Myogenin Protein Stability Is Decreased by BMP-2 through a Mechanism Implicating Id1*

Francès Viñals‡‡ and Francesc Ventura‡‡‡

From the Unitat de Bioquímica, Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, E-08907 L’Hospitalet de Llobregat, Spain.

Bone morphogenetic protein-2 (BMP-2) induces a switch in differentiation of mesenchymal cells from the myogenic to the osteogenic lineage. Here we describe that in C2C12 cells, BMP-2 decreases myogenin expression induced by des-(1,3)insulin-like growth factor-1 (des-(1,3)IGF-1) or ectopically expressed from a constitutive promoter, even in conditions where myogenin mRNA levels were unaffected. Addition of BMP-2 decreases myogenin protein half-life to 50%, whereas proteasome inhibitors abolish these effects. Forced expression of Id1, either by transient transfection or under the control of an inducible system, causes degradation of myogenin in the absence of BMP-2. In contrast, E47 overexpression blocks the inhibitory effect of BMP-2 on myogenin levels. Finally, expression of E47 in 293 cells stabilizes myogenin, an effect that is dependent on the heterodimerization mediated by their helix-loop-helix.

Our findings indicate that induction of Id1 not only blocks transcriptional activity but also induces myogenin degradation by blocking formation of myogenin-E47 protein complexes.

Mesenchymal cells differentiate into distinct cell types, such as adipocytes, osteoblasts, or myoblasts. Differentiation has two stages; first is the commitment to a particular cell lineage, and secondly, cells start to express the proteins that characterize their final phenotype. Commitment to a specific lineage depends on mutually exclusive factors. Thus, signals that induce a particular phenotype repress others. For example, signals that induce the osteoblastic phenotype, such as BMPs (bone morphogenetic proteins), repress myogenic differentiation in vitro (1, 2) and induce bone after implantation in muscle in vivo (3). The capacity of mesenchymal cells to differentiate into distinct cell types is a result of the expression and function of determination genes, which include distinct families of transcription factors known as master genes. Thus, the fate of the mesenchymal precursors depends on the expression of a specific combination of master genes: the myogenic basic helix-loop-helix (bHLH) family for myoblasts, the peroxisome proliferator-activated receptor-γ (PPAR-γ) and C/EBP enhancer-binding protein (C/EBP) families for adipocytes, and core binding factor α-1 (Cbfa-1) and Osterix for osteoblasts (4–6).

The myogenic bHLH family, also known as muscle regulatory factors (MRFs), is formed by four members, MyoD, myogenin, Myf5, and MRF4 (4, 7). These were identified by their ability to induce the differentiation of non-muscle cells into a muscle phenotype (8, 9). Whereas MyoD and Myf5 are involved in the initial determination state, myogenin and MRF4 participate in terminal differentiation (7). The MRFs, which belong to the superfamily of bHLH transcription factors, contain a conserved basic DNA-binding domain and a helix-loop-helix motif, which is essential for dimerization. MRFs heterodimerize with the E family of ubiquitously expressed bHLH factors (10, 11). Assembly of the heterodimer allows correct juxtaposition of the two basic regions, which leads to binding and activation of E-boxes (CANNTG), DNA motifs in the promoters of skeletal muscle-specific genes (12, 13).

Mesenchymal cells use redundant mechanisms to ensure that the muscle differentiation program is activated only at the appropriate moment. These mechanisms probably also control the timing of other mesenchymal differentiation programs with the concomitant block of muscle differentiation. The first checkpoint is the expression of the master genes for the myogenic program. Thus, myogenin is absent in proliferating myoblasts, and its expression is induced by factors that stimulate the myogenic program, such as the decrease in growth factors or presence of IGF-1 (9, 14, 15). Moreover, expression of MyoD and myogenin mRNA is suppressed by incubation with BMP-2 (16). The second level of control is a covalet modification, mainly phosphorylation of the transcription factors. Thus, myogenin is phosphorylated by protein kinase C (17) and calmodulin-dependent kinase II (CaMKII) (18, 19) in its DNA-binding domain, thereby inhibiting DNA binding. Similarly, overexpression of protein kinase A inhibits the transcriptional activity of MRF4 (20) and myogenin (21). The third level of control is through interaction with other proteins that regulate the
activity of transcription factors. For example, the hypophosphorylated form of the retinoblastoma protein (Rb) associates with MyoD and is required for the efficient transactivation of E-box-containing muscle-specific promoters (22). Moreover, several proteins that participate in the control of the cell cycle, such as Fos, Jun, or the adenosvir protein E1A, directly interact with MRFs and inhibit their transcriptional activity (23–25). Finally, the helix-loop-helix transcription factors can be negatively regulated by the Id family of proteins (26–28). Id members are helix-loop-helix factors that lack the basic region that allows DNA binding. Thus, Ids heterodimerize with the E-factors and inhibit their binding to DNA. Ids sequester ubiquitous E-factors (27, 29) and act as dominant negative regulators with respect to the tissue-specific helix-loop-helix proteins (MyoD, myogenin, etc.).

Here we describe a new posttranscriptional mechanism implicated in the inhibition of the myogenic program by BMP-2, the abrupt degradation of myogenin by a mechanism that involves the proteasome. This mechanism is dependent on the induction of Id proteins by BMP-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant BMP-2 was obtained from the Genetics Institute, and N-acetyll-Leu-Leu-norleucinal (LNL) was from Sigma. Des-(1,3)IGF-1 was from Angiela F. Schutzelle (Tubingen, Germany). Cell culture media, fetal bovine serum, glutamine, and antibiotics were obtained from Invitrogen. The other reagents were of analytical or molecular biology grade and were purchased from Sigma or Roche Applied Science.

**Cell Culture and Transfections**—C2C12 mouse cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate. Confluent cells were differentiated in DMEM containing 2% horse serum. Depleted of growth factors for 24 h in DMEM containing 0.1% fetal bovine serum.

Confluent cells were differentiated in DMEM containing 2% horse serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate. Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate. Confluent cells were differentiated in DMEM containing 2% horse serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate.

**C2C12 Cells**—C2C12 cells were transiently transfected after addition of differentiation medium with piresbleo-Id1 (the murine Id1 cDNA was obtained by PCR, sequenced, and subcloned into piresbleo from Clontech), pCDNA3-E47 (a generous gift from Dr. Pura Muñoz-Canoles), or pCDNA3 using polyethylenimine (PEI) (Aldrich) (30). Stable clones of C2C12 cells were generated by transfecting 10 µg of pCDNA3-myogenin or pcDNA3 and selection using G418 (400 µg/ml) for 2–3 weeks. We used three clones that expressed distinct levels of myogenin (Fig. 2, Clones 5, 7, and 8) or the C2C12-pCDNA3 control cells.

We generated an inducible clone of Id1 in C2C12 cells (C2C12-pTISN-Id1) following the Tet-Off protocol described by Chamard and Pogonec (31). First, we used two distinct vectors that code for tetracycline-regulated transactivator (tTA) and puromycin resistance under the control of a tTA-responsive promoter (tetO-CMV) to generate C2C12 cells that stably expressed the tTA. After selection of clones resistant to puromycin, we transfected Id1 cloned in the pTISN vector (which expressed neomycin resistance), where Id1 was under the control of the tTA-responsive promoter. After selection, clones expressed Id1 in inverse proportion to the tetracycline concentration in the culture medium. The concentration of tetracycline was 100 ng/ml in all experiments. To reverse the effect of tetracycline, cells were incubated for 12 h in the presence of tetracycline, rinsed five times with phosphate-buffered saline (PBS), and incubated in normal medium in the absence of tetracycline for the times indicated.

HEK-293 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells were transiently transfected using polyethylenimine. The expression vectors encoding full-length E47, E47 lacking the helix-loop-helix (E473HLL, which codes for amino acids 1–386), or E47 containing the helix-loop-helix (E471HLL, which codes for amino acids 487–649) were kindly provided by Dr. P. Muñoz-Canoles and tagged with Myc.

**Western Blot Analysis**—Cells were washed twice in cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 200 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 1 µg/ml leupeptin, 4 µg/ml apronin, 1% Triton X-100) for 15 min at 4 °C. Western blots were performed as described previously (2). The blots were incubated with polyclonal Id1 antibody (Santa Cruz Biotechnology), monoclonal antimyogenin 1F8 antibody (a generous gift from Dr. Pura Muñoz-Canoles, Centre de Regulació Genòmica, Barcelona), polyclonal E47 antibody (Santa Cruz Biotechnology), monoclonal anti-β-actin (Sigma), polyclonal anti-ERK2 (32), or monoclonal anti-Myc (Sigma) in blocking solution overnight at 4 °C.

**Northern Blot**—Total RNA from cells was extracted using the phenol/chloroform method (33), and Northern blot with 20 µg of RNA was performed as described previously (2). Blots were hybridized to the mouse myogenin cDNA or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA labeled with [α-32P]dCTP (Amersham Biosciences).

**Immunofluorescence Studies**—Cells were cultured on glass coverslips for 24 h and transfected with the plasmids indicated. 48 h after transfection, cells were rinsed three times with PBS and fixed in 3% paraformaldehyde for 20 min. After four washes with PBS, they were permeablized with PBS, 0.2% Triton X-100 for 5 min, rinsed four times with PBS, and blocked for 30 min at room temperature in PBS containing 2% bovine serum albumin. Coverslips were incubated with mouse monoclonal 1F8 antimyogenin antibody, rabbit polyclonal anti-Id1 antibody (Santa Cruz Biotechnology), or rabbit polyclonal anti-E47 antibody (Santa Cruz Biotechnology) in blocking solution for 1 h at room temperature, followed by Texas Red- or fluorescein isothiocyanate-conjugated anti-mouse or anti-rabbit antibody (Molecular Probes) for 1 h at room temperature. Finally, cells were incubated with Hoechst 33258 (Sigma) for 5 min at room temperature. Coverslips were mounted using Mowiol (Calbiochem), and immunofluorescence was visualized with a Nikon Eclipse E800 microscope.

**RESULTS**

**BMP-2 Induces the Degradation of Myogenin in C2C12 Cells**—C2C12 cells incubated for 3 days with a limited supply of growth factors (2% horse serum) or in the presence of 2 nm des-(1,3)IGF-1 (a potent myogenic inducer) (15, 34) differentiated to multinucleated myotubes. These myotubes expressed typical skeletal muscle markers, such as myogenin (Fig. 1A). Treatment with BMP-2 not only blocked myotube formation (2, 35) and myogenin expression (Fig. 1A) but also stimulated typical osteoblastic markers, such as osteocalcin or alkaline phosphatase (2, 36). Simultaneous treatment of cells with both factors abolished myogenin expression (Fig. 1A), whereas the osteoblastic markers were maintained (data not shown). Furthermore, the addition of des-(1,3)IGF-1 12 or 24 h after BMP-2 did not reverse its inhibitory effects, and levels of myogenin remained low. In contrast, addition of BMP-2 12 or 24 h after IGF-1 caused a progressive decrease in myogenin levels (Fig. 1A). Thus, the effects of BMP-2 on myogenin expression were dominant over those of des-(1,3)IGF-1.

BMP-2 inhibits transcription of the myogenin gene (16). To evaluate the time course of the inhibitory effect of BMP-2 on myogenin expression, we incubated C2C12 cells in the presence of BMP-2, des-(1,3)IGF-1, or both factors for 4 or 8 h. After a 4-h incubation, BMP-2 induced Id1, an inhibitor of the family of helix-loop-helix transcription factors (28, 29, 37), and its expression was maintained until 8 h (Fig. 1B). Des-(1,3)IGF-1 induced myogenin mRNA and protein at 4 and 8 h (Fig. 1C) but did not inhibit Id1 induction by BMP-2. Incubation in the presence of BMP-2 blocked the myogenin protein induction not only at 8 h but also at 4 h, in conditions where the myogenin mRNA induction was unaffected (Fig. 1, B and C).

To identify the mechanisms involved in the effect of BMP-2 on myogenin protein levels over short times, we first evaluated a possible effect of BMP-2 on the IGF-1 signaling pathways. C2C12 cells were preincubated in the presence of BMP-2 followed by the addition of des-(1,3)IGF-1 for 30 min or 1 h. We did not detect any difference in ERK1/2 or phosphatidylinositol 3-kinase stimulation by des-(1,3)IGF-1 in the presence of the presence of BMP-2 (data not shown). The observation that BMP-2 blocked myogenin protein induction at 4 h, independently of transcriptional regulation, led us to hypothesize that BMP-2 affects myogenin protein stability. To test this hypothesis, we generated stable clones of C2C12 that express myogenin under...
the control of an independent promoter (cytomegalovirus promoter of the vector pcDNA3). We generated three independent clones (Fig. 2A, Clones 5, 7, and 8) with distinct expression levels of the myogenin mRNA and protein. Myogenin transcription from these clones did not respond to BMP-2 (Fig. 2A), indicating the dependence of a limiting factor that controlled myogenin degradation by BMP-2. To confirm the data obtained by Western blot experiments, we performed immunofluorescence studies with antibodies against myogenin. As described previously (38), myogenin was immunolocalized in the nucleus of C2C12 cells (Fig. 2C). Incubation for 4 h with BMP-2 caused a significant decrease in the myogenin signal, without causing a clear effect of protein relocalization at the subcellular level.

**Fig. 1.** BMP-2 blocks myogenin induction by IGF-1. A, C2C12 cells were incubated for 3 days in differentiation medium containing 2% horse serum in the absence (DM) or presence of 2 nM BMP-2 (BMP) or 2 nM des-(1,3)IGF-1 (IGF). Where indicated, BMP-2 or des-(1,3)IGF-1 was added 12 h (+IGF or BMP 60h) or 24 h (+IGF or BMP 48h) after the addition of the differentiation medium containing the other cytokine. Finally, both factors were added simultaneously for 3 days (+B+I). After these incubations, cells were lysed, and Western blot was performed using antmyogenin and anti-ERK2 (as a loading control) antibodies. B, depleted C2C12 cells were incubated for 4 or 8 h in the absence (C) or presence of 2 nM BMP-2, 2 nM des-(1,3)IGF-1, or both (B+I). Myogenin, Id1, and ERK2 (as a loading control) were immunodetected as described. C, using the same protocol as in B total RNA was obtained and analyzed by Northern blot. After blotting, myogenin was detected by hybridization using a specific probe. 18 S ribosomal RNA is shown as a loading control.

**Fig. 2.** BMP-2 induces degradation of the myogenin protein. A, C2C12 clones stably overexpressing myogenin (Clones 5, 7, and 8) were incubated for 0, 4, 8, or 24 h in the presence of 2 nM BMP-2. After these times cells were lysed, and myogenin, Id1, or ERK2 (as a loading control) were immunodetected using specific probes. B, C2C12 cells overexpressing myogenin (Clones 5 and 8) or mock-transfected cells were incubated for 0, 4, 8, or 24 h in the presence of 2 nM BMP-2. Cells were lysed, and total RNA was obtained as described. After blotting, myogenin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as a loading control) was detected by hybridization using specific probes. C, cells of Clone 8 overexpressing myogenin were incubated in the absence (0h BMP-2) or in the presence for 4 h of 2 nM BMP-2 (4h BMP-2). After this time cells were fixed, and myogenin was immunolocalized.

**Myogenin Is Degraded by a Proteasome-dependent Mechanism—**To confirm an induction of the degradation of the myogenin protein by BMP-2, we incubated cells with BMP-2 for 1 h, and after this preincubation, we added cycloheximide, a protein synthesis inhibitor, and then harvested cells after a range of times. In control conditions myogenin had a short half-life of about 60 min (Fig. 3A), which is consistent with the findings of other studies (39). Addition of BMP-2 increased the rate of myogenin degradation, causing the half-life to fall to 30 min, confirming that the short term effect of BMP-2 was because of an increase in myogenin degradation.

The BMP-2-dependent decrease in myogenin protein levels may be explained by its degradation by a ubiquitin-proteasome system. Proteasome is one of the systems most often used by the cellular machinery to control the levels of subtly regulated proteins (40, 41). To assess this possibility, we preincubated cells in the presence of LLnL, a potent inhibitor of the 26 S proteasome (42). Addition of the inhibitor in the absence of BMP-2 caused a clear increase in the levels of myogenin (Fig. 3B) both in its major 36-kDa form and in a higher M protein, because of its phosphorylation (data not shown) (39, 43). These results indicate that myogenin was degraded in basal conditions by a proteasome-dependent mechanism. More importantly, addition of LLnL before incubation with BMP-2 abolished the effect of the cytokine, and the accumulation of
myogenin was at levels comparable with those obtained in the LLnL condition (Fig. 3B).

Id1 Is Sufficient to Mediate the BMP-2-induced Degradation of Myogenin—One of the mediators of the antimyogenic response of BMP-2 is the induction of the Id proteins that act as dominant negative inhibitors of the tissue-specific helix-loop-helix transcription factors (36, 37, 44). Given these effects, one possibility was that the induction of Id by BMP-2, by sequestering E-factors, not only blocked the transcriptional activity of myogenin but also served as an intermediate for the increase in myogenin degradation. To test this, we transiently transfected clones of C2C12 that expressed ectopic myogenin with Id1, and we immunolocalized both proteins. Cells that did not express Id1 clearly expressed myogenin in the nucleus (Fig. 4A). Id1 was localized in the nucleus and cytoplasm as described before (45, 46). The overexpression of Id1 in the absence of BMP-2 always caused a decrease in the levels of myogenin.

To further confirm these effects in a more controlled system, we used the Tet-Off system to generate an inducible Id1 expression system in C2C12 cells. Id1 expression in these cells was an inverse function of the tetracycline in the medium. Thus, in the presence of 100 ng/ml tetracycline in the medium for 12 h, Id1 expression was abolished (Fig. 4B). After removal of tetracycline, expression levels increased with time but did not exceed the physiological levels obtained after 4 h of stimulation with BMP-2 (Fig. 4B). In these conditions of Id1 expression and in the absence of other effects caused by the presence of BMP-2, the myogenin protein decreased in an inverse function of Id1. These results confirmed that Id1 was sufficient to cause the drop in the levels of myogenin working as a mediator for the effects of BMP-2.

E47 Protects Myogenin from Degradation Induced by BMP-2—The E family of transcription factors is essential for the transcriptional activity of the MRFs on the specific E-boxes of the muscle-specific gene promoters (12, 13). If Id1 promotes degradation of myogenin by releasing this factor from the heterodimer with the E-members, overexpression of an E-member should prevent the effect of BMP-2. To evaluate this, we transfected E47 expression constructs in C2C12 cells expressing ectopic myogenin. Incubation for 8 h with BMP-2 caused a large decrease in the myogenin expression in these cells (Fig. 5A). In contrast, most cells that overexpressed E47 (Fig. 5A, green nuclei) maintained the expression of myogenin and were refractory to the BMP-2 effect. 5% of the control cells that
expressed green fluorescent protein still maintained the myogenin expression after 8 h in the presence of BMP-2. In contrast, 90% of cells that overexpressed E47 maintained the myogenin expression. To biochemically confirm these data, mock or E47 expression plasmids were cotransfected with a plasmid that encoded puromycin resistance. After 24 h of transfection, puromycin was added to the culture medium to select cells that had incorporated the expression plasmids. The resistant cells were incubated for 0, 4, or 8 h in the presence of 2 nM BMP-2. Overexpression of E47 blocked myogenin degradation even 8 h after the addition of BMP-2 (Fig. 5). In contrast, myogenin was degraded by BMP-2 in the control cells that expressed pcDNA3. These results confirmed the hypothesis that Id was a mediator for the effects of BMP-2 observed through a mechanism that involved the sequestering of endogenous E-proteins.

To confirm these data obtained analyzing the endogenous myogenin protein in C2C12 cells, we expressed myogenin in HEK-293 cells. Basal levels of myogenin expression in these cells were low (Fig. 6A). As it was observed in C2C12 cells, incubation with LLnL caused a strong increase in the levels of myogenin. Co-expression of E47 with myogenin caused the stabilization of the protein in the absence of LLnL, confirming the stabilizing role of heterodimerization with E-proteins. In contrast, addition of a Myc tag to the N terminus of myogenin blocked its high degradation rate as it was highly expressed in the absence of LLnL (Fig. 6A). Finally, to analyze the importance of myogenin-E47 heterodimer formation in myogenin stability, we made similar assays using two E47 deletion mutants (E47/H9004HLH, defective in heterodimer formation (47), and E47/H11001HLH, which contains the bHLH). The E47 mutant unable to heterodimerize with myogenin was also defective in myogenin stabilization (Fig. 6B). In contrast, the mutant containing the bHLH stabilized myogenin even more that the wild type form of E47. All of these data indicated that myogenin protein stability was strongly enhanced in the heterodimer state with E-proteins, which would be competed by the presence of Id1.

**DISCUSSION**

BMP-2 induces the osteoblastic phenotype of mesenchymal cells and causes a concomitant blockade of the myogenic pro-
Myogenin Protein Stability Is Decreased by BMP-2

Myogenin function. The decrease in the levels of myogenin causes the blockade of the autostimulatory loop promoted by these myogenic bHLH factors (39, 50) and elicits the decrease in myoblast-specific markers. Moreover, these effects could promote cells already expressing myogenin to change their commitment and lose the myoblastic phenotype. Such effects constitute an essential feature in multipotent cells that express distinct sets of master genes and could trigger distinct differentiation pathways, such as muscle satellite cells (51).

Myogenin (Ref. 39 and this work) and other helix-loop-helix factors such as MyoD (50, 52, 53) and Id proteins (54, 55) have a short half-life of between 30 and 60 min. All of these proteins are degraded by mechanisms that involve ubiquitination and translocation to the proteasome. For instance, MyoD, Id1, and Id2 are degraded by the proteasome after its ubiquitination in the N-terminal residue without the participation of internal lysines (56–58). Recent results obtained with MyoD and Id1 indicate that the internal lysine-dependent pathway for protein ubiquitination and degradation seems to be more active in the cytoplasm, whereas the N-terminus-dependent pathway would be the most important in the nucleus (58, 59). Degradation of myogenin occurs mainly in the nucleus (59, 60) in contrast with other nuclear proteins that are degraded in the cytoplasm (61, 62). Furthermore, our results show a protection of myogenin degradation by the addition of a Myc6 tag to the N-terminal residue (Fig. 6A). Thus, it seems likely that myogenin would be degraded using the N-terminal pathway as is MyoD in the nucleus. Further studies would be required to confirm this hypothesis.

We have shown that overexpression of E47 is sufficient to overcome the degradation induced by BMP-2. Thus, this protein may be the limiting factor that accelerates a faster degradation of myogenin in clones that express lower amounts of myogenin (Fig. 2). The same effect was observed for Id proteins; overexpression of E47 attenuates the rapid elimination of Id proteins (55, 63). Moreover, Mash1, a neurogenic bHLH member, is degraded by the proteasome in the absence of its E-partner.2 Similarly, Id1 accelerates the degradation of MyoD in assays in vitro through direct interaction between them (53). However, myogenin does not interact directly with Id proteins (64), which discards the possibility of myogenin having a mechanism similar to that of MyoD. In the case of MyoD, its phosphorylation by the complex cdk2-cyclin E in Ser200 promotes the degradation of the transcription factor (52, 65), a mechanism implicated in the blockade of MyoD activity in proliferating myoblasts. A similar mechanism is not likely to be involved in the case of myogenin, which is absent in proliferating myoblasts (9, 14, 66) and lacks a residue equivalent to the Ser200 of MyoD. In fact, the hyperphosphorylated form of myogenin is more stable than the hypophosphorylated form (39), indicating that phosphorylation of myogenin by an unknown kinase would have protective effects rather than stimulate degradation.

A possible mechanism involved in the protection of myogenin from degradation would implicate the formation of a stable heterodimer with E-protein, whereas free myogenin, not bound to the E-protein, would be targeted to the proteasome. One possible explanation is that monomeric myogenin serves as a substrate for an E3-ubiquitin ligase. Formation of myogenin-E-protein heterodimers either could hinder the recognition sites for the E3-ligase directly or could promote posttranslational modifications that prevent this recognition. On the basis of our results, we propose a new posttranscriptional mechanism for the negative control of the myogenic program by BMP-2. This mechanism involves induction of Id1 by BMP-2, which, through its ability to block formation of protective complexes with E-proteins, not only blocks myogenin transcriptional activity but also is sufficient to increase monomeric myogenin, which is then rapidly degraded.

Acknowledgments—We thank the Genetics Institute for providing BMP-2, Dr. Pura Munoz-Canoves (Centre de Regulació Genòmica [CRG], Barcelona, Spain) for IF8 antibody, myogenin, and E47 plasmids, Dr. Jose Carlos Perales (University of Barcelona) for polyethyleneimine reagent and transfection methodology, Dr. Anna Bigas (Institut de Recerca Oncològica [IRO], Barcelona) for help with microscopy, Jean Claude Chambard (CNRS-Nice) for the Tet-Off methodology and plasmids, and Maria Moler, Ester Adanero, and Aurea Navarro for technical support.

REFERENCES

1. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. (1991) J. Cell Biol. 113, 681–687
2. Vinals, F., Lopez-Rovira, T., Rosa, J. L., and Ventura, F. (2002) FEBS Lett. 510, 99–104
3. Urist, M. R., DeLange, R. J., and Finerman, G. A. (1983) Science 220, 680–686
4. Molkentin, J. D., and Olson, E. N. (1996)Curr. Opin. Genet. Dev. 6, 445–453
5. Rosen, E. D., and Spiegelman, B. M. (2000) Annu. Rev. Cell Dev. Biol. 16, 145–171
6. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002) Cell 108, 17–29
7. Parker, M. H., Seale, P., and Rudnicki, M. A. (2003) Nat. Rev. Genet. 4, 497–507
8. Davis, H. L., Weintraub, H., and Lassar, A. B. (1997) Cell 91, 987–1000
9. Edmondson, D. G., and Olson, E. N. (1989) Genes Dev. 3, 628–640
10. Murre, C., McCave, P. S., Vaessen, H., Caudy, M., Jan L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauenschild, S. D., Lassar, A. B., Weintraub, H., and Baltimore, D. (1989) Cell 58, 537–544
11. Hu, J. S., Olson, E. N., and Kingston, R. E. (1992) Mol. Cell. Biol. 12, 1031–1042
12. Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985) Science 227, 134–140
13. Blackwell, T. K., and Weintraub, H. (1990) Science 250, 1104–1110
14. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) Cell 56, 607–617
15. Florini, J. R., Evton, D. Z., and Magri, K. A. (1991) Annu. Rev. Physiol. 53, 201–216
16. Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1997) Exp. Cell Res. 230, 342–351
17. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) Cell 71, 1311–1318
18. Blagden, C. S., Fromm, L., and Burden, S. J. (2004) Mol. Cell. Biol. 24, 1983–1989
19. Tang, H., Macpherson, P., Argetsinger, L. S., Cieslak, D., Suh, S. T., Carter-Su, C., and Goldman, D. (2004) Cell 116, 551–563
20. Hardy, S. K., Yong, K., and Konieczny, S. F. (1993) Mol. Cell. Biol. 13, 5943–5956
21. Li, L., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) Mol. Cell. Biol. 12, 4478–4485
22. Wu, G., Schneider, J. W., Cordorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) Cell 72, 309–324
23. Bengal, E., Ransone, L., Soderberg, R., Dwariki, V. J., Tapsfcott, S. J., Weintraub, H., and Verma, I. M. (1992) Cell 68, 507–519
24. Li, L., Chambard, J. C., Karin, M., and Olson, E. N. (1992) Genes Dev. 6, 676–680
25. Taylor, D. A., Kraus, V. B., Schwartz, J. J., Olson, E. N., and Kraus, W. E. (1993) Mol. Cell. Biol. 13, 4714–4727
26. Beneza, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61, 49–59

2 Vinals, F., Reiriz, J., Ambrosio, S., Bartrons, R., Rosa, J. L., and Ventura, F., EMBO J., in press.
27. Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. (1998) Trends Cell Biol. 8, 58–65
28. Ruzinova, M. B., and Benezra, R. (2003) Trends Cell Biol. 13, 410–418
29. Norton, J. D. (2000) J. Cell Sci. 113, 3897–3905
30. Abdallah, B., Hassan, A., Benoist, C., Goula, D., Behr, J. P., and Demeneix, B. A. (1996) Hum. Gene Ther. 7, 1947–1954
31. Chambard, J. C., and Pognonec, P. (1998) Nucleic Acids Res. 26, 3443–3444
32. McKenzie, F. R., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 13476–13483
33. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
34. Kaliman, P., Canicio, J., Shepherd, P. R., Beeton, C. A., Testar, X., Palacin, M., and Zorzano, A. (1998) Mol. Endocrinol. 12, 66–77
35. Chalaux, E., Lopez-Rovira, T., Rosa, J. L., Bartrons, R., and Ventura, F. (1998) J. Biol. Chem. 273, 537–543
36. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994) J. Cell Biol. 127, 1755–1766
37. Ogata, T., Wozney, J. M., Benezra, R., and Noda, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9219–9222
38. Brennen, T. J., and Olson, E. N. (1990) Genes Dev. 4, 582–595
39. Edmondson, D. G., Brennen, T. J., and Olson, E. N. (1991) J. Biol. Chem. 266, 21343–21346
40. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) Science 286, 579–582
41. Zhou, J., and Olson, E. N. (1994) Mol. Cell. Biol. 14, 6232–6243
42. Lopez-Rovira, T., Chalaux, E., Massague, J., Rossa, J. L., and Ventura, F. (2002) J. Biol. Chem. 277, 3176–3185
43. Jen, Y., Weintraub, H., and Benezra, R. (1992) Genes Dev. 6, 1466–1479
44. Tintignac, L. A., Leibovitch, M. P., Kitzmann, M., Fernandez, A., Ducommun, B., Meijer, L., and Leibovitch, S. A. (2000) Exp. Cell Res. 259, 300–307
45. Brunetti, A., and Goldfine, I. D. (1990) J. Biol. Chem. 265, 5960–5963
