The Nuclear Ubiquitin-Proteasome System Degrades MyoD

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Summary

Many short-lived nuclear proteins are targeted for degradation by the ubiquitin-proteasome pathway. The role of the nucleus in regulating the turnover of these proteins is not well defined, although many components of the ubiquitin-proteasome system are localized in the nucleus. We have used nucleoplasm from highly purified HeLa nuclei to examine the degradation of a physiological substrate of the ubiquitin-proteasome system (MyoD). In vitro studies using inhibitors of the system demonstrate MyoD is degraded via the ubiquitin-proteasome pathway in HeLa nucleoplasm. Purified nucleoplasm in vitro also supports the generation of high molecular mass MyoD-ubiquitin adducts. In addition, in vivo studies using leptomycin B to inhibit nuclear export, demonstrate that MyoD is degraded in HeLa cells by the nuclear ubiquitin-proteasome system.

Introduction

The ubiquitin-proteasome system is responsible for the degradation of many of the short-lived proteins in eukaryotic cells. The pathway targets proteins for degradation by the proteasome via covalent tagging of the substrate protein with a polyubiquitin chain. This is accomplished in three sequential steps. Ubiquitin, a 76 amino acid protein, is initially activated by E1, the ubiquitin-activating enzyme. Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which generally shuttles ubiquitin to a ubiquitin ligase (E3). The E3 is bound to the targeted substrate and catalyzes the covalent attachment of ubiquitin to the substrate. Once the first ubiquitin molecule is transferred to the substrate, a polyubiquitin chain is generated via a series of isopeptide linkages between a lysine residue of the attached ubiquitin and the carboxy-terminal glycine of the next ubiquitin molecule to be added. The multi-
ubiquitinated substrate protein is then degraded by the 26S proteasome in an ATP-dependent reaction (reviewed in Hershko and Ciechanover, 1998).

This highly selective proteolytic system is essential to the regulation of a wide range of nuclear proteins including cell cycle regulators, transcription factors, tumor suppressors and oncoproteins (Ciechanover and Schwartz, 1998; Tansey, 1999; Laney and Hochstrasser, 1999). While all of the components of the ubiquitin-proteasome system are found in the cytoplasm, the role of the nucleus in the degradation of nuclear proteins is not well understood although many of the components of the ubiquitin-proteasome system can also be found within the nucleus.

Among these components, ubiquitin is found in the nucleus (Schwartz et al., 1988). The 117 kDa isoform of the ubiquitin-activating enzyme (E1a) is found in the nucleus in a cell-cycle dependent manner (Trausch et al., 1993; Grenfell et al., 1994; Stephen et al., 1996). Ubiquitin-conjugating enzymes (E2) that are found in the nucleus include RAD6, an E2 involved in DNA repair (Watkins et al., 1993) and cdc34 (Song et al., 1998; Lisztwan et al., 1998; Reymond et al., 2000), an E2 associated with the SCF-type ubiquitin ligase complex (Skowyra et al., 1997; Feldman et al., 1997). Moreover, proteasomal subunits have been immunolocalized to both the cytoplasm and the nucleus throughout the cell cycle (Peters et al., 1994; Reits et al., 1997; Russell et al., 1999; Brooks et al., 2000).

These observations suggest that a functional ubiquitin-proteasome system may be operative within the nucleus. A functional nuclear ubiquitin-proteasome system may provide additional levels of regulation of intracellular proteolysis via the cellular targeting of a substrate as well as via compartment-specific activities of its components. Studies examining the degradation of TGF-β-activated Smad2 (Lo and Massaugue, 1999) and the dioxin receptor, a member of the basic helix-loop-helix/PER-ARNT-Sim family of transcription factors, (Roberts...
and Whitelaw, 1999) show that nuclear localization occurs prior to the ultimate ubiquitin-dependent degradation of these substrates. However, other studies have determined that nuclear export is required for the ubiquitin-dependent degradation of p27kip1 (Tomoda et al., 1999), cyclin D1 (Diehl et al., 1998), p53 (Freedman and Levine, 1998) and IκBα (Rodriguez et al., 1999) as well as the aryl hydrocarbon receptor (Davarinos and Pollenz, 1999). These results, which couple nuclear export with degradation of the substrate, suggest that export to the cytoplasmic ubiquitin-proteasome machinery may represent a common route for the degradation of nuclear substrates.

However, ubiquitin-dependent degradation of nuclear substrates in the cytoplasm envisions no role for the proteasome or the ubiquitin activating and conjugating enzymes that are localized to the nucleus. This raises questions about the role of the ubiquitin-proteasome components found in the nucleus and whether the nucleus will support ubiquitin-dependent degradation. To determine if the nucleus and its complement of components are sufficient to support ubiquitin-dependent degradation, we have developed a cell-free system based on highly purified HeLa nucleoplasm. We have evaluated both model substrates and MyoD as a physiological substrate using this system. In addition, we have extended these studies in vivo and taken advantage of the nuclear export inhibitor, leptomycin B (Kudo et al., 1999).

MyoD is a nuclear basic helix-loop-helix transcription factor that is pivotal in skeletal muscle differentiation (Weintraub et al., 1991). In addition, MyoD can act as a cell cycle inhibitor during G1 (Crescenzi et al., 1990; Sorrentino et al., 1990). While the cellular locus for the degradation of MyoD has not been established, in vivo (Song et al., 1998) and in vitro (Abu Hatoum et al., 1998; Breitschopf et al., 1998), studies including those using proteasomal inhibitors have shown that MyoD is a substrate of the ubiquitin-proteasome system. The present
results now demonstrate that highly purified nucleoplasm will degrade MyoD in a ubiquitin-proteasome dependent manner and that in vivo, nuclear export is not required for the degradation of MyoD, indicating that MyoD is degraded by a nuclear ubiquitin-proteasome system.

**Experimental Procedures**

*Cell Culture.* HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin) (Life Technologies). The cells were maintained at 37°C and 5% CO₂ in a humidified chamber and were harvested at 50-75% confluence. For large-scale culture, HeLa cells were maintained in suspension in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum and were harvested at 0.5 x 10⁶ cells/ml. Large-scale cultures were obtained from the Tissue Culture Support Center at the Washington University School of Medicine.

Transient transfections with MyoD were performed using Lipofectamine according to the manufacturer’s instructions (Life Technologies).

*Preparation of Extracts.* Rabbit reticulocyte lysate was prepared as previously described (Ciechanover et al., 1978). Nucleoplasm was obtained from HeLa cells via a procedure that is a modification of those of Dignam et al. (1983) and Blobel and Potter (1966). The preparation was monitored microscopically at each step. The HeLa cell pellet was rinsed twice in ice-cold phosphate-buffered saline and once in a hypotonic buffer (10 mM Tris-Cl, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT). The pellet resuspended in the hypotonic buffer was then dounced for 2-3 strokes with a “B” type pestle to ensure a single cell suspension. Thereafter the cells were swollen via a 45-minute incubation on ice. All buffer volumes were 25 ml/gm pellet (25 ml/liter suspension culture). The swollen cells were then dounced for 35 strokes.
with an “A” type pestle and the resulting crude nuclei were pelleted at 600g for 10 minutes. The pelleted nuclei were resuspended in 0.25 M sucrose in hypotonic buffer and incubated on ice for 10 minutes. Thereafter, 2.3 M sucrose in hypotonic buffer was added to yield a 1.62 M sucrose solution. The nuclei suspension was layered over a step gradient of 1.80 M and 2.30 M sucrose and centrifuged at 28K rpm for 1 hour in a SW 28 rotor (Beckman). The pellet of nuclei was resuspended in hypotonic buffer and rinsed once by centrifugation in the same buffer. The washed nuclei were then incubated in the hypotonic buffer without MgCl₂ on ice for 15 minutes. Following sonication, the resulting nucleoplasm was collected after centrifugation at 10,000 rpm for 10 minutes in an Eppendorf microfuge at 4°C and stored at –70°C after rapid freezing in liquid N₂. When MyoD was used as the substrate, the sonicated nuclei were incubated with 2.5 units DNase I/mg protein at room temperature for 20 minutes before the final centrifugation step. This step was necessary for removal of DNA, which is inhibitory in the ubiquitin-independent degradation of MyoD (data not shown). Control experiments showed no effect on substrate degradation when DNase I was added to reticulocyte lysate. A typical preparation of nucleoplasm (from 4 L of HeLa cell suspension culture) contained 4-6 mg/ml protein in a total volume of 1-1.5 ml. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

**Characterization of HeLa nucleoplasm.** Nuclei were evaluated for cytoplasmic protein contamination using both morphological and biochemical criteria. Nuclei were visualized under phase contrast at each step of the preparation and were considered substantially free of cytoplasmic remnants if the nuclear envelope appeared smooth and the nuclei did not clump. The washed nuclei were assayed for lactate dehydrogenase activity as a biochemical marker of cytoplasmic contamination (Stephen et al., 1996). Total activity was determined using nuclei...
volume based on total number of nuclei x 0.3 pl/nuclei (Weibel et al., 1969). HeLa homogenate was obtained from an aliquot of the disrupted HeLa cells prior to centrifugation. HeLa cytosol was the supernatant obtained from 100,000g-centrifugation of the homogenate (Stephen et al., 1996). Nucleoplasmic and cytoplasmic fractions were also evaluated by 12.5% SDS-PAGE and protein staining with Coomassie brilliant blue. In addition, nuclear protein contamination of the cytoplasm was assessed by western analysis of histones using a histone pan antibody (Roche Molecular Biochemicals, 25mg/ml, #1 492 519).

**Preparation of MyoD substrate.** Wild-type MyoD was cloned into the bacterial expression vector pT7-7 as previously described (Breitschopf et al., 1998). BL21(DE3) pLysS E. coli cells were used for expression of MyoD. Expression was induced with 0.5 mM IPTG at an absorbance of 0.6 at 600 nm and the cells were harvested after a five-hour induction at 30 °C. The cells were lysed by sonication in 20 mM HEPES, pH 7.9, 0.2 M KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 2 mM PMSF and nucleic acids were removed by precipitation with 0.3% polyethyleneimine. MyoD was precipitated from the resulting supernatant using 0.6 M ammonium sulfate as described (Thayer and Weintraub, 1993).

**Degradation Assays.** Degradation assays were performed according to the method of Hershko et al. (1983). Briefly, reactions were carried out in a final volume of 12.5 µl and contained either crude reticulocyte lysate (3 µl, ~550µg protein) or HeLa nucleoplasm (30µg protein) as the source of ubiquitin-proteasomal system components. The reaction mixtures contained 50 mM Tris-Cl, pH 7.6, 5 mM MgCl₂, 2 mM DTT along with an exogenous protein substrate (MyoD, 400 ng/reaction) supplemented with either an ATP-depleting system (0.5 µg hexokinase and 10 mM 2-deoxyglucose) or ATP-regenerating system (0.5 mM ATP, 10 mM phosphocreatine and 0.5 µg phosphocreatine kinase). The ATP-regenerating system maintains
the ATP level at approximately 1 mM. Degradation reactions were incubated at 37°C for 2 hours
and were terminated by boiling after the addition of an equal volume of 2X Laemmli sample
buffer (Laemmli, 1970). The reaction products were resolved by 10% SDS-PAGE and
electroblotted onto nitrocellulose. The immunoblots were incubated with a monoclonal anti-
MyoD antibody (1:250 dilution, Novacastra, #NCLMyoD1) and after incubation with a
secondary horseradish peroxidase-conjugated antibody, were detected by chemiluminescence
(Amersham). Statistical analysis of the western blot data was performed by Student t test after
the data was quantified using Un-Scan-It gel Version 5.1 software (Silk Scientific, Inc.).

For the inhibition studies, MG132 (N-carbobenzoxy-L-leu-Leu-Leucinal) (Peptide
Institute, Inc.) was diluted to 10 mM in DMSO and was added to the reactions containing the
ATP-regenerating system at 20 µM. A reaction containing an equal volume of the solvent
DMSO was run as a control. Methylated ubiquitin was prepared as previously described
(Hershko and Heller, 1985). Methylated ubiquitin and the ubiquitin K48R mutant (Boston
Biochem.) were each prepared as a 25 mg/ml stock in H2O and were added to the degradation
reaction containing the ATP-regenerating system at 5, 10 or 15 µg/12.5 µL reaction volume.
ATPγS (Sigma) was prepared as a 0.1 M stock in 10 mM Tris-Cl and the pH was adjusted to 7.0.

**Conjugation of MyoD in nuclear extract.** The MyoD cDNA was translated in the
presence of 35S-methionine using a transcription-translation coupled reticulocyte lysate system
(Promega). Prior to its addition to the conjugation assay, the labeled substrate-containing
mixture was treated with 9 mM N-ethylmaleimide (NEM; Sigma) for 10 minutes at room
temperature to inactivate components of the ubiquitin system. The NEM was neutralized with
excess (9 mM) NEM.
Conjugation assays were performed in a reconstituted cell-free system as described (Breitschopt et al., 1998). Briefly, the reaction mixture contained a final volume of 12.5 µl: 40 µg of nucleoplasmic proteins, or 40 µg of BSA as indicated, 5 µg ubiquitin and ~ 25,000 cpm of \textit{in vitro} translated and NEM-treated $^{35}$S-MyoD. Reactions were performed in the presence of the proteasome inhibitor ATP$_{\gamma}$S (4 mM) (Johnston and Cohen, 1981) and the isopeptidase inhibitor, ubiquitin aldehyde (0.5 µg) (Hershko and Rose, 1987). Conjugation assays were incubated at 37°C for 1 hour. Reactions were terminated by the addition of sample buffer and resolved by SDS-PAGE (10%). MyoD was visualized by phosphorimager (Molecular Dynamics/Fuji).

**Stability of MyoD in vivo.** At 24 hours following transfection, protein synthesis was inhibited by incubation with cycloheximide (final concentration of 100 µg/ml) (Kawazoe et al., 1998) along with either MG132 (10 µM) or leptomycin B (10 nM). MG132 was prepared as a 10 mM stock solution in DMSO and leptomycin B was prepared as a 10 µM solution in ethanol. Control experiments were carried out with either DMSO or ethanol alone. At the indicated time points, the cells were lysed in PBS with 0.5% Igepal, 1 mM EDTA, 1 mM DTT and 2 mM PMSF. The lysed cells were sonicated and cellular debris was removed by centrifugation at 13,000 rpm x 1 minute in an Eppendorf microfuge. Equal volumes of the supernatant at each time point were analyzed by 10% SDS-PAGE followed by western blotting as previously described for the degradation assays. The relative amounts of MyoD were analyzed using Un-Scan-It gel Version 5.1 software and the half-life was determined based on three separate experiments.

Pulse-chase analysis of MyoD stability was carried out in HeLa cells at 24 hours after transfection. Cells were transfected with pC1neoMyoD or the pC1neo vector as a control. The cells were pretreated for 2 hours with 10 nM LMB or an equal volume of ethanol for the (-)
LMB condition prior to metabolic labeling with $^{35}$S-cysteine (200 µCi/ml) (Amersham) for 1 hour in media devoid of exogenous cysteine. The cells were washed and chased in media containing unlabeled cysteine with or without 10 nM LMB and harvested after three hours. Cell lysates were prepared by resuspension of cells in lysis buffer (PBS, 0.5% Igepal, 1 mM DTT, 1 mM EDTA, 2 mM PMSF) followed by sonication. Cellular debris was removed by centrifugation and the resulting supernatant was pre-cleared overnight at 4°C with protein A-sepharose (RepliGen). Immunoprecipitations were performed by incubation with a polyclonal anti-MyoD antibody (1:200 dilution, Santa Cruz, #sc-760) followed by incubation with protein A-sepharose. The beads were extensively washed and the immunoprecipitates were analyzed by 10% SDS-PAGE followed by autoradiography.

Control experiments were carried out to confirm LMB’s biological activity. Herein, HeLa cells growing on coverslips were transiently transfected with GFP-IκBα (Sachdev et al., 2000). Twenty four hours after transfection, the cells were treated for 1 hour with 10 nM LMB and localization of the GFP- IκBα fusion protein was determined by indirect immunofluorescence (Gilmore and Temin, 1986) with anti-GFP antibody (1:500 dilution, Chemicon, #AB16901). The resulting images were photographed under 40X using an Olympus BX 60 microscope.

**Immunofluorescent localization of MyoD.** HeLa cells were transfected with pClneo myoD. 24h later cells were incubated with MG132 or LMB for 3h as described above. Thereafter, localization of MyoD was determined by indirect immunofluorescence with a polyclonal anti-MyoD antibody (1:500 dilution, Santa Cruz, #sc-760) and the resulting images were photographed under 40X using an Olympus BX60 microscope.
Results

Isolation of purified nuclei and preparation of nucleoplasm. In an effort to examine the role of the nucleus in the ubiquitin-proteasome dependent degradation of short-lived nuclear proteins, we established a cell free system that supports the ubiquitin-dependent degradation of exogenous protein substrates. This cell-free system is dependent upon highly purified nucleoplasm obtained from HeLa nuclei that were isolated by sucrose density centrifugation. The nuclei are essentially free of cytoplasmic contamination by both microscopic examination and lactate dehydrogenase (LDH) analyses (Figure 1A,C). A typical result for the LDH analysis yielded 1329 units/minute activity for the cytoplasm and 0.17 units/minute activity for the nuclei, for a relative contamination of 0.013%. In addition, SDS-PAGE and western analysis using histones as a marker indicate histones are the major protein species present in the nucleoplasm with essentially no contamination (undetectable by western blot scanning) of the post-nuclear supernatant (Figure 1B).

ATP-dependent degradation of MyoD in HeLa nucleoplasm. With this system, we first examined ATP-dependent degradation of lysozyme as a model protein substrate of the ubiquitin-proteasome system (Hershko et al., 1983). HeLa nucleoplasm served as the source of ubiquitin-proteasome components. All assays were also performed using rabbit reticulocyte lysate in control reactions. After establishing that our cell-free system supports the ATP-dependent degradation of lysozyme (data not shown), we next determined if a physiological substrate of the ubiquitin-proteasomal system could be degraded by the nucleoplasm. As seen in Figure 2B, MyoD is degraded by HeLa nucleoplasm in an ATP-dependent manner. The results from five similar experiments were quantified using Un-Scan-It gel Version 5.1 software and are reported here as total pixels corrected for background pixels. The results seen in the lower
portions of Figure 2B show the ATP-dependent degradation of MyoD in nucleoplasm. The difference between the degree of degradation for the ATP-containing and ATP-depleted reactions is significant at P<0.001. This is also the case for MyoD degradation in the reticulocyte lysate (Figure 2A). In addition, we observed a second immunoreactive band of slightly slower mobility in the (+) ATP reaction for the nucleoplasm (Figure 2B). This band was consistently present in reactions with nucleoplasm, was not observed with the reticulocyte lysate and may represent the phosphorylated form of MyoD described in Song et al. (1998).

**Nucleoplasm degrades MyoD in a ubiquitin and proteasome-dependent manner.** To determine if the degradation of MyoD that occurs in nucleoplasm is proteasome-dependent, we examined the effect of ATPγS and MG132. As seen in Figure 3, MyoD is not degraded in the presence of ATPγS alone. A > 5-fold molar excess of ATPγS is associated with a decrease in the ATP-dependent degradation of MyoD in nucleoplasm as well as in reticulocyte lysate (Figure 3, ATPγS).

The decrease in degradation in the presence of ATPγS is consistent with proteasome-mediated degradation since the ATPase activity at the 19S cap structure of the 26S proteasome is the ATP-dependent step in the ubiquitin-proteasome pathway requiring hydrolysis of the γ-phosphate (reviewed in Coux, Tanaka and Goldberg, 1996). To confirm involvement of the proteasome in nucleoplasm-supported MyoD degradation, we examined the effect of the proteasomal inhibitor, MG132. As seen in Figure 3, MG132 inhibited MyoD degradation in both HeLa nucleoplasm and reticulocyte lysate. These observations indicate that the nuclear proteasome is functional in the recognition and processing of a nuclear substrate.

To verify that the nucleoplasm-supported degradation of MyoD is ubiquitin dependent, we examined the specificity of the ATP-dependent degradation by using inhibitors directed at the
ubiquitin-substrate conjugation reactions. Proteins are tagged for recognition by the 26S proteasome via the conjugation of a polyubiquitin chain to the substrate (reviewed in Hershko and Ciechanover, 1998). This polyubiquitin chain is generated by isopeptide linkages between the carboxyl-terminus of each ubiquitin molecule with lysine 48 of the preceding ubiquitin. This process is competitively inhibited by methylated ubiquitin (Hershko and Heller, 1985) as well as the ubiquitin K48R mutant (Chau et al., 1989). Thus, methylated ubiquitin (MeUb) and ubiquitin K48R (Ub K48R) were added in increasing amounts to the degradation reaction in an effort to overcome the effect of endogenous ubiquitin. As seen in Figure 4, the ATP-dependent degradation of MyoD is competitively inhibited by MeUb and by UbK48R in either HeLa nucleoplasm or reticulocyte lysate. This competitive inhibition can be overcome by the addition of excess wild-type ubiquitin in either case. These results indicate that the ubiquitin-proteasome pathway is responsible for the ATP-dependent degradation of MyoD in HeLa nucleoplasm.

**Ubiquitin conjugation of MyoD in nucleoplasm.** Ubiquitin conjugation assays were performed with isolated nucleoplasm as a direct measurement of the ability of the nuclear ubiquitin system components to support conjugation (Figure 5). $^{35}$S-labeled MyoD is found in higher molecular mass forms typical of ubiquitin conjugate ladders. The MyoD-ubiquitin conjugate ladders occur only in the presence of nuclear extract and are increased by addition of the proteasome inhibitor, ATPγS (Johnston and Cohen, 1981) and ubiquitin aldehyde, an inhibitor of ubiquitin isopeptidases (Hershko and Rose, 1987). In addition, high molecular mass ubiquitin conjugates accompany nucleoplasm-mediated myoD degradation (Figure 6). These results support our conclusion that the nuclear ubiquitin-proteasome system is responsible for the degradation of MyoD.
MyoD is degraded in vivo in the presence of leptomycin B. To determine if the nucleus supports the degradation of MyoD in vivo, we examined the degradation of MyoD in HeLa cells in the presence of either MG132 or leptomycin B. Leptomycin B (LMB) inhibits the CRM-1 dependent nuclear export pathway through a covalent interaction at Cys-529 of CRM-1 (Kudo et al., 1999) and has been used to determine if nuclear export is required in the degradation of nuclear proteins. At 24-48 hours after transfection, MyoD is localized to the nucleus under control conditions as well as during incubation with MG132 and/or leptomycin B (Figure 7). As seen in figure 8, following inhibition of protein synthesis in HeLa cells, the half-life of MyoD in control cells is approximately 1.3 hours, i.e. there is an 80% decrease in MyoD levels during the 3 hour “chase” period. In the presence of MG132, there is only a 7% decrease in the level of MyoD (t½ = 28.4 hr). This is consistent with previous reports of the proteasome-dependent degradation of MyoD (Song et al., 1998; Abu Hatoum et al., 1998). In contrast, MyoD is not significantly stabilized in the presence of leptomycin B. The 63% decrease in the level of MyoD (t½ = 2.1 hr.) (Figure 8) in the presence of leptomycin B is comparable to that seen in the control cells. This result is confirmed in biosynthetic radiolabeling pulse-chase experiments (data not shown).

To ensure that leptomycin B was active in inhibition of nuclear export, we carried out a series of experiments using HeLa cells. Recent studies have shown that IκBα shuttles between the cytoplasm and nucleus (Arenzana-Seisdedos et al., 1997; Sachdev et al., 2000) and is degraded in the cytoplasm (Rodriquez et al., 1999). These studies have also shown that nuclear export and the subsequent degradation of IκBα alone or as a fusion protein with GFP is inhibited by LMB in HeLa cells (Sachdev et al., 2000; Rodriquez et al., 1999). We therefore transfected HeLa cells with GFP-IκBα and followed its fate with immunofluorescence in the presence or
absence of LMB (Figure 9). Our results demonstrate absence of GFP-\(\text{I}\kappa\B\alpha\) from the nucleus under control conditions and accumulation of GFP-\(\text{I}\kappa\B\alpha\) in the nucleus in the presence of LMB, confirming the LMB is biologically active.

**Discussion**

The ubiquitin-proteasome system is essential for the turnover of many short-lived regulatory proteins that are active within the nucleus. The selective degradation of these nuclear proteins is required for cellular processes such as cell cycle progression and transcriptional regulation. Cell cycle progression is marked by the ubiquitin-dependent degradation of nuclear substrates at the G1/S transition and during mitosis. The targeted nuclear proteins include cell cycle kinases and their regulators as well as proteins required for DNA replication (McGarry and Kirschner, 1998) and repair (Watkins et al., 1993; Desai et al., 1997). In addition, ubiquitin-dependent proteolysis of proteins that regulate sister chromatid adhesion serves as a trigger for exit from mitosis (reviewed in Nasmyth, 1999). Degradation of transcription factors and tumor suppressors by the ubiquitin-proteasome pathway is well established (Hershko and Ciechanover, 1998) and recently described examples include ligand-activated transcription factors such as the aryl hydrocarbon receptor (Roberts and Whitelaw, 1999; Davarinos and Pollenz, 1999; Rodriguez et al., 1999) and Smad2 (Lo and Massaque, 1999).

Studies examining the degradation of p53 (Freedman and Levine, 1998; Tao and Levine, 1999), p27\(^{kip1}\) (Tomoda et al., 1999), Cyclin D1 (Diehl et al., 1998), I\(\kappa\B\alpha\) (Rodriguez et al., 1999) and the aryl hydrocarbon receptor (Davarinos and Pollenz, 1999) have demonstrated that nuclear export is required for the turnover of a subset of nuclear proteins. For several of these proteins, ubiquitin-dependent degradation of the substrate appears to be linked to the nuclear export of associated “molecular chaperones”.
The Mdm2 oncoprotein targets p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997) and inhibiting the nuclear export of Mdm2 results in stabilization of p53 in the nucleus (Freedman and Levine, 1998). Jab1-mediated degradation of p27 also requires nuclear export. In this instance, inhibition of nuclear export blocks the ability of Jab1 to shuttle the phosphorylated form of p27 to the cytoplasm (Tomoda et al., 1999). Degradation of the aryl hydrocarbon receptor (AHR), which exists in the nucleus as an AHR-hsp90 complex, is inhibited by blocking nuclear export or by mutating the putative nuclear export sequence of AHR (Davarinos and Pollenz, 1999). From these examples, a model of nuclear protein degradation emerges in which the substrates are shuttled to the cytoplasmic ubiquitin-proteasome pathway after being tagged for degradation by a post-translation modification such as phosphorylation or via association with a molecular chaperone. Furthermore, studies with p27 (Tomoda et al., 1999), AHR (Davarinos and Pollenz, 1999) and Smad2 (Lo and Massague, 1999) indicate that nuclear proteasomal components may act in a modulating capacity in the export of nuclear proteins. The possibility that nuclear ubiquitin-proteasomal system may function to degrade the selected substrates is strengthened by several observations. Most, if not all, of the components of the ubiquitin-proteasome system necessary for the degradation of a substrate are found in the nucleus, including ubiquitin (Schwartz et al., 1988) and the 117 kD isoform of the ubiquitin-activating enzyme, E1 (Stephen et al., 1996). Several of the ubiquitin conjugation enzymes (E2) have been shown to be localized primarily to the nucleus. Cdc34 (Lisztwan et al., 1998; Goebel et al., 1994), an E2 associated with the SCF-type E3 complexes (Skowyra et al., 1997; Feldman et al., 1997) is of particular interest. Expression of dominant-negative deletions of Cdc34 result in the stabilization of p27kip1 in cell extracts (Pagano et al., 1995) and of MyoD in vivo (Song et al., 1998). The SCF-type E3-ubiquitin ligases have been shown to be responsible for the
destruction of a large number of proteins including Sic1p (Skowyra et al., 1997; Feldman et al., 1997) and E2F-1 (Marti et al., 1999) and function as a multisubunit complex (reviewed in Deshaïs, 1999). The Cdc53/Cul-1 subunit of SCF^{SKP2} is localized to the nucleus when expressed with the p45^{SKP2} F-box subunit of the E3 (Lisztwan et al., 1998). These subunits can be immunoprecipitated in a complex containing a third subunit of the E3 complex, p19^{SKP1} as well as Cdc34, suggesting that the active ubiquitin ligase complex can be formed within the nucleus. In addition, all of the components of the 26S proteasome are found in the nucleus (Reits et al., 1997; Enenkel et al., 1998; Wilkinson et al., 1998; Russell et al., 1999; Brooks et al., 2000).

Finally, studies using a folding mutant of the influenza virus nucleoprotein that is localized to the promyelocytic leukemia oncogenic (POD) domains in the nucleus indicate that the PODs are the site of concentration, and perhaps generation of polyubiquitin conjugates. Proteasomal subunits are also colocalized therein. Indirect evidence suggests that the ubiquitin-proteasome dependent degradation of the misfolded substrate occurs within the nucleus (Anton et al., 1999). A functional nuclear ubiquitin-proteasome system may provide additional levels of regulation of intracellular proteolysis via the cellular targeting of a substrate as well as via compartment-specific activities of its components.

In the present study, we developed a cell-free system based on HeLa nucleoplasm in order to examine the ability of isolated nucleoplasm to support ubiquitin-dependent degradation. This cell-free system allows for the direct examination of any specific nuclear component of the ubiquitin-proteasome pathway required for the turnover of nuclear substrates. It was essential to ensure that the nucleoplasm is not contaminated with cytoplasmic ubiquitin-proteasome components. We assessed cross-contamination of the cytoplasm and nucleoplasm with protein markers; lactate dehydrogenase activity (cytoplasmic marker) and histone content (nuclear
marker). Using this approach, we were able to obtain nucleoplasm that is essentially free of cytoplasmic contamination (i.e. < 0.02%).

We have established that HeLa nucleoplasm contains all the components of the ubiquitin-proteasome pathway necessary for degradation of a physiological nuclear substrate, MyoD. Recent studies have investigated the ubiquitin-dependent degradation of MyoD using both in vivo (Song et al., 1998; Kitzmann et al., 1999) and in vitro approaches (Abu-Hatoum et al., 1998; Breitschopf et al., 1998). In vitro studies (Breitschopf et al., 1998) using rabbit reticulocyte lysate have shown that MyoD is degraded by a mechanism involving attachment of ubiquitin at the N-terminal residue. In vivo studies indicate that phosphorylation at serine 200 is required for the proteasome-mediated degradation of MyoD (Song et al., 1998; Kitzmann et al., 1999). We demonstrate herein that degradation of MyoD in isolated nucleoplasm involves the 26S proteasome as shown by the stabilization of MyoD in the presence of ATPγS and MG132. We confirmed that the ATP-dependent degradation of MyoD in nucleoplasm is ubiquitin-dependent by using the polyubiquitin-conjugation inhibitors, methylated ubiquitin and ubiquitin K48R. Inhibition by each of these ubiquitin variants can be overcome by the addition of excess wild-type ubiquitin, indicating that ubiquitin activation and conjugation are required for the nucleoplasm-dependent degradation of MyoD. Moreover, in the nucleoplasm, high molecular mass MyoD-ubiquitin conjugates are formed in the presence of ATPγS, ubiquitin aldehyde and MG132. Taken together, our results demonstrate that the nuclear components of the ubiquitin-proteasome system are able to function in the recognition and degradation of a nuclear protein.

However, these results do not directly address the question of the cellular locus of MyoD degradation. To confirm that MyoD is degraded in the nucleus in vivo, we examined the stability of MyoD in the presence of leptomycin B. Biosynthetic radiolabeling pulse-chase experiments
demonstrate that MyoD is degraded in the presence or absence of leptomycin B. Furthermore, experiments in which protein synthesis is inhibited with cycloheximide show that although the addition of MG132 prolongs the half-life of MyoD from 1.3 to greater than 20 hours, the half-life of MyoD is relatively unchanged (1.3-2.1 hr) in the presence of leptomycin B (Figure 8). These results indicate that nuclear export is not required for the degradation of MyoD.

While the specific role(s) and importance of nuclear protein degradation are not known at present, it is clear that the ubiquitin-mediated degradation of various proteins is regulated by compartmentalized localization within the cell. Herein, the nucleus appears to be a dynamic organelle in terms of protein degradation. As discussed above, many of the components of the ubiquitin-proteasome system are localized to the nucleus, some in a temporally controlled manner. For example, while most of the subunits of the SCF complex are localized to both cytoplasm and nucleus, several F-box proteins (e.g., Cdc4 and Met30 in yeast (Rouillon et al., 2000)) localize specifically to the nucleus. This suggests that the subcellular compartmentalization of F-box proteins may be responsible for spatially-regulated SCF-mediated degradation (DeShaies, 1999). Individual substrates of the ubiquitin-proteasome system including several nuclear proteins (e.g., p53) are however degraded within the cytoplasm following chaperone-mediated export from the nucleus (see above). The precise signals which target an individual substrate for site-specific ubiquitin-proteasome mediated destruction remain largely unclear. Since submission of the present study, Blondel et al (2000) has shown that the yeast cyclin dependent kinase inhibitor Far1 is ubiquitinated and degraded in the nucleus via the nuclear SCF^{Cdc4} complex. Thus, these results together with the present results, demonstrate that a functional ubiquitin-proteasome system exists within the nucleus and allows for the regulated proteolysis of selected nuclear proteins. The signals which govern the targeting to the nucleus as
well as those processes which expose the determinants for ubiquitin-protein ligase (E3) recognition and polyubiquitination are yet to be fully defined. It is possible that turnover of MyoD, the focus of the present study, is regulated differently from other nuclear proteins given the unique requirement for ubiquitination at the N-terminal amino group rather than an internal lysine (Breitschopf et al., 1998). This will be the subject of future studies.

Acknowledgments

We thank Mark Hannick for the GFP-I\(\kappa\)B\(\alpha\) construct and Minoru Yoshida for the leptomycin B. This study was supported by National Institutes of Health (ALS), Israel-US Binational Foundation (AC, ALS), and the Israel Academy of Sciences and Humanities-Centers of Excellence Program, the German-Israeli Foundation for Scientific Research and Development (G.I.F.), the German-Israeli Cooperation Project (DIP), and a TMR grant from the European Community (AC).

References


2727-2730.


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**Figure Legends**

Figure 1. Purification of HeLa nucleoplasm.

(A) Nuclei were isolated from HeLa cells following disruption in a hypotonic solution followed by sucrose density gradient centrifugation. The purified nuclei were subjected to sonication and incubated with DNase I prior to centrifugation to obtain the nucleoplasm.

(B) The resulting nucleoplasm (N) along with the HeLa cytoplasm (C) (50 µg protein each lane) was analyzed by 12.5% SDS-PAGE followed by Coomassie staining and western blotting for histones.

(C) The purified nuclei (●), whole cell homogenate (○), cytoplasm (■), and cytosol (□) were assayed for lactate dehydrogenase activity using equivalent amounts of protein. Total activity was calculated as described in Experimental Procedures.

Figure 2. MyoD is degraded in an ATP-dependent manner by HeLa nucleoplasm.

MyoD (400 ng) was added to the reaction mixtures and degradation was assayed by western blotting with anti-MyoD as described in Experimental Procedures. The reaction mixtures contained an ATP-regenerating (+ATP) or ATP-depleting (to -ATP) system and were incubated for 2 hours at 37 °C except for the to reaction, which was kept at 4 °C during the incubation period. The results of five independent assays were quantified.

(*) indicates significance (<0.001).

Figure 3. Degradation of MyoD in HeLa nucleoplasm is dependent on the 26S proteasome.

Degradation reactions were carried out in the presence of either 5 mM ATPγS or 20 µM MG132. The ATPγS was added alone or in addition to the ATP-regenerating system (ATP reg). MG132
dissolved in DMSO was added to reactions containing the ATP-regenerating system. DMSO alone was run as a control.

Figure 4. Degradation of myoD in HeLa nucleoplasm is dependent on ubiquitin. Degradation reactions were carried out in the presence of the polyubiquitin chain terminators, MeUb and ubiquitin K48R. Each inhibitor was added in increasing amounts (5, 10, 15 µg/12.5 µl reaction) to reactions containing the ATP-regenerating system (ATP reg). Wild-type ubiquitin (20 µg) was added in two-fold excess over MeUb or Ub K48R (10 µg) in additional reactions. Both the t₀ and (ATP-) reactions contained the ATP-depleting system. The t₀ reactions were kept at 4 °C during the 2 hour incubation. Reticulocyte lysate or HeLa nucleoplasm served as the cellular fraction.

Figure 5. HeLa nucleoplasm supports the generation of myoD-ubiquitin conjugates. ³⁵S-labeled myoD was prepared in a coupled transcription-translation system as described in the text. The reaction was thereafter inactivated with NEM/DTT. Conjugation assays were performed in the absence or presence of HeLa nucleoplasm (NE), and in the absence or presence of the proteasome inhibitors ATPγS plus ubiquitin aldehyde (ATPγS), as described in the text. Lanes 1 and 4 were incubated at 4°C. High molecular mass myoD-ubiquitin conjugates resolved on SDS-PAGE are noted (conj). MyoD is indicated by the arrowhead.

Figure 6. High molecular mass ubiquitin conjugates accompany HeLa nucleoplasm-mediated myoD degradation. Degradation reactions containing 100 ng myoD and supported by either reticulocyte lysate (left) or nucleoplasm (right) were carried out in the presence of ATP and/or
MG132, plus ubiquitin aldehyde (Ubal) and ATPγS as described in the text. Each reaction mix was analyzed via SDS-PAGE and immunoblot with antibody to myoD. High molecular mass myoD-ubiquitin conjugates are noted (conj.). MyoD is noted by the arrowhead. Note that in the left lane (+ATP) the myoD band is weak as it is degraded efficiently.

Figure 7. Immunofluorescent localization of MyoD. HeLa cells were transfected with pClneo MyoD. 24h later cells were incubated with MG132 or LMB for 2h as described above. Thereafter, localization of MyoD was determined by indirect immunofluorescence with a polyclonal anti-MyoD antibody (Santa Cruz) and the resulting images were photographed under 40X using an Olympus BX60 microscope. A = control, B = MG132 (20µM), C = LMB (10µM).

Figure 8. MyoD is degraded in vivo in the presence of leptomycin B. (A) HeLa cells were transiently transfected with MyoD and 24 hours later were incubated with cycloheximide (100 µg/ml) for the indicated times. The relative amount of MyoD at each time point was determined by 10% SDS-PAGE followed by western blotting with anti-MyoD as described in Experimental Procedures. The reactions were carried out in the presence of MG132 (20 µM) or leptomycin B (10 nM) as indicated. (B) The relative amounts of MyoD were quantified from 3 independent experiments and the half-lives determined as described in Experimental Procedures.

Figure 9. Leptomycin B inhibits the nuclear export of GFP-IκBα. HeLa cells were transiently transfected with GFP-IκBα. 24 hours later the cells were incubated for 1 hour in the absence (A) or presence (B) of 10 nM leptomycin B. GFP-IκBα was localized by indirect immunofluorescence as described in Experimental Procedures.
Figure 2

A

B

Reticulocyte Lysate

Nucleoplasm

total pixels - background x 10^-2

ATP

t0  +  -

*
Figure 3

ATPγS

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Reticulocyte Lysate

Nucleoplasm

MG-132

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Reticulocyte Lysate

Nucleoplasm
Figure 4

Methylated Ubiquitin

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Reticulocyte Lysate

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Nucleoplasm

Ubiquitin K48R

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Conj.
Figure 6

Reticulocyte Lysate

Nucleoplasm

+Ubal
+MG132
+ATP

+ATPγS

Conj.
Figure 8

A

control
MG132
LMB

time (hr) 0 0.5 1 2 3

B

control MG132 LMB

total pixels - background

time (hr) 0 3 0 3 0 3

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Z Elizabeth Floyd, Julia S. Trausch-Azar, Eyal Reinsein, Aaron Ciechanover and Alan L. Schwartz

J. Biol. Chem. published online April 17, 2001

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