Regulated proteolysis has been postulated to be critical for proper control of cell functions. Muscle development, in particular, involves a great deal of structural adaptation and remodeling mediated by proteases. The transcription factor YY1 represses muscle-restricted expression of the sarcomeric α-actin genes. Consistent with this repressor function of YY1, the nuclear regulator is down-regulated at the protein level during skeletal as well as cardiac muscle cell differentiation. However, the YY1 message remains relatively unaltered throughout the myoblast-myotube transition, implicating a post-translational regulatory mechanism. We show that YY1 can be a substrate for cleavage by the calcium-activated neutral protease calpain II (m-calpain) and the 26 S proteasome. The calcium ionophore A23187 destabilized YY1 in cultured myoblasts, and the decrease in YY1 protein levels could be prevented by calpain inhibitor II and calpeptin. Treatment with the proteasome inhibitors MG132 and lactacystin resulted in the stabilization of YY1 protein, which is consistent with the finding that YY1 is readily polyubiquitinated in reticulocyte lysates. We further show that proteolytic targeting by calpain II and the proteasome involves different structural elements of YY1. This study thus illustrates two proteolytic pathways through which the transcriptional regulator can be differentially targeted under different cell growth conditions.

Many cellular processes are known to be controlled by short-lived proteins, including products of the proto-oncogenes, cell cycle regulators, and developmentally regulated transcription factors (1–3). The fast turnover of these regulatory proteins reflects a metabolic requirement for rapidly changing their concentrations and is presumably mediated by a complex interplay among various proteases and protease inhibitors. Selective degradation of transcriptional activators and repressors, in particular, may provide efficient regulatory mechanisms contributing to the rapid shut-off and turn-on of gene activity, respectively. The recent study of the pleiotropic transcription factor NF-κB has revealed that the ubiquitin-proteasome pathway can function not only in the complete degradation of proteins but also in the regulated processing of precursors into active transcription factors (4, 5). Calpains represent the other major class of nonlysosomal proteases functioning in a calcium-dependent fashion (6, 7). Interestingly, both the proteasome and calpains have been found to play a regulatory role in the function and/or stability of c-Fos and the tumor suppressor protein p53 (8–11). Thus, rapid degradation or processing of specific transcription factors can underlie a wide range of dynamic cellular and developmental processes.

Muscle development involves a great deal of structural adaptation and remodeling mediated by induced protein synthesis and degradation (12–14). Although protein turnover must be highly selective if it is to be developmentally useful, little is known concerning the regulatory mechanisms responsible for protein targeting and subsequent degradation during development. The expression of a lysosomal cysteine protease family, cathepsins, was found to increase during muscle differentiation (15). Myoblast fusions in chick and rats were both shown to require metalloendoprotease activity (16). Consistent with the observed calcium influx during myoblast membrane fusion (17), the activity of the calcium-activated neutral protease (calpain) is up-regulated during and required for myogenesis (14, 18). These findings suggest that temporal regulation of proteolytic events plays an important role in muscle development. In most cases, however, the endogenous protein substrates proteolyzed during differentiation have not been characterized, and the physiological relevance remains to be examined.

We have previously shown that YY1, a C2H2-type zinc finger DNA-binding protein (19), is capable of simultaneously activating and repressing the expression of the c-myc proto-oncogene and the sarcomeric α-actin gene, respectively (20). In chick embryonic myoblast culture, YY1 inhibits muscle-restricted transcription of the skeletal α-actin gene by excluding SRF, a positive MADS-box myogenic transcription factor, from the most proximal serum response element of the actin gene promoter (20, 21). In these studies, myoblasts rendered incapable of differentiation were found to contain higher levels of YY1 and c-Myc proteins compared with the differentiated myotubes. Given the established inhibitory effect of c-myc on myogenic differentiation (22) and the activating effect of YY1 on c-myc (20, 23), the down-regulation of YY1 was postulated to be essential for the expression of the sarcomeric α-actin genes. However, the YY1 down-regulation mechanism during myogenesis remains unclear. Here we report that YY1 is similarly down-regulated during the in vitro differentiation of cultured rat skeletal myoblasts and ventricular cardiomyocytes. In contrast to the down-regulation of YY1 protein, the YY1 message remains unaltered throughout the myoblast-myotube transition. We present both in vivo and in vitro evidence that calpains and the 26 S proteasome are involved in the in vivo stability of YY1. This finding illustrates a post-translational mechanism through which the repressor of myogenic transcription may be selectively inactivated by developmentally regulated proteolysis to facilitate muscle development.

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Proteolytic Regulation of the Zinc Finger Transcription Factor YY1, a Repressor of Muscle-restricted Gene Expression*
MATERIALS AND METHODS

**Muscle Cell Culture**—Primary skeletal and cardiac muscle cell cultures were prepared as described (24, 25). In brief, minced tissues were gently agitated in 15 ml of 0.05% trypsin + 1 mM EDTA at 4 °C overnight. Excessive trypsin solution was removed following overnight agitation, and tissue fragments further incubated at 37 °C for 10 min, after which 10 ml of minimal essential medium (MEM) containing 10% horse serum was added to inactivate trypsin. Collagenase (Worthington) and DNase I (Sigma) were then added to a final concentration of 1 mg/ml and 0.1 mg/ml, respectively, for another 20 min of incubation. Tissue fragments were then triturated three to four cycles with the addition of 10 ml of medium for each cycle. Skeletal myoblasts were plated in MEM + 10% fetal bovine serum on Primaria culture dishes (Falcon) at a density of 3,120 cells/mm². Cardiac myocytes were plated in MEM + 10% horse serum at a density of 1,040 cells/mm². Cultured muscle C2 and Sol8 myoblasts were maintained in MEM + 10% fetal bovine serum and 50 μg/ml gentamicin. Calpain inhibitor II and calpeptin were purchased from Calbiochem. MG132 was provided by Cecile Pickart (Johns Hopkins University). Lactacytin was purchased from E. J. Corey (Harvard University).

**Crude Myoblast Extracts**—Approximately 10 million Sol8 cells were harvested by scraping in phosphate-buffered saline, and cells were spun down and freeze-thawed once. The cell pellet was resuspended in 0.3 ml of ice-cold extraction buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1 mM NaCl, 10% glycerol, and 1 mM dithiothreitol). Cells were homogenized at 4 °C by 100 strokes in a period of 20 min. The cell lysate was briefly clarified (5 s), and the supernatant was collected and frozen in aliquots at −75 °C. This crude protein extract was used as the source of endogenous calpain.

**In Vitro Cleavage Assay**—The *in vitro* cleavage assay was done in a total volume of 20 μl. In each reaction, 2.5 μg of purified bacterially expressed YY1 was used as substrate. Either Sol8 myoblast lysate (3.3 μg) or purified calpain II (1–2 μg; from Calbiochem or Sigma) was used as the source of protease. Proteins were first assembled on ice, and CaCl₂ was added to initiate the cleavage reaction. Cleavage reactions were continued at 37 °C for 5 min for lysate or 20 min for purified calpain II. Reactions were terminated with SDS-PAGE sample buffer supplemented with β-mercaptoethanol. Samples were boiled for 5 min, electrophoresed by SDS-PAGE, and then probed by Western blot.

**Caseinolysis Assay**—To assess the specificity of protease inhibitors, a modified nonradioactive caseinolysis assay was used (26). Reactions were set up in 15 μl containing 10 mM Tris (pH 7.6), 60 mM KCl, 0.1 mM EDTA, 5% glycerol, 5 mM CaCl₂, and 60 μg of a-casein. An individual control reaction lacking CaCl₂ was also set up for each protease inhibitor. Reactions were initiated by adding various protease inhibitors and 1 μg of calpain II and allowed to proceed at room temperature for 60 min. 5 μl of 12.5 mM EGTA was added to stop the reaction, and 5 μl of sample was mixed with 0.2 ml of diluted Bio-Rad protein assay dye. Abs₅₀⁰ was measured by a Labystem Multiscan microplate reader. Calpain activity was expressed as decrease in Abs₅₀⁰ in the presence of calcium.

**In Vitro Protein Ubiquitination Assay**—*In vitro* ubiquitination of YY1 was carried out using rabbit reticulocyte lysates (a source of ubiquitination enzymes) purchased from Promega. Reactions were set up in 30 μl containing 22.5 μl of reticulocyte lysates and 0.1 μg of purified recombinant YY1 and incubated at 37 °C for 2 min. Samples were then processed for 10% SDS-PAGE and Western blot using an anti YY1 antibody as described below.

**Northern Blot**—Total RNA was isolated using the guanidinium thiocyanate method. Isolated RNA was dissolved in formamide, mixed with ethidium bromide (final concentration 0.1 μg/ml), and separated on a 1.2% agarose gel containing 37% formaldehyde. RNA was transferred to nylon membrane (Bio-Rad) and immobilized by UV cross-linking. Membrane was blocked in prehybridization solution (1% sodium dodecyl sulfate, 10% dextran sulfate, and 1 mM NaCl) for 5 h and then incubated with a random-primed YY1 cDNA probe at 55 °C overnight. Probed membrane was washed twice with 2× SSC at 55 °C followed by one wash with 0.2× SSC. Membrane was air-dried and processed for autoradiography.

**Western Blot**—Cells were harvested by scraping at the time points indicated and lysed in TNT (0.2 mM Tris, pH 8, 0.2 mM NaCl, 0.1% Triton X-100) supplemented with 1 mM dithiothreitol, and lysates were processed for SDS-PAGE. Proteins were electrotransferred to Immobilon-P membrane. The YY1 and SRF antibodies were described previously.

### RESULTS

**Developmental Down-regulation of YY1 Protein During Muscle Development**—Our previous studies using an avian embryonic muscle system showed that cycling myoblasts contain higher levels of YY1 protein but lower levels of SRF protein than post-mitotic, differentiated myotubes. To determine whether YY1 and SRF are also differentially regulated in mammalian cells, we set up primary muscle cell cultures prepared from newborn rats. Western blots shown in Fig. 1 (*top*) illustrated that the YY1 protein levels were the highest in replicating rat myoblasts (24 h) and were reduced between 24 and 48 h when myoblasts were progressing through cell fusion. It is possible that the residual YY1 protein detected after 24 h was derived from contaminating fibroblasts since we found that fibroblasts contain higher levels of YY1 (21). Fig. 1 (*middle*) shows that SRF protein levels were increased during myogenesis and were the highest in fully differentiated myotubes (96 h). The developmental pattern of YY1 protein was further examined in cultured rat ventricular cardiac myocytes (Fig. 2, *top*). The study indicated that YY1 was similarly down-regulated during *in vitro* cardiac differentiation. Cardiac fibroblasts maintained under the same differentiation medium (Fig. 2, *bottom*) exhibited similar YY1 protein contents throughout, suggesting a down-regulation mechanism unique to muscle cell differentiation.

**Constant Expression of YY1 mRNA from Cycling Myoblasts to Post-mitotic Myotubes**—Although YY1 is presumed to be expressed in many tissues where it may assume a constitutive rather than a regulatory role, our studies clearly show that YY1 protein is down-regulated during skeletal as well as ven-
tricular cardiac muscle cell differentiation. Since changes in YY1 protein content could be effected at multiple levels, we sought to examine the kinetics of YY1 mRNA synthesis. The temporal pattern of YY1 expression during skeletal muscle cell differentiation was analyzed by Northern hybridization in Fig. 3, which shows that YY1 mRNA levels did not significantly change from cycling myoblasts (24 h) to post-mitotic myotubes (72 h). Consistent with our finding, the YY1 mRNA levels were found to remain relatively constant during retinoic acid-induced F9 EC cell differentiation (27). Thus, the down-regulation of YY1 during muscle cell differentiation is most likely effected at the protein level.

Calpain II-mediated Proteolytic Cleavage of YY1 in Crude Myoblast Extracts—The findings demonstrated above prompted us to examine whether YY1 might be down-regulated by a protease-mediated mechanism during myogenesis. Calpains were first demonstrated in skeletal muscle homogenates (28), and its activity was found to be up-regulated and essential during myogenic differentiation. Of particular note is the down-regulation of YY1 protein during myoblast fusion (24–48 h; Fig. 1, top), which has long been known to be mediated by extracellular calcium influx (17). To examine the potential involvement of the Ca \(^2\)+-calpain system in the degradation of YY1, we set up an 

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Down-regulation of YY1 protein during ventricular cardiac myocyte differentiation. Cardiac ventricles were isolated from 2-day-old Sprague-Dawley rats. Cells were preplated to deplete fibroblasts and plated in MEM containing 10% fetal bovine serum and 20 mM araC. Cells were maintained in the growth medium for 3 days in the presence of araC, after which culture medium was changed to the differentiation medium (see text) without araC (0 h). Myocytes were harvested at the time points indicated, and lysates were processed for Western analysis. Top panel, cardiac myocyte proteins probed with YY1 antibody (CA); middle panel, cardiac myocyte proteins stained with Coomassie Blue; bottom panel, cardiac fibroblast proteins probed with YY1 antibody (FB). Fibroblasts were maintained in differentiation medium as control.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** The steady state levels of YY1 transcripts remain constant during muscle cell differentiation. Total RNA was isolated from 24-, 48-, and 72-hr time points. 10 μg of total RNA was loaded in each lane. Mouse YY1 cDNA was used as probe in Northern hybridization. Top panel, autoradiograph of probed membrane; bottom panel, total RNA on membrane stained by ethidium bromide (0.1 μg/ml) after an overnight capillary transfer.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Calcium-dependent proteolysis of YY1 by endogenous and purified calpain II. **In vitro** cleavage assays were done in a total volume of 20 μl. In each reaction, 2.5 μg of purified bacterially expressed YY1 (20) was used as a substrate. 3.3 μg of Sol8 myoblast lysate (left half) and 2 μg of purified calpain II (right half) were used as the source of protease. Proteins were assembled on ice, and CaCl\(_2\) was added to initiate the reaction. Reactions were carried out at 37°C for 5 min (crude lysate) or 10 min (pure calpain II) and were terminated with SDS-PAGE sample buffer supplemented with β-mercaptoethanol. Samples were boiled for 5 min, electrophoresed through a 10% gel, and processed for Western analysis. The full-length YY1 and the cleavage product are indicated by ▶ and ◀, respectively.

The finding indicates that YY1 may be a substrate of endogenous calpain II (or m-calpain), which requires micromolar levels of calcium ions for catalytic activity as opposed to calpain I (or μ-calpain), which requires micromolar levels of calcium ions. We further performed the assay using purified calpain II. Fig. 4 (right panel) again shows the appearance of the 40-kDa YY1 cleavage product triggered by the purified calpain II in the presence of 1 mM Ca\(^{2+}\). As predicted, calpain II caused no cleavage of YY1 in the absence of Ca\(^{2+}\) or at lower Ca\(^{2+}\) concentrations.

**Intracellular Stabilization of YY1 by Inhibitors of Calpains and the Proteasome**—We went on to determine whether YY1 protein might be stabilized in cells treated with specific calpain inhibitors. Primary myoblasts were used initially to examine the effect of two specific calpain inhibitors, calpeptin and calpain inhibitor II (29), on the intracellular level of YY1. However, the primary myoblasts were found to be exquisitely sensitive to the inhibitors, and the treatment resulted in a population consisting largely of fibroblasts (data not shown). This finding presumably indicates a vital role for calpains in maintaining the viability of cultured primary myoblasts. Sol8
A

myoblasts (30) were thus used in the subsequent inhibitor experiments. A panel of cell-permeable protease inhibitors, calpain inhibitor II (29), TLCK (inhibitor of trypsin-like serine proteases) (31), and MG132 (inhibitor of the 26 S proteasome) (32) were first examined for their inhibitory effects on calpain II activity using a quantitative in vitro assay described previously (26). Fig. 5 shows that whereas TLCK did not appreciably affect calpain II activity as expected, calpain inhibitor II and MG132, which are similar tripeptide aldehydes consisting of leucine-leucine-methioninal and leucine-leucine-leucinal, respectively, each exhibited a comparable potent dose-dependent inhibition of the caseinolytic activity of calpain II. Thus, MG132 appears to be capable of blocking the activities of both calpains and the proteasome.

We went on to determine whether the inhibitors might stabilize YY1 protein in the cell. Sol8 myoblasts were treated with the inhibitors, and Western blots were performed to examine the effect on YY1 protein levels. Inhibitor concentrations for the treatment were optimized such that minimal loss of cell viability was observed after treatment. Fig. 6A shows that whereas YY1 protein levels were not significantly altered by TLCK (54 μM) or calpain inhibitor II (40 μM), incubation of Sol8 myoblasts with 8 μM MG132 consistently increased YY1 protein content at least 2-fold. Prolonged treatment or treatment with higher doses of MG132 led to a rapid loss of myoblast viability (data not shown). Since autoproteolytic activation and catalytic activity of calpains require elevated calcium levels in myoblasts, an event typically associated with myoblast fusion during myogenesis (17), an effect of the calpain inhibitor on YY1 protein stability may require elevated calcium levels in the cell. To provide evidence along this line, intracellular calpains were stimulated by treating myoblasts with the calcium ionophore A23187, which has been shown to promote myogenesis (14) and activate calpains (33). Fig. 6B shows that YY1 protein levels were clearly reduced by A23187 in treated Sol8 myoblasts. Calpain inhibitor II as well as MG132 were both able to stabilize YY1 in A23187-treated Sol8 myoblasts. Together, these in vitro and in vivo results strongly suggest the involvement of the Ca<sup>2+</sup>-calpain circuit in signal-mediated intracellular proteolysis of YY1.

YY1 Is Also a Substrate of the 26 S Proteasome—That MG132, a well known inhibitor of the 26 S proteasome, was able to stabilize YY1 in myoblasts suggests that YY1 is likely to be a substrate of the proteasome as well. However, the results shown here together with those of others (29, 34) indicate that MG132 can inhibit both calpains and the proteasome. To resolve this issue, we further examined the effect of lactacystin, which is highly specific for the proteasome (35). We have shown previously that a putative polyubiquitinated YY1 species, which could not be demonstrated in actively growing myoblasts, could be detected in myoblasts deprived of serum (36). Thus, myoblasts maintained in growth medium as well as serum-free medium were treated with lactacystin for 30 h, and YY1 protein levels were examined by Western analysis. Fig. 7 shows that whereas lactacystin did not have a significant effect on the stability of YY1 in growing myoblasts (panel A), it increased YY1 protein levels severalfold in serum-starved myoblasts (panel B). To further provide biochemical evidence that YY1 is indeed a ubiquitination substrate, we carried out an in vitro experiment using rabbit reticulocyte lysates as an established source of ubiquitination enzymes (5). In the experiment presented in Fig. 8, purified YY1 was first incubated with reticulocyte lysates, and ubiquitination products were separated by SDS-PAGE and probed with YY1 antibody. A ladder of protein bands exhibiting slower mobilities relative to the free YY1 protein could be detected, which should correspond to YY1 proteins ligated to one or more ubiquitin moieties. The YY1 C-terminal truncation mutant N200, on the other hand, was not appreciably tagged by ubiquitin as indicated by the absence of ubiquitin conjugate ladders (Fig. 8). We thus conclude that amino acid sequences located within the zinc finger domains of YY1 contain recognition/degradation signals for the ubiquitin-dependent proteolytic pathway.

Targeting by Calpains and the Proteasome Involve Different Domains of YY1—That the C-terminal half of YY1, which is rich in PEST residues (Pro, Glu, Ser, and Thr), is required for protein ubiquitination is consistent with the notion that short lived proteins may be proteolytically targeted through these sequences (37). However, there is some controversy as to whether the PEST domain of PEST-containing proteins may be proteolytically targeted through these sequences (37).
after SDS-PAGE. Cleavage products were denoted by asterisks.

**DISCUSSION**

Several lines of evidence suggest that calpain II plays an important regulatory role in myogenic differentiation. The increase in calpain II activity coincides with the onset of myoblast differentiation, which is marked by cell membrane fusion and calcium influx (18). Exogenously added calpain II was found to promote myoblast fusion (40). Studies using calpain inhibitors also showed that inhibition of the endogenous calpain can suppress myogenesis (14, 41). Although possible roles of calpain II in muscle growth and differentiation have been postulated (6, 42), the biochemical mechanism underlying its myogenic function is still unclear. The work presented here offers a regulatory mechanism for calpain II through proteolytic cleavage of the multifunctional transcription factor YY1, which is capable of repressing myogenic transcription via its DNA-binding activity (43, 44). A direct in vivo evidence that YY1 can be proteolyzed by calpains is illustrated here by the A23187 experiment, showing stabilization of YY1 protein by calpain inhibitor II in A23187-treated myoblasts. Consistent with this “proteolytic” effect of A23187, this calcium ionophore was found to activate calpains (33) and promote myogenesis (17).

The 26 S proteasome system has been extensively studied and found to be involved in the degradation of several transcriptional regulators such as NF-kB (4), c-Jun (45), c-Fos (8), p53 (46), and the yeast MATa2 repressor (2). Our current and previous studies (36) together indicate that YY1 turnover is mediated by calpains as well as the proteasome. We found that MG132, which is generally used as an inhibitor of the proteasome (32), is able to stabilize YY1 in treated myoblasts. MG132 and calpain inhibitor II are both tripeptide aldehydes consisting of leucine-leucine-leucinal and leucine-leucine-methioninal, respectively. Thus, the ability of MG132 to inhibit calpains as shown here may not be unexpected. Since MG132 appears to be an inhibitor of both calpains and the proteasome, it is possible that stabilization of YY1 in vivo can be best observed under the condition where both proteolytic pathways are blocked concurrently. This notion is consistent with the findings here that the stabilization of YY1 in vivo by lactacystin can only be observed in serum-starved myoblasts that exhibit elevated proteasome activities (36). In cellular processes associated with signal-induced calcium influx (as mimicked by A23187 treatment), calcium-mediated calpain activation may become a major proteolytic driving force. This may explain why stabilization of YY1 by calpain inhibitors can only be observed in A23187-treated myoblasts.

The majority of short lived proteins are found to possess one or more domains rich in Pro, Gln, Ser, and Thr residues (PEST domain) (37). However, the mechanism that PEST regions confer susceptibility to rapid proteolysis remains unclear. The structural features or signals of transcription factors that cause them to be rapidly degraded in vivo remain to be further characterized. We present evidence that calpains and the proteasome utilize different structural elements for substrate targeting. Our finding supports the previous notion that PEST sequences do not influence substrate susceptibility to calpain proteolysis (38). Proteolytic targeting by the proteasome on the other hand involves the participation of PEST residues. These
differential targeting mechanisms of calpains and the proteasome presumably provide elements of specificity necessary for cellular regulation and allow for a more versatile developmental control module. Whereas the ubiquitin-dependent pathway is well known for its housekeeping function, we propose that the proteasome and calpains may differentially regulate the stability of a protein under different growth conditions and signaling pathways, as demonstrated by the use of A23187 and serum starvation here. Like YY1, the stability of c-Fos and p53 has previously been found to be co-regulated by calpains and the proteasome (8–11). Aside from the involvement of various proteases in controlling the level of transcription factors, the eukaryotic DNA-binding factor AEBP1 has been found to possess a protease activity (47). Thus, targeting proteases to a transcriptional machinery may represent a unique feature in gene regulation.

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