The Interferon-inducible p204 Protein Acts as a Transcriptional Coactivator of Cbfa1 and Enhances Osteoblast Differentiation*

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Bone formation and resorption start in the embryo and continue throughout life (1). In adult bone this process is termed bone remodeling and is accomplished by precise coordination of two cell types, osteoblasts, and osteoclasts. Its deregulation leads to metabolic bone disease, including osteoporosis and osteopetrosis (2). Bone morphogenetic proteins (BMPs)1, members of the transforming growth factor-β superfamily, have been shown to play central roles in the control of osteoblast differentiation and osteogenesis and to induce ectopic bone formation in vitro when implanted into muscular tissue (3, 4). BMPs signal through heteromeric complexes of transmembrane types I and type II serine/threonine kinase receptors that propagate signals to the Smad pathway. Smad proteins mediate BMP-induced signals from the cell surface to the nucleus. Three classes of Smads have been defined: receptor-regulated Smads (R-Smads), common-mediator Smads (Co-Smads), and inhibitory Smads (I-Smads), each of which has a distinct function. R-Smads (Smad1, -5, and -8) are phosphorylated by the type I BMP receptor in response to BMP signals. Once phosphorylated, R-Smads bind to a Co-Smad (Smad4) and enter the nucleus. After translocation into the nucleus, heteromeric Smad complexes regulate transcription of target genes by binding to their consensus DNA sequences, interacting with other transcription factors, and recruiting transcription coactivators or corepressors (5, 6).

Core binding factor α-1 (Cbfa1, also known as Runx2, FeBP2a A, Osf2, or AML3), a member of the runt family of transcription factors, is an essential transcription regulator of osteoblast differentiation and bone formation (7). During skeletal development, Cbfa1 was first observed in early mesenchymal condensations and then principally expressed in osteoblasts (8). Cbfa1 -/- mice exhibit a complete lack of ossification and die immediately after birth (9). Furthermore, 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 (7). Mutations in Cbfa1 are found in 65–80% of individuals with cleidocranial dysplasia (10–12). Cleidocranial dysplasia is a dominantly inherited skeletal dysplasia with high penetrance and variable expressivity characterized by hypoplastic clavicles, large fontanelles, dental anomalies, and delayed skeletal development (13). The phenotype suggests that the primary

1 The abbreviations used are: BMP, bone morphogenetic protein; Cbfa1, core binding factor α-1; Cbfb, core binding factor-β; RANKL, receptor-activated NF-κB ligand; Id, inhibitor of differentiation; IVT, in vitro translated; SBE, Smad-binding element; ALP, alkaline phosphatase; OCL, osteocalcin; pRb, retinoblastoma protein; HDAC, histone deacetylase; Smurf1, Smad ubiquitin regulatory factor 1; Mur, muris-terone; TAZ, transcription coactivator with PDZ-binding domain; GST, glutathione S-transferase; E3, ubiquitin-protein isopeptide ligase.

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defect is due to alteration of both intramembranous and endochondral bone formation. The molecular mechanisms underlying the pathogenesis of cleidocranial dysplasia are not completely defined. Some mutants of Cbfa1 abolish its DNA-binding activity (10–12), and others disturb the associations of Cbfa1 to its binding partners, including Smads (14). It was found that several Cbfa1-binding proteins, such as retinoblastoma protein (pRb) (15) and core-binding factor-β (Cbfb) (16, 17) may also play critical roles in bone development.

The interferons are cytokines with antimicrobial, immunomodulatory, and cell growth- and differentiation-regulatory activities (18, 19). Interferons inhibit the differentiation of osteoclasts by interfering with the RANKL-induced expression (RANKL: receptor-activated NF-κB ligand) of c-Fos, an essential transcription factor for the formation of osteoclasts (20, 21). The roles of interferons in the control of osteoblast differentiation, however, remain unclear.

The activities of interferon are realized by numerous interferon-inducible proteins. p204 is a member of the interferon-inducible p20 family of proteins that are encoded by genes in the gene 200 cluster (22, 23). p204 contains 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 nuclear export signal required for the translocation of p204 from the nucleus to the cytoplasm during myoblast differentiation (24). The C-terminal domain of p204 consists of two homologous, partially conserved 200-amino acid segments (a and b) in which pRb binding motifs are located. Overexpression of p204 is growth inhibitory, probably due in part to its inhibition of mRNA transcription by binding of p204 to the ribosomal DNA-specific upstream binding factor transcription factor, and inhibiting its sequence-specific binding to DNA (25, 26). The anti-proliferative activity of p204 has been attributed to the binding of p204 to pRb via its pRb-binding LXXL motifs (25, 27, 28). Overexpression of p204 has also been found to delay the progression of cells from the G0/G1 phase to the S phase (27). p204 was found to be an important regulator of myogenesis (24, 29). In the course of the fusion of cultured myoblasts to myotubes, p204 is induced, phosphorylated, and translocated from the nucleus to the cytoplasm. Overexpression of p204 accelerates myoblast fusion (24), which is probably due to p204 binding to inhibitor of differentiation (Id) proteins, including Id1, Id2, and Id3, and overcoming the inhibition of the MyoD activity by the Id proteins (29). Here we report that p204 is a novel regulator of osteoblast differentiation and discuss the molecular mechanism underlying the modulation of osteogenesis by p204.

**EXPERIMENTAL PROCEDURES**

**Preparation of Primary Murine Calvaria Osteoblasts**—Calvaria cells were obtained from mice 1–2 days after birth by sequential collagenase digestion. Calvariae were removed under aseptic conditions and incubated with continuous agitation in Dulbecco’s modified Eagle’s medium containing trypsin (0.5 mg/ml) and EDTA (1.5 mg/ml) at 37 °C for 15 min followed by incubation in Dulbecco’s modified Eagle’s medium containing 1 mg/ml collagenase for 20 min. The collagenase digests were then discarded and replaced with fresh collagenase solution; cells released between 20 and 40 min were collected by a sedimentation step (either 20–25% sucrose or 20–25% sucrose–luciferase) and washed in PBS to remove residual collagenase. After washing, the secondary antibody (horse/rat peroxidase dioxime-coujugated anti-rabbit immunoglobulin; 1:2000 dilution) was added, and bound antibody was visualized using an enhanced chemiluminescence system (Amersham Biosciences).

**Reporter Gene Assay**—To test whether Smads can activate p204-specific reporter genes, C2C12 cells grown to 50% confluence were transfected with 1 ng of pSVGal plasmid (internal control) and mammalian expression plasmids expressing Smad1, Smad5, and Smad4 (amounts indicated in Fig. 4). To examine whether p204 affects Cbfa1-dependent transcriptional activities, C2C12 cells were transfected with 1 ng of Cbfa1-specific reporter construct pEF-Cbfa1 expressing six OSE2 elements or pEF-cDNA in which a 160-bp segment (−147 to −13) from osteocalcin promoter was inserted into the upstream of luciferase, 1 ng of pSVGal plasmid, and 1 ng of mammalian expression plasmids (pEF1-Cbfa1 expressing Cbfa1, pCMV204 expressing p204, and pCMV204AS expressing anti-sense p204 or combinations of these). After transfection, the cultures were harvested and lysed. Luciferase assays were performed using 0.2 μg of cell extract bearing six OSE2 elements or a pEF-cDNA construct (10 mM Tris-HCl, pH. 7.9, 10% glycerol, 0.5% Tween 20), blots were incubated with rabbit polyclonal anti-p204 antisera (diluted 1:1000) for 1 h. After washing, the secondary antibody (horseradish peroxidase dioxime-coujugated anti-rabbit immunoglobulin; 1:2000 dilution) was added, and bound antibody was visualized using an enhanced chemiluminescence system (Biocon, Washington, D.C.).

**Alkaline Phosphatase and Osteocalcin Assays**—Previously generated inducible p204 stable and control lines (24) were cultured in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum in the presence of 2.5 μM muristerone (Mur) for 1 day and then treated with 300 ng/ml recombinant BMP-2. Three days later, cells were lysed for ALP assay, and medium was used for OCL assay. In brief, the ALP assay mixtures contained 0.1 mM 2-amino-2-methyl-1-propanol, 1 mM MgCl2, 8 μM p-nitrophenyl phosphate disodium, and cell homogenate (12.5 μg/ml). After 3-min incubation at 37 °C, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma). Each value was normalized to the protein concentration. The amount of OCL secreted into the culture medium was determined by enzyme-linked immunosorbtent assay using a mouse osteocalcin assay kit (Biomedical Technologies, Stoughton, MA) per the manufacturer’s protocol.

In Vitro GST Pull-down Assay—For examination of the binding of p204 to Cbfa1 in vitro, glutathione-Sepharose beads (50 μl) preincubated with either purified GST (0.5 μg) serving as control or GST-204 (0.5 μg) were incubated with 500 ng of cell extracts from C2C12 cells transfected with a pEF-Cbfa1 expression plasmid in 150 μl of buffer AM (10 mM Tris-HCl, pH 7.9, 10% glycerol, 100 mM KCl, and 0.5 mg/ml bovine serum albumin). Bead-protein complexes were then collected by sample buffer AM and centrifuged twice at 400 × g for 20 min. The binding of p204 to Cbfa1-expressing plasmids encoding intact Cbfa1 or
its various C- or N-terminal deletion mutants (see Fig. 9, A and B, for details) in 150 μl of buffer AM. The bound proteins were detected with anti-Cbfa1C (C-19, epitope mapping at the C terminus) or anti-Cbfa1N (S-19, epitope mapping near the N terminus) affinity-purified polyclonal antibodies (Santa Cruz Biotechnology).

Coimmunoprecipitation—Approximately 500 μg of cell extract proteins prepared from BMP-2-treated C2C12 cells was incubated with anti-pRB (25 μg/ml, positive control), anti-Cbfa1 (20 μg/ml), or control rabbit IgG (25 μg/ml) antibodies for 1 h, followed by incubation with 30 μl of protein A-agarose (PerkinElmer Life Sciences) at 4 °C overnight. After washing five times with immunoprecipitation buffer, bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (29). Released proteins were examined by Western blotting with anti-p204 antiserum, and the signal was detected using the ECL chemiluminescent system.

RESULTS

Expression of p204 in Native Osteoblasts—To test whether p204 is expressed in native osteoblasts, calvaria cells were obtained from mice 1–2 days after birth by sequential enzymatic digestion (30, 31), and cell lysates were used for Western blot analysis with anti-p204 antiserum (24). As shown in Fig. 1, p204 antiserum specifically recognized only one band in the cell lysates (lane 3) with a molecular mass identical to that of in vitro translated (IVT) p204 (lane 1). This indicates that p204 is present in native osteoblasts. To determine whether p204 is expressed in embryonic osteoblasts and to establish its subcellular localization, immunostaining for p204 was performed on tibial growth plates of mouse embryos on postcoital day 19. As shown in Fig. 2, p204 is expressed in native osteoblasts, and predominantly localized in the nucleus. In the growth plate, p204 is highly expressed in differentiating hypertrophic chondrocytes but is absent in both resting and proliferating chondrocytes; these results suggest that p204 plays an important role in bone longitudinal growth.

Increase in p204 Levels during Osteoblastic Differentiation of C2C12 Mesenchymal Cells as Induced by BMP-2—We next examined whether the p204 level changes in the course of osteoblast differentiation. The pluripotent murine mesenchymal cell line C2C12 (32), a well established cell line for in vitro differentiation assays, was used because it is capable of differentiating into different cell types (33–35). C2C12 cells were treated with exogenous BMP-2, and the differentiation of these cells into osteoblasts was observed (36–38); osteoblast markers used were increasing alkaline phosphatase (ALP) activity and osteocalcin (OCL) production. C2C12 cells cultured in 10-cm dishes in Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum were treated with 300 ng/ml exogenous recombinant BMP-2. Cultures were harvested at various time points, and a Western blot was performed on the cell extracts using antiserum to p204. The result showed that p204 was strongly induced during this process (Fig. 3): the p204 level was highest 1 day after BMP-2 induction and remained at a high level for at least 7 days. This suggests that the gene encoding p204 is an early response gene in the BMP-2-induced signaling pathway for osteoblast differentiation from mesenchymal stem cells.

Smads Activate p204-specific Reporter Genes—A 1.6-kb segment from the 5′-flanking region of the Ifi204 gene (24) was found to contain at least five Smad-binding consensus sequences (not shown) (24). This finding prompted us to test for the involvement of Smad-binding elements (SBEs) in the induction of p204 during osteoblast differentiation. Two reporter gene plasmids, 204–2SBE-luc and 204–5SBE-luc, were used in which segments with SBEs from the 5′-flanking region of Ifi204 (~1578 to ~1324 and ~1578 to ~38) were linked to the upstream end of a region encoding luciferase in the pGL3 vector (Fig. 4, A and B; the number preceding “SBE,” i.e. 2 or 5, indicates the number of SBEs in that plasmid). Transfection of the reporter plasmids into C2C12 cells resulted in luciferase expression, with the extent of expression increasing with the length of the p204 segment in the reporter (Fig. 4C). Cotransfection of the reporter plasmids with an expression plasmid encoding either Smad1 or Smad5, but not Smad4 (cDNA constructs kindly provided by Drs. Riko Nishimura and Regis O’Keefe), significantly increased the expression of each of the reporters (Fig. 4C). Although Smad4 alone did not activate the

FIG. 1. p204 is expressed in primary osteoblasts. In vitro translated (IVT) proteins using pBS204 (lane 1) and control vector (lane 2) as templates and cell lysates prepared from calvaria-derived primary osteoblasts (lane 3) were subjected to 10% SDS-PAGE and examined by Western blotting with anti-p204 antiserum. The p204 band is indicated by the arrow.

FIG. 2. Immunohistochemistry of p204 in tibial growth plates of postcoital day 19 mouse embryos. A, low power microphotograph of section stained with anti-p204 antiserum (red) and counterstained with Mayer’s hematoxylin (blue); immunostaining reveals positive nuclear staining in osteoblasts and hypertrophic chondrocytes (see insets in part C). B, “OS” and “HC” indicate osteoblasts and hypertrophic chondrocytes. S, resting chondrocytes; P, proliferating chondrocytes; H, hypertrophic chondrocytes; M, bone metaphysis. Bar = 100 μm.

FIG. 3. Increase in the p204 level in the course of BMP-2-induced osteoblastic differentiation of C2C12 cells. After incubation for the times indicated, the cells were lysed, and 40-μg protein samples were assayed for p204 by Western blotting with antiserum to p204.
two reporters, it boosted Smad1- and Smad5-activated p204-specific reporter genes (Fig. 4C).

Because p204 levels increased substantially in the course of BMP-2-induced osteoblastic differentiation (see Fig. 3), the effects of BMP-2 on the activity of the two p204-specific reporter genes bearing the 1.6 and 0.25 segments from the 5'-flanking region of the p204 gene were also tested. As revealed in Fig. 4D, exposure of the transfected C2C12 cells to BMP-2 clearly increased the expression of both p204-specific reporter genes tested.

Overexpression of p204 Enhances BMP-2-induced Osteoblastic Differentiation—To gain further understanding of the role of p204 in osteoblastic differentiation, we examined the effect of overexpression of p204 on osteogenesis in a previously generated p204-overexpressing stable cell line (24). A Western blotting assay was performed to verify the induced p204 expression (Fig. 5A), incubation with anti-p204 antiserum. A p204-specific band was present in the lane immunoprecipitated with anti-Cbfa1 antibodies, and the absence of p204 in the control IgG lane (negative control) (25, 28), or anti-Cbfa1 antibodies, and the p204 protein complex was detected with anti-Cbfa1 antibodies. As shown in Fig. 7A, GST-204 efficiently pulled down Cbfa1 protein (lane 2), whereas GST did not pull down Cbfa1 protein (lane 1), indicating binding of p204 to Cbfa1. Transfection of a construct expressing anti-p204 RNA significantly repressed the Cbfa1-dependent activation of the two reporter genes (Fig. 6B). In both experiments, the control vector (pEF1) showed only basal activity.

p204 Associates with Cbfa1 Both in Vitro and in Vivo—The findings, that p204 regulates Cbfa1-dependent reporter gene activity and that p204-binding protein (25), binds to Cbfa1 (15), suggested that p204 may also interact with Cbfa1.

To test whether p204 binds to Cbfa1 in vitro, we expressed Cbfa1 protein in HEK293 cells and expressed p204 as a GST fusion protein (GST-204). Affinity-purified GST and GST-204 immobilized on glutathione-Sepharose beads were incubated with cell lysates prepared from HEK293 cells transfected with the mammalian expression plasmid pEF1-Cbfa1 (39) and the protein complex was detected with anti-Cbfa1 antibodies. As shown in Fig. 7A, GST did not pull down Cbfa1 protein (lane 2), whereas GST-204 efficiently pulled down Cbfa1 protein (lane 3), indicating binding of p204 to Cbfa1.

To test whether p204 binds to Cbfa1 in vivo, we performed a coimmunoprecipitation assay (Fig. 7B). Extracts from C2C12 cells treated with 300 ng/ml BMP-2 for 2 days were first incubated with control IgG (negative control), anti-Rb antibodies (positive control) (25, 28), or anti-Cbfa1 antibodies, and the immunoprecipitated complexes were detected by Western blotting with anti-p204 antiserum. A p204-specific band was pres-
ent in the immunoprecipitated complexes brought down by either anti-Rb (lane 3) or anti-Cbfa1 (lane 4), but not by control antibodies, demonstrating that p204 specifically associates with Cbfa1 in vivo.

**Two Nonoverlapping Segments of p204 Bind to Cbfa1**—To identify the one or more p204 segments to which Cbfa1 binds, various segments of p204 were linked to GST (Fig. 8A) (25). Comparable amounts of purified GST fusion proteins were confirmed using Coomassie Blue-stained bands on 12.5% SDS-PAGE (Fig. 8B). Pull-down assays were performed involving immobilized p204 segments fused to GST and an extract from HEK293 cells transfected with a mammalian expression plasmid pEF1-Cbfa1 (39) and Western-blotted with antiserum to Cbfa1 (Fig. 9C). The assays revealed that Cbfa1 binds strongly to the p204 a1 and b1 segments, but not to the N-terminal domain, a2 and b2 segments (Fig. 8, A–C).

The N-terminal 88-Amino Acid Segment of Cbfa1 Binds to p204—A series of deletions of Cbfa1 constructs (Fig. 9A) encoding truncated Cbfa1 proteins in mammalian cells (generously provided by Drs. K. Ito and Y. Toshiyuki) (14). Pull-down assays involving the immobilized GST-204 and cell extracts prepared from HEK293 cells transfected with various Cbfa1 deletion mutants (Fig. 9B) were performed to determine the ability of the various Cbfa1 mutants to associate with p204. Immunoblotting using anti-Cbfa1N against the N-terminal domain of Cbfa1 shows that a series of C-terminal domain deletions of Cbfa1 bind GST-204, as does the full-length factor (Fig. 9C, right panel). However, immunoblotting using anti-Cbfa1C against the C-terminal domain of Cbfa1 revealed that deletion of the N-terminal 88 residues domain completely abolishes the interaction of the two polypeptides (Fig. 9C, left panel), suggesting that the N-terminal segment (amino acids 1–88) of Cbfa1 contains the molecular determinants for interaction with p204.

**Proposed Model to Explain the Role of p204 in Osteogenesis**—Based on the literature and our findings in this study, we propose a model for explaining the role of p204, specifically, its expression and function, in osteogenesis (Fig. 10). The binding of BMPs to type I and II receptors results in their phosphorylation, and the receptors in turn phosphorylate the MH2 domain of R-Smads (Smad1 and -5). Phosphorylated R-Smads bind to Co-Smad (Smad4), and the resulting complex enters the nucleus. Once inside the nucleus, Smads activate the transcription of target genes, including genes encoding the osteostatotic-specific factor Cbfa1 (40–42) and p204 (this study). It is possible that p204 binds to Cbfa1 either directly or indirectly via pRb as a bridge (15, 25). Both Rb and p204 act as cofactors of Cbfa1 to regulate the transcription of Cbfa1 target genes, including ALP and osteocalcin.

**DISCUSSION**

The interferon-inducible p204 was previously shown to regulate cell proliferation and myogenesis (24, 25, 29). The current study elucidates the role of p204 in osteogenesis and the molecular events underlying this process. p204 was detected in native osteoblasts and found to be induced in the course of osteoblast differentiation in vitro. This induction appears to be due to the transactivation of the gene encoding p204 (ifj204) by Smad transcription factors, including Smad1, -4, and -5. p204 functions as a positive regulator of osteogenesis, because overexpression of p204 enhanced the differentiation of pluripotent C2C12 cells to osteoblasts as triggered by BMP-2, at least in part, in consequence of the binding of p204 to Cbfa1 and acting as its coactivator.

p204 levels increased substantially in the course of BMP-2-induced osteoblast differentiation (Fig. 3). In accord with this finding, exposure to BMP-2 increase the expression of

![Fig. 5. Overexpression of p204 enhances BMP-2-induced osteoblast differentiation, as revealed by elevated ALP activity and OCL production.](http://www.jbc.org/)

**A.** Induction of p204 expression by Mur in the inducible p204 stable (ind.p204S) C2C12 line but not in the control line. Both lines were incubated in the presence (+) or absence (−) of 2.5 μM Mur for 48 h. The effects of this incubation on p204 level were determined by Western blotting. An arrow indicates the p204 band. The fold induction of p204 is shown. B, enhancement of BMP-2-induced ALP activity by overexpression of p204. Both lines described in part A were cultured in the presence of 2.5 μM Mur with (+) or without (−) 300 ng/ml recombinant BMP-2 for 72 h, and the cell lysates were used for determining ALP activity. C, enhancement of BMP-2-induced OCL production by overexpression of p204. Cultures were treated as described in B, and the medium was collected for measuring OCL production. Data are means of triplicate samples; error bars = standard deviations. For details, see “Experimental Procedures.”
two transfected p204-specific reporter genes in C2C12 cells (Fig. 4D); the extent of the increase (∼2 to 3-fold) was, however, much lower than that of the expression of p204 during BMP-2-induced osteoblastic differentiation. These findings suggest that 1) the putative BMP-responsive region, if present, is not located in its entirety in the 1.5-kb segment from the 5'-flanking region of *Ifi204* gene that was used to generate the 204-specific reporter genes (see Fig. 4A), but may locate elsewhere, and/or 2) the post-transcriptional processes (e.g. translation and protein turnover) are also involved in the increase in the level of p204 protein in the course of BMP-2-induced osteoblast differentiation.

A variety of Cbfa1-binding proteins that positively or negatively regulate osteoblast gene expression have been identified (15, 16, 43–50). It has been reported that Cbfa1 interacts with p300 histone acetylase (HAT) (43) as well as with histone deacetylases (HDACs), including HDAC3, -4, and -6 (46, 51), suggesting that chromatin remodeling is essential for the regulation of the osteoblast-specific genes. BMP-2 and its downstream signaling molecules Smad1 and Smad5 stimulate osteoblast differentiation. Smads interact with Cbfa1 both *in vitro* and *in vivo* and enhance the transactivating ability of the latter factor, and a Cbfa1 mutation in cleidocranial dysplasia exhibits impaired association with Smads (47). The E3 ubiquitin ligase Smurf1 (Smad ubiquitin regulatory factor 1) was reported to interact directly with both Smad1 and Cbfa1 and to mediate degradation of Cbfa1 in an ubiquitin- and proteasome-dependent manner (48, 49). Core-binding factor-β (Cbfb, also called PEBP2B), another member of the Runt family, was shown to interact and form heterodimers with Runx 1 and Cbfa1, which
are required for skeletal development (16, 52). TAZ (transcriptional coactivator with PDZ-binding domain) was identified as a Cbfa1-binding partner in a yeast two-hybrid assay; subsequent assays indicated that Cbfa1 affects the nuclear localization of TAZ, which is important for the regulation of osteoblast differentiation (50). The tumor suppressor retinoblastoma protein (pRb), which, along with several of its mutations, has been found in patients with osteosarcoma, also binds to Cbfa1 and functions as a direct transcriptional coactivator (15). Similar to pRb that inhibits cell proliferation and is also involved in skeletal muscle differentiation (24, 25, 29, 53–55), p204 was also found to physically associate with Cbfa1 and to act as a coactivator of Cbfa1 in osteoblast differentiation (Figs. 6 and 7).

Interestingly, the N-terminal 88-amino acid segment of Cbfa1 was found to be required for association with p204 (see Fig. 9), whereas the C-terminal region of Cbfa1 contains binding determinants for several known Cbfa1-associated proteins, including C/EBP and HDAC6 (45, 51).

Pluripotent mesenchymal cells, e.g. C2C12 cells, undergo differentiation from myoblasts to myotubes when triggered by extracellular signals for myogenesis (e.g. withdrawal of growth factors) (56) (Fig. 11, top pathway). Withdrawal of growth factors activates transcription factors required for myoblast formation, including MyoD and myogenin, which activate among others the expression of p204. p204 enhances MyoD activity by overcoming the inhibition of Id proteins on MyoD, which accelerates myotube

FIG. 8. Binding of Cbfa1 to the p204 a1 and b1 segments. A, schematics of GST-204 fusion protein and its deletion mutants used to map the sites of Cbfa1 binding to p204. Numbers, amino acid residues in p204; N, N-terminal domain; a1, a2, b1, and b2, segments encoded by single exons. The a1 and a2 segments constitute the a segment; the b1 and b2 segments constitute the b segment. Binding of Cbfa1 to the various GST-204 segments, as shown in part C, is indicated. B, expression of GST or GST linked to p204 or its segments. Samples (0.5 μg) of affinity-purified GST, GST-204, or its segments, as indicated, were examined by SDS-PAGE and Coomassie Blue staining. C, binding of Cbfa1 to p204 and its segments. Glutathione-Sepharose beads carrying GST, GST-204, or its segments, as indicated, were incubated with extracts expressing Cbfa1, and the bound Cbfa1 was detected by immunoblotting with anti-Cbfa1 antibodies.

FIG. 9. Identification of Cbfa1 segments binding to p204. A, schematic of Cbfa1 constructs used to map the binding of Cbfa1 to p204. Numbers, amino acid residues in Cbfa1; + and −, binding or lack of binding, respectively, of the various Cbfa1 mutants to p204, as shown in part C. B, the protein expression pattern of Cbfa1 and its deletion constructs in HEK 293 cells detected by anti-Cbfa1C antibody (against the C-terminal domain of Cbfa1, left panel) and anti-Cbfa1N antibody (against the N-terminal domain of Cbfa1, right panel). C, GST pull-down assay. GST-204 immobilized on glutathione-Sepharose beads was incubated with extracts expressing various Cbfa1 mutants as shown in B, and the proteins trapped by GST-204 were detected by Western blotting with anti-Cbfa1C antisum (left panel) and anti-Cbfa1N antisum (right panel).
formation (24, 29). In the case of C2C12 cells in which MyoD is expressed, overexpression of p204 alone is sufficient to trigger myogenesis (24, 29). The induction of myotube formation by p204 requires the expression of muscle-specific transcription factors, including MyoD. Thus, p204 up-regulation alone fails to induce myotube formation from 10T1/2 cells, which do not express MyoD (29). In the presence of extracellular signals for osteogenesis (e.g., BMPs), mesenchymal cells will differentiate into osteoblasts (Fig. 11, bottom pathway). For this process, transcription factors such as Smads and Cbfa1 are essential; BMP-2 and Smads can drive the expression of p204. p204 associates with and acts as a cofactor of Cbfa1, an essential transcription factor of osteogenesis, and thereby enhances the differentiation of osteoblasts. Mesenchymal cells undergo myotube formation or osteoblast differentiation in consequence of extracellular signals. Similarly to pRb (15, 55, 57), p204 appears to play an important role in both differentiation pathways by modulating the activity of appropriate transcription factors.

Given that both p204 and pRb bind to Cbfa1 and act as its cofactors (15, 25), it remains to be determined whether p204 and pRb synergistically regulate osteoblast differentiation and whether pRb is required for the p204-mediated regulation of osteogenesis. In the case of myogenesis, nuclear p204 becomes phosphorylated and is translocated into the cytoplasm (24). In the case of embryonic osteoblasts, the immunostaining with antiserum to p204 exhibits only nuclear localization (Fig. 2). This suggests that translocation of p204 to the cytoplasm (which is important during the differentiation of myoblasts into myotubes (24, 29)) is not involved in osteogenesis.

During the differentiation of myoblasts to myotubes, Id (i.e.
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inhibitors of DNA binding (differentiation) proteins form heterodimers with several muscle-specific transcription factors, including MyoD, and block their binding to DNA as well as skeletal muscle differentiation (58–61). p204 binds to Id-1, Id-2, and Id-3 and thereby overcomes the inhibitory effect of Ids on MyoD transcriptional activity and myotube formation (24, 29). Ids have been shown to be up-regulated in osteoblast differentiation and to inhibit BMP-2 activity (62–64); the level of Id1 reaches a maximum 1 h after BMP-2 treatment and it is rapidly reduced thereafter (34). p204 protein was also induced in BMP-2-triggered osteoblastic differentiation of C2C12 cells (Fig. 3), but its level, although declining slightly, remained high to the end of the 7-day testing period. Interestingly, constitutive overexpression of Id1, Id2, and Id3 was found to strongly inhibit osteoblast differentiation of C2C12 cells initiated by BMPs (36), and inducible overexpression of p204 enhanced BMP-2-stimulated osteoblast differentiation (this study). These results suggest that p204 might also associate with Id proteins and overcome their inhibitory activities during osteogenesis, similar to what it does during myogenesis.

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REFERENCES

1. Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. (1995) Cell 80, 371–378
2. Marks, S. C., Jr. (1989) Am. J. Med. Genet. 34, 43–54
3. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471
4. Miyazono, K., ten Dijke, P., and Heldin, C. H. (2000) Adv. Immunol. 75, 115–157
5. Attisano, L., and Wrana, J. L. (2002) Adv. Immunol. 79, 647–689
6. Miyazono, K., Kusunoki, K., and Itoh, H. (1997) J. Cell. Physiol. 187, 265–276
7. Ducy, P. (2003) Dev. Dyn. 219, 461–471
8. Ducy, P., Zhang, R., Geoffroy, V., Risdal, A. L., and Karsenty, G. (1997) Cell 89, 747–754
9. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bremou, R. T., Guo, Y. H., Inada, M., Sato, M., Okamoto, K., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) J. Biol. Chem. 272, 17182–17189
10. Mundlos, S., and Olsen, B. R. (1997) FASEB J. 11, 227–233
11. Zhou, G., Chen, Y., Zhou, L., Thirunavukkarasu, K., Hecht, J., Chayattay, D., Gelb, B. D., Pirinen, S., Berry, S. A., Greenberg, C. R., Karsenty, G., and Li, X. (1998) Hum. Mol. Genet. 8, 2311–2316
12. Lee, B., Thirunavukkarasu, K., Zhou, L., Pastore, L., Baldini, A., Hecht, J., Geoffroy, V., Ducy, P., and Karsenty, G. (1997) Nat. Genet. 16, 307–310
13. Jones, K. L. (1997) in Smith’s Recognizable Patterns of Human Malformation (Saunders, W. B., ed) pp 204–205, Philadelphia, PA
14. Xie, W. F., Zhang, X., and Sandell, I. L. (2000) Matrix Biol. 19, 501–509
15. Thomas, M. P., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001) Mol. Cell 8, 303–316
16. YOSHIDA, C. A., Furuchi, T., Fujita, T., Fukuyama, R., Kanatani, N., Koba, S., Takebe, T., Tsukada, M., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) J. Cell Biol. 139, 645–649
17. Mundlos, S., and Olsen, B. R. (1997) FEBS Lett. 416, 639–645
18. Attisano, L., and Wrana, J. L. (2002) Adv. Immunol. 79, 635–644
19. Ducy, P., Zhang, R., Risdal, A. L., and Karsenty, G. (1997) Cell 89, 747–754
20. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bremou, R. T., Guo, Y. H., Inada, M., Sato, M., Okamoto, K., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) J. Cell Biol. 139, 645–649
21. Xie, W. F., Zhang, X., and Sandell, I. L. (2000) Matrix Biol. 19, 501–509
22. Thomas, M. P., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001) Mol. Cell 8, 303–316
23. Yoshida, C. A., Furuchi, T., Fujita, T., Fukuyama, R., Kanatani, N., Koba, S., Takebe, T., Tsukada, M., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) J. Cell Biol. 139, 645–649
24. Mundlos, S., and Olsen, B. R. (1997) FASEB J. 11, 227–233
25. Zhou, G., Chen, Y., Zhou, L., Thirunavukkarasu, K., Hecht, J., Chayattay, D., Gelb, B. D., Pirinen, S., Berry, S. A., Greenberg, C. R., Karsenty, G., and Li, X. (1998) Hum. Mol. Genet. 8, 2311–2316
26. Lee, B., Thirunavukkarasu, K., Zhou, L., Pastore, L., Baldini, A., Hecht, J., Geoffroy, V., Ducy, P., and Karsenty, G. (1997) Nat. Genet. 16, 307–310
27. Jones, K. L. (1997) in Smith’s Recognizable Patterns of Human Malformation (Saunders, W. B., ed) pp 204–205, Philadelphia, PA
28. Xie, W. F., Zhang, X., and Sandell, I. L. (2000) Matrix Biol. 19, 501–509
29. Thomas, M. P., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001) Mol. Cell 8, 303–316
30. Yoshida, C. A., Furuchi, T., Fujita, T., Fukuyama, R., Kanatani, N., Koba, S., Takebe, T., Tsukada, M., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) J. Cell Biol. 139, 645–649
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