Inhibition of Osteoblast-specific Transcription Factor Cbfa1 by the cAMP Pathway in Osteoblastic Cells

UBIQUITIN/PROTEASOME-DEPENDENT REGULATION*

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The cAMP pathway, a major intracellular pathway mediating parathyroid hormone signal, regulates osteoblastic function. Parathyroid hormone (through activation of protein kinase A) has also been shown to stimulate ubiquitin/proteasome activity in osteoblasts. Since the osteoblast-specific transcription factor Osf2/Cbfa1 is important for differentiation of osteoblastic cells, we examined the roles of the cAMP and ubiquitin/proteasome pathways in regulation of Cbfa1. In the osteoblastic cell line, MC3T3-E1, continuous treatment with cAMP elevating agents inhibited both osteoblastic differentiation based on alkaline phosphatase assay and DNA binding ability of Cbfa1 based on a gel retardation assay. Cbfa1 inhibition was paralleled by an inhibitory effect of forskolin on Cbfa1-regulated genes. Northern and Western blot analyses suggested that the inhibition of Cbfa1 by forskolin was mainly at the protein level. Pretreatment with proteasome inhibitors prior to forskolin treatment reversed the effect of forskolin. Furthermore, addition of proteasome inhibitors to forskolin-pretreated samples resulted in recovery of Cbfa1 protein levels and accumulation of polyubiquitinated forms of Cbfa1, indicating a role for the proteasome pathway in the degradation of Cbfa1. These results suggest that suppression of osteoblastic function by the cAMP pathway is through proteolytic degradation of Cbfa1, involving a ubiquitin/proteasome-dependent mechanism.

The osteoblast-specific factor-2/core binding factor α-1 (Osf2/Cbfa1), hereafter called Cbfa1, is a transcription factor recently shown to be a master regulator of osteoblastic differentiation. Komori et al. (1) and Otto et al. (2) observed that targeted disruption of osteoblast-specific Cbfa1 in vivo blocks skeletogenesis, and that heterozygous mutations of Cbfa1 in both human and mice lead to cleidocranial dysplasia, an autosomal dominant disorder (2–4). As further evidence of its role in differentiation, Ducy and colleagues (5) showed that forced expression of Cbfa1 induced expression of principal osteoblast-specific genes such as bone sialoprotein, osteopontin, and osteocalcin in non-osteoblastic cells. Furthermore, Ducy et al. (5) and Banerjee et al. (6) demonstrated that disruption of Cbfa1 by antisense oligonucleotides in osteoblast cultures inhibited expression of osteoblastic differentiation markers and formation of mineralized nodules.

The cAMP pathway, the major mediator of PTH signaling in osteoblasts (7), can have either anabolic or catabolic effects (8, 9) depending on the cell line used, the stage of differentiation, culture conditions, and treatment duration (10–12). PTH/cAMP have been shown to regulate expression of several osteoblastic differentiation genes including Cbfa1-regulated osteoblast-specific genes such as osteopontin (13), type I collagen (14, 15), bone sialoprotein (16), and osteocalcin (11). Although sequences conferring cAMP responsiveness have been found in the promoters of osteocalcin (8) and bone sialoprotein (16), it remains largely unknown which transcription factors mediate the cAMP response in these and other osteoblastic differentiation gene promoters.

The 26 S proteasome, a multicatalytic protease complex, is a site of regulatory degradation for most proteins (17, 18). It is an ATP-dependent extralysosomal protease that is necessary for viability and essential for regulation of proliferation and differentiation of eukaryotic cells (18). It is ubiquitously distributed throughout the cell, in the nucleus, cytosol, endoplasmic reticulum, and even associated with the cytoskeleton (18). It catalyzes degradation of rate-limiting enzymes (e.g. ornithine decarboxylase), transcriptional regulators (e.g. IκB), cell-cycle proteins (e.g. cyclins), abnormal proteins, and turnover of membrane proteins (18). Substrate proteins are tagged with multiple copies of ubiquitin for recognition by the proteasome complex. The 26 S proteasome, through the cAMP pathway, has been shown to stimulate 26 S proteasomes (19), which account for 90% of the neutral regulatory proteolysis in eukaryotic cells (20). In yeast, the involvement of proteasomes in protein turnover has been studied using various mutants of the proteasome complex. In mammalian cells, which lack such mutants, its role has been studied mainly using peptide inhibitors (17, 18). Although many aspects of the ubiquitin/proteasome pathway have been elucidated, the endogenous substrates it regulates in osteoblastic cells remain largely unknown.

Since it has been shown that cAMP regulates osteoblastic differentiation, and that Cbfa1 is essential for osteoblastic differentiation, we hypothesized that cAMP regulation of osteoblastic differentiation occurs through Cbfa1 activity. In addition, since cAMP also stimulates ubiquitin/proteasome activity in osteoblasts, we examined the role of the ubiquitin/proteasome pathway in regulation of Cbfa1 by cAMP. In the present study, we tested the effect of cAMP elevating agents on Cbfa1 expression and function in MC3T3-E1, a non-transformed
osteoelastic cell line established from newborn mouse calvaria which has a capacity to undergo osteoelastic differentiation and mineralization in vitro. Results showed that continuous treatment of cAMP agonists inhibited Cbfa1 by proteolytic degradation involving the ubiquitin/proteasome-dependent mechanism.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, dibutyryl cAMP, dibutyryl cGMP, proteasome inhibitor 1 (PSI), MG-132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal), and MG-101 (N-acetyl-Leu-Leu-Nle-CHO) were purchased from Calbiochem (San Diego, CA). Parathyroid hormone (PTH), epidermal growth factor, β-glycerophosphate, and ascorbic acid were purchased from Sigma. [35S]CaCl₂, [γ-32P]dCTP, and [γ-32P]ATP were from Amersham Pharmacia Biotech. Anti-NF-κB antibody and mouse monoclonal anti-ubiquitin antibody (PIA16) were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Human osteopontin probe (21) for Northern analysis was obtained from American Tissue Culture Collection (Rockville, MD). Human 28 S rRNA probe was obtained from CLONTECH (Palo Alto, CA). Anti-Cbfa1 antibody (22) and the 336-base pair 5' Cbfa1 DNA binding probe were custom-ordered from Life Technologies, Inc. (Gaithersburg, MD).

Cell Culture—MC3T3-E1, a mouse preosteoblast cell line was obtained from Riken Cell Bank (Japan). MC3T3-E1 cells were grown in α-minimal essential medium (Irvine Scientific) supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 units/ml). One day after plating, subconfluent cells were treated with vehicle alone (Me[S]O) or 25 μM forskolin in differentiation media (α-minimal essential medium containing 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid). The medium was changed every 3–4 days, and fresh agents (e.g. forskolin) were added, as applicable, at that time.

Gel Retardation Assay—The oligonucleotide sequences used as probes or as competitors are as follows: mouse OSE2 wild-type (5'-CGTTCAGAAGGACA-3') (5); mouse OSE2 mut-4 5'-CACGTCTTCTCACCAGTGGTC-3' (5); murine OC 5'-CTCTCTCTTCAGTCACAGC-3' (5); and 5'-GGATGCTGTGATGATCATC-3' (4) murine Cbfa1 5'-GGAGGCAACAAGTCTTCTACG-3' and 5'-GGTGGTTGCGCAGTAGTCTC-3'.

Gel Retardation Assay—Nuclear extracts (5 μg) were run on 12% Tris glycine gel (Novex, San Diego, CA) and electrotransferred to nitrocellulose membrane overnight at 4 °C. The blots were probed with anti-Cbfa1 antibody at 1:500 dilution for 2 h at room temperature. The cbfa1 protein was detected by enhanced chemiluminescence according to manufacturer's recommendations (ECL, Amersham Pharmacia Biotech).

Immunoprecipitations—15 μg of nuclear extracts were diluted to 300 μl in a dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.025% sodium azide, 0.1% bovine serum albumin), and immunoprecipitated with 4–6 μl of anti-Cbfa1 antibody for 4–5 h at 4 °C. After which time, 50 μl of Protein A-Sepharose (CL-4B) (Amersham Pharmacia Biotech) was added and incubated overnight at 4 °C. The protein A-antibody-antigen complexes were pelleted and washed twice with the dilution buffer, once with buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.025% sodium azide), and once with buffer B (50 mM Tris-HCl, pH 6.8). The immunoprecipitated proteins were boiled for 5 min in a sample buffer and separated on a 12% Tris glycine gel. The ubiquitinated Cbfa1 was probed with mouse monoclonal anti-ubiquitin antibody at 1:200 dilution for 2 h at room temperature, and visualized by ECL. The data were from a representatve of two separate experiments.

RESULTS

Effect of Forskolin on Osteoblastic Differentiation and Mineralization—To examine the effect of forskolin on osteoelastic differentiation and mineralization, MC3T3-E1 cells were treated continuously with forskolin and alkaline phosphatase activity, as well as matrix mineralization, were measured over a period of 3, 7, 14, and 21 days. The results showed that alkaline phosphatase activity increased as cells progressed through differentiation in control cells, however, this increase was blocked by forskolin treatment (Fig. 1A). Matrix mineralization, as measured by incorporation of [35S]Ca, was also inhibited by forskolin (Fig. 1B). Von Kossa cytochemical stain also showed that forskolin treatment completely inhibited mineralization (data not shown).

Effect of Forskolin on Cbfa1 DNA Binding—Since Cbfa1 has been shown to be an important regulator of osteoelastic differentiation, we examined the effect of the cAMP pathway on Cbfa1 DNA binding via gel retardation assay. Nuclear extracts of MC3T3-E1 cells, treated with 25 μM forskolin, 1 mM dibutyryl cAMP (Bt₂cAMP), or 0.5 μM PTH, were incubated with probe containing the Cbfa1 DNA-binding site (5). Results showed that forskolin, Bt₂-cAMP, and PTH reduced Cbfa1 activity (Fig. 2A). In contrast, treatment of control cells with 1 mM dibutyryl cGMP (Bt₂cGMP) or 500 ng/ml epidermal growth factor produced little or no effect (Fig. 2A).

The specificity of Cbfa1 binding to DNA probe was examined using anti-Cbfa1 antibody (22). The addition of anti-Cbfa1 antibody resulted in a supershifted band and a decrease in the faster migrating complex (lanes 1–2 versus lanes 3–4, 1 μl of antibody and 5–6, 3 μl of antibody; Fig. 2B). The supershifted band was apparently specific to anti-Cbfa1 since an irrelevant antibody (anti-NF-κB antibody) had no effect (lanes 7–8, Fig. 2B). Cbfa1 DNA binding was also competed by excess unlabeled wild-type probe but not by probe containing mutation in the Cbfa1-binding site (5) (data not shown).

Effect of Forskolin on Expression of Osteoblastic Differentiation Genes—To examine whether forskolin induced changes in Cbfa1 DNA binding correlated with changes in expression of
downstream Cbfa1-regulated genes, we determined the effect of forskolin on expression of alkaline phosphatase, bone sialoprotein, osteopontin, and osteocalcin genes. Total RNA was isolated from control and forskolin-treated samples; osteopontin expression was determined by Northern analysis; alkaline phosphatase, bone sialoprotein, and osteocalcin expression were determined by RT-PCR. RNA isolated from 7-day cultured cells was used for analyses of alkaline phosphatase, bone sialoprotein, and osteopontin expression, whereas RNA isolated from 21-day cultured cells was used for analysis of osteocalcin expression. Results showed that forskolin also inhibited the expression of alkaline phosphatase, bone sialoprotein, osteopontin, and osteocalcin genes (Fig. 2).

**Effect of Forskolin on Cbfa1 Expression**—To determine whether the inhibition of Cbfa1 DNA binding by forskolin was due to reduced Cbfa1 protein level, nuclear extracts were prepared from cells treated with forskolin for 7 or 14 days and Cbfa1 protein level was determined by Western blot analysis. Results showed that the level of Cbfa1 in control samples, detected as two immunoreactive species of 60- and 65-kDa sizes (6), was reduced in both time points of forskolin-treated samples (Fig. 3A). To determine the earliest time required for this effect, Western analysis was repeated with nuclear extracts from cells treated with forskolin for 2–48 h. Results showed that in control samples, Cbfa1 levels increased over this time (compare lanes 1, 3, 5, and 7; Fig. 3A), whereas the Cbfa1 level was reduced in samples treated with forskolin for 48 h (lanes 7 versus 8; Fig. 3A). However, the reduction in Cbfa1 level was not observed prior to 32-h treatment with forskolin (Fig. 3A), indicating that degradation requires a lag time.

We next examined the inhibition of Cbfa1 by forskolin at the mRNA level. Cells were treated with forskolin for the indicated...
time, total RNA was isolated, and Cbfa1 transcript level was analyzed by Northern analysis using Cbfa1-specific probe (5). Results showed only minor variations in Cbfa1 expression levels with forskolin treatment, except in the samples treated for 7 days, in which the transcript level of Cbfa1 was moderately reduced (Fig. 3B). Nevertheless, these changes do not appear to account for the more dramatic change in Cbfa1 protein level.

**Mechanism of Cbfa1 Inhibition by Forskolin**—To determine whether the reduction in Cbfa1 protein level in the nucleus was due to its translocation to the cytoplasm, Western analysis was performed using both cytoplasmic and nuclear extracts. Results showed no increase in cytoplasmic Cbfa1 levels with forskolin treatment (data not shown).

Since it has been previously shown that PKA activation in osteoblastic cells stimulates ubiquitination of substrate proteins and proteolytic activity of proteasomes (19), a predominant mechanism of regulatory proteolysis (20), we examined the involvement of the ubiquitin/proteasome pathway in the forskolin-induced reduction of Cbfa1 protein level. Proteasome inhibitors (MG-101, MG-132, or PSI) were added to MC3T3-E1 cells, which had been pretreated with forskolin for 3 days. Nuclear extracts, prepared 6 h after addition of inhibitors, were immunoprecipitated with anti-Cbfa1 antibody. The results showed an accumulation of the polyubiquitinated Cbfa1 in forskolin-treated cells prior to proteasomal degradation, we immunoprecipitated Cbfa1 from the samples that had been treated as in panel A (pretreatment with forskolin for 3 days and subsequently treatment with proteasome inhibitors for 6 h). The immunoprecipitates were separated on a gel and probed with anti-ubiquitin antibody which is reactive to both ubiquitin and ubiquitinated substrate proteins. Results showed an accumulation of the polyubiquitinated Cbfa1 in forskolin-treated cells in the presence of proteasome inhibitors compared with the control samples (no forskolin and no inhibitor) or the samples treated only with forskolin (Fig. 4C). These results suggest that proteasome inhibition leads to accumulation of polyubiquitinated Cbfa1.

**DISCUSSION**

These findings indicate that continuous long-term exposure of osteoblastic cells to cAMP elevating agents inhibits DNA
binding of the osteoblast-specific transcription factor, Cbfal, and that this inhibition is through proteolytic degradation involving ubiquitin/proteasome-dependent mechanism. The decreased Cbfal DNA binding correlated with inhibition of several downstream Cbfal-regulated genes including alkaline phosphatase, bone sialoprotein, osteopontin, and osteocalcin (5, 28). Forskolin also decreased von Kossa staining and radiolabeled calcium incorporation into the matrix, presumably due to inhibition of alkaline phosphatase and other extracellular matrix synthesis.

Regulation of Cbfal has been reported at a variety of levels including transcriptional and post-transcriptional. Ducy et al. (5) showed that 1,25(OH)2D3 inhibits Cbfal mRNA levels, whereas bone morphogenetic protein 4/7 increases the mRNA level of Cbfal (5, 29). In contrast, PTH regulates the collagenase-3 promoter through cooperative interaction of two transcription factors, AP-1 and Cbfal, without directly affecting their individual binding affinities (30). Our results showed that forskolin regulated Cbfal more at the post-transcriptional level than transcriptional level. Similar regulation of Cbfal at the protein level has been reported with glucocorticoid treatment (28). Forskolin also decreased von Kossa staining and radiolabeled calcium incorporation into the matrix, presumably due to inhibition of alkaline phosphatase and other extracellular matrix synthesis. 

The induction of proteases has been reported in response to PTH stimulation in MC3T3-E1. Murray and colleagues (19, 32) previously showed that PTH, through the cAMP pathway, stimulates both calpains (calcium activated papain-like proteases I and II) and 26 S proteasome activities in osteoblasts, and that membrane-permeable protease inhibitors have been shown to attenuate the effects of PTH. They also noted that the 26 S proteasomal pathway, which accounts for more than 90% of the regulatory proteolysis, is 100- to 1000-fold higher than calpain activities (19). This proteolytic mechanism also appears to account for our observed decrease in Cbfal activity with forskolin treatment.

The results showed that after 3 days of continuous forskolin treatment (4 days in culture), a 6-h incubation with protease inhibitors is sufficient to restore Cbfal to ~50% of the control level. This recovery time may appear rapid relative to the time required for degradation. However this difference may be explained by 2 considerations. First, the data indicate that Cbfal levels increase over this time period in control cells, suggesting that baseline turnover may be different between onset of degradation (32–48 h) versus the time of treatment with proteasome inhibitors (96 h). Second, the data also suggest that a lag time (at least 32 h) is required prior to induction of proteasomal dependent Cbfal degradation induced by forskolin. A similar lag time for proteolytic degradation has been reported with the transcription factor YY1, another substrate of the proteasome pathway (33). One possible explanation for this lag time is de novo synthesis of one or more enzymes involved in the ubiquitin/proteasome pathway: in T lymphocytes, induction of the ubiquitin-conjugating enzyme, hUBC9 mRNA, which targets the transcription factor ATF2 for ubiquitin/proteasome pathway, requires a 24–48-h lag time after stimulation (34). Thus, this lag time for onset of Cbfal degradation (presumably required for enzyme synthesis) together with the increasing control levels of Cbfal over time may account for this rapid recovery in the presence of proteasome inhibitors.

Preceding degradation, there appears to be a small increase in Cbfal protein levels at 24–32 h in cells treated with forskolin. This transient increase may be due to a stimulatory effect of forskolin on Cbfal protein levels during the 32-h lag time prior to onset of degradation by the ubiquitin/proteasome pathway. This is in agreement with reports by other investigators that catabolic versus anabolic effects of PTH/cAMP on osteoblastic differentiation depend on exposure time (8–10).

Finally, our data indicate that Cbfal is a novel endogenous substrate for the proteasome in osteoblastic cells, in this case one that is induced by the cAMP pathway. These results provide new insight into the mechanistic basis of osteoblast inhibition by hormones such as PTH, through the cAMP pathway, namely proteasome-dependent degradation of Cbfal, the master regulator of osteoblastic differentiation.

Acknowledgments—We thank S. Jackson for suggestions, S. Kao and H. Huyhn for technical assistance, and A. Han and S. Munton for general assistance. The graphic illustrations were prepared in the Bio-medical Technology Research and Instructional Production facility, and we also thank its staff for assistance.

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Effect of the cAMP Pathway on Cbfal

28879