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

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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.

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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 adenosinergic 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-acetylated-Leu-Leu-norleucine (LLNL) was from Sigma. Des-(1,3)IGF-1 was from Angelika F. Schutzdeller (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% bovine serum albumin and 2% horse serum in the presence or absence of 2 nM BMP-2 or 2 nM des-(1,3)IGF-1 (a potent myogenic inducer) (15, 34) differentiating transfected cells with 2% bovine serum albumin. Coverslips were incubated with mouse monoclonal anti-β-actin (Sigma), polyclonal anti-ERK2 (32), or monoclonal anti-Myo (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 [32P]dCTP (Amersham Biosciences). The concentration of tetracycline was 100 ng/ml in all experiments. To reverse the effect of tetracycline, cells were incubated for 12 h

**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) differentiating transfected cells with 2% bovine serum albumin. Coverslips were incubated with mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology), polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology), monoclonal anti-β-actin (Sigma), polyclonal anti-ERK2 (32), or monoclonal anti-Myo (Sigma) in blocking solution overnight at 4 °C.

**Nordic 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 [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 permeabilized 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 anti-β-actin antibody, rabbit polyclonal anti-ERK1 antibody (Santa Cruz Biotechnology), or rabbit polyclonal anti-ERK3 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.

**Id1 Induction by BMP-2**—We generated an inducible clone of Id1 in C2C12 cells (C2C12-pTISN-Id1) following the Tet-Off protocol described by Chambard and Pognonec (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 (tet-O-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 expressing 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.

**BMP-2 Induces the Degradation of Myogenin in C2C12 Cells**—BMP-2 induces myogenin mRNA and protein at 4 and 8 h (Fig. 1C). 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 inhibited 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).

**Id1 Induction by BMP-2**—We generated an inducible clone of Id1 in C2C12 cells (C2C12-pTISN-Id1) following the Tet-Off protocol described by Chambard and Pognonec (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 (tet-O-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 expressing 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**—HEK-293 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells were transiently transfected using polyethyleneimine. The expression vectors encoding full-length E47, E47 lacking the helix-loop-helix (E47ΔHLH, which codes for amino acids 1–386), or E47 containing the helix-loop-helix (E47+HLH, which codes for amino acids 387–649) were kindly provided by Dr. P. Muñoz-Canoves 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 NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin, 4 μg/ml aprotinin, 1% Triton X-100) for 15 min at 4 °C. Western blots were performed as described previously.
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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. 2B). In all the cases BMP-2 caused a time-dependent decrease in the levels of myogenin protein, which was already observed 4 h after the addition of BMP-2 (Fig. 2A). The rate of myogenin decrease depended on the initial levels of myogenin expressed in each clone. Thus, in those that expressed low levels, the rate of decrease was faster than in those that expressed high myogenin levels (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.

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 L6L6, 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 form 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 L6L6 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

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**FIG. 3.** BMP-2 increases the degradation rate of myogenin. A, C2C12 cells incubated for 48 h in differentiation medium were maintained for 1 h in the absence (−BMP-2) or in the presence of 2 nM BMP-2 (+BMP-2). After this time the protein synthesis inhibitor cycloheximide (10 μg/ml) was added. At the times indicated, cells were lysed, and myogenin or Id1 was analyzed by Western blot. Values represent the mean ± S.E. of three independent experiments. B, C2C12 cells overexpressing myogenin (Clone 5) were preincubated for 15 min in the presence of 50 μM LLnL or vehicle. After this time cells were incubated for 4 h in the presence of only LLnL, 2 nM BMP-2, or both as indicated. Cells were lysed, and myogenin and Id1 were immunodetected by Western blot as described.

**FIG. 4.** Id1 overexpression is sufficient to induce myogenin degradation. A, C2C12 cells stably overexpressing myogenin (Clone 5) were transiently transfected with a plasmid coding for Id1. After 48 h, cells were fixed, and Id1 (green) and myogenin (red) were immunostained using appropriate antibodies. Nuclei from cells were stained using Hoechst 333258 (blue). Two images from two separate experiments are shown. B, C2C12-pTISN-Id1 cells were incubated for 12 h in the presence of 100 ng/ml tetracycline or its vehicle ethanol (0.1% final). After this time 2 nM BMP-2 was added (+BMP) for 4 h or not (−), or the cells were rinsed five times with PBS and incubated in the absence of tetracycline for 4 h (−T 4h) or 8 h (−T 8h). Cells were lysed, and myogenin, Id1, and β-actin were immunodetected by Western blot as described. A representative experiment is shown.
expressed green fluorescent protein still maintained the myogenin expression after 8 h of incubation 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. 5B). 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 Myc6 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 (E47HLH, which codes for amino acids 1–386), or E47 containing the helix-loop-helix (E47H+HLH, which codes for amino acids 387–649) all tagged with Myc. 24 h after transfection cells were treated or not with LLnL for 12 h. Cells were lysed, and myogenin, E47, and Id1 were analyzed by Western blot.

**DISCUSSION**

BMP-2 induces the osteoblastic phenotype of mesenchymal cells and causes a concomitant blockade of the myogenic pro-
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Myogenin is implicated in the blockade of MyoD activity 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.

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