The Retinoblastoma Protein Is an Essential Mediator of Osteogenesis That Links the p204 Protein to the Cbfa1 Transcription Factor Thereby Increasing Its Activity*

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Bone formation requires the coordinated activity of numerous proteins including the transcription factor core-binding factor α1 (Cbfa1). Deregeneration of Cbfa1 results in metabolic bone diseases including osteoporosis and osteopetrosis. The retinoblastoma protein (pRb) that is required for osteogenesis binds Cbfa1. We reported earlier that the p200 family protein p204, which is known to be involved in the differentiation of skeletal muscle myotubes, cardiac myocytes, and macrophages, also serves as a cofactor of Cbfa1 and promotes osteogenesis. In this study we established that sequestration of p204 expression by an adenovirus construct encoding p204 antisense RNA inhibited osteoblast-specific gene activation by Cbfa1 in an osteogenesis assay involving the pluripotent C2C12 mesenchymal cell line. Using protein-protein interaction assays we established that Cbfa1, pRb, and p204 form a ternary complex in which pRb serves as a linker connecting p204 and Cbfa1. Chromatin immunoprecipitation assays revealed the binding of such a p204–pRb–Cbfa1 transcription factor complex to the promoter of the osteocalcin gene. The pRb requirement of the stimulation of Cbfa1 activity by p204 was established in experiments involving p204 mutants lacking one or two pRb binding (LXCXE) motifs. Such mutants failed to enhance the Cbfa1-dependent transactivation of gene expression as well as osteogenesis. Furthermore, as revealed in reporter gene and in vitro osteogenesis assays p204 synergized with pRb in the stimulation of Cbfa1-dependent gene activation and osteoblast differentiation.

The differentiation of uncommitted mesenchymal cells to osteoblasts is a fundamental process in embryonic development and bone repair. The bone morphogenetic proteins (BMPs) are important regulators of this process (1, 2). They function by binding cell surface receptors, signaling by Smad proteins, and activating bone-specific genes including core binding factor α1 (Cbfa1) (3–5). This process is positively or negatively regulated by a variety of coactivators and corepressors (3, 5, 6). Cbfa1, also known as Runx2, PeBP2αA, Osf2, or AML3, is a member of the runt family of transcription factors. It is an essential transcription factor of osteoblast and bone formation (4). During skeletal development Cbfa1 was observed first in early mesenchymal condensations and is then principally expressed in osteoblasts (7). Cbfa1−/− mice exhibit a complete lack of ossification and die immediately after birth (8). Cbfa1 maintains osteoblastic function by regulating the expression of several bone-specific genes such as osteopontin and osteocalcin, and by controlling bone extracellular matrix deposition (4). Mutations in Cbfa1 are found in 65–80% of individuals with cleidocranial dysplasia (9–11). Some mutations of Cbfa1 abolish its DNA binding activity (9, 11, 12) and others disturb the association of Cbfa1 to its binding partners including Smads (13). It was found that several Cbfa1-binding proteins such as retinoblastoma protein (pRb) (14), p204 (15), and Core binding factor β (Cbfb) (16, 17) may also play critical roles in bone development.

Interferons are cytokines with antimicrobial, immunomodulatory, and cell growth and differentiation regulatory activities (18, 19). The activities of interferons are mediated by numerous interferon-inducible proteins. p204 is a member of the interferon-inducible p200 family proteins that are encoded by genes from the gene 200 cluster (20, 21). p204 consists of 640 amino acid residues. In the N-terminal domain (amino acids 1–216), there is a basic amino acid-rich nuclear localization signal and a canonical export signal required for the translocation of p204 from the nucleus to the cytoplasm during skeletal and cardiac muscle differentiation (22, 23). The C-terminal domain of p204 consists of two homologous, partially conserved 200-amino acid repeats with a high content of acidic residues (amino acids 217–640). The two repeats have been shown to serve as a functional domain for the function of p204. The two repeats contain multiple LXCXE motifs that are required for binding to several transcription factors. The p204 protein was shown to bind to pRb and Cbfa1 in vitro and in vivo (15). However, the mechanism by which pRb and Cbfa1 induce the differentiation of uncommitted mesenchymal cells to osteoblasts is not known.

BMPs are important regulators of this process and play a critical role in osteogenesis. BMPs also have been shown to regulate cell proliferation and differentiation of skeletal muscle myotubes, cardiac myocytes, and macrophages (22, 23). The C-terminal domain of p204 contains multiple LXCXE motifs that are required for binding to several transcription factors. The p204 protein has been shown to bind to pRb and Cbfa1 in vitro and in vivo (15). However, the mechanism by which pRb and Cbfa1 induce the differentiation of uncommitted mesenchymal cells to osteoblasts is not known.

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2 The abbreviations used are: BMP, bone morphogenetic protein; Cbfa1, core binding factor α1; Cbfb, core binding factor β; Id, inhibitor of differentiation; ALP, alkaline phosphatase; OCL, osteocalcin; pRb, retinoblastoma protein; HAT, histone acetylase; GST, glutathione S-transferase; co-IP, co-immunoprecipitation; pRb, retinoblastoma protein; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein.
acid segments (a and b) in which pRb binding motifs (e.g., LXCXE) are located. Overexpression of p204 is growth inhibitory due to its inhibition of ribosomal RNA transcription by binding of p204 to the ribosomal DNA-specific upstream binding factor transcription factor and inhibiting its sequence-specific binding to DNA (24, 25). The antiproliferative activity of p204 has been attributed to the binding of p204 to pRb by its pRb binding LXCXE motifs (24, 26, 27). However, the antiproliferative activity of p204 does not always depend on pRb (28).

Overexpression of p204 was found to delay the progression of cells from the G0/G1 phase to the S phase of the cell cycle (29). p204 was found to be an important regulator of both skeletal and cardiac muscle differentiation (22, 23, 30, 31). In the course of these processes p204 is induced, phosphorylated, and translocated from the nucleus to the cytoplasm (23, 30). Overexpression of p204 accelerates muscle formation (23). This is due at least in part to the binding of p204 to the inhibitor of differentiation (Id) proteins including Id1, Id2, and Id3, and overcoming the inhibition by the Id proteins of MyoD activity in the case of skeletal muscle differentiation, and Gata4 and Nkx2.5 activity in the case of cardiac myocyte formation (22, 31). A recent publication revealed the involvement of p204 also in macrophage differentiation (32). The p204 gene was isolated as a Macrophage Colony-Stimulating Factor (M-CSF)-responsive gene using a gene trap approach in the interleukin 3-dependent myeloid FD-Fms cell line. Moreover forced expression of p204 strongly repressed the interleukin-3 and M-CSF-dependent gene expression in stimulated Cbfa1 activity, pCMV-p204mt-a/b encoding the p204 mutant in which two Rb-binding sites located in the a and b segments of p204 were mutated. To determine whether both Rb-binding sites in p204 are needed for the p204 action in stimulating Cbfa1 activity, pCMV-p204mt-a/b (Rb-binding site in the a segment is mutated), pCMV-p204mt-b (Rb-binding site in the b segment is mutated), and pCMV-p204mt-a/b and pCMV-204 were used with the Cbfa1 expression construct in the reporter gene assay. The sample was processed as described above.

**Generation of Recombinant Viruses and Infection of C2C12 Cells—**The AdEasy adenoviral vector system (Invitrogen) was used to construct, according to the protocol of the manufacturer, adenoviruses expressing various proteins. The viral constructs include Ad-pRb, Ad-p204, Ad-p204mt-a Ad-p204mt-b, Ad-p204mt-a/b, and Ad-204AS (encoding 204 antisense RNA). All constructs were verified by DNA sequencing. The expression of all recombinant viruses in infected C2C12 cells was tested by Western blotting with appropriate antibodies. C2C12 cells were infected with recombinant Ad-Cbfa1 (kindly provided by Dr. R. Franceschi) and as specified with Ad-pRb, Ad-p204, Ad-p204mt-a Ad-p204mt-b, Ad-p204mt-a/b, and Ad-204AS (encoding 204 antisense RNA).
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infection the C2C12 cells were lysed for alkaline phosphatase (ALP) assay and the media were collected for osteocalcin (OCL) assay.

Alkaline Phosphatase and Osteocalcin Assays—Cells were sonicated in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM MgCl₂, 1% Triton X-100) supplemented with protease inhibitor mixture (Sigma) and centrifuged at 10,000 × g for 15 min. The supernatant fractions were collected and protein concentrations were determined using the Bio-Rad assay kit. ALP activities were assayed by a standard method with p-nitrophenyl phosphate as a substrate (Sigma). Enzyme activity was calculated using a p-nitrophenyl standard curve and expressed as micromoles of p-nitrophenyl phosphate/μg of protein. OCL in the culture medium was assayed by an enzyme-linked immunosorbent assay using a mouse osteocalcin assay kit (Biomedica Technologies, Stoughton, MA) according to the manufacturer’s protocol.

Transfection and Infection—To examine the interactions of p204, pRb, and Cbfa1 in vivo, 5 × 10⁶ C2C12 or Saos2 (pRb deficient) cells were transfected with 6 μg of expression plasmids (as indicated in Figs. 2, 4, and 6) and 10 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. To examine the interaction of p204, pRb, and Cbfa1 during osteogenesis 5 × 10⁶ C2C12 cells were infected with 1 × 10⁷ plaque-forming units of Ad-BMP2. The cells were incubated in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum for 3 days and thereafter resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM MgCl₂, 0.5% Nonident P-40, and 0.1% (w/v) SDS), supplemented with protease inhibitors, sonicated, and centrifuged to remove the cellular debris.

Co-immunoprecipitation and Western Blotting—Total cell extract proteins (300 μg) prepared from C2C12 cells transfected with pCMV204, pCMV/Rb, and pEF-Cbfa1 expression plasmids or infected with Ad-BMP2, from osteoblast MC-3T3-E1 or from osteocyte-like MLO-Y4 cells, were incubated with either rabbit polyclonal antibodies to p204 (25 μg/ml), rabbit antibodies to pRb (25 μg/ml), goat antibodies to Cbfa1 (25 μg/ml), or control rabbit IgG (25 μg/ml), respectively, at room temperature for 2 h. Immune complexes were collected with protein A-agarose (Amersham Biosciences), washed five times with lysis buffer, released by boiling in 40 μl of 2× SDS loading buffer for 10 min and examined by Western blotting with antibodies to p204, pRb, or Cbfa1. The signals were detected using the ECL chemiluminescent system.

To assay whether pRb is required for the binding of p204 to Cbfa1 in vivo, pRb-deficient Saos-2 cells were transfected with pCMV204 and pEF-Cbfa1 together with or without pCMV/Rb. Immunoprecipitation with antibodies to p204 or Cbfa1 was performed as described above. The immunoprecipitated proteins were subjected to 10% SDS-PAGE and detected by Western blot analysis using antibodies to p204, pRb, or Cbfa1.

GST Pull-down Assay in Vitro—To assay whether pRb is required for the binding of p204 to Cbfa1 in vitro, glutathione-Sepharose beads (50 μl) preincubated with purified recombinant GST-204 (500 ng) or GST (500 ng) (serving as negative control) were incubated with 50 μg of cell extract from Saos-2 cells, if so indicated, transfected with pCMV/Rb or C2C12 cells that had been transfected with a pEF-Cbfa1 expression plasmid (positive control). The beads were washed 5 times with washing buffer (0.3% Triton X-100 in phosphate-buffered saline). The bound proteins were denatured in sample buffer, released, and analyzed by 10% SDS-PAGE, and the Cbfa1 protein was detected by Western blotting with antibodies to Cbfa1.

Knockdown of pRb by Specific siRNA Silencing—For silencing the expression of Rb, 19 nucleotides (AAAGTTAACACCGATC of murine Rb were targeted with small interfering RNA (siRNA) using pSuper mammalian expression vector (OligoEngine, Seattle, WA) according to the manufacturer’s instructions. To generate siRNAs, equimolar amounts of complementary sense and antisense strands were mixed and annealed by slowly cooling to 10°C in 50 (or is it 100?) μl of reaction buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). The annealed oligos were inserted into the BglII/HindIII sites of pSuper vector. The resulting plasmids and the pSuper control vector were transfected into MC3T3-E1 cells using Lipofectamine 2000 reagent (Invitrogen) and the level of pRb was determined using Western blotting with anti-pRb antibody. MC3T3-E1 cells transfected with either pSuper-pRb or pSuper vector control (CTR) were cultured in minimal essential medium, a medium supplemented with 10% fetal bovine serum under standard cell culture conditions for 3–4 days and harvested for co-immunoprecipitation (co-IP) and chromatin immunoprecipitation (ChIP) assays.

ChIP—To assay whether a p204-pRb-pCbfa1 complex is associated with the osteocalcin promoter in vivo we performed ChIP assays. Using a ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) and murine C3H 10T1/2 progenitor cells transiently transfected with expression plasmids encoding Cbfa1, pRb, and p204. Murine C3H 10T1/2 progenitor cells were cultured in 10-mm dishes and treated with formaldehyde (1% final concentration) to cross-link the p204-pRb-Cbfa1 complex to the DNA. The cells were washed with cold, phosphate-buffered saline and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1). The lysates were sonicated to shear the DNA to a length between 200 and 1000 bp. The sonicated supernatant fraction was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM Tris-HCl, pH 8.1), and 150 mM NaCl) and incubated with antibodies to Cbfa1 (Stratagene, La Jolla, CA), pRb (Santa Cruz Biotechnology, Santa Cruz, CA), p204, or preimmune serum with rotation at 4°C overnight. To collect the DNA-protein complexes a salmon sperm DNA-protein A-agarose slurry was added to the mixture and incubated with rotation at 4°C for 1 h, and the DNA-protein-agarose was pelleted by centrifugation. After extensive washing of the pellet with a series of buffers (Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Immune Complex Wash Buffer, and 1× TE Buffer (10 mM Tris, pH 8.1, 1 mM EDTA)) the pellet was dissolved in 250 μl of elution buffer (1% SDS, 0.1 M NaHCO₃, 0.01 mg/ml herring sperm DNA), and centrifuged to remove the agarose. The supernatant fraction was treated with 20 μl of 5 × NaCl and heated to 65°C for 4 h to reverse the p204-pRb-Cbfa1-DNA cross-linking. After treatment with EDTA and proteinase K, the supernatant fraction was extracted with phenol-chloroform and precipitated with ethanol to recover the DNA. For amplification of the osteocalcin promoter region, 10% of the chromatin-immunopre-
precipitated DNA was PCR amplified using as forward primer 5'-H11032-CTGCAATCACCAACCACAGC, and as reverse primer 5'-H11032-CTGCACCCTCCAGCATCCAG. 40 cycles of PCR (at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) were performed. The PCR products were analyzed by electrophoresis in a 2% agarose gel. The validity of the ChIP assay was strengthened by interrogating the input and ChIP DNAs with two pairs of negative control primers located roughly 2 kb upstream and downstream from the Cbfa-1-binding site in the osteocalcin promoter. The sequences of the upstream primer pair were: forward primer, 5'-H11032-CCCTCCAAACAAATCATGG, and reverse primer, 5'-H11032-AGGAGGAAGTATGCTCCACTGT. The sequences of the downstream primer pair were: forward primer, 5'-H11032-ATGCTGGGGACCCCTGATAA, and reverse primer, 5'-H11032-GTTTTGGGGCTGGAGAAATG.

RESULTS

Lowering the Level of Endogenous p204 Inhibits the Cbfa1 Transcription Factor-mediated Osteogenesis—The pluripotent mesenchymal C2C12 cells are a well established model for studying osteogenesis in vitro (35–37). Our earlier data (15) demonstrated that overexpression of p204 enhances the BMP2-induced osteogenesis of C2C12 cells (38). We now tested whether endogenous p204 is required for the Cbfa1-mediated expression of osteocalcin, a marker gene for osteoblasts. For this purpose we generated a recombinant adenovirus encoding p204 antisense RNA (Ad-204As). As shown in Fig. 1A, infection of C2C12 cells with Ad-204As, but not control Ad-GFP, decreased the expression of endogenous p204 in a dose-dependent manner. As revealed in Fig. 1, B and C, infection of C2C12 cells with the recombinant adenovirus encoding Cbfa1 (Ad-Cbfa1) led to enhanced expression of ALP and a robust production of osteocalcin. The expression of ALP and production of osteocalcin were strongly inhibited by coinfection with Ad-p204As, but not control Ad-GFP, decreased the expression of endogenous p204 in a dose-dependent manner. As revealed in Fig. 1, B and C, infection of C2C12 cells with the recombinant adenovirus encoding Cbfa1 (Ad-Cbfa1) led to enhanced expression of ALP and a robust production of osteocalcin. The expression of ALP and production of osteocalcin were strongly inhibited by coinfection with Ad-p204As, but not control Ad-GFP, in a dose-dependent manner. These results indicated the requirement for p204 of Cbfa1-triggered osteogenesis in C2C12 cells.

p204 Synergizes with pRb in C2C12 Cells in the Stimulation of Cbfa1-dependent Gene Activation and Osteoblast Differentiation—Both p204 and pRb bind Cbfa1 and act as its cofactors (15). We tested whether these two cofactors synergistically enhance the activity of Cbfa1 in assays of the expression in C2C12 cells of two Cbfa1-specific reporter genes: p6OSE2LUC and p-147luc. As shown in Fig. 2 transfection of plasmids encoding p204 or pRb resulted in an approximately
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**FIGURE 3.** pRb and p204 synergistically stimulated the Cbfa1-mediated osteoblast differentiation as revealed by increased ALP activity and OCL production.

A, adenovirus encoding pRb (Ad-pRb) and adenovirus encoding p204 (Ad-p204) expressed pRb and p204 proteins, respectively, in C2C12 cells. C2C12 cells were infected with Ad-GFP (serving as a control), Ad-pRb, or Ad-p204, and the levels of pRb and p204 protein were determined by Western blotting. The pRb and p204 bands are indicated with arrows. B, overexpression of p204 and/or pRb enhanced the Cbfa1-mediated ALP activity. C2C12 cells were infected with Ad-Cbfa1 without or together with Ad-pRb, Ad-p204, or both as indicated, and ALP activity was assayed in the cell lysates 4 days after infection. C, overexpression of p204 and/or pRb enhanced the Cbfa-mediated OCL production. Cultures were treated as described in B and the medium was collected for assaying the level of OCL.

**FIGURE 4.** pRb was required for the enhancement by p204 of the Cbfa1-dependent activation of gene expression.

A, schematic structures of wild type and mutant p204 proteins. The N-terminal domain (N-term), the two partially conserved 200-amino acid domains (types a and b) as well as the pRb binding motifs LXGXE are indicated. In the p204mt-a mutant the LXGXE in the a domain was substituted by LXGKX in the b domain was substituted by LXGKX and in the p204mt-a/b double mutant the LXGXE motifs in both a and b domains were replaced by LXGKX. B, p204 mutants failed to enhance the Cbfa1-dependent expression of the Cbfa1-specific reporter construct p-147luc. C2C12 cells were transfected with a plasmid encoding Cbfa1 together with pCMV vector, or the pCMV204, pCMV204mt-a, pCMV204mt-b, or pCMV204mt-a/b plasmids. 48 h after transfection the cultures were harvested and the medium was collected for assaying the level of OCL.

B, p-147luc

C, luciferase activity

Cbf1

p204

p204mt-a

p204mt-b

p204mt-a/b

p6OSE2

p147

1.2

2

3.5

2–3-fold increase in the Cbfa1-dependent activity that is in accord with an earlier report (15). Cotransfection of plasmids encoding p204 and pRb, however, led to an approximately 9–10-fold increase in reporter gene activity. These data revealed the synergy between p204 and pRb in the stimulation of Cbfa1-dependent transcription.

We next explored whether p204 cooperates with pRb in the Cbfa1-dependent osteogenesis of C2C12 cells. We generated adenovirus constructs encoding pRb (Ad-pRb) and p204 (Ad-p204) and infected with these C2C12 cells (shown in the Western blots in Fig. 3A). The effects of the infection of C2C12 cells with Ad-p204, Ad-pRb, or both were tested on the Cbfa1-dependent expression of ALP and OCL, i.e. proteins that are expressed in osteoblasts. In C2C12 cells infected with an adenovirus construct encoding Cbfa1 (Ad-Cbfa1) infection with Ad-p204 or Ad-pRb increased the production of ALP slightly (between 1.5–2-fold), whereas coinfecion with Ad-p204 and Ad-pRb resulted in a stronger increase (approximately 4-fold) (Fig. 3B). OCL expression in Ad-Cbfa1-infected C2C12 cells was increased upon infection with Ad-p204 or Ad-pRb approximately 2- and 7-fold, respectively, whereas coinfecion with both Ad-p204 and Ad-pRb resulted in an approximate 20-fold increase (Fig. 3C). These results indicate that pRb synergizes with p204 also in the promotion of Cbfa1-dependent osteoblastic differentiation.

Association of p204 with pRb Is Required for the p204-mediated Stimulation of Cbfa1-dependent Transactivation—As noted, p204 contains an LXGXE pRb binding motif in both the a and b domains (Fig. 4A, top scheme). We generated p204 derivatives with mutations in the pRb binding motifs in the a domain (Ad-p204mt-a), the b domain (Ad-p204mt-b), or both domains (Ad-p204mt-a/b) (Fig. 4A, three lower schemes). In C2C12 cells infected with Ad-Cbfa1 and trans-
fected with a p-147luc reporter construct infection with Ad-p204 stimulated luciferase activity approximately 3.2-fold (over Cbfa1 alone), whereas p204 in which either of the two pRb binding motifs was mutated failed to stimulate luciferase activity (Fig. 4B). These results indicate that both LXCE motifs are needed for stimulating Cbfa1-dependent transcription.

Unexpectedly mutant p204 in which both LXCE sequences were altered strongly inhibited Cbfa1-dependent gene activation (Fig. 4B). It is conceivable that this double mutant might inhibit the association of endogenous p204 with Cbfa1.

The above results suggest that both pRb binding motifs in p204 are required to allow proper interaction between p204 and endogenous pRb that is required for the synergistic stimulation of Cbfa1-mediated transcription.

**Functioning pRb Is Needed for the p204-dependent Enhancement of Cbfa1-dependent Transcription**—We tested the need of functional pRb for the enhancement by p204 of the Cbfa1-dependent transcription in experiments with human Saos-2 osteosarcoma cells that are known to be deficient in pRb activity (39). As revealed in Fig. 4C, transfection of a p204 expression plasmid failed to stimulate the Cbfa1-mediated expression of either the p6OSE2luc or the p-147luc reporter construct in these cells lacking pRb.

**pRb Is Required for p204-mediated Enhancement of Osteoblast Differentiation**—To establish whether pRb is required for enhancement of Cbfa1-mediated osteogenesis in the osteoblast-like MLO-Y4 cells. Lysates from MLO-Y4 were processed and examined as in A. D, p204, pRb, and Cbfa1 formed a ternary complex in the osteocyte-like MLO-Y4 cells. Lysates from MLO-Y4 were processed and examined as in A.

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**Figure 5.** pRb was required for the enhancement of Cbfa1-induced osteoblast differentiation by p204. A, Western blotting assays of the expression of a p204 mutant encoded by adenovirus Ad-p204mt-a/b. C2C12 cells were infected with Ad-p204mt-a/b or Ad-p204 (serving as a control) and the expressions of p204mt-a/b and p204 were assayed by Western blotting with anti-p204 antibodies. B, mutant p204 (p204mt-a/b) failed to enhance the Cbfa1-mediated ALP activity in C2C12 cells. C2C12 cells were infected with Ad-Cbfa1, if so indicated together with Ad-p204mt-a/b or Ad-p204, incubated for 4 days and the cell lysates were assayed for ALP activity. C, mutant p204 (p204mt-a/b) failed to enhance the Cbfa1-mediated production of OCL. Cultures were treated as described in B and the medium was assayed for OCL production.

**Figure 6.** p204, pRb, and Cbfa1 formed a ternary complex in vivo. A, p204, pRb, and Cbfa1 formed a complex in the transfected C2C12 cells. Cell lysates prepared from C2C12 cells transfected with mammalian expression plasmids encoding p204, pRb, and Cbfa1 were incubated with control IgG (lane 2) or antibodies to p204 (lane 3), pRb (lane 4), or Cbfa1 (lane 5). The cell lysate (positive control, lane 1), and the immunoprecipitates from the cell lysates were examined by Western blotting with antibodies to p204, pRb, and Cbfa1. B, p204, pRb, and Cbfa1 formed a ternary complex during osteogenesis. Lysates from C2C12 cells infected with Ad-BMP-2 were incubated with antibodies to p204 (lane 1), pRb (lane 2), Cbfa1 (lane 3), or control IgG (lane 5), followed by incubation with protein A-agarose. The cell lysate (positive control, lane 4) and the immunoprecipitates from the cell lysates were examined as in A. C, p204, pRb, and Cbfa1 formed a ternary complex in the osteoblast MC3T3-E1 cells. Lysates from MC3T3-E1 were processed and examined as in A. D, p204, pRb, and Cbfa1 formed a ternary complex in the osteocyte-like MLO-Y4 cells. Lysates from MLO-Y4 were processed and examined as in A.
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Figure 7. pRb was an essential linker for the association of p204 and Cbfa1. A and B, p204 associated with Cbfa1 in C2C12 cells, but not in pRb-deficient Saos-2 cells (Co-IP assay). Cell lysates prepared from C2C12 cells, Saos-2 cells transfected with the eukaryotic expression plasmids for p204, and Cbfa1 for Saos-2 cells transfected with the expression plasmids for p204, Cbfa1, and pRb were incubated with control IgG, or antibodies to p204 (A) or Cbfa1 (B), as indicated, and the immunoprecipitated protein complexes and cell lysates (positive control) were examined by Western blotting with antibodies to Cbfa1 (A) or p204 (B). C, p204 failed to associate with Cbfa1 in vitro in the absence of functional pRb (GST pull-down assay). Purified GST (lanes 2, 5, and 8) or purified GST-204 fusion protein (lanes 3, 6, and 9) immobilized on glutathione-Sepharose beads were incubated with a lysate of C2C12 cells expressing Cbfa1 (positive control, lane 1), a lysate of Saos2 cells expressing Cbfa1 (lane 4), and a lysate of Saos2 cells expressing p204 and functional pRb (lane 7). The protein complexes retained by the beads with GST or GST-204 were assayed by immunoblotting with antibodies to Cbfa1.

Figure 8. Knockdown of pRb abolished the association of p204 and Cbfa1. A, siRNA against pRb dramatically suppressed pRb expression, assayed by Western blot. Cell lysates were prepared from MC3T3-E1 cells transfected with pSuper-pRb encoding a siRNA against pRb and the total protein was analyzed by Western blot with anti-pRb antibody. Tubulin was used as an internal control. B and C, p204 failed to associate with Cbfa1 in pRb-knockdown MC3T3-E1 cells, whereas the interaction was rescued when pRb was re-expressed (co-IP assay). Cell lysates prepared from MC3T3-E1 cells (lanes 1–3), MC3T3-E1 cells transfected with pSuper-pRb (lanes 4–6), or MC3T3-E1 cells transfected with pSuper-pRb first and then with pCMV-pRb (lanes 7–9), were incubated with control IgG, or antibodies to p204 (B) or Cbfa1 (C), as indicated, and the immunoprecipitated protein complexes and cell lysates (positive control) were examined by Western blotting with antibodies to Cbfa1 (B) or p204 (C).

whether these three proteins form a ternary complex, first we prepared cell lysates from C2C12 cells transfected simultaneously with mammalian constructs encoding p204, pRb, and Cbfa1. Immunoprecipitation of the cell lysates with antibodies to either one of the three proteins, but not control IgG, immunoprecipitated all three proteins (Fig. 6A). The relevance of these observations for osteoblast differentiation was revealed using lysates from C2C12 cells induced to undergo osteogenesis in vivo and in vitro (Fig. 6B). These results revealed that endogenous p204, pRb, and Cbfa1 also form a ternary complex in vivo.

pRb Is Required for the Association of p204 and Cbfa1 Both in Vivo and in Vitro—Functional studies revealed that pRb is required for the p204-mediated enhancement of Cbfa1-dependent transcription and osteoblast differentiation. Furthermore, as noted above, p204, pRb, and Cbfa1 form a ternary complex in the course of osteogenesis. These findings prompted us to determine whether pRb is needed for the association of p204 and Cbfa1. For this purpose we first performed co-IP assays using lysates from BMP2-treated C2C12 cells (serving as a positive control) and pRb-deficient Saos-2 cells transfected with the p204 and Cbfa1 expression plasmids. As shown in Fig. 7A anti-p204 antibodies efficiently immunoprecipitated Cbfa1 from a lysate of C2C12 cells but not from Saos-2 cells, although Cbfa1 is expressed in Saos-2 cells, as revealed by immunoprecipitation (not shown). These findings were verified with a co-IP assay (Fig. 7B). In this case, anti-Cbfa1 antibody, but not the control IgG, immunoprecipitated p204 from lysates of C2C12 cells but not from pRb-deficient Saos-2 cells. Intriguingly, the association between p204 and Cbfa1 was rescued in the Saos-2 cells transfected with a pCMV-pRb expression plasmid (Fig. 7, A and B, lanes 7–9). These results revealed that pRb is an essential mediator for the interaction between p204 and Cbfa1 in vivo.

The interaction between p204 and Cbfa1 in the presence or absence of functional pRb was also examined in GST pull-down assays in vitro (Fig. 7C). Briefly, affinity purified GST and purified GST-p204 that were immobilized on glutathione-Sepharose beads were incubated with cell extract, prepared from either BMP2-treated C2C12 cells or Saos-2 cells transfected with a Cbfa1 expression plasmid together with or without a pRb expression plasmid. After washing the glutathione-Sepharose beads the retained proteins were resolved by 10% SDS-PAGE, and detected by Western blotting. GST-p204 but not control GST pulled down Cbfa1 protein from a lysate from BMP-treated C2C12 cells and from Cbfa1- and pRb-double trans-
**FIGURE 9.** The p204-pRb-Cbfa1 transcription factor complex was associated with the osteocalcin promoter (ChIP assay). A, the p204-pRb-Cbfa1 complex was associated with the promoter of a transfected OCL-specific reporter construct p-147luc in vivo. 10T1/2 cells were transfected with the p-147luc reporter construct and expression plasmids encoding Cbfa1, pRb, and p204. After cross-linking with formaldehyde, cell lysates were prepared, sonicated, and subjected to immunoprecipitation with control IgG (lane 3) or antibodies to Cbfa1 (lane 4), pRb (lane 5), or p204 (lane 6). Purified DNA from the cell lysate (input DNA), positive control, lane 2) and DNA recovered from the immunoprecipitates were amplified by PCR and examined by gel electrophoresis. Size markers are indicated in lane 1. B, Cbfa1 transcription factor complex was bound to the segments located at 2–3 kb upstream or downstream of the Cbfa1-binding site. 10T1/2 cells were transfected with pCMVRb, which localized the Cbfa1 binding site to the OCL promoter in osteoblast MC3T3-E1 cells. MC3T3 cells were processed and DNA was examined as in A. C, pRb expression by this siRNA affects the interaction of p204 and Cbfa1 in vivo also requires the presence of functional pRb. The requirement of pRb for the binding of p204 to Cbfa1 was further tested in experiments involving silencing by siRNA. We identified a 19-nucleotide gene-specific sequence for pRb and generated a siRNA construct encoding an siRNA targeting this sequence in the Rb gene. As revealed in Fig. 8A, this construct dramatically reduced the endogenous pRb level in MC3T3-E1 osteoblasts. Next we examined whether the suppression of pRb expression by this siRNA target affects the interaction of p204 and Cbfa1 in co-IP assays using lysates from MC3T3-E1 cells (serving as a positive control), from MC3T3-E1 cells transfected with pSuper-pRb (MC3T3-E1/pSuper-pRb) and from MC3T3-E1/pSuper-pRb cells transfected with a pCMVRb construct. As shown in Fig. 8, B and C, anti-p204 or anti-Cbfa1 antibodies efficiently immunoprecipitated Cbfa1 or p204 from the cell lysate of MC3T3-E1 cells (lanes 1–3), but not from pRb-knockdown MC3T3-E1 cells (lanes 4–6), clearly indicating that pRb is needed for the association of p204 with Cbfa1. Furthermore, interaction between p204 and Cbfa1 was restored when pRb was re-expressed in MC3T3-E1/pSuper-pRb cells by transfection of pCMVRb (Fig. 8, B and C, lanes 7–9). Collectively, this set of assays indicated that pRb is strictly required for the binding of p204 to Cbfa1.

The Cbfa1-pRb-p204 Transcription Factor Complex Is Bound to the Osteocalcin Promoter in Vivo—To test whether the p204-pRb-Cbfa1 complex associates with the osteocalcin promoter upstream or downstream from the Cbfa1-binding site were not co-immunoprecipitated by the anti-Cbfa1 antibodies (Fig. 9B), indicating the sequence-specific binding of Cbfa1 to DNA. Similar ChIP assays were performed using C2C12 cells induced to osteogenic differentiation by infection with an adenovirus construct encoding BMP-2 (Ad-BMP2, generously provided by T.-C. He), MC3T3-E1 osteoblast cells as well as osteocyte-like MLO-Y4 cells. As revealed in Fig. 9, C–E, the products of the PCR analyses with the same primers as in Fig. 9A of the DNA samples in the immunoprecipitates with antibodies to Cbfa1, pRb, or p204 but not with the IgG control were of uniform and appropriate size. These results, together with the demonstration that Cbfa1, pRb, and p204 form a ternary complex in vivo (Figs. 7 and 8), indicate that the endogenous p204-pRb-Cbfa1 transcription factor complex is associated with the osteocalcin promoter.

The following results established that, as expected, endogenous pRb was required for the formation of the p204-pRb-Cbfa1 complex on the osteocalcin promoter in MC3T3-E1 osteoblasts. In a ChIP assay, using a lystate from MC3T3-E1 cells, in which pRb expression was inhibited by transfection with pSuper-pRb, the gene segment involving the Cbfa1 binding site was co-immunoprecipitated by anti-Cbfa1, but not by anti-p204 (Fig. 10A). However, in a ChIP assay, using a lystate from MC3T3 cells, in which pRb expression was inhibited by transfection with pCMVRb, anti-p204 did co-

in vivo we performed ChIP assays. These can identify the chromatin segments to which particular transcription factors are bound in vivo. ChIP was first carried out using 10T1/2 cells transfected with an OCL-specific reporter construct (p-147luc) and expression plasmids encoding Cbfa1, pRb, and p204. After cross-linking the proteins to the DNA they were associated with using formaldehyde, we lysed the cells, sheared the chromatin by sonication, and immunoprecipitated protein-DNA complexes using control IgG (negative control) as well as anti-Cbfa1, anti-pRb, or anti-p204 antibodies. We examined the DNA samples purified from the various immunoprecipitates by PCR with primers that span the Cbfa1 binding site in the OCL promoter. The PCR products of the DNA samples purified from the immunoprecipitates with antibodies to Cbfa1, pRb, or p204 (but not that obtained from immunoprecipitation with the IgG control) were identical and of the expected size (Fig. 9A). Note that segments located at 2 kb both
**DISCUSSION**

p204 is a multifunctional protein involved in the control of cell proliferation and the differentiation of numerous types of cells and tissues including skeletal muscle myotubes, cardiac myocytes, macrophages, chondrocytes, and osteoblasts. We have reported earlier that p204 associates with the key osteogenic transcription factor Cbfa1 and serves as its coactivator (15). Overexpression of p204 stimulates osteogenesis triggered by BMP2.

Here we established that inhibition of the induction of p204 expression (by 204 antisense RNA) decreased the Cbfa1-dependent formation of the osteoblast protein osteocalcin (Fig. 1). We also demonstrated that p204 and pRb, known to bind each other, synergistically stimulated Cbfa1-dependent gene expression and thereby promoted osteogenesis (Figs. 2 and 3).

p204 controls cell proliferation and differentiation by associating with various proteins. By associating, e.g. with UBF1 and/or pRb, p204 acts as an inhibitor of cell proliferation (24, 26, 27). By associating with the Id proteins and accelerating their degradation, p204 promotes the differentiation of skeletal muscle myotubes and cardiac myocytes (22, 30, 31). p204 also mediates the differentiation of macrophages, although the proteins with which it interacts in the process remain to be identified (32).

As noted, Cbfa1 (also known as Runx2, Osf2, PeBP2α, or AML3) a member of the Runt family of transcription factors, is a central regulator of osteoblast differentiation and bone formation. A variety of Cbfa1-binding proteins that positively or negatively regulate osteoblast gene expression have been identified (14, 17, 40–47). It has been reported that Cbfa1 interacts with histone acetylase (44), as well as with histone deacetylase, including histone deacetylase 3, 4, and 6 (43, 48). This suggests that chromatin remodeling is essential for regulating the osteoblast-specific genes. BMP2 and its downstream signaling proteins Smad1 and Smad5, as well as Cbfa1 stimulate osteoblast differentiation. Smads interact with Cbfa1 both in vitro and in vivo and enhance the transactivation of gene expression by Cbfa1. A Cbfa1 mutation in cleidocranial displasia impairs its association with Smads (45). Smurf1 (Smad ubiquitin regulatory factor), an E3 ubiquitin ligase, was reported to interact directly with both Smad1 and Cbfa1, and to mediate the degradation of Cbfa1 in a ubiquitin- and proteasome-dependent manner (46, 47). Core binding factor β (Cbfb, also called PEP2B), another protein of the Runt family, forms heterodimers with Runx1 and Cbfa1, and this is involved in skeletal development (13, 49). TAZ (transcriptional coactivator with PDZ binding domain) was identi-
fied to bind Cbfa1 in a yeast two-hybrid assay. Cbfa1 affects the nuclear localization of TAZ and this affects osteoblast differentiation (40). Although there is no direct interaction between activating transcription factor 4 and Cbfa1 the two proteins can be associated in vivo. Activating transcription factor 4 and Runx2 activate the expression of osteocalcin via cooperative interactions. A groucho homolog, Grg5, was isolated as a Runx2-interacting protein in a genetic screen. Grg5 modulates Runx2 activity during postnatal development in mice (50, 51). Stat1 is one of the mediators of type I and type II interferon responses. Unphosphorylated Stat1 in the cytoplasm binds Cbfa1, blocks its translocation to the cytoplasm, and thereby inhibits Cbfa1 activity and bone remodeling in postnatal mice (52). HES, a basic loop helix transcription factor interacts with Cbfa1, and thereby antagonizes the binding of Cbfa1 to mammalian corepressors of the groucho family including Grg5. This suggests that groucho and HES modulate the functioning of mammalian Runt-related proteins (53).

As described earlier pRb, whose mutants have been found in osteosarcoma patients, binds Cbfa1 and acts as its direct transcriptional coactivator (14). p204, which binds pRb, is involved osteosarcoma patients, binds Cbfa1 and acts as its direct transcriptional coactivator (14). The ternary complex (this study). In this ternary complex both p204 and pRb are the genes encoding the osteoblast-specific Cbfa1 factor (60–62) and p204 (15). Serving as a bridge pRb links p204 and Cbfa1 and activates the expression of osteocalcin via cooperative interactions. A groucho homolog, Grg5, was isolated as a Runx2-interacting protein in a genetic screen. Grg5 modulates Runx2 activity during postnatal development in mice (50, 51). Stat1 is one of the mediators of type I and type II interferon responses. Unphosphorylated Stat1 in the cytoplasm binds Cbfa1, blocks its translocation to the cytoplasm, and thereby inhibits Cbfa1 activity and bone remodeling in postnatal mice (52). HES, a basic loop helix transcription factor interacts with Cbfa1, and thereby antagonizes the binding of Cbfa1 to mammalian corepressors of the groucho family including Grg5. This suggests that groucho and HES modulate the functioning of mammalian Runt-related proteins (53).

As described earlier pRb, whose mutants have been found in osteosarcoma patients, binds Cbfa1 and acts as its direct transcriptional coactivator (14). p204, which binds pRb, is involved

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