Osteoblast differentiation and bone formation is stimulated by bone morphogenetic protein (BMP)-2 and its downstream signaling molecules Smad1 and -5 and the osteoblast-specific transcription factor core-binding factor α1 (Cbfa1). Proteolytic degradation of Smad1 and Cbfa1 is proteasome-dependent, and intracellular concentrations of Smad1 and Cbfa1 are enhanced by inhibition of the 26 S proteasome. Smad1 degradation is mediated by the E3 ubiquitin ligase Smurf1 (Smad ubiquitin regulatory factor 1), but the specific E3 ligase responsible for Cbfa1 degradation has not been identified. Because Cbfa1 interacts with Smurf1, whose degradation is mediated by Smurf1, we examined the effect of Smurf1 on Cbfa1 degradation in osteoblast precursor cells. Smurf1 interacts directly with Cbfa1 and mediates Cbfa1 degradation in a ubiquitin- and proteasome-dependent manner. Because Smurf1 controls the intracellular concentrations of several key molecules in the bone formation cascade, we examined the effect of a mutant form of Smurf1 in osteoblasts and found that expression of mutant Smurf1 markedly enhanced osteoblast differentiation. Smurf1 therefore appears to be an important regulatory factor in osteoblast differentiation and a potential molecular target for identification of bone anabolic agents.

Bone formation is regulated by a number of growth regulatory factors, key signal transduction molecules, and critical transcription factors. Important among these are the bone morphogenetic proteins (BMPs), which promote normal appositional bone growth and induce ectopic bone formation experimentally (1). BMP-2 and -4 act through a complex serine-threonine kinase receptor mechanism to activate the signal transduction molecules Smad1 and -5, which in turn activate downstream target genes with other transcription factors. The mechanism of action and regulation of BMP signaling molecules during osteoblast differentiation are not fully defined.

Cbfa1 is a transcription factor that belongs to the runt-domain gene family and plays an essential role in osteoblast differentiation, bone development, and postnatal bone formation (2–5). Its expression and activity are regulated by many bone-derived growth factors, including BMPs (2, 6). BMPs may stimulate osteoblast differentiation by activating Cbfa1 through Smad1, as evidenced by reports that Smad1, the downstream effector of BMP signaling, directly interacts with Cbfa1 (7), but precise signaling mechanisms remain unclear.

The activities of signaling proteins and transcription factors are regulated at both the transcriptional and post-translational levels. Recent reports (8–10) demonstrate that both Smad1 and Cbfa1 undergo ubiquitin-proteasome-mediated degradation. Protein ubiquitination involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin ligases (11), which play a crucial role in defining substrate specificity and subsequent protein degradation by the 26 S proteasomes. Smurf1 is a member of the Hect family of E3 ubiquitin ligases and has been found to interact with the BMP-activated Smad1 and -5, thereby triggering their ubiquitination and degradation (8).

Hect domain proteins represent a major subclass of E3 ligases and contain a conserved cysteine located at the carboxyl-terminal of the Hect domain that is capable of forming a thioester bond with ubiquitin (8, 12). Another motif often found in the Hect family of E3 ligase is the WW domain, which contains two highly conserved tryptophans and a conserved proline in an -30-amino acid region (8, 13). The WW domains have a preference for binding to small proline-rich sequences, PXXY motifs, and different WW domains possess different substrate specificity. Although Cbfa1 has been reported to be degraded through the ubiquitin-proteasome pathway (10), the specific E3 ubiquitin ligase for Cbfa1 has not been identified.

In the present studies, we examined the relationship between Smad1, Cbfa1, and Smurf1 in osteoblast precursor cells. Smad1 interacts directly with Cbfa1 in these cells, and both Smad1 and Cbfa1 are required for BMP-2 signaling. The intracellular concentrations of both Smad1 and Cbfa1 are dependent on osteoblast osteosomal function. Smurf1 mediates Cbfa1 degradation in a ubiquitin proteasome-dependent manner and therefore is a powerful regulator of osteoblast differentiation.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Myc-tagged Smad1 expression plasmid was obtained from Dr. R. Nishimura (Department of Biochemistry, Osaka University, Osaka, Japan), and FLAG-tagged Smurf1 and mutant Smurf1 (C710A) cDNA plasmids in SK vector were obtained from Dr. G. Thomsen and cloned into pcDNA3 expression vector. Cbfa1 cDNA was

2 Other names for Cbfa1/Runx2 are PEBP2α-A, AML-3, and Osf-2.
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FIG. 1. Smurf1 mediates Cbfa1 degradation in osteoblast precursor cells. a, Smurf1 mediates Cbfa1 degradation. Dose-dependent reductions of steady-state levels of Cbfa1 protein were detected by Western blot using an anti-FLAG antibody. Different amounts of Smurf1 expression plasmid (0.125–2 μg/well) were co-transfected into FLAG-Cbfa1 (F-Cbfa1) expression plasmid into C2C12 cells in 6-well culture plates and processed for Western blot analysis using an anti-FLAG antibody. b, Smurf1 mediates endogenous Cbfa1 degradation. 2T3 cells were transfected with empty vector or Smurf1 expression plasmid (0.5 and 1 μg/well, 6-well plate) and cultured for 48 h. Cbfa1 expression was detected by Western blot using a polyclonal antibody against human Cbfa1. Transfection of Smurf1 reduced Cbfa1 protein levels. c, Smurf1 mediates Cbfa1 ubiquitination. Ubiquitinated Cbfa1 protein ladders were detected by an anti-HA antibody. M-Cbfa1 (M-Cbfa1), FLAG-Smurfl (F-Smurfl) and HA-ubiquitin (HA-UB) expression plasmids (5 μg/dish, 10-cm culture dish) were co-transfected into C2C12 cells, and Cbfa1 protein was immunoprecipitated by an anti-Myc antibody. d, Smurf1-mediated Cbfa1 degradation is proteasome-dependent. Cbfa1 degradation induced by Smurf1 was detected by Western blot using an anti-FLAG antibody. Smurf1 expression plasmid (1 μg/well in 6-well plate) was co-transfected with FLAG-Cbfa1 plasmid into C2C12 cells. Treatment with proteasome inhibitor PS1 (1 μM) completely abolished the effect of Smurf1 on Cbfa1 degradation. e, Interaction of Cbfa1 with Smurf1 is demonstrated in the upper panel. ΔSmurf1 was detected by Western blot after Myc-Cbfa1 (M-Cbfa1) and FLAG-ΔSmurf1 (G710A) expression plasmids (5 μg/dish, 10-cm culture dishes) were co-transfected into C2C12 cells and Cbfa1 protein was immunoprecipitated by an anti-Myc antibody. M-Cbfa1 in total cell lysates is shown in the lower panel. f, Smurf1 regulates Cbfa1 biological activity. Expression of Cbfa1 stimulated luciferase activity of the Cbfa1 reporter gene, 6×OSE2-OC/pGL3, following Cbfa1 expression plasmid and 6×OSE2-OC/pGL3 reporter construct co-transfection into C2C12 cells. Expression of Smurf1 almost completely inhibited the activity of Cbfa1, whereas expression of ΔSmurf1 had no significant effect on Cbfa1-induced luciferase activity of the reporter. *, p < 0.05; Student’s t test (control versus Cbfa1); #, p < 0.05; Student’s t test (Cbfa1 versus Cbfa1 + Smurf1).

amplified by RT-PCR based on mouse Cbfa1 cDNA sequences (GenBankTM accession number AF010284) using RNA extracted from 2T3 osteoblast precursor cells and then cloned into p3×FLAG-CMV vector (Sigma). The 6×OSE2-OC-pGL3 reporter construct was generated by PCR as described by Ducy et al. (2). The constitutively active type 1B BMP receptor (cbBMPR-IB) expression plasmid was obtained from Dr. X. Cao (Department of Pathology, University of Alabama at Birmingham, Alabama).

Cell Culture and Transfections—C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium, and 2T3 osteoblast precursor cells were cultured in α-minimal essential medium supplemented with 8% fetal calf serum. The cDNA expression plasmids were transiently transfected into these cells using LipofectAMINE Plus reagents (Invitrogen) in a 10-cm-diameter culture dish for immunoprecipitation assay, 6-well culture plates for Western blotting analysis, and 24-well plates for the luciferase assay. 5, 1, and 0.2 μg of expression plasmid were used for these transfections. After transfection (48 h), cells were lysed with lysis buffer. In proteasome inhibitor experiments, transfected cells were incubated with the proteasome inhibitors for 2, 6, or 24 h, the proteasome inhibitor was removed, and the cells were then cultured an additional 24 h.

Immunoprecipitation and Immunoblotting—Expression plasmids were transfected into C2C12 cells separately or together for immunoprecipitation. In Western analysis, the total amounts of transfected plasmids in each group were equalized by addition of empty vector cDNA. 48 h after transfection, cells were washed three times with phosphate-buffered saline and solubilized in lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% doc, and 50 mM Tris buffer, pH 7.5). For Western blotting, 0.1% SDS was included in the lysis buffer. The protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) were added to the lysis buffer. Cell lysates were centrifuged for 10 min at 4 °C at 10,000 × g and incubated with anti-Myc antibody for 4 h at 4 °C, followed by immunoprecipitation with protein G-agarose (Roche Applied Science) at 4 °C overnight. Immunoprecipitates were washed with lysis buffer five times, added to 1× reducing buffer containing 0.5 M β-mercaptoethanol, and boiled for 3 min. The immunoprecipitation and Western blotting samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with anti-FLAG or anti-Myc antibody, and visualized with horseradish peroxidase-coupled anti-mouse IgG antibody (Amersham Biosciences) with an enhancement by ECL detection kits (Amersham Biosciences). A monoclonal antibody against human Smad1 and a polyclonal antibody against human Smurf1 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. A polyclonal antibody against human Cbfa1 was purchased from Oncogene Research Product, Cambridge, MA.

Luciferase Assay—Oligonucleotides containing 6 copies of Cbfa1 response element, OSE2, (6×OSE2) and 12 copies of Smad1 binding element (12×SBE) were synthesized in the DNA laboratory at The University of Texas Health Science Center at San Antonio. The oligonucleotides were cloned in front of the osteocalcin basal promoter (−155/+1), which was amplified by RT-PCR, and cloned in pGL3 vector. C2C12 and 2T3 cells were co-transfected with Cbfa1 expression plasmid and 6×OSE2-OC-pGL3 reporter plasmid in the presence or absence of Smurf1, or Smurf1 expression after forskolin treatment. 2C212 cells were treated with 4, 20, and 100 μM forskolin and cultured for 4 days. Smurf1 was detected by Western blot using a polyclonal antibody against human Smurf1. Treatment with forskolin slightly increases Smurf1 expression in C2C12 cells. b, transfection of ΔSmurf1 inhibits forskolin-induced Cbfa1 degradation. FLAG-Cbfa1 expression plasmid was transfected into C2C12 cells with empty vector or ΔSmurf1, treated with 25 and 50 μM forskolin, and cultured for 4 days. Cbfa1 was detected by Western blot using an anti-FLAG antibody. A significant reduction in Cbfa1 protein levels was found when C2C12 cells were treated with forskolin. Transfection of ΔSmurf1 inhibits forskolin-induced Cbfa1 degradation.

FIG. 2. Smurf1 is involved in forskolin-induced Cbfa1 degradation. a, Smurf1 expression after forskolin treatment. C2C12 cells were plated at 60% confluency, transfected transiently with Smurf1 or mutant Smurf1 expression plasmids using LipofectAMINE Plus reagents (Invitrogen), and normalized by β-galactosidase activity.
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RESULTS AND DISCUSSION

Smurf1 Mediates Cbfa1 Degradation in Osteoblasts—Because Cbfa1 interacts with Smad1 protein (7) whose degradation is mediated by Smurf1 (8), we examined the effect of Smurf1 on Cbfa1 degradation in myoblast/osteoblast precursor C2C12 cells and osteoblast precursor 2T3 cells. FLAG-tagged Cbfa1 expression plasmid was co-transfected with different amounts of Smurf1 expression plasmid into C2C12 and 2T3 cells. Expression of Smurf1 greatly reduced steady-state levels of Cbfa1 protein and Cbfa1, and ubiquitin expression plasmids in C2C12 cells. After transfection, the medium was collected; the cells were washed twice with ice-cold phosphate-buffered saline, and cell lysates were extracted with 0.05% Triton X-100. ALP activity in cell lysates was measured using a Sigma ALP assay kit (Sigma), and osteocalcin in the medium was measured with a mouse osteocalcin immunoradiometric assay (IRMA) kit (Immutopics, Inc., San Clemente, CA).

Calvarial Periosteal Bone Formation Assay—Proteasome inhibitor 1 (PS1) was injected subcutaneously over the parietal bone of the calvariae of 2-month-old ICR Swiss mice (14). Mice were sacrificed 14 days after commencing treatment (20 g of phosphate-buffered saline) alone or PS1 (0.2, 1, and 5 mg/kg/day, daily for 5 days). Mice were sacrificed 14 days after commencing injections, and calvarial bones were removed. The bones were decalciﬁed in 14% EDTA, bisected coronally midway between the coronal and lambdoid sutures, dehydrated through graded alcohols, and embedded in paraffin. Transverse 3-μm-thick sections were cut and stained with hematoxylin and eosin. Newly formed bone was identiﬁed historically using an E400 microscope (Nikon Inc., Melville, NY) linked to a color video monitor (PVM-14M2MDU Trinitron, Sony Corp., Japan) and image capture techniques.

Smurf1 Mediates Cbfa1 Degradation—Smurf1 mediates Cbfa1 degradation in osteoblasts because Cbfa1 interacts with Smad1 protein (7) whose degradation is mediated by Smurf1 (8). Using epitope-tagged proteins in co-immunoprecipitation and Western blot analyses, we found that Cbfa1 co-precipitates with Smurf1 (Fig. 1c, d). A putative PY motif, presumably bound with the WW domain of Smurf1 protein, was found in the carboxyl-terminal of Cbfa1, suggesting that Smurf1 directly binds Cbfa1 and mediates its degradation.

Smurf1 Mediates Smad1 Degradation in Osteoblasts—Altogether, these results demonstrate that Smurf1 mediates Smad1 degradation. Dose-dependent reductions in steady-state levels of Smad1 protein were detected by Western blot using an anti-FLAG antibody. Different amounts of Smurf1 expression plasmid (0.0625–1 μg/well, 6-well culture plates) were co-transfected with FLAG-Smad1 (F-Smad1) expression plasmid into C2C12 cells. Expression of Smurf1 induced a signiﬁcant reduction in protein level of Smad1, and expression of ΔSmurf1 had only a minor effect on protein level of Smad1. Smurf1 and ΔSmurf1 expression plasmids (0.5 μg/well) were co-transfected with FLAG-Smad1 expression plasmid (1 μg/well) into C2C12 cells in 6-well culture plates. Smurf1-mediated Smad1 degradation is proteasome-dependent. Smad1 protein was detected by Western blot using an anti-FLAG antibody. Expression of Smurf1 reduced protein levels of Smad1, and treatment with the proteasome inhibitor epoxomicin (12.5, 25, and 50 nM) reversed the effects of Smurf1 on Smad1 degradation in a dose-dependent manner.

Cbfa1 to activate a speciﬁc reporter gene, 6×OSE2-OC-Luc (15), in C2C12 cells. A Cbfa1 expression plasmid and 6×OSE2-OC-Luc reporter construct were co-transfected into C2C12 cells in the presence and absence of Smurf1 or ΔSmurf1. Expression of Cbfa1 signiﬁcantly increased activity of 6×OSE2-OC-Luc reporter, whereas co-transfection of Smurf1 with Cbfa1 signiﬁcantly increased luciferase activity of the reporter gene. In contrast, co-transfection of ΔSmurf1, lacking in catalytic activity, had no signiﬁcant effect on Cbfa1-induced luciferase activity of the reporter gene (Fig. 1f). These results demonstrate that Smurf1 mediates Cbfa1 degradation and leads to a decrease in Cbfa1 activity. That there was no signiﬁcant effect of ΔSmurf1 on Cbfa1-induced luciferase activity of the reporter gene may be because activation of the reporter gene by expressed Cbfa1 had already plateaued.

Because it has been reported that Cbfa1 degradation is CaM-dependent in osteoblasts (10), in the present studies we also treated C2C12 cells with forskolin, an agent that stimulates adenylyl cyclase and examined expression of endogenous Smurf1, Cbfa1 degradation, and the effect of ΔSmurf1 on forskolin-induced Cbfa1 degradation. Although forskolin treatment increased endogenous Smurf1 level only slightly (Fig. 2a), Cbfa1 protein level was signiﬁcantly reduced by forskolin; this reduction was blocked in cells transfected with ΔSmurf1 (Fig. 2b). These results suggest that Smurf1 may be involved in the CaM-induced Cbfa1 degradation.
in COS cells transfected with a Smad1 expression plasmid, the effects of Smurf1 on Smad1 degradation in osteoblasts, and in particular degradation of endogenous Smad1 protein by proteasomal-dependent mechanism in osteoblasts, have not been examined. In the present studies, we confirmed the effect of Smurf1 on Smad1 degradation in osteoblast precursor cells and examined the regulatory roles of proteasome inhibitors on Smad1 protein. We first examined the effect of Smurf1 on steady-state protein levels of Smad1 in C2C12 and 2T3 cells and found that Smurf1 mediates Smad1 degradation in a dose-dependent manner (Fig. 3a). The effect of Smurf1 on Smad1 degradation is ubiquitin-proteasome-dependent because the catalytic mutant of Smurf1 (C710A) had only minor effects on Smad1 degradation (Fig. 3b) and the proteasome inhibitor epoxomicin reversed the effect of Smurf1 on Smad1 degradation in a dose-dependent manner (Fig. 3c). Similar effects were observed when other structurally different proteasome inhibitors such as PS1, lactacystin, and MG-132 were utilized (data not shown). To further determine the effects of proteasome inhibitors on the steady-state protein levels of endogenous Smad1, the C2C12 cells were treated with different proteasome inhibitors for 6 h. The treatment with proteasome inhibitors increased Smad1 levels 2–6-fold (Fig. 3d), demonstrating that Smad1 is under Smurf1-mediated ubiquitin-proteasome regulation in osteoblasts.

Chf1a Is Required for Activation of BMP-2 Signaling—Chf1a has been reported to interact with Smad1, but details of the transactivation mechanism of these two signaling molecules have not been clearly defined. Therefore, we examined whether Chf1a is specifically required for BMP signaling by first confirming the interaction of Chf1a with Smad1 in osteoblast precursor cells. Epitope-tagged Chf1a and Smad1 expression plasmids were co-transfected into C2C12 and 2T3 cells. Co-precipitation of Chf1a and Smad1 was detected in both cells (Fig. 4a). To determine the role of Chf1a in BMP signaling, we generated multiple copies of Smad1 response element (OSE2) and cloned those upstream of an osteocalcin and a SV40 basal promoter. Expression of Chf1a completely blocked the effect of caBMPR-IB on the BMP-2 reporter. Treatment with BMP-2 (100 ng/ml) and expression of caBMPR-IB stimulated the BMP-2 signaling reporter, 12×SBE-OCpGL3 reporter construct with or without Chf1a expression plasmid and the 12×SBE-SV40/pGL3 reporter construct with or without Chf1a expression plasmid. BMP-2 or expression of caBMPR-IB had no effect on the 12×SBE-ΔOC/pGL3 reporter with in which the Chf1a binding site has been deleted. Luciferase activity was normalized to β-galactosidase activity.

Fig. 4. Chf1a is required for activation of BMP-2 signaling. a, Chf1a interacts with Smad1. Chf1a protein was detected by Western blot using an anti-FLAG antibody (upper panel) after FLAG-Chf1a (F-Chf1a) and Myc-Smad1 (M-Smad1) expression plasmids (5 μg/dish, 10-cm culture dishes) were co-transfected into C2C12 cells and Smad1 protein was immunoprecipitated by an anti-Myc antibody. Protein levels of F-Chf1a and M-Smad1 in total cell lysates, detected by Western blot, are shown in the lower panel. b, BMP-2 and caBMPR-IB (caIB) stimulate the BMP signaling reporter. Treatment with BMP-2 or expression of caBMPR-IB significantly stimulated luciferase activity of the BMP signaling reporter, 12×SBE-OCpGL3, but had no effect on 12×SBE-SV40/pGL3 reporter gene activity when these reporter constructs were transfected into C2C12 cells. Luciferase activity was measured 24 h after transfection and normalized to β-galactosidase activity. *, p < 0.05, Student’s t test (BMP-2 versus control). #, p < 0.05, Student’s t test (caIB versus pCMV5). c, ΔChf1a blocks BMP signaling. Expression of caBMPR-IB activates the 12×SBE-OCpGL3 reporter gene in C2C12 cells. Expression of ΔChf1a completely blocked the effect of caBMPR-IB on the BMP signaling reporter. Luciferase activity was normalized to β-galactosidase activity. *, p < 0.05, Student’s t test (caIB versus pCMV5). #, p < 0.05, Student’s t test (caIB + ΔChf1a versus caIB). d, binding of Chf1a to its response element is required for activation of the BMP-2 reporter. Treatment with BMP-2 (100 ng/ml) and expression of caBMPR-IB stimulated the BMP-2 signaling reporter, 12×SBE-OC-pGL3, but had no effect on the 12×SBE-ΔOC/pGL3 reporter in which the Chf1a binding site has been deleted. Luciferase activity was normalized to β-galactosidase activity. *, p < 0.05, Student’s t test (BMP-2 versus control). #, p < 0.05, Student’s t test (caIB versus pCMV5).
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ΔOC) completely lost its response to BMP-2 or caBMPR-IB (Fig. 4d). These results suggest that Cbfa1 is required for BMP-2 and Smad1 to activate downstream target genes in osteoblasts.

Function of Smurf1 in Osteoblast Differentiation—To examine the function of Smurf1 in osteoblast differentiation, a Smurf1 or ΔSmurf1 expression plasmid was transfected into C2C12 cells, and ALP activity and osteocalcin production were measured. Expression of Smurf1 significantly reduced ALP activity as well as osteocalcin production in C2C12 cells (data not shown). In contrast, transfection of ΔSmurf1 increased basal as well as BMP-2-induced ALP activity and osteocalcin production in these cells (Fig. 5, a and b). Similar results were obtained when the same expression plasmids were transfected into 2T3 cells (data not shown). These results suggest that Smurf1 mediates Cbfa1 and Smad1 degradation and inhibits function of Cbfa1 and Smad1. ΔSmurf1 acts in a dominant-negative fashion to inhibit the degradation of Cbfa1 and Smad1.

To establish a correlation between Smurf1-mediated Smad1 degradation and osteoblast differentiation, we analyzed changes in endogenous Smad1 protein after C2C12 and 2T3 cells were transfected with mutant Smurf1 or treated with proteasome inhibitors. We found that proteasome inhibitors increase endogenous Smad1 levels 2–6-fold (Fig. 3d). Transfection of ΔSmurf1 causes a 5-fold increase in protein levels of endogenous Smad1. Overexpression of Smad1 (6-fold higher than endogenous Smad1) significantly enhanced ALP activity and osteocalcin production in C2C12 cells (Fig. 5, a and b). These results suggest that enhanced Smad1 levels by ΔSmurf1
or proteasome inhibitors promote osteoblast differentiation.

Proteasome Inhibitors Induce Osteoblast Differentiation and Bone Formation—To further determine the role of proteasome inhibitors in osteoblast function and bone formation, we examined the effects of PS1, a synthetic proteasome inhibitor, on ALP activity and the BMP signaling reporter in C2C12 cells and on periosteal bone formation in rodents in vivo. We found that in C2C12 cells, treatment with PS1 increased ALP activity (Fig. 6a) and luciferase activity of the 12SBE-OC-Luc reporter (Fig. 6b) in a dose-dependent manner. Increasing concentrations of PS1 (0.2, 1, and 5 mg/kg/day) were injected daily for 5 days into subcutaneous tissues over the calvariae of mice. The mice were sacrificed 2 weeks after the injections, and calvariae were processed for histology. PS1 induced significant new bone formation (Fig. 6, c and d). Similar effects were also obtained when epoxomicin, a naturally occurring proteasome inhibitor, was administered in vivo (data not shown). Moreover, these proteasome inhibitors administered systemically stimulated bone formation in intact and ovariectomized mice (16). These results demonstrate that inhibition of Smurf1 and proteasome degradation can lead to increased osteoblast function and suggest that part of the mechanism of proteasome inhibitor on new bone formation may be due to regulation of intracellular protein levels of Cbfa1 and Smad1.

We have demonstrated for the first time that 1) E3 ubiquitin ligase Smurf1 mediates Cbfa1 degradation through the ubiquitin-proteasome pathway, 2) Cbfa1 is required for BMP-2 signaling, and 3) expression of mutant Smurf1 stimulates osteoblast differentiation. These findings suggest that Smurf1 plays an important role in osteoblast differentiation by regulating Cbfa1 and Smad1 function.

Cbfa1 is a critical transcription factor in osteoblast differentiation and function (2). BMPs have been shown to stimulate osteoblast differentiation in vitro and in vivo (1), in part by up-regulating Cbfa1 expression (2, 6) and/or through direct interaction with Smad1 and Cbfa1 (7). Smad1 is a downstream mediator of BMP receptors and plays a central role in BMP receptor signaling (17). In the present studies, we found that Cbfa1 is required to activate BMP-2 signaling. It has been reported (18, 19) that Smad1 and Cbfa1 binding sites co-localize in the promoters of several bone-related genes. Taken together with our results, this suggests that Smad1 and Cbfa1 co-activate downstream target genes in osteoblasts.

Expression of mutant Smurf1 inhibits forskolin-induced Cbfa1 degradation in C2C12 cells, suggesting that Smurf1 may be involved in Cbfa1 degradation induced by a cAMP-dependent protein kinase pathway. Parathyroid hormone (PTH) induces osteoclastic degradation of protein substrates in osteoblasts (20), and it has recently been reported that the anabolic effect of PTH requires Cbfa1-dependent signaling (21). We speculate that PTH may induce Cbfa1 degradation via aCAMP-dependent signaling and that Smurf1 is involved in this process. However, this remains to be determined.

To maintain efficient signal transduction and gene activation, cells must precisely regulate levels of signaling proteins and transcription factors during cellular processes. Under physiological conditions, Smurf1 may mediate degradation of the Cbfa1-Smad1 protein complex, but further investigation is required for unequivocal proof. Because Smurf1 mediates degradation of two critical proteins in the BMP signaling pathway and overexpression of Smurf1 modulates osteoblast differentiation and function, we propose that Smurf1 plays an important role in bone formation in vivo. It has been shown that Smurf mutations in Drosophila lead to enhanced decapentaplegic (a homologue of BMP-2/4) signaling and downstream target gene expression (22). Our findings suggest that Smurf1 may be a critical protein regulating Cbfa1 and Smad1 functions and, ultimately, osteoblast differentiation and bone formation.

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