Bone Morphogenic Protein 2 Activates Protein Kinase D to Regulate Histone Deacetylase 7 Localization and Repression of Runx2

The transcriptional activity of Runx2 is determined by associations with co-repressors including histone deacetylase 7 (HDAC7). We previously found that bone morphogenic protein 2 (BMP2) induces export of HDAC7 from the nucleus. In this study we demonstrate that BMP2 specifically stimulates redistribution of HDAC7 but not HDAC 4, 5, or 6. HDAC7 subcellular redistribution in mesenchymal cells requires Crm1-mediated nuclear export, is associated with increased HDAC7 serine phosphorylation, and requires conserved serines in the HDAC7 amino terminus. The protein kinase D (PKD) inhibitor Go6976 blocked both basal and BMP2-directed HDAC7 nuclear export. Protein kinase D1 (PKD1) associated with HDAC7 in a BMP2-enhanced manner, and a constitutively active form of PKD1 stimulated HDAC7 nuclear export. Furthermore, active PKD1 inhibited repression of Runx2-mediated transcription by HDAC7. Suppression of HDAC7 was not sufficient to rescue BMP2 induction of osteoblast marker genes in Go6976-treated cells, indicating that PKD-dependent factors beyond attenuation of HDAC7-repressive activity are required for osteoblast differentiation. These results establish a novel pathway by which BMP signaling regulates Runx2 activity via PKD-dependent inhibition of HDAC7 transcriptional repression.

Bone morphogenetic proteins (BMPs) are central regulators of skeletal biology (1, 2). These secreted proteins promote bone formation and maintenance by binding to receptor complexes and stimulating multiple intracellular pathways. BMP signaling has been implicated in numerous aspects of skeletal development including lineage specification, growth, and differentiation of osteoblasts and chondrocytes as well as limb and dermal patterning. They are used clinically to enhance bone healing in cases of nonunion fractures and spinal fusions (3). Their osteoinductive potential is also strikingly demonstrated in cases of fibrodysplasia ossificans progressiva, a disease characterized by progressive ectopic formation of bone that is caused by aberrant activation of the BMP signaling pathway, most commonly by activating mutations in the BMP receptor complex (4). BMPs elicit their cellular responses through a number of signal transduction pathways (5, 6). Best known are the SMADs, a group of transcription factors that are phosphorylated by BMP receptor complexes, translocate to the nucleus, and alter gene transcription, frequently in association with other transcription factors. BMP signaling also activates a number of kinase cascades including mitogen-activated protein kinase pathways (7–11) and protein kinase D (PKD) (9, 12).

Histone deacetylases (HDACs) are a large family of related co-repressor proteins (13–15). HDACs repress transcription by catalyzing the removal of acetyl-groups from histones, leading to an inactive chromatin state and reduced transcription, but they also deacetylate non-histone substrates. Class IIa histone deacetylases, HDACs 4, 5, 7, and 9, are composed of a carboxyl-terminal deacetylase catalytic domain and an amino-terminal domain that mediates diverse protein-protein interactions (13, 14) including sequence-specific transcription factors, which recruit the HDACs to target gene promoter regulatory elements.

An important mechanism for regulating the activity of class IIa HDACs is their coordinated shuttling between the nucleus and cytoplasm. Phosphorylation of conserved serine residues in their amino-terminal domain by CaMK (16–19) and PKD (20–22) leads to association with 14-3-3 chaperone proteins (16–19, 23–25) and Crm1-dependent nuclear export (16, 26–28). Sequestration of HDACs in the cytoplasm presumably relieves target genes from HDAC repressive actions, thereby facilitating gene expression. This is best understood in the case of the association of class IIa HDACs with MEF2 transcription factors. In the contexts of myogenesis (18, 19, 29, 30), vascular formation (31, 32), pathological cardiac hypertrophy (22, 33–37), and T and B cell maturation (20, 21, 38, 39), various extracellular stimuli including endothelin-1 and vascular endothelial growth factor cause phosphorylation and nuclear export of HDACs, thus permitting expression of MEF2 target genes.

We recently discovered that HDAC7 directly binds to and represses the activity of Runx2 (40). Runx2 is a master transcriptional regulator of skeletal biology (41–43) that functions...
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as either a transcriptional activator or repressor, depending upon its interaction with other transcription factors and transcriptional co-activators and co-repressors (44–46). We also showed that BMP2 induced a transient redistribution of HDAC7 from the nucleus to the cytoplasm in multipotent C2C12 mesenchymal cells (40). In this report, we show that the redistribution of HDAC7 by BMP2 is mediated by PKD and that this interaction modulates HDAC7 repression of Runx2 activity.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 200 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. MC3T3-E1 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% nonessential amino acids.

Plasmids—Plasmids encoding FLAG-tagged HDAC7 (40) and hemagglutinin-tagged Runx2 (47) were previously described. Point mutations at HDAC7 serine residues were generated with the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. Dr. Yoji Shimizu (University of Minnesota) kindly provided the plasmids encoding GFP-tagged PKD1 constructs (48).

Immunofluorescence—Cells were grown on glass coverslips and transfected with Lipofectamine (Invitrogen). In some experiments, cells were pretreated with nuclear export or protein kinase inhibitors for 30 min before being treated with 300 ng/ml BMP2 for the indicated times in the continued presence of the inhibitors. Nuclear export and protein kinase inhibitors were purchased from Calbiochem and used at the following concentrations: leptomycin B, 100 nM; Go¨6976, 5 μM; GF109203X, 5 μM; KN93, 5 μM; H-89, 10 μM; LY294002, 10 μM; U0126, 10 μM, SB203580, 3 μM; SP600125, 20 μM. After treatment, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 in phosphate-buffered saline for 5 min, blocked for 30 min in immunofluorescence buffer (3% bovine serum albumin, 20 mM MgCl₂, 0.3% Tween 20 in phosphate-buffered saline), and incubated with anti-FLAG antibody (Sigma-Aldrich, clone M2) in immunofluorescence buffer. Cells were washed three times with 0.1% Triton X-100 in phosphate-buffered saline, incubated for 30 min with Alexa-conjugated secondary antibodies at 1:800 (Invitrogen), washed 3 times, and mounted in 90% glycerol, 0.4% N-propylgallate. Images were obtained using an Olympus Fluoview 500 confocal microscope and processed using Adobe Photoshop.

For quantitation of nuclear-cytoplasmic distributions, the predominant localization of HDAC7-FLAG was scored in 50–75 cells per slide with the investigator blind to the experimental treatment. In experiments where GFP-tagged PKD1 was co-transfected, HDAC7 localization was only scored in cells that were also positive for GFP fluorescence. Statistical significance of HDAC7 localization and redistribution in cell populations was determined by the χ² test.

Immunoprecipitations—C2C12 cells were grown in 60-mm dishes and transfected with the indicated expression plasmids using Lipofectamine. 24 h after transfection, whole cell lysates were prepared in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% EDTA, 0.5% Nonidet P-40 supplemented with Complete Protease Inhibitor Tablets (Roche Applied Science) and phosphatase inhibitor mixture (Sigma)). Lysates were sonicated, precleared by centrifugation (12,000 rpm at 4 °C), and immunoprecipitated overnight with anti-FLAG-M2-agarose (Sigma) or with anti-HDAC7 polyclonal antibody (Abcam) and collected by protein A/G-plus-agarose beads (Santa Cruz Biotechnology) for 30 min. Immunoprecipitates were washed three times with lysis buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membrane (Millipore). Proteins were detected by immunoblotting with antibodies against GFP (B2, Santa Cruz), FLAG (M2, Sigma), phosphoserine (PSR45, Sigma), or HDAC7 (Ab12175, Abcam) followed by horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using ECL-plus chemiluminescent substrate (GE Healthcare).

Reporter Assays—C2C12 cells were transfected using Lipofectamine (Invitrogen) in 12-well plates with 200 ng of p6OSE2-luc, 100 ng of pRL-null, 300 ng in pCMV5-HA-Runx2, 450 ng of HDAC7, and 100 ng of PKD1 expression plasmids as indicated. pcDNA3.1 was added to maintain a uniform amount of total DNA per transfection. Luciferase activity was measured 24 h after transfection using the Dual-Luciferase Assay System (Promega). Each transfection was performed in triplicate and normalized to Renilla luciferase activity.

Osteoblast Differentiation—C2C12 cells stably expressing a short hairpin RNA (shRNA) against HDAC7 or a negative control shRNA were previously described (40). HDAC7 protein levels in control and HDAC7-suppressed cells were measured by densitometry of immunoblots using NIH ImageJ software and normalized to α-tubulin. Confluent cultures were grown in duplicate in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 300 ng/ml of BMP2 supplemented with DMSO (vehicle) or 2.5 μM Go6976 for 3 days. Alkaline phosphatase activity was measured by incubating cell lysates in 0.6 M 2-amino-2-methyl-1-propanol, 2.4 mM MgCl₂, 9.6 mM p-nitrophenylphosphate at 37 °C for 30 min. Reactions were stopped by the addition of 2 N NaOH. The activity was determined as absorbance at 410 nm and normalized to protein content. RNA was harvested using Trizol reagent (Invitrogen) according to the manufacturer’s directions and analyzed by real-time reverse transcription-PCR. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and analyzed by the Pfaffl method (49).

RESULTS

BMP2 Induces Nuclear Export of HDAC7, but Not HDAC4, -5, or -6 in Osteoblast-lineage Cells—We previously reported that BMP2 induces transient redistribution of overexpressed and endogenous HDAC7 from the nucleus to cytoplasm in C2C12 multipotent progenitor cells (40); however, the mechanism was not characterized at that time. We began by determining whether this also occurs in MC3T3-E1 pre-osteoblasts, which are committed to the osteoblast lineage, whereas C2C12 are multipotent and can be induced to differentiate into the myogenic lineage by low serum (50) or into the osteogenic lineage by BMP2 (51). When FLAG-tagged HDAC7 is overex-
were therefore interested in whether BMP2 influences HDAC localization. The distribution of FLAG-tagged HDAC proteins in C2C12 and MC3T3-E1 cells treated with 300 ng/ml BMP2 for 45 min was determined by anti-FLAG immunofluorescence staining. A, C2C12 and MC3T3-E1 cells were transfected with HDAC7-FLAG constructs and subjected to immunofluorescence staining against the FLAG epitope. Shown are examples of cells where HDAC7 exhibits mainly nuclear (left), both nuclear and cytoplasmic (center), and mainly cytoplasmic distributions (right). B–C, transient redistribution of FLAG-tagged HDAC7 from nucleus to cytoplasm induced in C2C12 (B) and MC3T3-E1 (C) cells treated with 300 ng/ml BMP2 for the indicated times. *, p < 0.05 versus 0 min BMP2. D and E, BMP2 effect on class II HDAC localization. The distribution of FLAG-tagged HDAC proteins in C2C12 (D) and MC3T3-E1 (E) cells treated with 300 ng/ml BMP2 for 45 min was determined by anti-FLAG immunofluorescence. *, p < 0.01 between untreated and BMP2-treated cells. F, BMP2 induces Crm1-mediated HDAC7 nuclear export. C2C12 cells were pretreated with 100 nM leptomycin B or ethanol (vehicle) for 30 min before stimulation with 300 ng/ml BMP2 in the continued presence of the vehicle or leptomycin B. *, p < 0.01 versus vehicle, no BMP2; **, p < 0.01 versus LMB, no BMP2. B–F, black bars represent nuclear localization, gray bars indicate nuclear plus cytoplasmic localization, and white bars represent cytoplasmic localization.

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PKD regulates HDAC7 subcellular localization. Before BMP2 treatment, HDAC7 was distributed between the nucleus and cytoplasm in both C2C12 and MC3T3-E1 cells (Fig. 1, D–E) as described above. In contrast, HDAC5 was observed solely in the nucleus in 100% of C2C12 cells and 77% of MC3T3-E1 cells. Conversely, HDAC6 was confined to the cytoplasm in 97% of C2C12 and 86% of MC3T3-E1 cells. After BMP2 treatment, HDAC7 was redistributed from the nucleus to cytoplasm, whereas no significant change in the distribution of either HDAC5 or HDAC6 was evident. In unstimulated cells, HDAC4 was divided between nuclear and cytoplasmic compartments. Upon BMP2 treatment of MC3T3-E1 cells, the percentage of cells with HDAC4 in the nucleus only or in both the nuclear and cytoplasmic compartments was decreased, whereas the percentage with HDAC4 primarily in the cytoplasm increased from 36 to 66% (Fig. 1E). However, we were unable to demonstrate a clear or consistent effect of BMP upon HDAC4 localization in C2C12 cells (Fig. 1D and data not shown). Because HDAC7 showed the strongest and most reproducible redistribution in response to BMP2, we chose to focus our further investigations on it.

To determine whether the altered nuclear-cytoplasmic distribution of HDAC7 induced by BMP2 involved nuclear export, we pretreated C2C12 cells with the nuclear export inhibitor leptomycin B (LMB) for 60 min before BMP2 treatment. Pretreatment with LMB enhanced exclusive nuclear localization of HDAC7 before BMP2 treatment from 17 to 76% of cells (Fig. 1F). LMB also reduced the ability of BMP2 to cause redistribution of HDAC7-FLAG. After 40 min of BMP2 stimulation, HDAC7 was expressed exclusively in the cytoplasm in only 3% of LMB-treated cells as compared with 43% of control cells. The ability of LMB to inhibit HDAC7 redistribution from BMP2 suggests that the altered subcellular distribution involves Crm1-dependent nuclear export. Collectively, these data show that BMP2 regulates nuclear-cytoplasmic trafficking of HDAC7 to control its distribution in osseous cells.

BMP2 Regulates HDAC7 Subcellular Localization through Kinase Signaling—Phosphorylation of serine residues in the HDAC7 amino terminus by protein kinases, including CaMKII, was observed to be phosphorylated in either cell line, it shows a range of subcellular distributions: primarily nuclear, distributed between nucleus and cytoplasm, and primarily cytoplasmic (Fig. 1A). BMP2 stimulated the redistribution of HDAC7 away from the nucleus and toward the cytoplasm in C2C12 (Fig. 1B) and MC3T3-E1 (Fig. 1C) cells. Thus, although HDAC7 was strictly nuclear in 15–20% of cells in untreated populations, it was exclusively nuclear in just 5% of cells after 40 min in BMP2. This effect was transient, with the maximal effect most commonly observed between 40 and 60 min, followed by a return toward basal conditions by 90 min.

Nuclear-cytoplasmic shuttling of other HDACs including class IIa HDACs 4 and 5 and HDAC6, a class IIb HDAC, has been reported in response to various stimuli (14, 15, 28). We were therefore interested in whether BMP2 influences the localization of these HDACs.
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(16) and PKD (16, 20, 21, 31, 39), regulates its nuclear–cytoplasmic distribution in response to extracellular stimuli. We hypothesized BMP2 regulates HDAC7 localization by a similar mechanism and that inhibition of the kinase(s) that transduces the signal from BMP2 to HDAC7 should block BMP2-responsive export of HDAC7. To test this hypothesis and identify the kinase(s) involved, we pretreated C2C12 cells with various kinase inhibitors (Table 1) for 30 min before stimulation with BMP2. The localization of HDAC7 was examined both at the end of the pretreatment and after 45 min of BMP2 treatment. The distributions of HDAC7 in cells treated with PKD, CaMK, protein kinase C (PKC), or JNK inhibitors were significantly different ($p < 0.001$) from the distribution in vehicle-treated cells, although the PKD and CaMK inhibitors gave the clearest effect. Thus, in the absence of BMP2 (Fig. 2A), KN93, a CaMK inhibitor, and Gö6976, an inhibitor of PKD and calcium-dependent PKC isoforms, each increased the nuclear localization of HDAC7 as compared with DMSO (vehicle) from 19% to 66 and 67%, respectively. By comparison, a broad inhibitor of PKC isoforms (GF109203X) and inhibitors of p38 (SB203580) or JNK (SP600125) produced weaker shifts (from 19% to 37–41%) in HDAC7 distribution toward the nucleus in the absence of exogenous BMP2. Inhibitors of protein kinase A (H-89), phosphoinositide 3-kinase (LY294002), or MEK (U0126) decreased the number of cells in which HDAC7 was only located in the cytoplasm but did not increase the number of cells where HDAC7 was only in the nucleus. Thus, broader distribution across both compartments was increased. These data indicate that PKD and CaMK are regulators of HDAC7 localization.

We next tested the effects of the inhibitors on BMP2-induced HDAC7 nuclear export. Similar to Fig. 1, BMP2 induced export of HDAC7 from the nucleus within 45 min when pretreated with DMSO (vehicle) (Fig. 2B). Although several of the inhibitors produced statistically significant changes in HDAC7 distribution, Gö6976 most effectively inhibited BMP2-stimulated redistribution of HDAC7. Thus, although 48% of control cells (Fig. 2B) showed exclusive cytoplasmic staining of HDAC7 after BMP2 treatment (up from 20% in untreated cells, Fig. 2A), only 12% of cells pretreated with Gö6976 before BMP2 exposure had exclusive cytoplasmic staining of HDAC7. Cells pretreated with Gö6976 were the only population with a greater percentage of cells having HDAC7 exclusively nuclear than exclusively cytoplasmic after BMP2 treatment. In cells pretreated with all the other inhibitors, HDAC7 was located in the cytoplasm of 26–42% of cells. The ability of Gö6976, but not GF109203X, to strongly inhibit BMP2-dependent HDAC7 nuclear export suggests that PKD rather than PKC is the kinase involved in transducing the BMP2 signal to HDAC7. These data indicate that CaMK and PKD are both involved in limiting the amount of HDAC7 in the nucleus in the absence of BMP2 stimulation but that only PKD regulates HDAC7 subcellular distribution in the presence of BMP2.

HDAC7 Localization and Activity Is Regulated by PKD and Serine Phosphorylation—BMP2 activates PKD in osteoblast-like cells (9, 12). PKD1 and PKD3 induce phosphorylation-dependent nuclear export of HDAC7 during T- and B-cell maturation (20, 21, 39) and of HDAC5 in response to cardiac hypertrophic signals (22). Based on this literature and our initial results with BMP2 and Gö6976, we further investigated the role of PKD in transmitting BMP2 signals to HDAC7. We began by examining the effect of Gö6976 on the kinetics of BMP2-induced HDAC7 nuclear export. Pretreatment with Gö6976 for 30 min increased the percentage of cells that exclusively expressed HDAC7 in the nucleus from 13 to 48% (Fig. 3A).

### Table 1: Kinase inhibitors used in this study

| Inhibitor | Specificity | Concentration |
|-----------|-------------|---------------|
| KN93      | CaMK II    | 5 μM          |
| Gö6976    | PKD, PKCα, PKCβ | 5 μM         |
| GF109203X | PKCα, PKCδ, PKCγ, PKCδ, PKCe | 5 μM         |
| H89       | Protein kinase A | 10 μM         |
| LY294002  | Phosphoinositide 3-kinase | 10 μM         |
| U0126     | MEK1, MEK2 | 10 μM         |
| SB203580  | p38         | 3 μM          |
| SP600125  | JNK         | 20 μM         |

FIGURE 2. Effects of protein kinase inhibitors on BMP2-induced nuclear export of HDAC7. C2C12 cells were transfected with HDAC7-FLAG, pretreated with kinase inhibitors for 30 min, and fixed for immunofluorescence (A) or stimulated with 300 ng/ml BMP2 in the continued presence of the inhibitors for 45 min before fixation (B). The subcellular localization of HDAC7-FLAG was determined by anti-FLAG immunofluorescence and scored by epifluorescence microscopy with the investigator blind to the experimental treatment. The results shown are the combined data from 2–4 independent experiments in which ~50–75 cells were blindly scored and which each gave similar results. *, $p < 0.001$ versus DMSO, no BMP2; **, $p < 0.001$ versus DMSO + BMP2.
After G6976 pretreatment, the ability of BMP2 to induce nuclear export of HDAC7 was diminished at all time points (Fig. 3A). For example, HDAC7 was nuclear in only 34% (2% nuclear only + 32% nuclear and cytoplasmic) of vehicle-treated cells at 40 min after BMP2 exposure, but it was at least partially nuclear in 80% (49% nuclear only) of cells pretreated with G6976 after BMP2 stimulation. At no time were there more cells with HDAC7 exclusively cytoplasmic than exclusively nuclear in the presence of G6976. Similar results were obtained in MC3T3-E1 cells (supplemental Fig. 2A) where HDAC7 was exclusively nuclear in less than 5% of cells after 40 min with BMP2, but it was exclusively nuclear in ~38% of cells pretreated with G6976. These data indicate that PKD is an intermediate molecule during BMP2-dependent HDAC7 redistribution in osseous cells.

We next examined the serine phosphorylation status of HDAC7 in cells stimulated with BMP after pretreatment with G6976 or the DMSO vehicle. A low level of serine-phosphorylated HDAC7 was detected in unstimulated cells (Fig. 3B). BMP2 increased serine phosphorylation of HDAC7 within 60 min. Pretreatment with G6976 diminished basal and BMP2-induced HDAC7 serine phosphorylation. This result indicates that phosphorylation of HDAC7 after BMP2 requires PKD activity.

HDAC7 becomes phosphorylated at four serine residues (Ser-155, -181, -318, and -446) by protein kinases such as PKD and CaMK, leading to export from the nucleus (20, 21, 39). To determine whether export of HDAC7 in response to BMP2 requires these serines, we mutated all four of them to alanine and called the resulting construct HDAC74A. This mutant HDAC7 polypeptide was expressed at levels similar to wild-type HDAC7 by Western blotting (supplemental Fig. 1). If phosphorylation of these residues is required for BMP2-induced nuclear export, then HDAC74A localization should be insensitive to BMP2 signaling. As shown in Fig. 3C, HDAC74A was exclusively localized to the nucleus in 98% of cells as compared with 24% of cells expressing wild-type HDAC7. The HDAC74A protein was resistant to BMP2-stimulated nuclear export as 0% of cells expressing it showed exclusive cytoplasmic staining 40 min after BMP2 treatment, whereas 100% of cells

**FIGURE 3.** BMP2 and PKD stimulate phosphorylation-dependent HDAC7 nuclear export. A, pretreatment of C2C12 cells with 5 μM G6976 for 30 min inhibits BMP2-dependent HDAC7 nuclear export. The distribution of HDAC7 was determined by anti-FLAG immunofluorescence with the investigator blind to the experimental treatment of each slide. *, p ≤ 0.02 versus DMSO, no BMP2; **, p ≤ 0.05 versus G6976, no BMP2. B, BMP2 enhancement of phosphorylated HDAC7 requires PKD activity. C2C12 cells were pretreated with DMSO vehicle or 5 μM G6976 before being stimulated by 300 ng/ml BMP2 for 60 min, lysed, and subjected to anti-HDAC7 immunoprecipitation (IP). Proteins were examined by immunoblotting against phosphoserine and HDAC7. C, conserved serine residues are required for HDAC7 nuclear export. C2C12 cells were transfected with wild-type HDAC7 or HDAC74A (in which serines 155, 181, 318, and 446 were converted to alanines) before treatment with 300 ng/ml BMP2. HDAC7-FLAG localization was determined by anti-FLAG immunofluorescence. *, p ≤ 0.0001 versus HDAC7, no BMP2; **, p = 0.01 versus HDAC74A, no BMP2. D, C2C12 cells were co-transfected with plasmids encoding wild-type or HDAC74A-FLAG, GFP, and PKD1-GFP plasmids and subjected to immunofluorescence labeling. The localization of HDAC7 was examined by confocal microscopy and scored only in cells also positive for GFP fluorescence. *, p = 0.0001 versus HDAC7 + GFP; **, p ≤ 0.0001 versus HDAC74A + GFP.
expressing HDAC7 in the nucleus from 98 to 83%, with the constitutively active PKD1 reduced the percentage of cells co-transfected with plasmids encoding wild-type or HDAC7-FLAG, to cause HDAC7 nuclear export. C2C12 and MC3T3-E1 cells were co-transfected with HDAC7-FLAG and either wild-type or a constitutively active PKD1 expression construct, to phospho-orylate the HDAC7 amino terminus in thymocytes (20, 21). Constitutively active PKD1-GFP and HDAC7-FLAG. 24 h post-transfection cells were treated with 300 ng/ml BMP2 for 40 min, lysed, immunoprecipitated with anti-FLAG-agarose beads, resolved by SDS-PAGE, and immunoblotted against FLAG and GFP.

PKD1 associates with HDAC7. A, representative images of C2C12 cells co-transfected with plasmids encoding wild-type or HDAC74A-FLAG, GFP, and PKD1-GFP plasmids and subjected to immunofluorescence labeling showing localization of HDAC7 (red) and GFP or PKD1-GFP (green). Co-localization appears as yellow in the Merge row. B, BMP2 enhances the co-immunoprecipitation (IP) of PKD and HDAC7. C2C12 cells were transfected with PKD1-GFP and HDAC7-FLAG. 24 h post-transfection cells were treated with 300 ng/ml BMP2 for 40 min, lysed, immunoprecipitated with anti-FLAG-agarose beads, resolved by SDS-PAGE, and immunoblotted against FLAG and GFP.

results indicate that activation of PKD1 is sufficient to induce nuclear export of HDAC7 in osteoblast-like cells. PKD1 and PKD3 both directly interact with and phosphorylate the HDAC7 amino terminus in thymocytes (20, 21). Consistent with these reports, wild-type PKD1-GFP and constitutively active PKD1ΔPH-GFP co-localized with HDAC7 in C2C12 cells (Fig. 4A). Although FLAG-tagged HDAC7 did not co-localize with GFP, it was readily detected in close proximity to PKD1-GFP in cytoplasmic foci (Fig. 4A, Merge, second and third columns). Interestingly, although both PKD1-GFP and PKD1ΔPH-GFP were detected only in the cytoplasm when transfected alone or with wild-type HDAC7-FLAG, HDAC74A sequestered both of the PKD1-GFP constructs into large bodies within the nucleus (Merge, fourth column, Fig. 4A and supplemental Fig. 2C). The association between HDAC7 and PKD1 was confirmed by co-immunoprecipitation studies (Fig. 4B). HDAC7-FLAG complexes in unstimulated cells contained a relatively low amount of PKD1-GFP. BMP2 treatment for 40 min before lysis increased in the amount of PKD1-GFP in HDAC7-FLAG complexes. Taken together, these data demonstrate that PKD1 associates with HDAC7 in vivo in response to BMP2 signaling and suggest that BMP2 induced nuclear export of HDAC7 is mediated by phosphorylation of HDAC7 in the nucleus by PKD.

PKD1 Inhibits HDAC7 Repression of Runx2 Transcription— We previously showed that HDAC7 represses Runx2 transcriptional activity (40). To determine the functional significance of the interaction between PKD1 and HDAC7, we asked whether PKD1 inhibited the ability of HDAC7 to repress Runx2 (Fig. 5). Runx2 activated the p6OSE2-luc reporter 29-fold above the level from empty pcDNA3.1 (not shown), and this was enhanced 17% by PKD1ΔPH. Repression of Runx2 activity by wild-type HDAC7 was inhibited by PKD1ΔPH. Thus, HDAC7 repressed Runx2 activity to 12% that of Runx2 alone, whereas co-transfection of HDAC7 and PKD1ΔPH gave 53% of the activity of Runx2 alone or 45% the activity of Runx2+PKD1ΔPH. In contrast, inhibition of Runx2 activity by
HDAC7 was insensitive to PKD1 PH. In the absence of PKD1 PH, HDAC7 reduced activation by Runx2 to 7% of Runx2 alone. With PKD1 PH, HDAC7 repressed reporter activity to 5% of Runx2 alone or 4% of Runx2/PKD1 PH. Similar results were obtained with wild-type PKD1. These data demonstrate that activated PKD1 inhibits HDAC7 repressive activity toward Runx2 and that this activity is dependent upon HDAC7 phosphorylation and/or nuclear export.

Reduced HDAC7 Expression Does Not Rescue Gö6976 Inhibition of Osteoblastic Differentiation—PKD inhibition with Gö6976 or antisense nucleotides prevented induction of osteoblast markers by BMP2, indicating that PKD activity and events downstream of PKD are necessary for BMP2-stimulated osteoblastic differentiation (9, 12). To determine whether HDAC7 is a major effector of PKD activity after BMP2 stimulation, we compared the effects of BMP2 and Gö6976 on C2C12 cells sta-

FIGURE 6. Suppression of HDAC7 does not rescue Gö6976 inhibition of BMP2-stimulated osteoblastogenesis. A, immunoblotting against endogenous HDAC7 in control shRNA and HDAC7 shRNA C2C12 cell lines. The membrane was re-blotted with α-tubulin antibodies to determine relative protein amounts in each lane. B, alkaline phosphatase (ALP) activity in shRNA cell lines after 3 days of differentiation. C–E, relative expression of mRNAs for alkaline phosphatase (C), osteocalcin (OCN, D), and Runx2 (E) were determined by real-time reverse transcription-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. *, p ≤ 0.05. These results are representative of three experiments.

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difference in the inhibitory effect of Gö6976 between HDAC7-suppressed and control cells treated with BMP2. Thus, suppression of HDAC7 is not sufficient to restore BMP2-stimulated expression of the osteoblast markers examined when PKD activity is inhibited.

**DISCUSSION**

Runx2 is one of the principal regulators of skeletal formation and function. Its transcriptional activity is determined through interactions with co-regulators including HDAC co-repressors (28, 40, 52–54). We showed that osteogenic induction of C2C12 cells with BMP2 led to a redistribution of both endogenous and overexpressed HDAC7 from the nucleus to the cytoplasm (40). In this report we describe a mechanism by which BMP2 controls the subcellular localization of HDAC7, finding that PKD1 regulates HDAC7 localization and activity in response to BMP2.

We began by showing that BMP2 stimulates HDAC7 redistribution in C2C12 cells as well as MC3T3-E1 committed preosteoblasts. This effect is blocked by LMB, an inhibitor of Crm1-dependent nuclear export. We previously showed that nuclear localization of HDAC6 was increased in osseous cells after leptomycin B treatment (28). Thus, Crm1 appears to regulate the localization of several class II HDACs in osteoblasts. Nuclear-cytoplasmic shuttling of class Ila HDACs is regulated by phosphorylation of conserved serines, and the localization of each of these HDACs can be regulated by the same kinases, e.g. PKD and CaMK. Despite these similarities, we find that under identical conditions HDAC4, HDAC5, HDAC6, and HDAC7 each exhibit distinct subcellular distributions and display different responsiveness to BMP2, indicating that their localization and presumably their activities are differentially regulated, most likely by distinct sets of regulatory factors.

The impaired responsiveness of the serine-mutant HDAC7 protein (HDAC74A) to BMP2 stimuli suggested that BMP2 regulates HDAC7 localization through phosphorylation-dependent means. In addition to the well known SMAD pathways, BMPs also activate various kinase pathways such as mitogen-activated protein kinases (7–10, 55), PKD1 (9), and the phosphoinositide 3-kinase/Akt pathway (56). Of these potential mediators of the BMP2 signal, only the PKD inhibitor had a clear effect of HDAC7 localization. Although PKC is often cited as an upstream activator of PKD, BMP-stimulated activation of p38 and JNK by PKD in osteoblastic cells was reported to be independent of PKC (9). The inability of GF109203X to inhibit HDAC7 nuclear export in our assays supports a model of PKC-independent activation of PKD by BMP2 in osteoblasts. Inhibition of CaMK activity affected HDAC7 export under basal conditions, but its activity was not required for BMP2-mediated export. It is possible that autocrine and/or serum-derived stimuli other than BMP2 affect HDAC7 localization through CaMK.

Previous studies in a variety of model systems lend indirect support for a pathway from BMP2 through PKD to HDAC7. BMP2 is a known activator of PKD1 during osteoblast differentiation (9, 12). During thymocyte selection, activation of the T-cell receptor causes PKD-dependent nuclear export of HDAC7, allowing expression of Nur77 (20, 21, 38). Similarly, during B-cell maturation PKD1 and PKD3 affect signal-dependent phosphorylation and export of HDAC7 and HDAC5 (39), and PKD releases HDAC5 and HDAC7 from the nucleus during cardiac hypertrophy (22, 57). Our results are significant in that they provide direct evidence for connecting a pathway from BMP2 to PKD to HDAC7 to Runx2 and regulation of gene expression (Fig. 7). It is possible, indeed highly probable, that the activities of other transcription factors are affected by BMP2-dependent export of HDAC7.

BMP signaling promotes Runx2 activity through multiple mechanisms (41, 42, 58). BMPs increase transcription of Runx2 (59, 60). Moreover, SMADs directly associate with Runx2 in a region that is essential for Runx2 function (61, 62). SMADs associate with HDACs and recruit HDACs into Runx2 complexes (63). It is currently unknown whether SMADs influence Runx2-HDAC7 interactions. Deacetylation of the Runx2 protein by HDAC4 and HDAC5 leads to its degradation by ubiquitin-mediated proteolysis, but BMP signaling inhibits Runx2 deacetylation by these HDACs and protects it from proteolysis.
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(52). We have been unable to document a reproducible effect of HDAC7 on Runx2 protein levels (data not shown). Rather, our data suggest a distinct mechanism by which BMP2 signaling regulates Runx2 activity by removing HDAC7 from the nucleus. The mechanism by which HDAC7 represses Runx2 activity remains poorly understood but does not require deacetylase catalytic activity (40).

Göö6976 remained a potent inhibitor of BMP2-induced differentiation in HDAC7-suppressed cells. Thus, although HDAC7 suppression enhanced BMP2-stimulated osteoblast maturation, it was not sufficient to restore osteoblast differentiation, as measured by a limited set of markers, in the absence of PKD signaling. Other events downstream of BMP2 and PKD are also necessary. It is interesting that known downstream effectors of BMP2 signaling display different kinetics, with phosphorylated SMADs detected within 5 min of BMP stimulation and sustained for many hours (9). Redistribution of HDAC7 is first detected after activation of SMADs and returns to basal conditions by ~90–120 min. In contrast, BMP2-dependent stimulation of p38 and JNK activities in MC3T3-E1 cells is not detected until 1–2 h after stimulation (9). It remains unclear whether there is functional significance to the sequential activation of SMADs, inhibition of HDAC7 activity, and activation of p38 and JNK pathways.

HDAC inhibitors (64–66) and BMP2 (3) are both currently utilized therapeutically in different contexts. Our results establish a connection between BMP signaling and HDACs in the regulation of Runx2. As key molecular regulators of skeletal biology, understanding how these molecules interact furthers our ability to understand how they control cellular behavior during bone growth, maintenance, and regeneration as well as in various pathological states.

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