Enhancer of Polycomb1 Acts on Serum Response Factor to Regulate Skeletal Muscle Differentiation

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Skeletal muscle differentiation is well regulated by a series of transcription factors. We reported previously that enhancer of polycomb1 (Epc1), a chromatin protein, can modulate skeletal muscle differentiation, although the mechanisms of this action have yet to be defined. Here we report that Epc1 recruits both serum response factor (SRF) and p300 to induce skeletal muscle differentiation. Epc1 interacted physically with SRF. Transfection of Epc1 to myoblast cells potentiated the SRF-induced expression of skeletal muscle-specific genes as well as multinucleation. Proximal CArG box in the skeletal α-actin promoter was responsible for the synergistic activation of the muscle-specific genes (6). For example, SRF is known to mediate skeletal muscle differentiation by interacting with myocyte enhancer factor-2 (MEF2), a MADS box transcription factor (4). Serum response factor (SRF) is an important transcription factor for cell survival and differentiation in diverse tissues (5). Likewise, in skeletal muscle cells, it is well known that SRF is responsible for the transcriptional activation of muscle-specific genes (6). For example, SRF mRNA and nuclear SRF protein levels increase more than 40-fold when primary skeletal myoblasts are withdrawn from the cell cycle, which precedes the up-regulation of the muscle-specific proteins. However, the dominant-negative SRF mutant blocks the transcription of muscle-specific genes such as skeletal α-actin during myogenesis (6), which suggests that SRF is an important prerequisite for the initiation of muscle differentiation.

Recently, we reported that enhancer of polycomb1 (Epc1) mediates skeletal muscle differentiation by interacting with homeodomain only protein (Hop or Hod) and that muscle regeneration is impaired in Hop null mice (7). Epc1 was first described to enhance the phenotypes of homozygotic mutations of the polycomb group gene in Drosophila (8). Previous reports elucidated that Epc1 may act as a transcription co-factor as a binding partner of other transcription factors such as E2F6 (9) or RET finger protein (10). Although Epc1 is known to associate with other transcriptional modulators, the biological role of Epc1 and its regulatory mechanism in mammalian tissues remains to be described.

In this study, we postulated that Epc1 induces muscle differentiation by interacting with SRF, an important modulator of muscle differentiation (11). Thus, we studied the mechanism of action of Epc1 in association with SRF and p300 and also investigated the muscle phenotypes of mice in which Epc1 expression was disrupted. The Epc1-mediated enhancement of SRF-dependent muscle gene expression is mediated by its...
interaction with p300, which possesses histone acetyltransferase activity. Epc1 heterozygous mice show a delay in myoblast differentiation, which suggests that Epc1 is a novel modulator of skeletal muscle differentiation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pCMV-myc-mouse Epc1 was described previously. pCMX-p300 was kindly provided by Prof. Debabrata Chakravarti (Northwestern University, Chicago). pCGN-HA-SRF was kindly provided by Prof. Jonathan A. Epstein (University of Pennsylvania, Philadelphia). For the skeletal α-actin promoter-reporter assay, −450 to +26 base pairs from the transcription start site were amplified from mouse genomic DNA and subcloned into pGL3 basic vector (Promega, Madison, WI). The CAI+G-far (−419 to −410) and near (−97 to −88) mutations of the skeletal α-actin promoter were generated by site-directed mutagenesis by using the QuickChange kit (Stratagene Inc., La Jolla, CA). si-p300 was purchased from Dharmacon Inc. (Chicago). All plasmids were confirmed by sequencing.

Antibodies, Cell Cultures, and Transfection Study—Epc1 antibody was described previously (7). Antibodies were used to recognize MyoD (C-20, Sc-304), myogenin (M-225, Sc-576), SRF (G-20, Sc-335), c-Myc (9E10, Sc-40), and p300 (c-20, Sc-585) (all of the above were from Santa Cruz Biotechnology, Santa Cruz, CA); anti-acetyl-histone H3 (06–599), anti-acetyl-histone H4 (06–866), and anti-histone H3 (05–499) (from Upstate Biotechnology, Lake Placid, NY); skeletal α-actin (catalogue No. 18-272-196320, Genway Biotech, Inc., San Diego); and anti-histone H4 (catalogue No. 2592, Cell Signaling Technology Inc., Danvers, MA). H9c2, COS-7, 293T, 10T1/2, and C2C12 cells were obtained from the Korean Cell Line Bank and were maintained with Dulbecco’s modified Eagle’s medium containing 10 or 15% fetal bovine serum for C2C12 cells. Anti-sense Epc1 cells were described previously (7).

For transient transfection of SRF and Epc1, pCGN-HA-SRF and/or pCMV-myc-Epc1 was introduced to 293T cells by use of FuGENE 6 transfection reagent (Roche Diagnostics) and to C2C12 or H9c2 cells by use of Lipofectamine Plus reagent (Invitrogen). Promoter analysis, immunoprecipitation, Western blot, fluorescent immunocytochemistry, and RT-PCR were described previously (7). The antibodies for Western blot analysis were anti-HA (1:500), Epc1 (1:200), p300 (1:1000), and actin (1:2000). The primer sequences for the RT-PCR reaction will be provided upon request. RT-PCR amplification products were confirmed by sequencing.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed using a commercially available assay kit according to the manufacturer’s instructions (Upstate Biotechnology). Briefly, cells were treated with 1% formaldehyde for 10 min. The sonicated chromatin was immunoprecipitated with specific antibodies and then recovered with protein A-agarose/salmon sperm DNA beads. After reversing the cross-links, chromatin was subjected to proteinase K digestion, and the DNA was purified. A 197-bp fragment corresponding to nucleotides −192 to +5 of the rat skeletal α-actin promoter was amplified by PCR.

Sequential chromatin immunoprecipitation was performed as described (12). Epc1 precipitates were then eluted with standard elution buffer, except for washing with high salt solution (500 mM NaCl, 2 mM EDTA, 0.1% SDS), and the chromatin was recruited by anti-SRF antibody.

Histone Acetyltransferase Activity—Histone acetyltransferase assays were performed as described previously (13). After cells were lysed with radioimmune precipitation assay buffer and immunoprecipitated with specific antibody, protein A-agarose beads were added. Two µg (50 pmol) of histones (Roche Applied Science) and [14C]acetyl-CoA (50 µCi/µl, 1000 pmol/µl, Amersham Biosciences) were added to the precipitates and incubated for 30 min at 30°C. Reaction products were separated by 14% SDS-PAGE and analyzed by use of a quantitative imaging device (PhosphorImager, GE Healthcare).

Generation of Epc1 Knock-out Mice—The experimental protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Gene-trapped embryonic stem cells (XB645) were purchased from University of California-Davis after confirmation of the preceding sequences by rapid amplification of cDNA ends (5'-RACE). The gene trap vector (pGTOps) was inserted in the coding region in the first exon of the mouse Epc1, and the gene trap location was confirmed by sequencing (Seegene Inc., Seoul, South Korea). The 129/Ola mouse embryonic stem cell clones were injected into C57BL/6 blastocysts and then subsequently transferred into pseudopregnant foster mothers. The resulting male chimeric mice were bred to C57BL/6 females to obtain het-
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RESULTS

Epc1 Interacts with SRF—To investigate whether Epc1 binds to SRF, we first performed co-immunoprecipitation by transfecting pCMV-myc-Epc1 and pCGN-HA-SRF. SRF pulled down Epc1 (Fig. 1A) in 293T cells. Inversely, Epc1 could also recruit SRF (Fig. 1B). Physical interactions of endogenous Epc1 and SRF were confirmed in C2C12 cells (Fig. 1C). *In vitro* association between Epc1 and SRF was tested by a glutathione S-transferase (GST)-pulldown assay. *In vitro* translated SRF was recruited by GST-Epc1 (Fig. 1D). Both proteins were colocalized in the nucleus of COS-7 cells (supplemental Fig. S1).

Forced Expression of Epc1 and SRF Induces Muscle Differentiation—Next, the biological significance of the interaction of Epc1 and SRF was investigated. First, we measured the transcript level of Epc1 in serum-deprived C2C12 cells. As we described in the previous report using H9c2 cells (7), Epc1 was transiently up-regulated in 3–6 days after serum deprivation (data not shown). MyoD is an important MRF that induces muscle determination and differentiation (14). We examined the expression of MyoD after cotransfection of SRF and Epc1. Epc1 strengthened the expression of MyoD induced by SRF (Fig. 2, A and C). Likewise, cotransfection of Epc1 and SRF synergistically induced the expression of myogenin (Fig. 2, B and D), a critical determinant of skeletal muscle differentiation and myotube formation (15). The forced expression of SRF induced a significant increase in the number of multinucleated cells. Although Epc1 did not induce multinucleation, interestingly, it significantly potentiated the SRF-mediated myoblast differentiation (Fig. 2E).

We studied whether Epc1 potentiates the SRF-mediated expression of the muscle-specific structural genes as well as transcription factors in C2C12 myoblast cells after transient transfection of pCGN-HA-SRF or pCMV-myc-Epc1. Both SRF and Epc1 were expressed appropriately, as shown in Fig. 3A. The expression level of skeletal α-actin, a primary component of skeletal muscle thin filament (16), was increased by transfection of SRF, which was further potentiated by cotransfection of Epc1. In addition, myogenin expression was increased by transfection of both genes (Fig. 3, A and B).

The transcript levels of MyoD and of muscle-specific genes such as muscle creatine kinase (17) were also further increased by co-transfection of Epc1 and SRF (Fig. 3, C and D). However, the transcript levels of myf-6 (Fig. 3C) or myf-5 (data not shown), which do not contain a CArG box in their promoter region (18), were not altered by transfection of SRF and Epc1. The changes in the amounts of transcripts of those muscle-specific genes were evaluated quantitatively by real-time PCR after three independent experiments (Fig. 3D).
Epc1 Activates the Skeletal α-Actin Promoter in an SRF/SRE-dependent Manner—Like many skeletal muscle-specific genes, skeletal α-actin has an SRE in the promoter region (19). To investigate whether SRF/SRE is responsible for the action of Epc1, we performed a transfection assay with the minimal promoter of skeletal α-actin (20), which contains CArG boxes. Transfection of SRF transactivated the promoter by 2.5-fold over the mock-transfected basal level. Interestingly, cotransfection of Epc1 potentiated the activity by 6-fold, which suggests that the synergistic activation is mediated by the transcriptional regulation of the minimal promoter region (Fig. 4A). The mouse skeletal α-actin proximal promoter has two SREs designated as CArG-near and CArG-far boxes (19). To test which CArG box is required for the synergistic action of SRF and Epc1, mutations were introduced into either the near or the far CArG box (Fig. 4B, left diagrams). Whereas Epc1 and SRF could synergistically activate the skeletal α-actin-luciferase reporter with the CArG-far mutation, the potentiation was completely abolished by the CArG-near or double CArG mutations (Fig. 4B and supplemental Fig. S2).

Epc1 Binds to the SRE in the Skeletal α-Actin Promoter—To examine whether Epc1 binds to the SRE together with SRF, we performed chromatin immunoprecipitation assays with H9c2 cells. In contrast with mouse, rat skeletal α-actin has one CArG box with a sequence identical to the CArG-near of mouse skeletal α-actin (Fig. 4C). Epc1 antibody successfully pulled down the DNA fragments spanning the SRE in the promoter region, whereas it failed to recruit the fragments containing the exon 3 and exon 5 regions (Fig. 4D). As expected, the reduction of Epc1 expression caused a decrease in the binding of Epc1 to the SRE when the Epc1 was pulled down with anti-Epc1 antibody (Fig. 5A). Epc1 knockdown resulted in a decrease in SRF binding to the SRE (Fig. 5B (third lane) and E). These results indicate that Epc1 is required for the binding of SRF to the SRE.

Epc1 homologues mediate epigenetic regulation to enhance the role of the polycomb group genes (21) by recruiting histone acetyltransferase activity in Drosophila (22). The NuA4 com-
plex, which contains Epc1, is known to acetylate histones, and the acetylation is mediated by Tat-interacting protein 60 (Tip60), a MYST family histone acetyltransferase (22). These previous reports raise the possibility that Epc1 may induce the acetylation of histone residues to activate downstream genes, although little is known about Epc1-mediated histone modification in either mammalian cells or skeletal muscles.

First, we measured the histone H3 and H4 acetylation status of the SRE of the skeletal α-actin promoter by chromatin immunoprecipitation. As shown in Fig. 5, the residues of both histones H3 and H4 associated with the SRE were acetylated. However, the acetylation was greatly reduced in antisense H9c2 cells, which suggests that Epc1 is necessary for the histone acetylation of the SRE.

**Epc1 Recruits p300 to Acetylate Histones**—We performed a histone acetyltransferase assay by using the GST fusion protein of Epc1 in the cell-free condition, but we did not see any intrinsic activity (data not shown). Thus, we used Epc1 immunoprecipitates for the histone acetyltransferase assay and clearly observed the acetylation of histone (Fig. 6C, second lane).

The lack of intrinsic activity led us to postulate that histone acetylation was mediated by other histone acetyltransferase proteins. To answer this, immunoprecipitation assays were carried out to observe the physical interaction of Epc1 and other histone acetyltransferase proteins. First, we examined whether Epc1 interacts with Tip60 by transfecting pCMV-myc-Epc1 or pcDNA3.1-HA-Tip60; exogenous Tip60 interacted with Epc1 (data not shown). However, a previous report that Tip60 is barely detected in the skeletal muscle tissue (23) raises the question of whether the interaction between Tip60 and Epc1 really takes place in skeletal muscle cells. Indeed, we were not able to detect Tip60 in H9c2 or C2C12 cells or in adult mouse muscle tissue (data not shown). Thus, we postulated that a histone acetyltransferase other than Tip60 may participate in the Epc1-mediated histone acetylation. We immunoprecipitated Epc1 and several histone acetyltransferase proteins such as p300/CREB-binding protein (CBP)-associated factor (PCAF) and p300 were examined. Among them, p300, which is required for skeletal muscle gene transcription (24), was successfully detected (Fig. 6A). Reduction of Epc1 in antisense H9c2 cells

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**FIGURE 4. Epc1 binds to the SRE in the skeletal α-actin promoter.** A, Epc1 potentiated SRF-induced transactivation of the minimal promoter of skeletal α-actin. Values are the fold increase in luciferase activity relative to activation of the reporter alone. *, p < 0.05; **, p < 0.01 compared with mock-transfected group; @, p < 0.01 compared with SRF-transfected group. B, diagram showing the proximal region of the skeletal α-actin promoter and promoter mutants. Epc1 potentiates SRF-mediated transactivation when the CArG-near box of the skeletal α-actin promoter is intact. C, schematic diagram showing the promoter of rat skeletal α-actin; the arrows show the PCR primers for the chromatin immunoprecipitation assay. D, chromatin immunoprecipitation (ChIP) assay to show the binding of the Epc1-containing complex to the SRE in the skeletal α-actin promoter in H9c2 cells. Chromatin was prepared from H9c2 cells as described under “Experimental Procedures” and was immunoprecipitated with anti-Epc1 antibody. Note that exon 3 and exon 5 regions were not recruited by Epc1. E, quantification of results from three independent chromatin immunoprecipitation assays. Error bars represent S.E. F, sequential chromatin immunoprecipitation assay to show whether Epc1 and SRF can bind simultaneously to the SRE. The Epc1 precipitates (fourth lane) were subjected to immunoprecipitation with anti-SRF antibody and the SRE regions were amplified by PCR (sixth lane). See “Experimental Procedures” for detailed protocols.
resulted in a reduction in the interaction with p300. The physical interactions between endogenous Epc1 with endogenous p300 were also confirmed in C2C12 cells (Fig. 6B) and in H9c2 cells (data not shown).

The involvement of p300 in the acetylation of the Epc1-containing complex was further confirmed by measuring histone acetyltransferase activity after overexpression or knockdown of p300. When pCMX-p300 was transfected, histone acetylation was enhanced in the Epc1-containing complex precipitates in a dose-dependent fashion (Fig. 6C, second to fourth lane). Transfection of si-p300 successfully reduced the protein amounts of p300 (Fig. 6D). Additionally, treatment with si-p300 significantly reduced the histone acetyltransferase activity in Epc1 immunoprecipitates (Fig. 6E, fifth lane).

Skeletal Muscle Differentiation Is Impaired in Epc1 Heterozygous Mice—To investigate the biological role of Epc1, we generated Epc1 knock-out mice with gene-trapped embryonic stem cells (Fig. 7A). The homozygous mice died in embryo (Fig. 7B). However, Epc1 heterozygous mice survived for more than 6 months without apparent pathological phenotypes (data not shown). Epc1 expression was reduced in the skeletal muscles of the heterozygous mice (Fig. 8A). The expression of desmin was also significantly reduced in the mice (Figs. 7C (right panel) and 8A). In the Epc1 heterozygous mice, the expression of skeletal α-actin was reduced (Fig. 7D). Under the higher magnification, we observed that skeletal α-actin was well demarcated along with sarcomeric striation in the wild type. In contrast, the demarcation was not prominent in heterozygous group (Fig. 7E).

The transcript levels (Fig. 8A) and protein amounts (Fig. 8B) of several muscle-specific genes or transcription factors in skeletal muscles were also significantly less than those in wild type mice. We examined whether the muscle differentiation of the myoblasts was impaired. Although differentiation of isolated myoblasts by serum deprivation elevated the expression of differentiation markers in the wild type, gene expression was not induced as much in myoblasts from heterozygous mice (Fig. 8C). The reduction in expression of the muscle-specific transcription factors resulted in a delay in the differentiation of the myoblasts, as evaluated by counting the multinucleated cells after serum starvation (Fig. 8D).
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**DISCUSSION**

In our previous report, we observed that Epc1 is involved in skeletal muscle differentiation, which raised the possibility that Epc1 may interact with other muscle-specific transcription factors to regulate differentiation. We have shown here that Epc1 interacts with SRF, a key regulator of diverse muscle differentiation and cell survival (1, 25). We have demonstrated that Epc1 binds to SRF and activates SRF/SRE-dependent genes by recruiting p300, which induces skeletal muscle differentiation (Fig. 9). Epc1 is required for the full activation of those muscle-specific genes.

Many proteins that regulate muscle differentiation, such as GATA4, Nkx2.5, YY-1, TEF-1, and myocardin (26, 27), are abruptly induced by removing serum, which implicates the significance of SRF in the regulation of those proteins. SRF, a MADS box transcription factor related to MEF2, is required for the differentiation of skeletal, cardiac, and smooth muscle cells (1, 28–30). SRF-dependent genes have similar consensus sequences in their proximal promoter regions called CArG boxes, and the binding of SRF to the promoter region activates downstream genes (26, 27). Likewise, many SRF-dependent muscle-specific genes have one or more CArG boxes. MyoD is an example of a transcription factor involved in the SRF-dependent regulation of skeletal muscle differentiation; SRF modifies the transcription of MyoD by its binding to the functional CArG element in the distal regulatory region in the promoter (31). Likewise, skeletal α-actin has an SRE in the proximal promoter region (19).

Therefore, we postulated that the potentiation of SRF-transactivation by Epc1 is mediated by the CArG box and observed that the minimal promoter of skeletal α-actin containing the SRE was required for Epc1-mediated transactivation. In this study, we observed that Epc1 binds to SRF to enhance the muscle differentiation in the myoblast cell lines.

Mouse skeletal α-actin has two CArG boxes, designated CArG-near and CArG-far, both of which can bind to SRF (32). By disrupting the CArG box sequences with site-directed mutagenesis, we showed that the mouse CArG-near box was responsible for Epc1-mediated potentiation. This finding was further supported by the results of the chromatin immunoprecipitation assay using anti-Epc1 antibody in H9c2 cells. The SRE in the skeletal α-actin promoter was successfully amplified from the immunoprecipitates with anti-Epc1 antibody, which suggests that the Epc1-containing complex binds to the SRE. Interestingly, when we used anti-SRF antibody, the reduction in Epc1 expression in the Epc1 knockdown cells resulted in a decrease in the binding of SRF to the SRE. These results suggest that the Epc1-SRF-containing complex binds the SRE and that Epc1 is required for the full activation of SRF-dependent downstream genes.

Numerous transcriptional coactivator complexes possess histone acetyltransferase activity, which is mediated by histone acetyltransferase enzymes such as p300, a functional homologue of CREB-binding protein (CBP), PCAF, and Tip60 (3, 33–35). Generally, the histone acetyltransferase-containing complexes induce transactivation of downstream genes by introducing structural modifications in the nucleosome of the promoter region (36). In yeast, a homologue of human EPC1, Esa1, has been discovered as a component of the picNuA4 complex; this complex contains histone acetyltransferase activity that is mediated by Tip60 (21). In addition, Tip60 is known to interact with SRF and to regulate early cardiac development (37) by activating atrial natriuretic factor (38).

However, in this study, although Tip60 could bind to Epc1, it was not present in adult skeletal muscle. Instead, an alternate histone acetyltransferase, p300, which is abundant in skeletal muscle, was successfully recruited by Epc1. It is widely accepted that histone acetyltransferase activity mediated by p300 is necessary for myogenesis, either by forming a complex to activate the E-box gene family (39) or by association with SRF to induce CArG box-dependent genes (31, 40). In addition, p300-mediated histone acetyltransferase activity is required for myogenesis from embryonic stem cells (41), suggesting p300 as a regulator of the MRFs. Thus, we have shown that p300 is a component of the SRF-Epc1 complex that activates skeletal muscle-specific genes.
Our observations in the present study of the phenotypes in Epc1 heterozygous mice clearly support the involvement of Epc1 in skeletal muscle maturation. Although Epc1 heterozygous mice can achieve normal growth without apparent alterations in muscle structure, the expression levels of the muscle-specific transcription factors were decreased and the cytoskeletal proteins were distorted. Also, the differentiation of isolated myocytes by serum deprivation was significantly impaired, which suggests a requirement for Epc1 in the maturation processes. It is interesting that skeletal α-actin arrangement was also disrupted in Epc1-heterozygous mice muscles. Considering that SRF also plays an important role in the regulation of cytoskeleton dynamics as well as in the expression of those cytoskeletal proteins (42), Epc1 also seems to be required for the SRF-mediated regulation of cytoskeletons, although this will require further evaluation.

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Of course, it is not clear whether Epc1 plays a pivotal role in SRF-mediated transcriptional regulation and cytoskeletal arrangement.
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Skeletal muscle gene activation

FIGURE 9. Diagram showing the mechanism of Epc1/SLF/p300 interaction in the transcriptional regulation of SRF-dependent muscle-specific genes.

through its intrinsic enzymatic activity or its function of transcriptional activity. Rather, Epc1 could be a bridging molecule that works as a simple component of the Epc1-SRF-p300 complex. However, at least in part, Epc1 participates in muscle differentiation as a modulator of SRF.

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