β-gal (where X is a short fused peptide not encoded by the native molecule; Chau *et al.*, 1989), one and two lysines, respectively, that reside in proximity to the degradation signal, are required for

ubiquitination. In striking contrast, ubiquitination of the ζ chain of the T cell receptor is independent of any particular Lys residue and proceeds as long as one residue is present in the cytosolic tail of the molecule (Hou *et al.*, 1994). Similarly, no single specific lysine residue is required for ubiquitination of c-Jun (Treier *et al.*, 1994).

The basic helix–loop–helix (bHLH) protein MyoD is a tissue-specific transcriptional activator that acts as a master switch for muscle development. It interacts with a consensus binding sequence in the enhancer and promoter regions of many genes in the differentiating muscle cell. Expression of the MyoD protein in a large number of cells is sufficient to initiate the myogenic program and to convert these cells into skeletal myoblasts (Davis *et al.*, 1987; Weintraub *et al.*, 1991). Additional members of the muscle-specific transcription factors include myogenin, Myf5 and MRF4 (Weintraub, 1993; Olson and Klein, 1994). MyoD forms heterodimers with other proteins belonging to the bHLH group, such as the ubiquitinously expressed E2A, E12 and E47 (Murre *et al.*, 1989). These dimers are probably the transcriptionally active forms of the factor. Association of MyoD with HLH proteins of the Id family (inhibitors of differentiation that lack the basic domain) inhibits its DNA binding and biological activities (Benezra *et al.*, 1990; Sun *et al.*, 1991).

MyoD is a short-lived protein with a half-life of ~45 min (Thayer *et al.*, 1989; Abu-Hatoum *et al.*, 1998). Degradation of MyoD is mediated by the ubiquitin system both *in vitro* and *in vivo*. Furthermore, the process is regulated by its consensus DNA binding site. Addition of Id1 destabilizes the MyoD–E47–DNA complex and renders the protein susceptible to degradation (Abu Hatoum *et al.*, 1998).

Here we show that degradation of MyoD by the ubiquitin system involves a novel mechanism. The NH_2 group of the N-terminal residue of the protein, rather then internal lysine(s), serves as an essential and sufficient conjugation site necessary for subsequent degradation of the protein.

Results

A lysine-less MyoD is conjugated and degraded in an ATP- and ubiquitin-dependent manner in vitro

We have shown that the rapid turnover of MyoD is mediated by the ubiquitin–proteolytic system *in vitro* and *in vivo* (Abu Hatoum *et al.*, 1998). To analyze specific ubiquitination sites, we used site-directed mutagenesis to substitute systematically all the lysine residues with arginines. MyoD contains nine lysine residues, most of them located within the N-terminal domain of the molecule. The nine residues are in positions 58, 99, 102, 104, 112, 124, 133, 146 and 241. The various proteins were generated either by expression in bacteria followed by subsequent purification, or by *in vitro* translation in a eukaryotic system using rabbit reticulocyte lysate and [^{35}S]methionine. Degradation of the proteins was monitored in a reconstituted cell-free system containing, in most cases, ubiquitin-supplemented Fraction II and ATP. Proteins were detected by Western blot analysis or PhosphorImaging. Figure 1A shows that progressive substitution of lysine residues (lanes 3–6) does not affect the efficiency of degradation of a bacterially expressed protein (compare with lanes 1 and 2). Surprisingly, even a MyoD species that lacks all Lys residues is still degraded efficiently in an ATP-dependent manner (lanes 7 and 8). Similar results were obtained using a lysine-less protein translated *in vitro* in a eukaryotic system (Figure 1B). To demonstrate involvement of the ubiquitin system in the process, we followed the degradation of wild-type (wt) and lysine-less MyoD in the absence and presence of ubiquitin (Figure 1C and D). Like the degradation of the wt protein (Figure 1C), degradation of the lysine-less MyoD is completely dependent upon the addition of ubiquitin (compare lanes 2 and 5). Furthermore, addition of methylated ubiquitin that cannot form polyubiquitin chains and serves as a chain terminator (Hershko and Heller, 1985), inhibits degradation of lysine-less MyoD. The inhibition can be alleviated by the addition of an excess of free ubiquitin (Figure 1C and 1D, compare lanes 3 and 4). The data strongly suggest that polyubiquitination of lysine-less MyoD is necessary for degradation of the protein. Furthermore, they imply that the modification occurs on internal Lys residues of ubiquitin. To demonstrate directly polyubiquitinated lysine-less MyoD, we used *in-vitro*-translated ^{35}S-labeled protein in a partially reconstituted system. As can be seen in Figure 2, lysine-less MyoD generates high-molecular-mass ubiquitinated adducts. It should be noted that these conjugates are of somewhat lower molecular mass than those of the wt MyoD. This can be attributed to the role that the internal lysine residues also play in the process (see also below).

Ubiquitin-mediated degradation of MyoD in vivo can proceed efficiently in the absence of internal lysine residues

To investigate the physiological relevance of the observations in the cell-free system, we followed the fate of the different MyoD lysine mutated proteins *in vivo*. Figure 3 shows a series of pulse–chase experiments in COS-7 cells transiently transfected with the different MyoD cDNAs, in which we examined the stability of the different proteins. In agreement with our *in vitro* data, the lysine-less MyoD protein is degraded efficiently. However, we could observe a progressive increase in the half-life of the proteins of up to ~2-fold with the gradual substitution of the lysine residues (Figure 3A and B). While the half-life of wt MyoD was ~50 min, that of lysine-less MyoD was ~2 h (Figure 4 A and B). Interestingly, we found that the stability of MyoD is not affected by any specific lysine residue, and it is the total number of these residues that determines the half-life of the protein (data not shown). To identify the system involved in the proteolysis of lysine-less MyoD *in vivo*, transfected cells were incubated in the presence of inhibitors of the proteasome and of lysosomal degradation. Chloroquine, a general inhibitor of lysosomal proteolysis, and E-64, a cysteine protease inhibitor that affects lysosomal, and also certain cytosolic, proteases, had no effect on the stability of the lysine-less MyoD (Figure 5A, lanes 8–11, and Figure 5B). In striking contrast, the proteasomal inhibitors clasto-lactacystin β-lactone and MG132 block degradation of the lysine-less protein significantly (Figure 5A, lanes 4–7, and Figure 5B).

To demonstrate the intermediacy of ubiquitin conjugates in the degradation of lysine-less MyoD, we incubated COS-7 cells, transiently transfected with either wt or

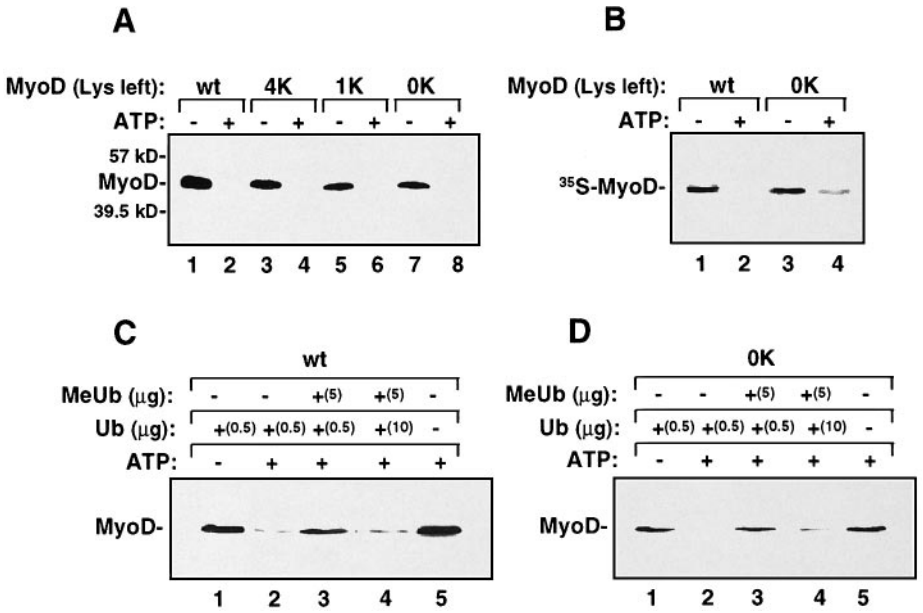

Fig. 1. ATP- and ubiquitin-dependent degradation of wt and mutated MyoD proteins in a cell-free reconstituted system. (**A**) Western blot analysis of ATP-dependent degradation of bacterially expressed purified MyoD. Lanes 1 and 2, wt MyoD (wt); lanes 3 and 4, a MyoD protein in which the last four C-terminal lysines were left (4K); lanes 5 and 6, a MyoD protein in which only the last C-terminal lysine was left (1K); and lanes 7 and 8, lysine-less MyoD (0K). (**B**) ATP-dependent degradation of ^{35}S-labeled wt (lanes 1 and 2) and lysine-less (lanes 3 and 4) MyoD. (**C** and **D**) Polyubiquitination is required for the degradation of wt (wt; C) and lysine-less (0K; D) MyoD. Ubiquitin and methylated ubiquitin (MeUb) were added at the indicated concentrations. Reactions were carried out in the presence of ubiquitin and Fraction II in the absence or presence of ATP as indicated and as described in Materials and methods. Detection of the proteins was carried out using Western blot analysis (A, C and D) or PhosphorImager analysis (B). Ub denotes ubiquitin and MeUb denotes methylated ubiquitin. ^{35}S-MyoD denotes ^{35}S-labeled MyoD.

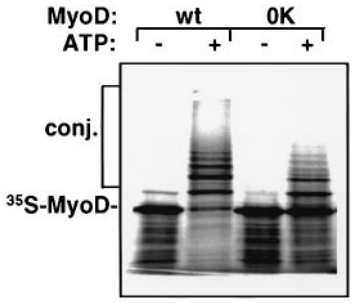

Fig. 2. *In vitro* conjugation of wt and lysine-less (0K) MyoD. ^{35}S-labeled proteins translated in reticulocyte lysate *in vitro* were incubated in a cell-free reconstituted system containing ubiquitin, ubiquitin aldehyde and Fraction II as described in Materials and methods. Notes are as described in the legend to Figure 1. The weak band seen above the main protein band of translated MyoD is, most probably, the phosphorylated form of the protein left over from the biosynthetic step. We do not see this band in the bacterially expressed protein, as the appropriate kinase is not expressed in *E.coli*. This band collapses following treatment with alkaline phosphatase (Abu Hatoum *et al.*, 1998).

lysine-less MyoD cDNAs, with MG132, and followed generation of ubiquitin–MyoD adducts. As can be seen in Figure 6A and B, immunoprecipitation with anti-MyoD antibody followed by Western blot analysis with anti-ubiquitin antibody reveals accumulation of high-molecular-mass compounds in cells transfected with either wt (A) or lysine-less (B) MyoD. Re-probing of the stripped nitrocellulose membrane with anti-MyoD antibody reveals a similar pattern of conjugates of the lysine-less protein (Figure 6C, lane 3). Similar analyses of mock-transfected cells (Figure 6, lanes 1 in all panels) clearly demonstrate the specificity of both the anti-MyoD and anti-ubiquitin antibodies.

Is a free NH_2 terminus of MyoD targeted by ubiquitin and essential for degradation of the protein?

Based on the results described above, it was clear that polyubiquitination is essential for targeting MyoD for degradation. The lack of internal Lys residues, the only known targets for ubiquitin modification, made it important to identify the functional group that can serve as an attachment site for ubiquitin. Chemically, several groups can generate covalent bonds with ubiquitin. Ser and Thr can participate in ester bond formation, while Cys can generate a thiol ester bond. However, these bonds are unstable. The stability of the adducts makes it highly unlikely that any of these modifications is the one we observe. A likely candidate is the free amino group of the N-terminal residue of the protein which can generate a stable peptide bond with the C-terminal Gly residue of ubiquitin. Edman degradation of the N-terminal residue has demonstrated that the N-terminal residue of bacterially expressed MyoD as well as that of the *in-vitro*-translated and the cellularly expressed proteins is the free, unmodified initiator Met. The proteins are not modified and the N-terminal residue is not acetylated (see also Discussion). To demonstrate a role for the free N-terminal amino group in the degradation of MyoD, we chemically modified this group. Initially, we blocked this group in the lysine-less MyoD protein by reductive methylation (see Materials and methods). As can be seen in Figure 7A (lanes 7–9), blocking of the α-NH_2 group, which is the only free amino group left in the molecule, stabilizes the protein completely (compare with lanes 4–6).

Whereas in the lysine-less MyoD, a free α-NH_2 group appears to be sufficient for ubiquitination and subsequent degradation, it is not clear whether it also plays a physio-

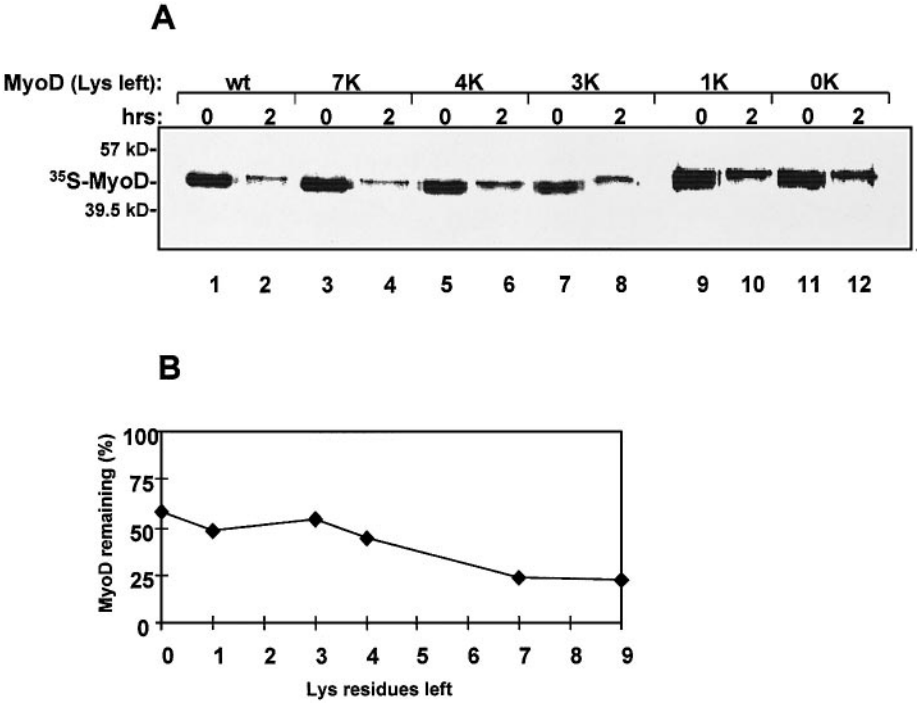

Fig. 3. *In vivo* degradation of MyoD-mutated proteins in which the lysine residues were progressively substituted with Arg. (**A**) COS-7 cells, transiently transfected with MyoD cDNA, were pulse labeled (1 h) with [^{35}S]methionine and chased (2 h) as described in Materials and methods. Labeled MyoD was immunoprecipitated from aliquots containing equal amounts of labeled proteins as described in Materials and methods. Lanes 1 and 2, wt MyoD (wt); lanes 3 and 4, a MyoD protein in which the last seven C-terminal lysines were left (7K); lanes 5 and 6, a MyoD protein in which the last four lysines were left (4K); lanes 7 and 8, a MyoD protein in which the last three lysines were left (3K); lanes 9 and 10, same as before, but with only the last C-terminal lysine left (1K); lanes 11 and 12, lysine-less MyoD protein (0K). (**B**) Quantitative (PhosphorImaging) analysis of the degradation data of the different MyoD proteins following a chase period of 2 h. The number of remaining Lys residues in the different MyoD proteins was plotted against the amount of residual protein following the chase period.

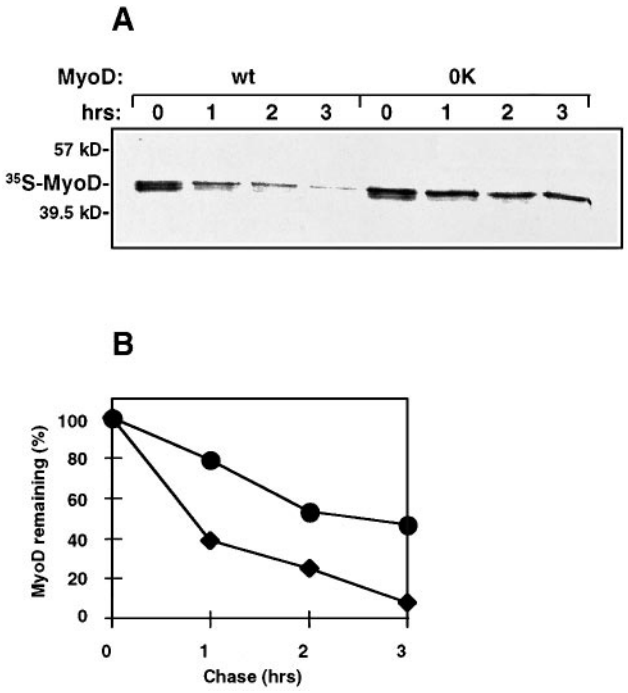

Fig. 4. Time course of degradation of wt and lysine-less MyoD *in vivo*. (**A**) The half-lives of wt (wt) and lysine-less (0K) MyoDs in COS-7 cells were determined in a pulse–chase labeling experiment as described in Materials and methods and in the legend to Figure 3. (**B**) Quantitative analysis of the data depicted in (A). Filled diamonds denote wt and filled circles denote lysine-less MyoD.

logical role in targeting the wt molecule, which has nine available lysine residues. In order to investigate the role and biological relevance of the free α-NH_2 group in the targeting of wt MyoD, we selectively blocked it by carbamylation with potassium cyanate at low pH. This procedure does not modify ε-NH_2 groups of internal lysine residues (Hershko *et al.*, 1984). Automated Edman degradation along with fluorescamine determination of the extent of remaining free NH_2 groups confirmed accurately that the modification affected only the N-terminal group (see Materials and methods). The modified protein was subjected to *in vitro* degradation and conjugation assays in fractionated reticulocyte lysate. In contrast to lysine-less MyoD, the N-terminally carbamylated protein is stable (compare Figure 7A, lanes 10–12, with lanes 1–6 and 16–18), and cannot be ubiquitinated (compare Figure 7B, lanes 5–6 with lanes 1–4). Thus, a free and exposed NH_2 terminus of MyoD appears to be an essential site for degradation, most probably because it serves as an attachment site for the first ubiquitin moiety. As an additional control, we selectively modified the internal Lys residues of wt MyoD by guanidination with *O*-methylisourea. The modification, which does not affect the N-terminal group (see Materials and methods), generates a protein that is essentially the chemically modified counterpart of the lysine-less MyoD that was generated by site-directed mutagenesis. As can be seen in Figure 7A (lanes 13–15), and similar to the lysine-less protein (Figure 7A, lanes 4–6 and 16–18), this MyoD protein is degraded efficiently in the cell-free system in a ubiquitin- and an ATP-dependent mode.

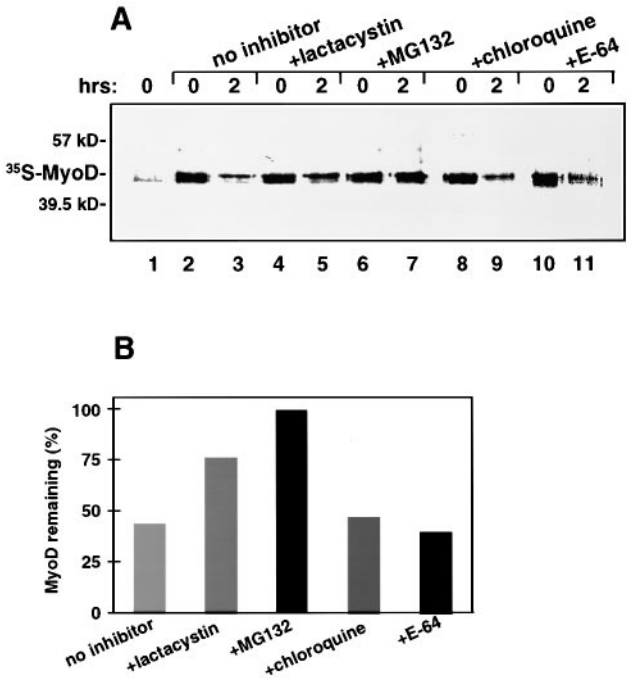

Fig. 5. Sensitivity of lysine-less MyoD (0K) degradation to different inhibitors. (**A**) Degradation of lysine-less MyoD in COS-7 cells was monitored in a pulse–chase experiment in the absence (lanes 2 and 3) and presence of the proteasome inhibitors clasto-lactacystin β-lactone (lanes 4 and 5) and MG132 (lanes 6 and 7), the general inhibitor of lysosomal proteolysis chloroquine (lanes 8 and 9) and the lysosomal cysteine protease inhibitor E-64 (lanes 10 and 11). Cell lysis and immunoprecipitation were carried out as described in Materials and methods and in the legends to Figures 3 and 4. Lane 1 presents mock-transfected COS-7 cells. ^{35}S-MyoD denotes immunoprecipitated and ^{35}S-labeled MyoD protein. (**B**) Quantitative analysis of the data depicted in (A) after a chase period of 2 h in the absence and presence of the different inhibitors. Quantities are relative to the amount of protein at time 0.

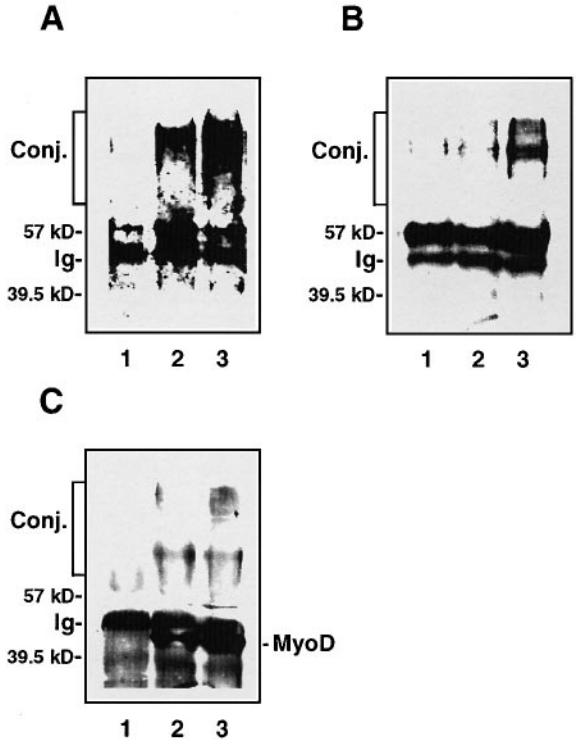

Fig. 6. Detection of ubiquitin–MyoD conjugates in COS-7 cells. COS-7 cells were transiently transfected with 10 μg of expression vector containing either wt (A) or lysine-less MyoD (B and C) cDNA. Following 48 h of transfection, cells were incubated for additional 2 h in the presence or absence of MG132. Following lysis, equal amounts of protein, as determined by the Bradford (1976) method, were subjected to immunoprecipitation with anti-MyoD antibody and ubiquitin conjugates were identified using Western blot analysis and anti-ubiquitin antibody (A and B) or anti-MyoD antibody (C) as described in Materials and methods. (**A**) wt MyoD was precipitated with a specific antibody and conjugates were identified on the blotted nitrocellulose membrane using anti-ubiquitin antibody. Lane 1: mock-transfected cells. The experiment was performed in the presence of MG132. Lane 2: cells transfected with wt MyoD; the protein was detected in the absence of MG132. Lane 3: same for lane 2, except that the experiment was performed in the presence of MG132. (**B**) Conjugates of lysine-less MyoD. The experiment was performed, reaction mixtures resolved and data presented in an identical manner as in (A). (**C**) Conjugates of lysine-less MyoD as detected with anti-MyoD antibody. Following stripping of the nitrocellulose membrane (B), proteins were re-detected with anti-MyoD antibody. Details are as described for (B). Conj. denotes conjugates and Ig indicates the heavy chain of the Ig molecule. The band detected at 57 kDa (A and B) has not been identified.

Proteins with acidic NH_2-terminal residues are degraded by the ubiquitin system only following conversion of the acidic residue to a basic residue by the addition of an arginine moiety. The reaction utilizes charged $tRNA^{Arg}$ for arginylation, and destruction of the tRNA by ribonuclease treatment inhibits degradation of acidic N-termini proteins (Ferber and Ciechanover, 1987). While the N-terminal residue of MyoD is Met and not Asp, Glu, Asn or Gln, and an N-terminal Lys transferase (unlike Arg transferase) has not been described, a formal possibility still existed that the N-terminal residue is modified by lysine, that is then targeted via modification of its ε-NH_2 group. While such 'lysinylation' would have been interesting in itself, we ruled it out by showing that RNase A does not affect the degradation of either the wt or the lysine-less MyoD (Figure 7C, lanes 1–8).

As mentioned above, sequencing of the N-terminal residue of the bacterially expressed, the *in-vitro* translated, and the metabolically labeled, immunoprecipitated, eukaryotic-cell-expressed MyoDs revealed that in all three cases the N-terminal initiator is Met. According to the N-end rule, methionine is a 'stabilizing' residue. Furthermore, while according to the 'N-end rule', recognition of the protein is mediated via binding of its N-terminal residue to the E3 enzyme while ubiquitination occurs on internal Lys residues, here we demonstrated clearly that the N-terminal group of MyoD is targeted by ubiquitin. Thus, for these two reasons it is clear that targeting of MyoD cannot possibly traverse the 'N-end rule' pathway. To rule out the even more remote possibility that the initiator Met is removed and a 'destabilizing' basic or acidic residue is exposed that targets the protein for 'N-end rule'-mediated conjugation, we tested the effect of two 'N-end rule' inhibitory peptides, Arg-Ala and Phe-Ala (Reiss *et al.*, 1988) on the degradation of MyoD *in vitro*. These peptides had no effect on the degradation of the wt and lysine-less proteins (Figure 7C, lanes 9–16).

As a preliminary analysis the role of the free N-terminal residue of MyoD in targeting of the protein *in vivo*, we added a myc tag to the N-terminal residue of the wt protein. As can be seen in Figure 8, the myc-tagged protein is stable both *in vitro* and *in vivo*. It should be

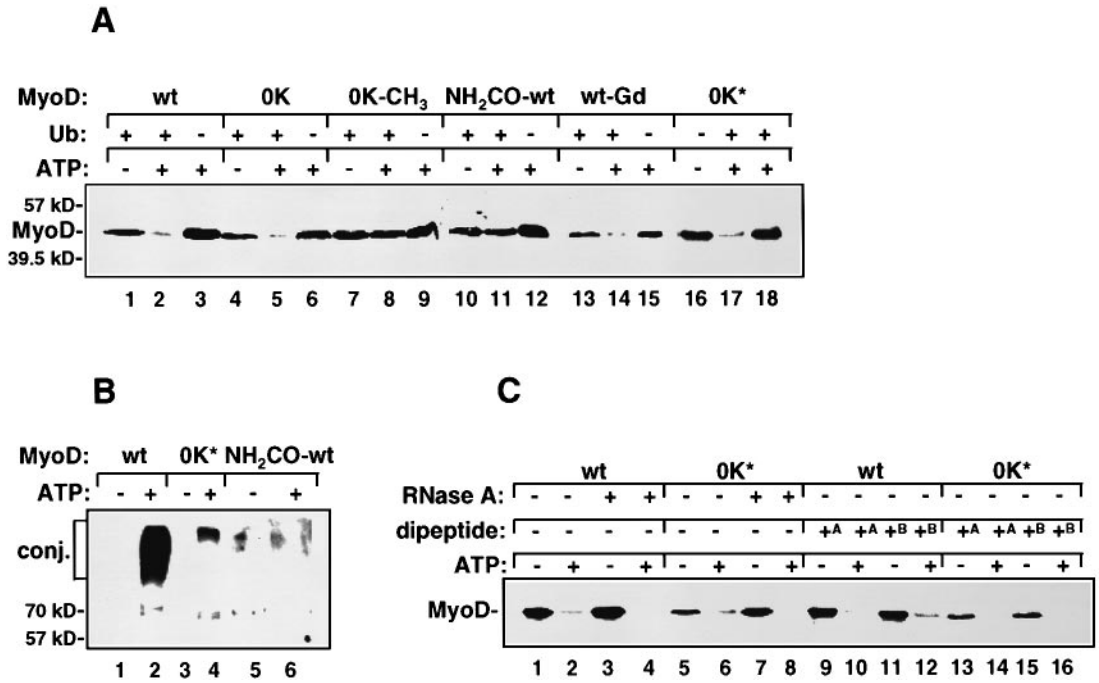

Fig. 7. Selective modification of the α-amino group of the initiator methionine in lysine-less and wt MyoD results in a stable protein that is not conjugated and degraded *in vitro*. (**A**) Ubiquitin-and ATP-dependent degradation of the various MyoD derivatives was monitored in a reconstituted cell-free system via Western blot analysis as described in Materials and methods. Lanes 1–6: ATP- and ubiquitin-dependent degradation of bacterially expressed wt (wt) and lysine-less (0K) MyoD. Lanes 7–9: the α-amino group of lysine-less MyoD was methylated (0K-CH_3). Lanes 10–12: the α-amino group of wt MyoD was selectively modified by carbamylation (NH_2CO-wt). Lanes 13–15: the ε-NH_2 groups of internal Lys residues of wt MyoD were selectively modified by guanidination (wt-Gd). Lanes 16–18: a lysine-less MyoD protein containing CGC (see Discussion) as a codon for Arg (0K*). (**B**) *In vitro* conjugation of wt (wt), lysine-less (0K*) and carbamylated (wt-NH_2CO) MyoDs. Equal amounts of bacterially expressed MyoD proteins were incubated in a cell-free reconstituted system containing ubiquitin, ubiquitin aldehyde and Fraction II as described in Materials and methods. MyoD proteins were immunoprecipitated with anti-MyoD antibody and ubiquitin conjugates were identified using Western blot analysis as described in Materials and methods. Lanes 1 and 2: wt MyoD (wt). Lanes 3 and 4: bacterially expressed, lysine-less MyoD protein containing CGC as codon for Arg (0K*). Lanes 5 and 6: carbamylated wt MyoD (wt-NH_2CO). Conj. denotes conjugates. (**C**) Sensitivity of degradation of wt and lysine-less MyoDs to RNase A and 'N-end rule' inhibitory peptides. Degradation of wt (wt) and lysine-less (0K*) MyoD was monitored in ubiquitin-supplemented Fraction II as described in Materials and methods. Ribonclease A (lanes 1–8) and one of the dipeptides Arg-Ala (+A; lanes 9, 10, 13 and 14) or Phe-Ala (+B; lanes 11, 12, 15 and 16) was added to the reaction mixtures as indicated and as described in Materials and methods.

noted that the two first N-terminal residues of the myc tag, Met and Glu, are identical to the first two N-terminal residues in MyoD. In addition, the myc tag contains also a Lys residue. Nevertheless, the myc tag cannot promote degradation of MyoD, and stabilizes it. Thus, it is clear that a specific sequence in the N-terminal domain of MyoD is essential for the preferential ubiquitination of the protein at the α-NH_2 group.

Discussion

The muscle-specific transcriptional activator MyoD is a short-lived protein that is degraded via the ubiquitin–proteasome pathway both *in vivo* and *in vitro*. Interestingly, the protein is specifically stabilized by its cognate DNA-binding sequence (Abu-Hatoum *et al.*, 1998). To dissect further the mechanisms involved in MyoD degradation, it was important to identify the ubiquitination site(s) and to determine its (or their) specificity. We progressively converted all the Lys residues of the molecule and substituted them with Arg. Surprisingly, a MyoD construct that lacks completely lysine residues is still degraded efficiently, both *in vivo* and *in vitro*, in a ubiquitin- and proteasome-dependent manner (Figures 1–7). In accordance with this result, a chemically modified protein in which all the internal lysines had been modified, was also degraded efficiently *in vitro* in a ubiquitin-dependent mode (Figure 7). We suspected that the protein is targeted via attachment of the first ubiquitin moiety to the free NH_2 terminal group of the initiator Met. To test this hypothesis, we specifically modified the N-terminus by carbamylation. Selective blocking of the α-NH_2 terminus of wt MyoD, as well as of the lysine-less protein, stabilized the two proteins (Figure 7). Likewise, fusion of a myc tag to the N-terminal residue also stabilized it (Figure 8). These findings strongly suggest that in the case of MyoD, the N-terminus is not just an additional ubiquitin acceptor site, but rather an essential targeting signal. It should be noted, however, that the internal Lys residues govern, to a certain extent, the stability of MyoD, though they are not essential. *In vitro*, some residual lysine-less protein still remains after 2 h of incubation (Figure 1B), and the ubiquitin adducts of the lysine-less protein do not attain the high molecular mass attained by their wt counterparts (Figure 2; see, however, Figures 6 and 7B). *In vivo*, the half-life of the lysine-less species is somewhat (~2-fold) longer than that of the wt protein (Figure 4). It is possible that ubiquitination at multiple site facilitates recognition by the proteasome, though it is not absolutely necessary for this process to occur. All MyoD constructs used in this study show physiological activity using a CAT assay

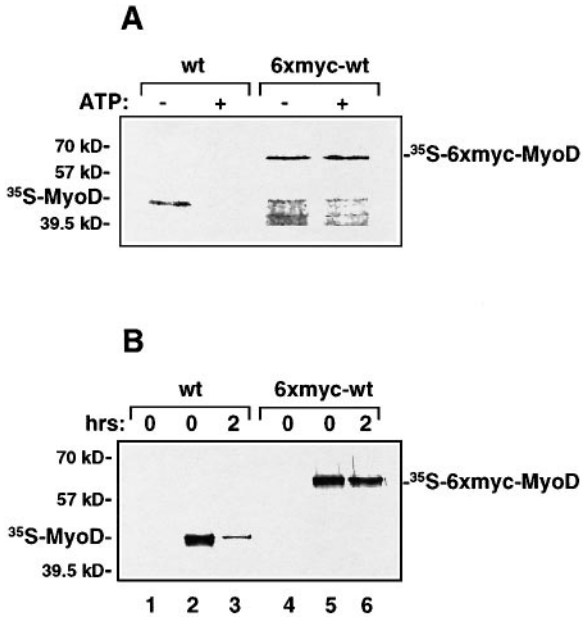

Fig. 8. *In vitro* and *in vivo* stability of wt MyoD with an N-terminal extension. (**A**) Degradation of ^{35}S-labeled wt MyoD (^{35}S-MyoD) and a wt MyoD with a six-myc tag fused to its N-terminal residue (^{35}S-6× myc–MyoD) was monitored in ubiquitin-supplemented Fraction II as described in Materials and methods. (**B**) Degradation of ^{35}S-labeled wt MyoD (^{35}S-MyoD; lanes 2 and 3) and N-terminally extended wt MyoD (^{35}S-6× myc–MyoD; lanes 5 and 6) was monitored in a pulse–chase experiment in transiently transfected COS-7 cells. Lanes 1 and 4: mock-transfected cells were treated identically to the MyoD-transfected cells. Cell lysis and immunoprecipitation were carried out as described under Materials and methods, and in the legends to Figures 3–5.

test (not shown). This finding suggests that the minor effects on stabilization of the altered proteins cannot be attributed to gross changes in their three-dimensional structure and are probably due to the role that the Lys residues play in modulating the stability.

All of our *in vivo* experiments were carried out in intact eukaryotic cells. Most of the cell-free assays used proteins that were translated in a eukaryotic system or expressed in bacteria. A certain concern was raised about the bacterially expressed lysine-less protein. For the transformation of wt MyoD into a complete lysine-less MyoD protein, we initially used AGG as a codon for Arg. AGA and AGG are rare codons in *Escherichia coli*, and misincorporation of Lys at AGA (but not at AGG) has been recently documented for bacterially expressed eukaryotic proteins (Calderone *et al.*, 1996; Forman *et al.*, 1998). To make sure that the AGG codon did not lead to any misincorporation, we subjected intact wt and lysine-less MyoD proteins as well as peptides derived from these proteins to mass spectroscopy and a variety of enzymatic analyses (see Materials and methods). No misincorporation of Lys at AGG could be detected in any of these methods. Independently, we re-mutated all the Lys residues using the bacterially most frequently used Arg codon, CGC, which does not misincorporate Lys for Arg. As expected, data from cell-free assays using this protein were identical to those obtained using the AGG construct (Figure 7A–C).

For the purpose of clarification, a clear distinction should be made between recognition via the 'N-end rule' and the novel finding that the N-terminal residue is essential for targeting a protein for conjugation and subsequent degradation. According to the 'N-end rule', the protein is recognized and bound to its cognate ubiquitin ligase, E3α or E3β, via the N-terminal residue, and ubiquitination occurs, as for all other known substrates of the system, on an internal Lys residue. In the case of MyoD, the substrate is probably bound to the ligase via an internal, as yet unidentified motif, whereas the essential ubiquitination occurs at the N-terminus. Yet, to rule out the unlikely possibility that MyoD is routed indirectly to the 'N-end rule' pathway we used two 'N-end-rule' inhibitors. Neither Arg-Ala nor Phe-Ala, two N-end-rule inhibitory peptides, nor RNase A, which inhibits 'N-end rule'-mediated degradation of acidic N-terminal proteins, affected degradation of MyoD, ruling out the possibility that N-terminal 'lysinylation' or removal of the Met that exposes an otherwise 'N-end-rule' 'destabilizing' residue, target the protein for 'N-end-rule'-mediated degradation. An interesting finding involves the identity of the N-terminal residue of MyoD. Edman degradation has revealed that it is the initiator Met, a 'stabilizing' residue according to the 'N-end rule'. Thus, MyoD belongs to a relatively small group (~20%) of cellular proteins that have free, unmodified N-terminal residues. In ~80% of cellular proteins, the N-terminal residue is acetylated. Despite the fact that the N-terminal residue is 'stabilizing', the protein is extremely unstable. Thus, even for proteins with free N-termini, the 'N-end rule' does not apply and its physiological roles are still an enigma.

An interesting case involves the degradation of the T cell receptor α chain (TCRα). Following substitution of all the Lys residues, the protein is still degraded by the proteasome (Yu *et al.*, 1997). However, no highly ubiquitinated mass adducts of this construct were observed. Since the C-terminus of this Type I membrane protein protrudes into the cytosol and cannot be ubiquitinated, it is possible, that like ornithine decarboxylase (Murakami *et al.*, 1992), lysine-less TCRα is degraded in a proteasome-dependent, but ubiquitin-independent, manner. It is still possible that ubiquitin moieties are attached to the lysine-less TCRα at internal non-lysine residue(s), or to the N-terminal amino acid following its release from the membrane. Theoretically, ubiquitin can be attached to Ser or Thr residues, generating an ester bond, or to a Cys residue, generating a thiol ester bond. However, these conjugates have never been demonstrated. Furthermore, such putative adducts should be extremely labile and will not withstand the boiling conditions in SDS–PAGE sample buffers. Therefore, they cannot be the sample buffer-resistant ubiquitin adducts that we observe during the proteolysis of lysine-less MyoD (see Results). It should be noted, however, that the wt αTCR (unlike the lysine-less) chain is degraded in a ubiquitin-dependent mode, as demonstrated by the detection of ubiquitin adducts of this protein (Yang *et al.*, 1998). In a different example, Hershko and colleagues (1984) have shown that selective blocking of ε-NH_2 groups of internal Lys residues block the degradation of lysozyme only partially. Here, the N-terminal residue of the protein could serve as a ubiquitination site, and the lack of identification of ubiquitin adducts can be attributed to a technical problem such as their rapid removal by isopeptidases. Recently, it has been shown that Us9, a lysine-less herpes simplex virus 1

protein, is modified by a single ubiquitin moiety (Brandimarti and Roizman, 1997). Moreover, modified Usc9 associates with the proteasome. However, the ubiquitinated protein is stable and not degraded. The chemical nature of the modification as well as its physiological role are still obscure, though it is highly likely that the single ubiquitin moiety is attached to the N-terminal residue of the protein.

What sort of structural feature or sequence targets MyoD via its N-terminal residue and what is the mechanism involved in this novel modification? We postulate that the signal must reside in the N-terminal domain of the molecule, since extension of the protein by a six-myc tag stabilizes the protein. Interestingly, the first two amino acids of the myc tag, ME, are identical to those of MyoD, and the tag also contains an internal Lys residue. Thus, the identical first two residues and the six added Lys residues are not sufficient to destabilize the protein. At this stage, the signal that renders the NH_2 terminus an essential ubiquitination acceptor and at the same time suppresses the acceptor capability of the internal Lys residues is still an enigma. It will be interesting not only to identify the structural motif in MyoD that makes it the first exception, but also the E3 enzyme involved in this unique linear modification. It is not clear, at present, whether 'classical' E3s that add ubiquitin to ε-NH_2 groups of internal lysines are involved in this modification, or whether a new species of ligase(s) is involved. As for the mechanism of targeting, following attachment of the first ubiquitin in a linear fashion, a polyubiquitin tree is synthesized, most probably on an internal Lys^{48} residue of the first ubiquitin moiety, and targets the protein for recognition and degradation by the proteasome. Evolutionarily speaking, it is possible that the proteasome has evolved in a manner such that in the case of MyoD, and probably in that of other, yet to be identified proteins, only N-terminally linked polyubiquitin chains are recognized.

As for the generality of this observation, this is still a mystery. It is clear that the protein must have a free and exposed N-terminal residue. Approximately 20% of cellular proteins are not modified at this residue. For others, removal of the N-terminal modifying group can expose a free residue that can be then targeted by ubiquitin.

Our data suggest that conjugation of MyoD occurs via a novel ubiquitin modification linked to the NH_2 terminus of the protein.

Materials and methods

General materials

Materials for SDS–PAGE were from Bio-Rad. Hexokinase was from Boehringer Mannheim. L-[^{35}S]methionine was obtained from New England Nuclear (NEN). Ubiquitin, chloroquine, E-64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane], Arg-Ala, Phe-Ala, bestatin and fluorescamine were purchased from Sigma. Diethylaminoethyl (DEAE) cellulose (DE52) was from Whatman. Tissue culture sera and media were purchased from Biological Industries, Kibbutz Bet Haemek, Israel or from Sigma. Clasto-lactacystin β-lactone and MG132 (*N*-carbobenzoxyl–Leu–Leu–Leucinal) were purchased from Calbiochem. Antibodies against MyoD were from Novocastra (monoclonal) or Santa Cruz (polyclonal). Anti-ubiquitin polyclonal antibody was raised in rabbits and is described elsewhere (Abu-Hatoum *et al*., 1998). Restriction and modifying enzymes were from New England Biolabs. Immobilized protein A was from Pharmacia. Reagents for ECL were from Pierce and Amersham. Oligonucleotides were synthesized by Biotechnology General, Rehovot, Israel. All other reagents used were of high analytical grade.

Plasmids and expression of MyoD

wt MyoD cDNA was subcloned into the *Hin*dIII–*Nde*I site of the bacterial expression vector pT7-7. This vector was used for both *in vitro* translation (under the control of T7 RNA polymerase) and bacterial expression of MyoD. For expression in mammalian cells, cDNA encoding wt MyoD was subcloned into the pCI-neo vector (Promega). The pT7-7 vector containing wt MyoD cDNA was linearized with *Cla*I. Following fill-in with Klenow enzyme, the vector was further cut with *Xba*I and the cDNA was ligated into a *Sma*I–*Xba*I-linearized pCI-neo vector. cDNA encoding wt MyoD in a pCS2+MT vector that contains also a six-myc tag fused to the N-terminal residue of the protein was generated in the laboratory of the late Dr Harold Weintraub and was obtained from Dr Stephen Tapscott. All mutated species of MyoD were generated by site-directed mutagenesis using the QuikChange^{TM} kit (Stratagene). Sequences of all constructs were confirmed by either the manual (Amersham) or automatic (Applied Biosystems) dideoxy method.

BL21(DE3)/pLysS *E.coli* cells were used for bacterial expression of MyoD. Following induction with IPGT, cells were lysed and nucleic acids were removed by precipitation with 0.3% polyethyleneimine. MyoD (~90–95% pure) was precipitated by 0.6 M ammonium sulfate as described previously (Thayer and Weintraub, 1993).

Cell lines and transfection

COS-7 cells were transiently transfected with wt and the various MyoD constructs using the DEAE–dextran method (Guan and Rose, 1984) or the Superfect kit (Qiagen).

Stability of proteins in vivo

Cellular stability ($t_{1/2}$) of MyoD proteins was monitored in pulse (1 h)–chase labeling experiments followed by immunoprecipitation. The proteasome inhibitors, MG132 (100 μM) and clasto-lactacystin β-lactone (10 μM), were present throughout the experiment. The lysosomal inhibitor chloroquine (100 μM) and the cysteine protease inhibitor E-64 (28 μM) were added during the chase period. Following labeling, cells were harvested (time 0: pulse) or were further incubated for the indicated periods (chase). Cells were lysed, and using anti-MyoD antibody, the labeled protein was immunoprecipitated from aliquots that contained an equal amount of labeled proteins (as determined by precipitation on a filter with trichloroacetic acid). Immunocomplexes were collected using immobilized protein A. Following SDS–PAGE (10%), proteins were visualized by a PhosphorImager (Fuji, Japan).

Demonstration of MyoD–ubiquitin conjugates in vivo

To identify high-molecular-mass conjugates of MyoD, COS-7 cells, transiently transfected with MyoD cDNA, were incubated with MG132 for 2 h. Cells were lysed as described (Govers *et al.*, 1997) and protein concentrations were determined by the Bradford method (Bradford, 1976). Equal amounts of protein were subjected to immunoprecipitation with anti-MyoD antibody. The immunoprecipitate was resolved via SDS–PAGE (10%) and transferred onto nitrocellulose paper, and the conjugates were detected using anti-ubiquitin antibody and the ECL method. When indicated, the membrane was stripped and incubated with anti-MyoD antibody.

In vitro translation

cDNAs were first linearized with *Cla*I and subjected to run-off transcription using T7 RNA polymerase (Promega) as described previously (Ciechanover *et al.*, 1991). Purified and isolated mRNAs were translated in rabbit reticulocyte lysate (Promega) in the presence of L-[^{35}S]methionine according to the manufacturer's instructions.

Preparation and fractionation of crude reticulocyte lysate

Reticulocytes were induced in rabbits and lysates were prepared as described (Hershko *et al*., 1983). The lysate was fractionated over DEAE cellulose onto unadsorbed material (Fraction I) and high-salt eluate (Fraction II), as described previously (Hershko *et al.*, 1983).

Conjugation and degradation assays

Conjugation and degradation assays in reconstituted cell-free systems were performed essentially as described previously (Orian *et al.*, 1995; Abu-Hatoum *et al.*, 1998). Briefly, reaction mixtures contained in a final volume of 12.5 μl: Fraction II (50 μg protein), 5 μg (or as indicated) ubiquitin and either bacterially expressed [either 200 ng (for degradation)

or 800 ng (for conjugation)] or ~40 000 c.p.m. of *in vitro* translated MyoD. Reactions were carried out in the presence of either 0.5 mM ATP plus an ATP-regenerating system (10 mM phosphocreatine and 0.5 μg phosphocreatine kinase), or 0.5 μg hexokinase and 10 mM 2-deoxyglucose to deplete endogenous ATP. Degradation assays that were performed in the presence of the dipeptides Arg-Ala or Phe-Ala (10 mM) also contained 20 ng/μl bestatin. Where indicated, 0.5 μg of Ribonuclease A was added to the mixtures monitoring degradation of MyoD. Conjugation assays also contained 0.5 μg of the isopeptidase inhibitor ubiquitin aldehyde (UbAl; Hershko and Rose, 1987). Conjugation reactions were incubated for 30 min at 37°C, whereas degradation mixtures were incubated for 2 h at the same temperature. Reactions were terminated by the addition of 7.0 μl 3-fold concentrated sample buffer, and, following boiling, were resolved via SDS–PAGE (10%). Conjugation reactions that were performed with bacterially expressed MyoD were first subjected to immunoprecipitation with anti-MyoD antibody and then resolved via SDS–PAGE (10%). Depending on the nature of the MyoD protein resolved, gels were dried and exposed to a PhosphorImager screen, or blotted onto nitrocellulose paper, and proteins were detected by ECL following incubation with anti-MyoD antibody or anti-ubiquitin antibody. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Protein modifications

All modifications were carried out essentially as described by Hershko and colleagues (1984) using bacterially expressed MyoD (~1 mg protein) as a substrate. For reductive methylation (that blocks all NH_2 groups), MyoD was dissolved in 6 M urea and 50 mM HEPES pH 7.4. Sodium cyanoborohydride and formaldehyde were added to a final concentration of 10 mM. The reaction mixture was incubated for 2 h at room temperature and the reagents were removed by dialysis against water.

Carbamylation (that selectively blocks the α-NH_2 group) was carried out in 0.2 M potassium phosphate, pH 6.0/6 M urea/50 mM potassium cyanate. Following incubation for 8 h at 37°C, the reaction was stopped by the addition of Gly-Gly to a final concentration of 150 mM. The pH was adjusted to 8.1 with 30% (w/v) K_2HPO_4, pH 11, and the mixture was incubated for an additional 1 h at 37°C. The latter treatment releases carbamyl groups bound to non-amine residues within the protein. Reagents were removed by dialysis against water.

Guanidination (that selectively blocks ε-NH_2 groups of internal Lys residues) of MyoD was carried out in 0.3 M *O*-methylisourea for 2 days at 4°C, followed by dialysis for 2 days against four changes of 50 mM KPi, pH 7.0 and two changes of ddH_2O.

The degree of modification of amino groups was determined by the fluorescamine method (Böhlen *et al.*, 1973), which monitors the content of free, primary amine groups in proteins. Fluorescence measurements (Luminescence Spectrometer LS-5B, Perkin Elmer) were carried out using an excitation wavelength of 390 nm and an emission wavelength of 475 nm. wt and lysine-less MyoDs were used as a standard, and the results were normalized to 10 (wt MyoD has 10 free amino groups, nine in internal Lys residues and one at the N-terminus). According to measurements, the following number of primary amino groups were detected in the different proteins: wt MyoD (wt), 10; lysine-less MyoD (0 K), 1.5; methylated lysine-less MyoD (0K-CH3), 0.2; carbamylated wt MyoD (NH_2CO-wt), 7.8; and guanidinated wt MyoD (wt-Gd), 1.2. To confirm that the carbamylated MyoD is blocked at the N-terminus, the protein was subjected to an automated Edman degradation reaction. No signal could be detected in three successive cycles. The wt protein yielded the expected MEL sequence.

Confirmation of complete substitution of all lysine residues in the lysine-less MyoD protein

To verify that all lysine residues were indeed substituted with Arg, the wt and lysine-less MyoD proteins were subjected to three independent analyses: (i) the bacterially expressed intact proteins were subjected to molecular mass determination; (ii) the proteins were cleaved with CNBr and the masses of the three resulting peptides were determined; and (iii) the proteins were proteolyzed by V8 and Lys-C.

Initially, MyoD proteins were purified by HPLC on a C-8 column (AQUAPORE RP-300, Applied Biosystems) and eluted with a linear gradient of 15–65% Acetonitrile (ACN) in 0.1% TFA at 1% per min.

Determination of the mass of the intact proteins in Sinapinic acid was determined using the Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF 2E; Micromass, Manchester, UK).

For determination of the mass of internal peptides that span the complete sequence of the protein, wt and lysine-less MyoD proteins were cleaved with CNBr (Sigma) in 70% formic acid. Mouse MyoD has three internal Met residues, two of them are adjacent. Thus, CNBr cleavage yields three peptides. Masses of the resulting peptides were determined by LC-MS as follows: peptides were resolved by HPLC on a C-8 column with a linear gradient of 15–65% ACN in 0.05% TFA at 1.25%/min and analyzed by electrospray ion-trap mass spectrometer (LCQ, Finnigan) in the positive ion mode. Peptides masses were also determined by MALDI-TOF mass spectrometer (REFLEX II, Bruker).

In an independent approach, the proteins were digested with Lys-C (Boehringer Mannheim) or *Staphylococcus aureus* V8 protease (Promega). The resulting peptides were analyzed by LC-MS as described above, except that we used a C18 column. Peptides were eluted with a linear gradient of 4–65% ACN in 0.05% TFA, at 1%/min. In the wt protein, Lys-C is expected to yield 10 peptides, whereas if all Lys residues are substituted, the enzyme will not act, and the altered MyoD will remain intact. In the wt protein we identified eight peptides (a di- and a tripeptide that result from digestion at the carboxyl sides of two Lys residues, with one or two amino acid residues in between, respectively, were not identified). As expected, the lysine-less MyoD remained intact and was not cleaved.

For the intact proteins and the cyanogen bromide and V8 cleavages (there are five V8 Lys-containing peptides in MyoD), the mass spectrometry analysis indicates that the replacement of Lys with Arg residues in the lysine-less MyoD protein was complete: the expected difference in the masses of the intact proteins and the different peptides reflect exactly the additional mass attributed to the Arg residues.

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