Phosphophoryn Regulates the Gene Expression and Differentiation of NIH3T3, MC3T3-E1, and Human Mesenchymal Stem Cells via the Integrin/MAPK Signaling Pathway*

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Extracellular matrix proteins (ECMs) serve as both a structural support for cells and a dynamic biochemical network that directs cellular activities. ECM proteins such as those of the SIBLING family (small integrin-binding ligand glycoprotein) could possess inherent growth factor activity. In this study, we demonstrate that exon 5 of dentin matrix protein 3 (phosphophoryn (PP)), a non-collagenous dentin ECM protein and SIBLING protein family member, up-regulates osteoblast marker genes in primary human adult mesenchymal stem cells (hMSCs), a mouse osteoblastic cell line (MC3T3-E1), and a mouse fibroblastic cell line (NIH3T3). Quantitative real-time PCR technology was used to quantify gene expression levels of bone markers such as Runx2, Osx (Osterix), bone/liver/kidney Alp (alkaline phosphatase), Ocn (osteocalcin), and Bsp (bone sialoprotein) in response to recombinant PP and stably transfected PP. PP up-regulated Runx2, Osx, and Ocn gene expression. PP increased OCN protein production in hMSCs. Furthermore, an α5β1 integrin-blocking antibody significantly inhibited recombinant PP-induced expression of Runx2 in hMSCs, suggesting that signaling by PP is mediated through the integrin pathway. PP was also shown to activate p38, ERK1/2, and JNK, three components of the MAPK pathway. These data demonstrate a novel signaling function for PP in cell differentiation beyond the hypothesized role of PP in biomineralization.

The tissue microenvironment consists of soluble and immobilized growth factors, ECM molecules, and cells that orchestrate the tissue-specific cell growth, differentiation, and survival that are crucial for tissue development, morphogenesis, and remodeling (1–4). A particular group of ECM proteins, the SIBLING protein family consisting of bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP-1), and dentin matrix protein 3 (DMP-3), also called dentin sialophosphoprotein (DSPP) (5), may have important signaling roles. Although not extremely similar in protein sequence, members of the SIBLING family share common traits such as RGD integrin-binding domains and casein kinase II phosphorylation sites (5). As it was shown more extensively in systems other than bone or dentin, the ECM plays an important role in growth factor function (1, 4, 6, 7). This cooperative/synergistic process can involve a convergence of intracellular signaling pathways triggered by ECM proteins and growth factors and becomes important in tissue regeneration.

Reports in the literature show evidence of type I collagen involvement in the ECM-directed differentiation of bone cells (8, 9). Type I collagen regulates BMP-2 activity via an interaction with α1β1 integrins (8, 10). BMP-7 and type I collagen demonstrated a synergistic activation of Ocn and Bsp gene expression via the MAPK pathway for accelerated osteogenesis (11). Type I collagen is the major protein constituent of the bone and dentin ECM, comprising ~90% of the total protein matrix (9, 12, 13). The remaining ~10% is composed of non-collagenous proteins (NCPs), which have proposed functions in the formation of mineralized tissues (12, 14–16). Bone and dentin formation share many properties, among which are several common ECM proteins (13, 15). OCN, MEPE, osteonec-tin (ON), OPN, BSP, BMP-1 and DMP-3 are all acidic NCPs that possess Ca2+ and hydroxyapatite (HA) binding properties (16–20). Initiation, nucleation, and inhibition of HA crystal growth is rigorously controlled by the NCPs (13, 21–24). However, little is known regarding the role of these ECM proteins in signaling related to tissue morphogenesis. OPN (25), MEPE (26), and DMP-1 (15) have been shown to regulate gene expression; thus there is building interest in the NCPs, specifically the SIBLINGS, and how they might also contribute to intracellular activities and synergize with growth factors (5).

DMP-1, a SIBLING protein and NCP localized in dentin and bone ECM, was shown to stimulate mouse embryonic mesenchymal stem cells toward an osteoblastic lineage (15). Overexpression of DMP-1 in C3H101/2 and MC3T3-E1 cells resulted in osteopontin, Ocn, Osterix; PBS, phosphate-buffered saline; PP, phosphophoryn; rBMP-2, recombinant human BMP-2; SIBLING, small integrin-binding ligand glycoprotein; qPCR, quantitative real-time PCR; rPP, recombinant PP; tPP, transgenic PP.
in up-regulation of Runx2, Ocn, Bsp, Alp, On, and Opn and increased mineralized nodule formation (15). DMP-1 is a highly acidic, phosphorylated protein consisting mainly of glutamic acid, serine, and aspartic acid residues (27). In situ hybridization experiments have shown that DMP-1 is expressed by hypertrophic chondrocytes, osteoblasts, and odontoblasts (28). Another dentin ECM protein, phosphophoryn (PP), a cleavage product of DSPP (16), has been implicated as a regulator of mineral crystal formation (16, 18, 19, 29). DSPP is localized to chromosome 4, linking mutations in the gene to dentinogenesis imperfecta type II (15, 16). Although initially thought to be tooth-specific, the Dspp message is also localized in mouse calvaria and rat tibia, although at a much lower levels (30). PP is the most abundant NCP in dentin ECM, comprising ~50% of the ECM protein sector (16). Like other proteins in the bone/dentin microenvironment, PP is highly phosphorylated and anionic in character (18). PP is exceedingly rich in aspartic acid and serine residues, and ~85–90% of the serine residues are phosphorylated in the endoplasmic reticulum (31–34). The majority of the protein sequence consists of (DSS), repeats, where n could be as high as 24 (35, 36). Odontoblasts secrete PP along Ca2+ and 25-(OH)2 vitamin D3 was obtained from Biomol (Plymouth Meeting, VA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin, streptomycin were obtained from the American Type Culture Collection (Manassas, VA). Mesenchymal stem cell medium. MC3T3-E1 (clone 4) and NIH3T3 cells were purchased from Pierce Biotechnology. Anti-phospho-p38, anti-phospho-ERK1/2 and anti-phospho-JNK were purchased from Cell Signaling Inc. (Beverly, MA). Western Lightning chemiluminescence reagents were purchased from PerkinElmer Life Sciences.

**EXPERIMENTAL PROCEDURES**

**Materials—**Vectors for the production of recombinant PP (pGEX) and stable transfection (pShooter-ER) were obtained from Amersham Biosciences and Invitrogen, respectively. BL21 cells were obtained from Invitrogen. Luria-Bertani medium, ampicillin and isopropyl-1-thio-galactopyranoside were purchased from PerkinElmer Life Sciences. Western Lightning chemiluminescence reagents were purchased from PerkinElmer Life Sciences.

**Cell Culture—**hMSC, MC3T3-E1, and NIH3T3 cells were obtained from BioWhittaker, Inc. (Walkerville, MD). Human mesenchymal stem cell medium, mesenchymal cell growth supplement, l-glutamine, penicillin, and streptomycin were obtained from BioWhittaker, Inc. and added to the medium according to the manufacturer’s specifications to prepare complete mesenchymal stem cell medium. MC3T3-E1 (clone 4) and NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin/streptomycin, and tretinoin-EDTA were obtained from Invitrogen. 1, 25-(OH)2 vitamin D3 was obtained from Biomol (Plymouth Meeting, MA). The antibody to αβ3 integrin (anti-αβ3) was obtained from Chemicon (Temecula, CA). An OCN ELISA kit was obtained from Zymed Laboratories Inc. PBS, ALP assay kits, alizarin red S, and cetylpyridinium chloride (CPC) were obtained from Sigma Diagnostics, Inc. Total protein assay kits were obtained from Bio-Rad. RNeasy Kit and RNase I were obtained from Qiagen (Valencia, CA). The Ribogreen kit was obtained from Molecular Probes (Eugene, OR). All quantitative real time PCR reagents, primers, and probes were purchased from Applied Biosystems (Foster City, CA). Protease inhibitors were purchased from Pierce Biotechnology. Anti-phospho-p38, anti-phospho-ERK1/2 and anti-phospho-JNK were purchased from Cell Signaling Inc. (Beverly, MA). Western Lightning chemiluminescence reagents were purchased from PerkinElmer Life Sciences.

**Generation of Recombinant PP and Transfected Cell Line—**Isolated mouse genomic PP was used as a template to amplify exon 5 by PCR. The primers used were designed with Sall and XbaI at the 5’-ends of the gene-specific sequence (bold letters). In addition we inserted five random bases 5’ to the restriction site to allow Sall and XbaI digestions. The primers used were 5’-CTAATGTCGACATGAGTGGCAGCC-GTTGAGA-3’ (forward) and 5’-GATTCTCTAGATGACCCCGCAT-CATTCAATTG3’ (reverse).

**Transfection** experiments have shown that DMP-1 is expressed by hypothetic chondrocytes, osteoblasts, and odontoblasts (28).

**PP treatment using a recombinant form of PP (non-phosphorylated) in three independent cell types, hMSC, MC3T3-E1, and NIH3T3. We also created a stably transfected NIH3T3 cell line that secretes PP. Our rationale was to assess the signaling role of PP that could be phosphorylated by the stably transfected fibroblasts. Here, we report that PP, an NCP and SIBLING protein, drives cell differentiation through integrin signaling and activation of the MAPK pathway.

**Phosphophoryn Signals via the Integrin/MAPK Pathway**

**FIG. 1. Isolated rPP was electrophoresed on a 12% SDS-polyacrylamide gel and stained with Stains-all. Lane 1, bacterial lysate; lane 2, first wash following the addition of glutathione-Sepharose 4B; lane 3, last wash of the column; lane 4, GST fusion protein cleaved on the column by thrombin and the cloned protein eluted. A strong band was visible at ~55 kDa that corresponds to the correct mass of PP. Std, standard.**
qPCR. Total RNA content was photometrically analyzed with a Tecan Spectrafluor plate reader with excitation at 485 nm and emission at 595 nm. RNA concentrations were calculated based on a standard curve of control ribosomal RNA.

Quantitative Real Time PCR—Cells were harvested from the culture treatments at the time points described above. After extraction and quantification of RNA, quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step RT-PCR Master Mix. 10–30 ng of total RNA were added per 50-μl reaction with sequence-specific primers and probes purchased from Applied Biosystems. qPCR assays were carried out using Taqman® one-step RT-PCR Master Mix. 10–30 ng of total RNA were added per 50-μl reaction with sequence-specific primers and probes purchased from Applied Biosystems. qPCR assays were designed by and purchased from Applied Biosystems. Gene expression values were calculated based on the comparative CT method (separate tubes) detailed in Applied Biosystems User Bulletin Number 2 (42). For each primers/probe set, validation experiments demonstrated that the efficiencies of target and reference gene amplification were approximately equal; the absolute value of the slope of log input amount versus CT was >0.1. Target genes were normalized to the reference housekeeping gene at 18S.

Gene expression values were calculated based on the comparative ΔΔCT method (separate tubes) detailed in Applied Biosystems User Bulletin Number 2 (42). For each primers/probe set, validation experiments demonstrated that the efficiencies of target and reference gene amplification were approximately equal; the absolute value of the slope of log input amount versus CT was >0.1. Target genes were normalized to the reference housekeeping gene at 18S. Fold differences were calculated as described above and analyzed via qPCR for Ocn gene expression. For the OCN ELISA, cells were cultured in rhBMP-2- or rPP-containing media for 28 days. For the final 48 h of culture, cells were cultured in medium without serum added. Conditioned medium was collected and stored at −80 °C until use. The OCN ELISA was performed according to the manufacturer’s instructions. OCN concentration (nanograms per milliliter) was calculated from a standard curve and normalized to the total protein of the cell lysate as determined by the Bio-Rad protein assay.

MAP Kinase Activation—hMSCs and NIH3T3 cells were cultured in triplicate as specified above, except that the cells were cultured over-

Table 1

| Gene   | Accession number | Forward primer | Reverse primer | Tagman® probe |
|--------|------------------|----------------|----------------|---------------|
| Human Runx2 | NM_004348 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_004348 |
| Human Ocx | AF477981 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | AF477981 |
| Human Alp | NM_001826 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_001826 |
| Human Ocn | NM_008711 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_008711 |
| Human Bsp | NM_004867 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_004867 |
| Mouse Runx2 | NM_009820 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_009820 |
| Mouse Omx | NM_130458 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_130458 |
| Mouse Alp | XM_124424 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | XM_124424 |
| Mouse Ocn | NM_007541 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | NM_007541 |
| Mouse Bsp | L20232 | 5′-ACGCAATGATGCAACTCTTTCT-3′ | 5′-GAGAACTTCTTTCTTTCTTTCT-3′ | L20232 |

Fig. 2. qPCR analysis. Cells were cultured in basal media or with rhBMP-2 (hMSC, 100 ng/ml; MC3T3-E1 and NIH3T3, 50 ng/ml) or 50 ng/ml rPP. qPCR analysis was performed on total RNA at the indicated time points. Gene expression levels were calculated over control by the CT method. A, Runx2 on day 2. B, Ocx on day 4. For Ocx only, hMSC cultures were supplemented with 100 nM dex. Bars equal mean ± S.E.; n = 3. *p < 0.05.

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TABLE 1

Sequences for target gene primers and probes

Runx2

| NCBI/Bankit Number | Accession number | Forward primer | Reverse primer | Tagman® probe |
|--------------------|------------------|----------------|----------------|---------------|
| NM_004348 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_004348 |
| AF477981 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | AF477981 |
| NM_001826 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_001826 |
| NM_008711 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_008711 |
| NM_004867 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_004867 |
| NM_009820 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_009820 |
| NM_130458 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_130458 |
| XM_124424 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | XM_124424 |
| NM_007541 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | NM_007541 |
| L20232 | 5′-TTAGCGGCACTCCAACTGCCC-3′ | 5′-CCTTGTCCTTCTTGCTCTTCT-3′ | L20232 |
RESULTS

**PP-regulated Expression of Osteoblastic Transcription Factors**—We quantified the expression of several osteoblast gene markers in response to PP treatment in three cell types. The time points reported represent the highest change in gene expression for each target gene compared with control. For all experiments, the negative control consisted of a basal medium unless otherwise noted. rBMP-2 was used as a positive control. rPP up-regulated *Runx2* gene expression 2.5-fold over the basal medium control (p < 0.05) in both hMSC and MC3T3-E1 but not in NIH3T3 cells after 2 days in culture (Fig. 2A). Traditionally, hMSCs are cultured in medium containing dexamethasone to guide the cells toward osteoblastic lineage (41). We did not initially include dexamethasone in our cultures to avoid masking any changes that PP or rBMP-2 may have on gene expression (47, 48). It was our observation that changes in *Runx2* and *Ocn* gene expression were not detected by qPCR when dexamethasone was added in combination with PP or rBMP-2 (data not shown). On the other hand, *Ox* is not expressed in hMSCs in basal media. A baseline expression of *Ox* is required to quantify a "fold over control" by qPCR. Therefore the addition of dexamethasone was necessary for the gene expression analysis of *Ox* in hMSCs only. For MC3T3-E1 and NIH3T3, dexamethasone was not needed because they both express basal levels of *Ox*. rBMP-2 up-regulated *Ox* above control in all three cell types (19-fold in hMSC, 10-fold in MC3T3-E1, and 15-fold in NIH3T3; p < 0.05). *Ox* gene expression was up-regulated 8-fold over control by rPP in NIH3T3 after 4 days in culture and was not affected in either hMSC or MC3T3-E1 (Fig. 2B). It is of interest to note that PP up-regulated *Runx2* in hMSC and MC3T3-E1 but not in NIH3T3 cells. *Ox* expression had the opposite expression pattern, i.e. PP up-regulated *Ox* expression in NIH3T3 but not in hMSC or MC3T3-E1 cells. These data concur with recent evidence that *Ox* and *Runx* could be activated independently (49).

*Bsp* gene expression was not affected by rPP in MC3T3-E1, whereas our positive control, rBMP-2, increased *Bsp* gene expression 20-fold over control (data not shown). In NIH3T3 cells and hMSCs up to 21 days in culture we neither detected a baseline of *Bsp* gene expression in the control nor a change when rPP or rBMP-2 were added (data not shown). *Bsp* is usually expressed at later stages of differentiation, peaking just before matrix mineralization (40). We repeated this experiment in the presence of dexamethasone to determine whether dexamethasone would induce *Bsp* gene expression. We detected a low level of *Bsp* expression, but rBMP-2 and rPP did not enhance further expression (data not shown).
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PP-Induced Ocn Gene Expression and Protein Production—PP up-regulated Ocn gene expression over control in all three cell types (4-fold in hMSCs, 6-fold in MC3T3-E1 cells, and 3-fold in NIH3T3 cells; p < 0.05) (Fig. 3A). PP increased OCN protein release ~10-fold above negative controls (p < 0.05) for both hMSC (40 versus 4 ng/ml) and MC3T3-E1 (10 versus 1 ng/ml) cells (Fig. 3B). There was no change in OCN protein release in NIH3T3 cells due to PP treatment. These data raise the following question. Is the role of PP during matrix mineralization due to the physical properties of PP and/or a capacity to signal the expression of other proteins that could be involved in mineralization process?

Phosphophoryn Increased ALP Activity in hMSCs—To further examine the function of the PP in osteogenic lineage progression we examined ALP activity, which is a common phenotypic marker for osteogenesis. ALP activity was measured over a time course of 7–28 days. Fig. 4 shows the highest increase in ALP activity in hMSC at day 14. In contrast, rPP-treated groups did not increase ALP activity in MC3T3-E1 and NIH3T3 cells or in the stably transfected NIH3T3 cells over the time course examined. The positive control, rhBMP-2, increased ALP activity over basal medium control for all three cell types.

Phosphophoryn Increased Calcium Deposition in hMSCs—When added in combination with 100 nM dex and 10 mM β-glycerophosphate in hMSCs, both rhBMP-2 and rPP demonstrated increased alizarin red staining after 28 days in culture (Fig. 5A). Without dex, no alizarin red is detected. Upon quantification of alizarin red stain with 10% CPC, dex alone, dex with rhBMP-2, and dex with rPP exhibited increased alizarin red staining over cultures that did not contain dex (Fig. 5B). Furthermore, rhBMP-2 and rPP demonstrated increased alizarin red staining in cultures supplemented with dex compared with hMSC cultured with dex alone. These data reinforce the above-posed question as to the role of PP in signaling and/or mineral deposition. We therefore investigated the signaling mechanism of PP via integrin/β3 interactions.

PP Regulates Gene Expression via the αvβ3 Integrin—PP has an RGD domain; therefore, we hypothesized that PP may be functioning in osteoblastic gene expression via binding to the αvβ3 integrin on the cell surface. As demonstrated above, rPP stimulated Runx2 gene expression in hMSCs. We therefore decided to test our integrin interaction hypothesis on hMSCs using a blocking antibody to the αvβ3 integrin. Upon the addition of the integrin blocking antibody, Runx2 gene expression due to rPP was decreased by ~60% compared with an uninhibited control (Fig. 6). Runx2 gene expression was not inhibited by anti-αvβ3 in the rhBMP-2-treated hMSCs, as BMP-2 acts via it own specific receptors (types I and II). We suggest that PP functions to up-regulate bone-specific gene markers via binding to the αvβ3 integrin and the triggering of intracellular signaling pathways.

PP Regulates Gene Expression via the MAPK Pathways—To further study the signaling pathway following PP interaction with the αvβ3 integrin receptor, we investigated the involvement of the MAPK pathway and, more specifically, the activation of p38, ERK1/2, and JNK. As shown in Fig. 7, the p38 activation was apparent within 10 min of exposure to rPP in both NIH3T3 and hMSC cells. The ERK1/2 pathway seems to be active only in NIH3T3 cells, whereas the hMSCs were not activated compared with the control cells. When JNK was assessed for its activation, it was evident that there was a positive response at 10 min. These data clearly show that rPP is signaling via the MAPK pathway as demonstrated by the phosphorylation of p38, ERK1/2, and JNK. We believe that the gene activation shown in this paper by quantitative PCR is due to the activation of the MAPK pathway and the translocation of its components to the nucleus where they activate transcription of target genes.
CONT.

JNK (phos-Jnk) hMSC and NIH3T3 were cultured with rPP for 10, 20, 30, and 60 min. Cell lysates were harvested and subjected to SDS-PAGE and probed for phosphorylated p38 (phos-p38), phosphorylated ERK1/2 (phos-Erk), and phosphorylated JNK (phos-Jnk) by Western blotting. Cont., control.

DISCUSSION

Previous studies have suggested that PP functions in mineralization because of its calcium binding properties and its highly acidic and anionic character (18, 21, 22, 24, 37, 38). However, to date, PP has not been investigated as a signaling molecule that might regulate differentiative gene expression. Although well defined in several cell systems (1, 4, 6, 7), ECM-directed cell differentiation in the bone microenvironment has not been well documented and is limited to type I collagen (8, 11). We demonstrate here that PP, as a non-collagenous ECM protein and a SIBLING family member, is another component of the complex cascade of signaling events that leads to the formation of highly organized mineralized tissues.

We reason that PP functions in the formation of mineralized tissues in two principal roles (Fig. 8). First, PP binds to integrin receptors on the cell surface via its RGD domain, activating the MAPK signaling pathways that culminate in a mature osteoblast characterized by expression of early and late differentiative marker genes such as Runx2, Oax, and Ocn. Second, PP is localized within the ECM of bone and dentin and functions as a regulator of calcium phosphate deposition and crystal growth. The preference for one function over another is unknown but may be related to phosphorylation state. We propose that the mineralization process could probably be the following: 1) the result of the physiochemical properties of PP independent of cells as PP binds to HA by its highly acidic and anionic residues; 2) due to the signaling role of PP by regulating other bone/dentin genes; or 3) a combination of these two roles. We suspect that PP has both an autocrine and a paracrine effect on local cells. Progenitor (hMSC) and differentiated cells (NIH3T3) alike may be responsive to PP in the microenvironment. As such, PP could induce differentiation in progenitors and provide maintenance support for the differentiative pathway of mature osteoblasts/odontoblasts and even fibroblasts.

Signaling via integrins/MAPK activates many of the same pathways as growth factors, converging for an enhanced effect (1,6–8,11). Non-collagenous ECM proteins could provide key signals that potentiate growth factor activity (1) for an enhanced therapeutic outcome. The literature reports that ECM-directed signals, transduced by integrins, play indispensable roles in the regulation of tissue-specific gene expression of primary osteoblasts (3, 50–52). As a regulator of bone/dentin gene expression via the MAPK pathway, PP might activate these intracellular signaling pathways in concert with or independent of growth factors, resulting in optimized growth factor activity. At this time, the timing of signaling by PP versus BMP-2 is unknown. Our data demonstrate that PP and BMP-2 stimulate many of the same target genes, suggesting dual roles that may synergize inside the cell as an amplified signal.

Runx2 gene expression was induced by rPP in hMSC at a level equal to that induced by rhBMP-2. Runx2 was also up-regulated by rPP in MC3T3-E1 but not in NIH3T3. Runx2 has been shown to function in an early commitment to the osteogenic lineage as a transcriptional regulator of other osteoblast genes (53–55). It is reasonable that Runx2 was only stimulated in cells that could be considered multipotent (hMSC) and osteoblast-like (MC3T3-E1). Perhaps the NIH3T3 cells, which are fibroblastic, lack other factors that are necessary for inducible Runx2. Up-regulation of Runx2 was moderate (~2–3-fold) for either rhBMP-2 or rPP across all three cell lines. It has been documented that enhancement of downstream RUNX2 targets is regulated by “activated” RUNX2 (i.e., phosphorylated RUNX2) and not due to increases in Runx2 message per se (56, 57). Runx2 is an important regulator of osteogenic and chondrogenic differentiation (54), and induction by PP suggests a role in progression of mesenchymal stem cells and osteoprecursors toward a more mature cell.

Oax was not induced by rPP in either hMSC or MC3T3-E1 cells. However, in PP-transfected NIH3T3 cells Oax was up-regulated compared with the basal control. Furthermore, rhBMP-2 induced Oax gene expression more potently than did tPP in NIH3T3. Interestingly, although Runx2 was not activated by rPP in NIH3T3, Oax was induced. Conversely, in

FIG. 7. Activation of the MAPK pathway. hMSC and NIH3T3 were cultured with rPP for 10, 20, 30, and 60 min. Cell lysates were harvested and subjected to SDS-PAGE and probed for phosphorylated p38 (phos-p38), phosphorylated ERK1/2 (phos-Erk), and phosphorylated JNK (phos-Jnk) by Western blotting. Cont., control.

FIG. 8. Current model of the mechanism of action for phosphophoryn. PP binds to the αβ integrin via its RGD domain. Differentiative gene expression is activated via the MAPK pathway initiated by integrin binding of PP.

hMSCs Runx2 gene expression was increased, but not Osx by rPP. rhBMP-2 induced Osx in all three cell lines with an exceptional induction in hMSC. To date, there is no conclusive evidence that Osx is a direct target of Runx2 (i.e. Osx may be activated via RUNX2-independent pathway(s)) (49). Up-regulation of Runx2 and Osx, the transcriptional units of osteogenesis, indicates progression of the osteogenic lineage and increases the likelihood for activation of downstream phenotypic changes. The connection between Osx and Runx2 remains unclear at this time. It is interesting that Osx was inducible by PP in a fibroblastic cell line but not in mesenchymal stem cells or osteoblasts. This finding suggests a uniqueness of Osx that could be related to the differentiative state of the cell. Moreover, the varied response shown in the activation of Runx2 and Osx across cell types becomes unified in the activation of downstream markers such as Ocn.

An increase in OCN protein release was detected in both hMSCs and MC3T3-E1 cells. We were unable to detect an increase in OCN protein release due to rPP or tPP in NIH3T3 cells. We speculate that NIH3T3 cells, like fibroblasts, do not have the capacity to produce the OCN protein or secrete it, although they do express the Ocn gene inducible by either rhBMP-2 or tPP. However, secretion of the OCN protein suggests that hMSCs and MC3T3-E1 cells have differentiated into more mature osteoblast-like cells. PP increased ALP activity only in hMSCs. We also analyzed Alp gene expression in the presence of dexamethasone, and the outcome was a slight increase in Alp gene expression due to rPP, validating our ALP activity assay result (data not shown). PP also enhanced the final outcome of osteoblastic differentiation, i.e. matrix mineralization. In the presence of dexamethasone, rPP-treated hMSCs deposited significantly more calcium as evidenced by alizarin red stain.

Although neither an increase in ALP activity nor an enhancement of mineralization occurred in MC3T3-E1 cells due to rPP, it is possible that the rPP plays different roles at various stages of maturation, as was evidenced by the varied responses in gene expression across cell types. Furthermore, whereas tPP induced expression of Osx and Ocn in NIH3T3 cells, the recombinant form of the protein had no effect on any gene examined in NIH3T3 cells. We speculate that NIH3T3 cells that are genetically re-programmed to produce PP may phosphorylate the protein. The dose, phosphorylation state, or other native post-translational modifications of PP may have important implications in its function; NIH3T3 cells treated with rPP did not form a mineralized matrix, whereas PP-transfected NIH3T3 cells have shown extensive mineral deposition in vitro (data not shown). There was no change in matrix mineralization due to rPP in MC3T3-E1 (data not shown). The matrix mineralization of hMSCs could be explained by their inherent capacity to differentiate into the osteogenic lineage when provided the proper signals. These data raise many interesting questions such as the following two. Does the phosphorylation state of the protein have a role in the signaling function of PP? Will this novel role as a signaling protein affect matrix mineralization? The difference of response of rPP and tPP in the NIH3T3 cells suggests that the degree of phosphorylation of PP is likely a key regulator of the function of PP. The extracellular maintenance of the phosphorylation state of PP is unknown and could provide an additional level of control over its dual functions as a matrix mineralization regulator and a signaling molecule. Matrix mineralization by PP could be a cell-independent event based on its characteristics of acidity, anionic character, and HA binding properties in addition to degree of phosphorylation. However, the role of PP in gene expression of bone- and dentin-specific genes depends on cell surface receptors such as integrins and the activated pathways intracellularly.

Because αβ3 integrins are also crucial for cell attachment and migration, the role of PP as a signal transducer via the MAPK pathway may be critical in the proper formation of mineralized tissues and possibly in abnormal pathologies such as cancer. Recently, it was shown that DMP-1 is expressed in lung cancer (58). The authors postulate that SIBLING protein family members may function in metastasis to bone, as αβ3 integrins are over-expressed in tumor cells at metastatic sites (59). Therefore, metastatic tumor cells bind to SIBLING proteins in bone ECM via their αβ3 integrin receptors. Two other SIBLING proteins, BSP and OPN, may also contribute to tumor cell survival by recruiting complement factor H to the cell surface, protecting them from lysis by the alternate complement pathway (60). Our data agree with this concept that SIBLING proteins elicit their actions by interactions with integrin receptors. Clearly, SIBLING protein family members are more than static ECM proteins; they play dominant and crucial roles in both normal and pathological tissue morphogenesis.

In conclusion, we have demonstrated that PP not only stimulates differentiative gene expression in a variety of cell types including mesenchymal stem cells, pre-osteoblasts, and non-ossous fibroblasts but also enhances matrix mineralization, the end-stage of osteoblast differentiation. The evidence demonstrated here suggests that cellular responses to PP differ with cell type and origin and may be based on the method of PP delivery, the dosage, and the post-translational modifications of PP. We suggest that the non-collagenous ECM proteins such as the SIBLING proteins of the bone/dentin microenvironment have important and specific functions related to cell fate, mineralization, and tissue morphogenesis. Future studies will be focused on understanding the degree to which PP functions in these two distinct yet related roles. Specifically, the phosphorylation state of PP will be investigated as it pertains to the function of PP in either or both of its roles as a regulator of dentinogenesis/osteogenesis. Elucidating the intricate signaling pathways of ECM proteins that orchestrate the development of mineralized tissues will support the design of novel tissue-regenerative therapies. In turn, PPs may possess the ability to harness growth factor activity and, together, enhance their signaling capacities.

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