EGLN3 Prolyl Hydroxylase Regulates Skeletal Muscle Differentiation and Myogenin Protein Stability*

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EGLN3, a member of the EGLN family of prolyl hydroxylases, has been shown to catalyze hydroxylation of the α subunit of hypoxia-inducible factor-α, which targets hypoxia-inducible factor-α for ubiquitination by a ubiquitin ligase complex containing the von Hippel-Lindau (VHL) tumor suppressor. We now report that EGLN3 levels increase during C2C12 skeletal myoblast differentiation. EGLN3 small interference RNAs and EGLN3 antisense oligonucleotides blocked C2C12 differentiation and decreased levels of myogenin, a member of the MyoD family of myogenic regulatory factors, which plays a critical role in myogenic differentiation. We also report that EGLN3 interacts with and stabilizes myogenin protein, whereas VHL associates with and destabilizes myogenin via the ubiquitin-proteasome system. The effect of VHL on myogenin stability and ubiquitination can be reversed, at least in part, by overexpression of EGLN3, suggesting that its binding to myogenin may prevent VHL-mediated degradation. These data demonstrate a novel role for EGLN3 in regulating skeletal muscle differentiation and gene expression. In addition, this report provides evidence for a novel pathway that regulates myogenin expression and skeletal muscle differentiation.

EGLN3 (also known as PHD3, HPH1, and SM 20) is a member of a family of genes that are homologs of the Caenorhabditis elegans gene egl-9 (1). The rat homolog of EGLN3 is a growth factor-responsive gene originally identified as “SM-20” in a differential screen of cultured smooth muscle cells (2). EGLNs contain a highly conserved domain common to the superfamily of 2-oxoglutarate-dependent dioxygenases (3). EGLN3 encodes an intracellular prolyl hydroxylase that is involved in the cellular response to oxygen availability by hydroxylating the α subunits of transcription factor hypoxia inducible factor (HIF)2 (4, 5). Prolyl hydroxylation of HIF-α targets it for polyubiquitination by the von Hippel-Lindau (VHL)-containing E3 ubiquitin ligase complex and proteasomal degradation (6–8) and, thus, regulates the expression of hypoxia-responsive genes (9). EGLN3 interacts with E3 ubiquitin ligases Siah1a and Siah2, the mammalian homologs of Drosophila Seven in Absentia protein (10). Siah1a and Siah2 target EGLN2 and -3 for ubiquitination and subsequent proteasomal degradation (10).

In addition to regulating cellular response to oxygen availability, EGLN3 appears to play a role in cellular homeostasis. p53-mediated growth arrest and apoptosis in RAS-transformed rat embryo fibroblasts is associated with up-regulation of EGLN3 (11). Induction of apoptosis in cultured rat sympathetic neurons by withdrawal of nerve growth factor is also associated with up-regulation of EGLN3 (12), and overexpression of EGLN3 in these neurons and in PC12 cells results in apoptotic cell death (13–15). EGLN3 has also been identified as a c-Myc-responsive gene (16, 17) and shown to mediate anti-proliferative effects of angiotensin II (18).

We have previously reported that EGLN3 is expressed in adult skeletal muscle and is induced during differentiation of mouse C2C12 skeletal myoblasts; in contrast, EGLN1 and -2 are constitutively expressed in C2C12 (19). EGLN3 is also developmentally regulated in skeletal muscle (20). EGLN3 staining is first observed at E8.5 in the dermomyotomal cells of the rostral somites, and its expression progresses is in a rostral to caudal pattern, with highest levels seen in the muscle primordial and mature muscles. These studies prompted us to consider the possibility that EGLN3 might be involved in the regulation of skeletal muscle differentiation. The formation of skeletal muscle is mainly orchestrated by a family of myogenic regulatory factors (MRFs), including MyoD, Myf-5, myogenin, and MRF4 (21, 22). A wide array of proteins can interact with and regulate the myogenic potential of these MRFs. We now show that EGLN3 regulates differentiation of C2C12 myoblasts and enhances levels of myogenin, a critical determinant of skeletal muscle differentiation. In addition, we demonstrate that EGLN3 is a novel binding partner for myogenin and that it antagonizes VHL-mediated ubiquitination and degradation of the myogenin protein. These studies suggest that EGLN3 plays an important role in mediating skeletal muscle differentiation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies included anti-EGLN3 (Novus), anti-myogenin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-troponin T (Sigma), anti-VHL (BD Pharimgen and Santa Cruz Biotechnology), anti-Myc (Sigma), anti-FLAG (Sigma), anti-HA (Santa Cruz), anti-β-actin (Sigma),...
anti-α-tubulin (Sigma), anti-lamin A/C (Cell Signaling), and anti-ubiquitin (Santa Cruz Biotechnology). Cycloheximide was purchased from Sigma. Protease inhibitors were supplied by Sigma and Roche Applied Science.

Expression Constructs—The PCR-amplified full-length EGLN3 coding sequence with or without a C-terminal FLAG tag (DYKDDDDK) was subcloned into pcDNA3.1 (Invitrogen). The constructs were verified by DNA sequencing. The mouse myogenin expression vector was a gift from Dr. R. Mech. The GST-tagged myogenin expression vector was provided by Dr. S. Burden. Wild-type and mutant VHL expression plasmids were obtained from M. Schoell. Wild-type and mutant ubiquitin expression plasmids were supplied by Dr. Y. Jin.

Cell Culture and Transfection—HEK 293 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Murine C2C12 skeletal muscle myoblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum and antibiotics (growth medium). To induce myogenic differentiation, C2C12 myoblasts at ~70–90% confluency were shifted from growth medium into Dulbecco’s modified Eagle’s medium containing 2% horse serum and antibiotics (differentiation medium). Cells were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

siRNAs and Antisense Oligonucleotides—Nucleotides corresponding to bases 359–377 and 621–640 of the mouse EGLN3 coding region were chosen as targeting sequences for siRNAs. SMARTpool EGLN3 siRNA and VHL siRNA were purchased from Dharmacon and Ambion. siRNAs were transfected into cells by using the Lipofectamine 2000 reagent. Nucleotides corresponding to bases 10–29, 273–286, and 709–730 of the mouse EGLN3 coding region were chosen as the targeting sequences for antisense oligonucleotides. The antisense and corresponding sense oligonucleotides were purchased from Invitrogen and directly applied to C2C12 cells.

RT-PCR—Total RNA was extracted from C2C12 cells using an RNasy Mini Kit (Qiagen) and utilized to synthesize the first strand cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). To obtain the complete open reading frame of mouse EGLN3, PCR amplification was conducted using Pfu polymerase (Stratagene) under the following conditions: 1 cycle (94 °C for 2 min), 30 cycles (94 °C for 45 s, 58 °C for 45 s, and 72 °C for 90 s), and 1 cycle (72 °C for 10 min). To examine the expression of myogenin and glyceraldehyde-3-phosphate dehydrogenase mRNA in differentiated C2C12 cells, RT-PCR was conducted using a one-step RT-PCR kit (Eppendorf) under the following conditions: 1 cycle (52 °C for 50 min), 1 cycle (94 °C for 2 min), 22 cycles (94 °C for 22 s, 60 °C for 22 s, and 68 °C for 50 s), and 1 cycle (72 °C for 10 min). Reverse transcriptase was omitted from the reaction as a control. Primer sequences are available on request.

Northern Blot Analysis—Total RNA was isolated from cultured cells using an RNasy Mini Kit (Qiagen). Agarose gel electrophoresis and transfer to nitrocellulose were performed as previously described (2). Hybridization of 32P-labeled probes was conducted at 68 °C by using a Quick-Hyb™ hybridization mixture (Clontech) according to the manufacturer’s instructions. Equal loading of lanes was verified by staining the 18 S and 28 S ribosomal RNA with ethidium bromide.

Cellular Fractionation—All fractionation and centrifugation steps were performed at 4 °C using ice-cold buffers. C2C12 cells grown in differentiation medium were harvested, washed with PBS, and resuspended in Buffer A (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitors). Cells were incubated on ice for 15 min and then homogenized using a Dounce homogenizer. The nuclei were separated by centrifugation at 500 × g for 5 min. The supernatant was collected as cytosolic fraction. The pellet, containing the nuclei, was washed with PBS and then resuspended in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% glycerol, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 50 mM NaF, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture, 1 μg/ml pepstatin) for 10 min on ice, centrifuged, and collected the supernatant (nuclear fraction). The cytosolic and nuclear fractions were analyzed using Western blot.

Western Blotting and Immunoprecipitation—Cells were harvested in Triton X-100-based lysis buffer or radioimmunoprecipitation assay buffer as described previously (23). The whole cell lysates were clarified, and the soluble fractions were recovered and quantified using the Bio-Rad DC protein assay kit (Bio-Rad). For immunoprecipitation, precleared lysates (~300–500 μg of extracts) were incubated with specific antibody, or as a negative control normal mouse or rabbit IgG (Santa Cruz Biotechnology) for 2 h at 4 °C, followed by the addition of Protein A/G plus agarose beads (Santa Cruz Biotechnology). The incubation was continued for 2 h or overnight at 4 °C with constant agitation. After extensive washing with lysis buffer as described above, the immunoprecipitated materials were eluted in SDS-PAGE loading buffer (125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 10% 2-mercaptoethanol, 0.02% bromphenol blue), heated for 5 min, fractionated by SDS-PAGE (4–20% gel, Life Thera-peutics), and subjected to Western blot analysis as described (23).

GST Pulldown Assays—Expression and purification of the myogenin GST fusion protein (GST-myogenin) was conducted as described previously (23). GST alone or the fusion protein was immobilized on a glutathione resin that had been preblocked in PBS containing 0.5% nonfat milk and 0.05% bovine serum albumin, and incubated 4 h at 4 °C with the precleared cell lysates from transfected HEK 293 cells (23). After extensive washing, the complex was eluted with SDS-PAGE sample buffer, run on 12% polyacrylamide gels, visualized by Coomassie Blue staining, or detected by Western blotting.

Myogenin Decay Assays—HEK 293 cells transfected with expression vectors (as indicated in results), were exposed to the protein synthesis inhibitor cycloheximide (30 μg/ml final concentration). At the times indicated, total cell lysates were subjected to Western blotting using an antibody to myogenin. The blots were stripped and re-probed with an anti-β-actin antibody to confirm the equal protein loading. Gels were scanned and densitometry, employing a 256 grayscale, was performed using Image 2.0 software (Wayne Rasband, National Institutes
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The density of the myogenin band was normalized to β-actin and expressed as a percentage of the density measured at time 0. Statistical significance ($p < 0.05$) was determined using a two-tailed Student’s $t$ test.

Ubiquitinylation Assays—Cells were transiently transfected with plasmids encoding myogenin, His-tagged ubiquitin, and VHL and then lysed in 6 M guanidinium-HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM Tris, 10 mM imidazole, 10 mM β-mercaptoethanol (pH 8). Ni$^{2+}$-nitrilotriacetic acid-agarose beads were added to the lysates, and the mixture was incubated with rotation at room temperature for 4 h. The beads were successively washed and eluted as described previously (24). The eluate was subjected to Western blot analysis with anti-myogenin antibody. A ubiquitination assay was performed as described previously (23). Briefly, cells were transfected with the plasmids as indicated. Cells were harvested in the Triton-based lysis buffer. Cell lysates were subjected to immunoprecipitation using an anti-myogenin antibody, followed by the Western blot analysis with anti-ubiquitin antibody (Santa Cruz Biotechnology).

Immunoﬂuorescence—Cells were ﬁxed in 4% paraformaldehyde for 15 min at room temperature, permeabilized in PBS (pH 7.2) containing 0.1% Triton X-100 for 5–10 min at room temperature, and then blocked in PBS with 1% bovine serum albumin. Cells were incubated with an anti-MF20 monoclonal antibody (Developmental Studies Hybridoma Bank) at 4 °C overnight (to stain myosin heavy chain, a differentiation marker) followed by incubation with Texas Red-conjugated anti-mouse IgG (Santa Cruz Biotechnology) for 45 min at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (0.1 μg/ml). At least 800 nuclei were counted from several random ﬁelds. The fusion index was calculated as follows: (the number of nuclei within MF20-positive myocytes containing three or more nuclei/total number of nuclei analyzed) × 100. Statistical signiﬁcance ($p < 0.05$) was determined using a two-tailed Student’s $t$ test.

RESULTS

EGLN3 Is Up-regulated during C2C12 Differentiation—Mouse C2C12 skeletal muscle myoblasts have been widely used as an in vitro model to investigate the regulatory mechanisms underlying skeletal muscle differentiation (25). To examine the proﬁle of EGLN3 expression during C2C12 differentiation, we ﬁrst conducted Northern blot analysis. As shown in Fig. 1A, EGLN3 mRNA was up-regulated as early as 24 h after C2C12 cells were grown in differentiation medium to induce cell differentiation, as judged by troponin I, a biochemical marker of skeletal muscle differentiation. This ﬁnding is consistent with our previous report (19). To determine whether EGLN3 protein is increased upon cell differentiation, extracts from C2C12 myoblasts...
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shifted from growth medium to differentiation medium were analyzed on Western blots. As shown in Fig. 1B, EGLN3 protein was increased during differentiation as assessed by myogenin, an early biochemical marker of skeletal muscle differentiation (26–28).

EGLN3 Positively Regulates Gene Expression and Differentiation of Skeletal Muscle—To determine the role of EGLN3 in regulation of gene expression and differentiation of skeletal muscle, an expression vector encoding EGLN3 was introduced into C2C12 cells. These cells displayed elevated levels of EGLN3 expression (Fig. 2A). Overexpression of EGLN3 led to a considerable increase in the expression of myogenin, and to a lesser extent, troponin T (Fig. 2A), another differentiation marker and a downstream target of myogenin (29–31). Importantly, overexpressing EGLN3 promoted earlier differentiation as evidenced by induction of more and larger myotubes at 48 h (Fig. 2C).

To ascertain the role of endogenous EGLN3 in regulating myogenin expression and differentiation, siRNAs specific for EGLN3 and control siRNAs were transfected into C2C12 cells. EGLN3 siRNAs efficiently decreased EGLN3 and myogenin expression (Fig. 2B). Concomitantly, treatment of cells with EGLN3 siRNA led to impairment of differentiation (Fig. 2D). Moreover, the fusion index revealed that treatment of C2C12 cells with EGLN3 siRNA resulted in a marked reduction in fusion capacity (Fig. 2E). In addition, an EGLN3-antisense oligonucleotide markedly diminished C2C12 cell differentiation, whereas no effect was seen with a sense oligonucleotide (Fig. 2F). Identical results were achieved with two additional antisense oligonucleotides (data not shown).

EGLN3 Interacts with and Stabilizes Myogenin—Because EGLN3 is known to interact with several proteins, we examined whether EGLN3 associated with myogenin. COS-7 cells were transfected with plasmids encoding myogenin and/or FLAG-tagged EGLN3. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody (Ab) or mouse IgG (as a control), followed by immunoblotting with an anti-myogenin Ab. Myogenin was present in the anti-FLAG immunoprecipitates but was absent from the control precipitates (Fig. 3A). Identical results were achieved with HEK (human embryonic kidney) 293 cells (data not shown). To further examine this interaction, myogenin with a GST tag at its N terminus (GST–myogenin) was bacterially expressed and purified. A GST pulldown assay was then performed by incubation of the GST–myogenin fusion protein or GST alone (as a control) with extracts derived from HEK 293 cells expressing EGLN3. As shown in Fig. 3B, EGLN3 was retrieved by the GST–myogenin fusion protein, but not by GST alone, suggesting that EGLN3 specifically interacts with myogenin. To determine whether endogenous EGLN3 and myogenin can interact, extracts from C2C12 cells were immunoprecipitated with an anti-myogenin Ab and then immunoblotted with antibodies to EGLN3 or myogenin. GST or GST fusion proteins (arrows) were visualized by Coomassie Blue staining. C, proteins isolated from proliferating (Prol) or differentiating (Dif) C2C12 cells were immunoprecipitated (IP Ab) with antibodies to myogenin or control mouse immunoglobulin (mIg), and then analyzed by immunoblotting (IB) with anti-EGLN3. Also indicated on the figure is the immunoglobulin light chain (LC). C, C2C12 cells were induced to differentiate. Cytosolic (C) and nuclear (N) fractions were prepared and subjected to Western blot analysis using the antibodies as indicated.
myogenin transcript levels (Fig. 4B). To examine myogenin stability, myogenin levels were measured in cells incubated with the protein synthesis inhibitor cycloheximide. As shown in Fig. 4C, EGLN3 increased the stability of the Myc-tagged myogenin protein. By densitometric analysis of Western blots \((n = 3)\), the half-life of Myc-tagged myogenin increased from \(\sim 60\) min to 120 min in the presence of EGLN3. To further reveal the role of endogenous EGLN3 in the stability of myogenin, we evaluated the impact of EGLN3 siRNA on the steady-state level of myogenin. Consistent with the finding shown in Fig. 4C, knockdown of EGLN3 led to a markedly decreased stability of endogenous myogenin (Fig. 4D).

VHL Interacts with Myogenin—The ubiquitin-proteasome pathway is the principal system for proteolysis of short-lived proteins in mammalian cells (32). We hypothesized that EGLN3 might stabilize myogenin by interfering with the polyubiquitination and degradation of myogenin, and that the VHL

VHL Destabilizes Myogenin Protein via the Ubiquitin-proteasome Pathway—Because VHL interacts with myogenin, we tested the possibility that VHL can catalyze the assembly of polyubiquitin (Ub) chain on the myogenin protein using a ubiquitination assay. Myogenin was transfected into HEK 293 cells with His-tagged Ub in the presence or absence of VHL. Lysates were incubated with Ni\(^{2+}\)-nitrilotriacetic acid beads to isolate the ubiquitinated proteins, and the eluates were examined by Western blotting with antibodies to myogenin and VHL. Consistent with the finding shown in Fig. 4C, knockdown of EGLN3 led to a markedly decreased stability of endogenous myogenin (Fig. 4D).

To determine whether VHL regulated the abundance of myogenin, HEK 293 cells were co-transfected with myogenin and increasing amounts of VHL. Western blot analysis demonstrated that VHL down-regulated expression of myogenin protein containing ubiquitin ligase complex was involved. We therefore determined if VHL interacted with myogenin. HEK 293 cells were transfected with myogenin and/or VHL. Total cellular extracts were immunoprecipitated with an anti-VHL Ab and then immunoblotted with anti-myogenin. Myogenin was detected in the anti-VHL precipitates (Fig. 5A). An identical result was achieved in the reciprocal co-immunoprecipitation experiment in which extracts were immunoprecipitated with an anti-myogenin and then probed with anti-VHL (Fig. 5B). To determine if endogenous myogenin and VHL interacted, extracts prepared from differentiated C2C12 cells were immunoprecipitated with an anti-VHL Ab and then immunoblotted with anti-myogenin. As shown in Fig. 5C, myogenin was precipitated by anti-VHL Ab but not by the IgG control. To demonstrate the subcellular localization of VHL and myogenin in differentiated C2C12 cells, we performed cytosolic and nuclear fractionation. Interestingly, subcellular fractionation showed that VHL was primarily observed in the cytosolic fraction with minor portion being found in the nuclear fractions. Myogenin was present in both the cytosolic and nuclear fractions (Fig. 3D). Together, our results suggest that the interaction between VHL and myogenin occurs primarily in the cytoplasm.
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FIGURE 5. Interaction of pVHL with myogenin. A, HEK 293 cells were transfected with myogenin and/or HA-tagged VHL (VHL/HA). Extracts were immunoprecipitated (IP) with anti-VHL. The immunoprecipitates (left panel) and extracts (right panel) were examined by immunoblotting (IB) with the indicated antibodies. HC, IgG heavy chain; LC, IgG light chain. Arrow denotes myogenin. B, HEK 293 cells were transfected with Myc-tagged myogenin in the presence or absence of VHL/HA. Extracts were immunoprecipitated (IP) with anti-Myc. The immunoprecipitates (top panel) and extracts (bottom panels) were immunoblotted (IB) with the indicated antibodies. C, extracts from differentiated C2C12 cells were immunoprecipitated (IP Ab) with anti-VHL or mouse IgG (mIg), and the precipitates were immunoblotted (IB) with anti-body to myogenin. The arrow denotes myogenin. All experiments were performed three times.

protein in a concentration-dependent manner (Fig. 6B). Like with EGLN3, this appeared to be due to changes in protein stability, as shown in a cycloheximide chase experiment (Fig. 6C). To determine if polyubiquitination of myogenin by VHL is essential for myogenin degradation, a vector expressing VHL C162F, a mutant that is unable to interact with elongin C/Cullin2 and thus lacks activity to ubiquitinate the target protein (33), was expressed in HEK 293 cells. Unlike wild-type VHL, the mutant exhibited no effect on myogenin expression (Fig. 6D), suggesting that polyubiquitination of myogenin is necessary for its degradation by VHL.

To determine if the effect of VHL on myogenin is mediated by the proteasome, cells were transfected with myogenin and VHL in the presence of MG132, a widely used proteasome inhibitor (23). Treatment of cells with MG132 blocked the effect of VHL, resulting in accumulation of myogenin protein (Fig. 6E).

The dominant negative Ub mutant UbK48R, in which Lys-48 is replaced by Arg, has been shown to inhibit the elongation of polymerized Ub chain and proteasomal degradation of proteins (23). As shown in co-transfection experiments, the UbK48R mutant stabilized myogenin protein and rescued VHL-mediated degradation of myogenin (Fig. 6F). To evaluate the effect of knockdown of VHL on the endogenous myogenin expression, we transfected C2C12 cells with VHL siRNA. VHL siRNA efficiently deleted the expression of VHL (Fig. 6G, middle panel, lane 4). VHL siRNA treatment increased myogenin protein level (Fig. 6G, upper panel, lane 4). In contrast, C2C12 cells transfected with control siRNA or no siRNA exhibited no change in VHL or myogenin expression (Fig. 6G, lanes 2 and 3). Taken together, knockdown of VHL increased myogenin expression. These results strongly suggest that VHL accelerates polyubiquitination and therefore proteasomal degradation of myogenin.

EGLN3 Prevents Myogenin from Ubiquitination and Degradation—To test the possibility that EGLN3 stabilizes myogenin by shielding it from VHL-triggered ubiquitination and degradation, co-transfection experiments were performed with combinations of myogenin, EGLN3, and HA-tagged VHL. Extracts were immunoprecipitated with anti-HA and then immunoblotted with anti-myogenin Ab. As shown in Fig. 7A, the VHL–myogenin interaction was markedly reduced by co-expression of EGLN3.

To determine if EGLN3 could inhibit ubiquitination of myogenin by VHL, cells were transfected with myogenin and Ub in the presence or absence of VHL and EGLN3. Lysates were immunoprecipitated with an anti-myogenin Ab and then probed with an anti-Ub Ab. As shown in Fig. 7B, the VHL-enhanced ubiquitination of myogenin was blocked by co-expression of EGLN3.

Most importantly, VHL-mediated decrease in myogenin levels was reversed by co-transfection of EGLN3 (Fig. 7C). These data suggest that EGLN3 stabilizes myogenin, at least in part, by preventing VHL-mediated ubiquitination and degradation of myogenin.

**DISCUSSION**

This study demonstrates that the prolyl hydroxylase EGLN3 regulates skeletal muscle differentiation by binding to and controlling the stability of myogenin. It also demonstrates that VHL interacts with and negatively regulates the stability of myogenin via the ubiquitin-proteasome pathway. The effect of EGLN3 on myogenin stability appears to be due to its ability to block the effect of VHL on myogenin. This study thus suggests that EGLN3 is involved in a novel pathway that controls skeletal muscle differentiation.

EGLN3 is a member of a recently described family of HIF prolyl hydroxylases, including EGLN1, EGLN2, and EGLN3 (4, 5). All three members have intrinsic enzymatic activity and regulate the prolyl hydroxylation of the α subunits of HIF (6–8). Each member displays differences in substrate specificity (4, 34), tissue distribution (19, 35), patterns of subcellular localization (36, 37), and inducibility (2, 4, 11, 13, 38). EGLN1 appears to be the primary HIF prolyl hydroxylase in the cell types examined thus far (39).

Although there is considerable information concerning the biochemical activity of the EGLNs, their biological roles remain to be elucidated. EGLN3 is induced in sympathetic neurons after nerve growth factor withdrawal and stimulates apoptosis when overexpressed in pheochromocytoma cells (12–15). The induction of neuronal apoptosis requires prolyl hydroxylase activity and is specific for EGLN3 (14). Interestingly, several studies have demonstrated additional binding partners for the EGLNs, including Siah1a/2, Tric, ING4, OS-9, and Morg1 (10, 40–43). None of these has been shown to be a substrate for the EGLN prolyl hydroxylase, nor has the biological consequence of their binding to the EGLNs been fully elucidated. Taken together, these findings suggest that EGLN3 may participate in diverse cellular processes depending upon the cell type and the
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![Image](60x368 to 396x733)

**FIGURE 6.** VHL destabilizes myogenin via the ubiquitin-proteasome pathway. A, HEK 293 cells were transfected with vectors expressing His-tagged ubiquitin (His-Ub), myogenin, or VHL. Cell lysates were incubated with Ni²⁺-nitrilotriacetic acid beads to pull down the ubiquitinated proteins and the eluates immunoblotted (IB) with anti-myogenin. The ubiquitinated myogenin is indicated as (Ub)n-myog. Right panel, whole cell lysates were blotted with the indicated antibodies. B, Western blot analysis of extracts from HEK 293 cells transfected with myogenin and various amounts of VHL/HA. The blots were stripped and reprobed with β-actin antibody. C, HEK 293 cells were transfected with myogenin alone or with VHL and then treated with 30 μg/ml cycloheximide for the times indicated. Extracts were immunoblotted (IB) with anti-myogenin. The blots were stripped and reprobed with anti-β-actin. D, myogenin was transfected into HEK 293 cells together with wild-type (WT) or mutant (mut) VHL or the empty vector (−). Extracts were immunoblotted (IB) with anti-myogenin and anti-VHL. The blots were stripped and re-probed with anti-β-actin. E, myogenin was transfected into HEK 293 cells alone or with VHL/HA. Some cells were then treated the proteasome inhibitor MG132 (10 μM) for 6 h before harvesting. Extracts were immunoblotted (IB) with anti-myogenin or anti-HA. The blots were stripped and reprobed with an anti-β-actin. F, wild-type (WT) or the K48R mutant (mut) of ubiquitin (Ub) were transfected into HEK 293 cells together with myogenin with or without HA-tagged VHL. Cell lysates were immunoblotted (IB) with the indicated antibodies. The blots were stripped and probed with anti-β-actin. G, C2C12 cells were transfected with control siRNA (lane 3) or VHL siRNA (lane 4). As the controls, cells were mock transfected (without siRNA in transfection mixture, lane 2) or untransfected (lane 1). Cells were then cultured in differentiation medium for 48 h. Whole cellular extracts were examined by Western blot using the indicated antibodies. NS, nonspecific.

extracellular cues. The current study demonstrated that myogenin is also a binding partner for EGLN3. More importantly, we demonstrate that the binding of EGLN3 to myogenin has important biological consequences, including stabilization of myogenin and regulation of skeletal muscle differentiation. It remains to be determined whether EGLN3 is competent to catalyze hydroxylation of myogenin.

Skeletal muscle specification and differentiation are mainly orchestrated by a family of MRFs, including MyoD, Myf5, myogenin, and MRF4 (21, 22). The MRFs heterodimerize with a ubiquitously expressed basic helix-loop-helix proteins called “E” proteins, and the resultant heterodimers then bind to the consensus sequence (CANNTG) designated “E box” present in the promoters and enhancers of muscle-specific genes, hence activating muscle gene transcription (44, 45). Genetic studies and other experiments have established that myogenin is instrumental in controlling myogenesis (46, 47). Myogenin acts downstream of MyoD and Myf5 to activate muscle differentiation genes. Myogenin null mice survive fetal development, but die on birth due to a severe deficiency in skeletal muscle (46, 47). Myoblasts form and migrate to their correct embryonic locations, but most do not fuse to form myofibers. Despite the importance of myogenin for skeletal muscle differentiation, little is known about how the steady-state levels of this key molecule are regulated.

The HIF-1 pathway is involved in the regulation of multiple cellular processes, such as cell survival, apoptosis, cell cycle progression, differentiation, and tumor progression. A recent study by Yun et al. (49) has indicated that no significant changes in skeletal muscle differentiation are observed when C2C12 cells are infected with retrovirus containing oxygen-insensitive or constitutively active form of HIF-1α. Mason et al. (50) have demonstrated that targeted deletion of HIF-1α in skeletal muscle affects the energy metabolism of muscles, whereas the development of skeletal muscle seems to be highly comparable to that of the wild-type control. These studies suggest that the HIF-1 might not play a critical role in myogenin, and MRFX (21, 22). The MRFs heterodimerize with a ubiquitously expressed basic helix-loop-helix proteins called “E” proteins, and the resultant heterodimers then bind to the consensus sequence (CANNTG) designated “E box” present in the promoters and enhancers of muscle-specific genes, hence activating muscle gene transcription (44, 45). Genetic studies and other experiments have established that myogenin is instrumental in controlling myogenesis (46, 47). Myogenin acts downstream of MyoD and Myf5 to activate muscle differentiation genes. Myogenin null mice survive fetal development, but die on birth due to a severe deficiency in skeletal muscle (46, 47). Myoblasts form and migrate to their correct embryonic locations, but most do not fuse to form myofibers. Despite the importance of myogenin for skeletal muscle differentiation, little is known about how the steady-state levels of this key molecule are regulated.

The HIF-1 pathway is involved in the regulation of multiple cellular processes, such as cell survival, apoptosis, cell cycle progression, differentiation, and tumor progression. A recent study by Yun et al. (49) has indicated that no significant changes in skeletal muscle differentiation are observed when C2C12 cells are infected with retrovirus containing oxygen-insensitive or constitutively active form of HIF-1α. Mason et al. (50) have demonstrated that targeted deletion of HIF-1α in skeletal muscle affects the energy metabolism of muscles, whereas the development of skeletal muscle seems to be highly comparable to that of the wild-type control. These studies suggest that the HIF-1 might not play a critical role in myogenin.
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The best established function of VHL to date is as a substrate recognition component of an E3 ubiquitin ligase complex. VHL is composed of two domains, termed α and the β (52). The α domain binds to elongin C, which in turn nucleates a complex encompassing elongin B, Cullin-2 (Cul2), and Rbx 1 (53–57). The complex termed VCB-Cul2 has been shown to function as an E3 ubiquitin ligase (54, 58). VHL confers substrate specificity to the E3 ubiquitin ligase complex (52). The β domain is required to interact with the α subunit of the HIF protein, which was the first substrate identified to be ubiquitinated by VHL-containing ubiquitin ligase (59–61). VHL E3 ligase complex has been shown to bind to and catalyze ubiquitination of an array of proteins exemplified by the seventh subunit of RNA polymerase II (62), atypical protein kinase, TBP-1, FIH-1, α-tubulin (63), the large subunit of RNA polymerase II (64), and the subunit of the HIF protein, which was the first substrate identified to be ubiquitinated by VHL-containing ubiquitin ligase (59–61). VHL E3 ligase complex has been shown to bind to and catalyze ubiquitination of an array of proteins exemplified by the seventh subunit of RNA polymerase II (62), atypical protein kinase, TBP-1, FIH-1, α-tubulin (63), the large subunit of RNA polymerase II (64), and the subunit of the HIF protein, which was the first substrate identified to be ubiquitinated by VHL-containing ubiquitin ligase (59–61).

In summary, this study demonstrates that EGLN3 stabilizes the myogenic regulatory factor myogenin, partially by antagonizing VHL-mediated ubiquitination and degradation of myogenin. Further elucidation of the underlying mechanisms should provide important insights into the regulation of skeletal muscle differentiation and the biological role of EGLN3.

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