Dentin Matrix Protein 1 (DMP1) Signals via Cell Surface Integrin*

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Dentin matrix phosphoprotein 1 (DMP1) is a non-collagenous, acidic extracellular matrix protein expressed chiefly in bone and dentin. We examined the DMP1 ability to engage cell-surface receptors and subsequently activate intracellular signaling pathways. Our data indeed show that the presence of extracellular DMP1 triggers focal adhesion point formation in human mesenchymal stem cells and osteoblast-like cells. We determine that DMP1 acts via interaction with $\alpha v \beta 3$ integrin and stimulates phosphorylation of focal adhesion kinase. Further biochemical characterization confirms the activation of downstream effectors of the MAPK pathways, namely ERK and JNK, after DMP1 treatment. This activation is specifically inhibitable and can also be blocked by the addition of anti-$\alpha v \beta 3$ integrin antibody. Furthermore, we show that extracellular treatment with DMP1 stimulates the translocation of phosphorylated JNK to the nucleus and a concomitant up-regulation of transcriptional activation by phosphorylated c-Jun. The evidence presented here indicates that DMP1 is specifically involved in signaling via extracellular matrix-cell surface interaction. Combined with the published DMP1-null data (Feng, J. Q., Ward, L. M., Liu, S., Lu, Y., Xie, Y., Yuan, B., Yu, X., Rauch, F., Davis, S. I., Zhang, S., Rios, H., Drezner, M. K., Quarles, L. D., Bonewald, L. F., and White, K. E. (2006) Nat. Genet. 38, 1310–1315) it can be hypothesized that DMP1 could be a key effector of ECM-osteocyte signaling.

The extracellular matrix (ECM)[2] is a complex and dynamic network of proteins known to be involved in tissue compartmentalization, controlling differentiation events and mediation of communication from one cell to another (2). Cell-ECM interactions are known to be critical to many biological processes, including embryonic development and morphogenesis, cell growth and differentiation, tissue regeneration and cellular homeostasis (3). In mineralizing tissues such as bone and den-tin, the organic ECM comprises both a structural, collagenous component, and a second component of non-collagenous proteins. These non-collagenous proteins are thought to be essential regulators of the mineralization process, and recent evidence suggests that they are also likely involved in regulating cellular morphogenesis and differentiation (4). One such non-collagenous protein, known as DMP1, is a member of the family known as small integrin binding ligand N-linked glycoproteins (SIBLINGs). DMP1 is an acidic, highly phosphorylated protein normally present as two separate proteolytic fragments, a 37-kDa N-terminal species and a 57-kDa C-terminal species.

Expressed chiefly in the ECM of bone and dentin, DMP1 is unique in its content of a large number of acidic domains (5) These properties have been shown to be important in DMP1 affinity for Ca^{2+} and the ability to induce in vitro mineralization (6). Because of these physicochemical properties, DMP1 has been extensively studied, initially for its role in biomineralization, but more recently several roles have emerged underscoring its importance in bone biology. 1) Overexpression of DMP1 is sufficient to induce the differentiation of mesenchyme-derived cells to functional odontoblast-like cells and enhance mineralization (7–9). 2) DMP1 can be endocytosed via the GRP-78 receptor and transported into the nucleus to act as a transcriptional regulator for the phosphophoryn gene (10, 11). 3) the DMP1 role in the regulation of phosphate homeostasis and mineral metabolism has been underscored by the characterization of null mice displaying a recessive hypophosphatemic rickets and hypomineralized bone phenotype as well as morphological changes in osteocytes including irregular, buckled cell membranes and an absence of dendritic extensions (1, 12). These DMP1 null mice also showed a loss of osteocyte dendritic processes, which is normally a hallmark feature of these cells, and an irregular, buckled osteocyte cell membrane. Furthermore, the osteocyte lacunae were larger, randomly oriented, and lacking lamina limitans. Based on these data, we hypothesized that osteocytes require DMP1 to maintain their phenotype via cell-matrix interaction through a surface receptor(s). Our hypothesis was further supported by evidence suggesting that DMP1 has the ability to strongly bind the H factors, integrin $\alpha v \beta 3$ and CD44 (13). In this manuscript we focus our efforts on cell-matrix interactions and also the elucidation of intermolecular mechanisms involved in DMP1 signaling. We propose that defining these interactions shall deepen our knowledge of the nature of DMP1 involvement in cell differen-

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[2] The abbreviations used are: ECM, extracellular matrix; SIBLING, small integrin binding ligand N-linked glycoprotein; DMP1, dentin matrix phosphoprotein 1; rDMP1, recombinant DMP1; DMP1-F, full-length DMP1; DMP1-C, C-terminal DMP1; FA, focal adhesion; FAK, FA kinase; hMSC, human mesenchymal stem cell.

tiation, phosphate homeostasis, and the maintenance of the osteocyte phenotype.

Integrins are known to associate with proteins present in the ECM (14). Engagement with an ECM protein can induce integrin clustering, thereby enabling integrin pairs to associate with cytosolic ligands and perpetuate signals across the plasma membrane. The clustered integrins then engage in actin filament recruitment (15) and concomitant assembly of cytoskeletal-associated signaling molecules, initiating the formation of focal adhesions (15). These focal adhesions (FAs) comprise many known proteins, including vinculin, F-actin, focal adhesion kinase (FAK), paxillin, etc. Vinculin acts as the major link between the FA core and actin filaments and has been shown to regulate integrin clustering (16). Previous work has implicated the mitogen-activated protein kinase (MAPK) pathway as an avenue through which various bone and dentin non-collagenous extracellular matrix proteins affect intracellular signals. Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in mediating control over a variety of cellular activities such as gene expression, cellular differentiation, mitosis, and cell survival (17). MAPK cascades activate in response to extracellular stimuli and comprise a known set of players, including the extracellular signal-related kinases (ERK1/2), Jun N-terminal kinases (JNK1/2) (also known as stress-activated protein kinase (SAPK)), and p38 protein (p38). ERK1/2 is known to be activated by the MAP kinase kinase MEK1/2. Indeed, the work of Franceschi and co-workers (18) showed the importance of the MAP kinases to bone differentiation and formation. We have thus turned our effort toward establishing direct evidence of DMP1 ability to activate MAPK signaling; we hypothesize that MAPKs could facilitate the role of DMP1 as a pleiotropic regulator of an array of cellular processes.

MATERIALS AND METHODS

Cell Culture—MC3T3-E1 cells were cultured in α-minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin. Human mesenchymal stem cells (hMSC) (Tulane University, Center for Gene Therapy) were cultured in α-minimal essential medium (Cambrex) containing 15% fetal bovine serum, 4 mm L-glutamine and 1% penicillin/streptomycin. MDPC23 cells were cultured α-minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured at 37 °C with 5% CO_2.

Generation of Recombinant DMP1—The full-length (DMP1-F) and C-terminal DMP1 (DMP1-C) cDNAs in a pcDNA3 vector were provided by Dr. Jian Q Feng (Baylor College of Dentistry, Dallas, TX). The PGEX-4T-2-DMP1 was constructed by inserting DMP1 fragments into the EcoRI sites of pcDNA3. PGEX-4T-2-DMP1 plasmids were transformed into the bacterial host BL21, and the recombinant DMP1 C-terminal (rDMP1-C) and DMP1 full-length (rDMP1-F) protein were purified using the Amersham Biosciences GST system (GE Healthcare) according to the manufacturer's recommendations. The purified rDMP1 was then stored at −80 °C until use.

Microscopy to Detect FAs—Lab-Tek chamber slides (Nalge NUNC International) were coated first with poly-L-lysine for 30

min and then with rDMP1-F/C for the experimental group. The chamber was placed under ultraviolet light for 30 min before hMSC cells were plated in serum-free medium. The cells were fixed 20 h later and stained by immunofluorescence to detect the presence of focal adhesions using the actin cytoskeleton/focal adhesion staining kit (Chemicon International). All procedures were performed according to the manufacturer's recommendation. A fluorescent microscope (Nikon TE2000-E) was used to image the presence of focal adhesions.

Detection of MAP Kinase and FAK Activation—hMSCs were plated at 70% confluence. Cells were serum-deprived for 16 h before rDMP1-F/C treatment. Cells were treated in triplicate with 250 ng/ml rDMP1-C and rDMP1-F and assessed at different time points (5, 10, 20, and 30 min and 1, 2, and 3 h) for MAPK activation. MAPK inhibitors (10 μM U0126 (Calbiochem)) were added to cells 1 h before the addition of rDMP1-F/C. Cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) in the presence of protease inhibitor (Pierce) then sonicated at 4 °C. The concentration of cell lysates was determined by using the BCA bicinchoninic kit (Pierce). 10–20 μg of total protein were subjected to 10% SDS-PAGE gel.

Western Blotting for FAK and MAPK Activation—Western blots were then performed, and the gels were blotted onto polyvinylidene difluoride membranes and probed with the following antibodies: anti-phosphorylated-ERK (p-ERK), ERK, p-JNK, JNK, p-MEK, p-FAK, FAK, and β-actin (all antibodies were purchased from Cell Signaling Technology, Inc. and used at a 1:1000 dilution). Membranes were then exposed to X-Omat film (Eastman Kodak Co.), and the bands were obtained by chemiluminescent detection (PerkinElmer Life Sciences) of horseradish peroxidase-linked second antibody (Cell Signaling Technology, Inc).

FAK Activation—Cells were cultured in poly-L-lysine (Sigma)-coated 24-well plates and serum-deprived for 16 h before they were treated with rDMP1-F for 15 min. Cells were fixed in 4% paraformaldehyde for 15 min. Fixed cells were rinsed and incubated in a blocking buffer (2% donkey serum in PBS) for 1 h. Cells were incubated in anti p-FAK antibody at 4 °C overnight (Cell Signaling Technology, Inc), washed, and incubated with secondary antibody (Invitrogen) for 1 h before visualization under a fluorescent microscope (Nikon TE2000E).

Integrin Blocking—hMSCs were seeded on 6-well plates and allowed to reach ~70% confluence in basal medium. Cells were serum-deprived for 16 h then treated with 20 μg/ml anti-αvβ3 antibody or IgG control (Chemicon International, Inc.) for 1.5 h at 37 °C. Cells were then incubated in serum-free medium containing 250 ng/ml rDMP1-C and -F for 10 min, 30 min, and 1 h. Cell lysates were obtained and stored at −80 °C until analysis. For the blocking control experiment, cells were preincubated with either anti-integrin antibody or an IgG1 isotype control then for 30 min in the presence of 250 ng/ml rDMP1-F. Cells were assessed for JNK activation by Western blot.

DMP1-Integrin Co-immunoprecipitation—To determine whether DMP1 forms a complex with αvβ3 integrin, MC3T3 cells were cultured in 6-well plates for 24 h and treated with 0.5 μg/ml recombinant DMP1 for 30 min at 37 °C. The cells were

then thoroughly washed with PBS and lysed in radioimmune precipitation assay buffer containing a mixture of protease and phosphatase inhibitors (Roche Applied Science) on ice for 30 min, and the lysates were cleared of debris by centrifugation at $7700 \times g$ for 15 min at 4 °C. Four micrograms of either monoclonal anti-$\alpha v \beta 3$ or 5 μl of polyclonal α-DMP1 antibody was added to precleared cell lysates for 2 h followed by mixing with 75 μl of protein A/G-agarose beads in a rotator for 2 h. The beads containing immune complex were washed 5 times with lysis buffer, resuspended in 40 μl of 1× SDS-sample buffer, boiled for 5 min, and resolved on 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane for 3 h and probed with either monoclonal anti-$\alpha v \beta 3$ or polyclonal α-DMP1 antibodies as indicated (19). The signals were detected using the most sensitive chemiluminescent substrate system (Thermo Scientific Inc.).

Immunocytochemistry for Visualization of Nuclear Translocation—Cells were cultured in serum-free medium for 16 h before treatment with rDMP1C/F for 30 min at 37 °C and 5% CO_2. All subsequent steps were carried out at room temperature. Cells were rinsed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. Fixed cells were rinsed twice in wash buffer (PBS, 0.05% Tween 20) and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then rinsed twice with wash buffer and incubated in a blocking buffer (2% donkey serum in PBS) for 1 h. Incubation with primary antibody was carried out for 1 h (anti p-JNK and JNK at 1:100 dilution, anti-p-ERK, and ERK at 1:50 dilution with blocking buffer). Cells were then washed and incubated with an Alexa 488-conjugated secondary antibody (donkey anti-rabbit) for 1 h and visualized under a fluorescent microscope.

Transcriptional Activation—Cells were seeded in triplicate and allowed to reach 70–80% confluence. They were then transfected with plasmids 5×GAL-Luciferase and as GAL4-c-Jun (a gift from Anning Lin University of Chicago) using Lipofectamine 2000. Plasmid transfections were performed according to the manufacturer's recommendation. 24 h after the cell transfection, the cells were treated with rDMP1 for 24 h and then lysed. Luciferase expression was quantified using an automated luminometer, and results were normalized.

Statistical Analysis—The data were analyzed to test for significant mean differences in luciferase activity among the concentration groups using a one way analysis of variance. The post hoc pairwise comparisons were performed using the Tukey Honestly Significant Difference procedure. Significance was considered to be less than $p = 0.05$.

RESULTS

Generation of Recombinant DMP1—To facilitate analysis of the DMP1 signaling potential, we synthesized recombinant DMP1 proteins using both full-length (rDMP1-F) and C-terminal (rDMP1-C) plasmid DNA clones. The recombinant proteins were then analyzed by SDS-PAGE with Stains-All (20). To establish that the protein was in-frame and correctly synthesized, we also performed Western blot analysis using specific antibodies against the DMP1 N-terminal and DMP1 C-termi-
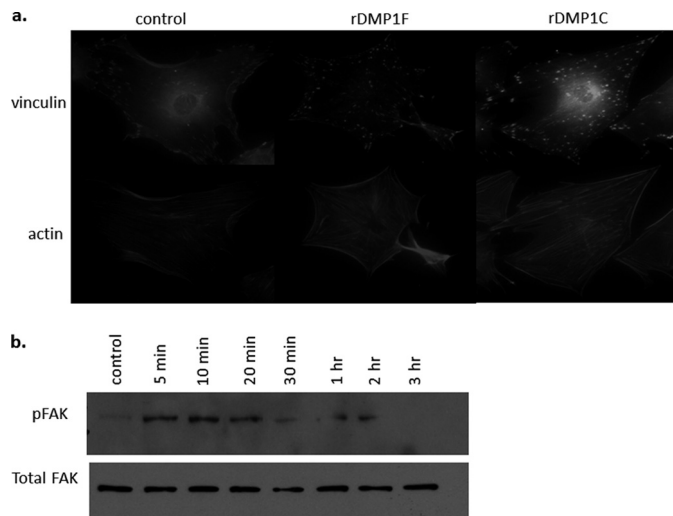

FIGURE 1. **Extracellular rDMP1F and rDMP1C induce the formation of focal adhesions and activation of FAK in hMSC cells.** *a*, hMSC cells were plated on poly-L-lysine (*control*), rDMP1F, or rDMP1C-coated surfaces. Vinculin clustering was seen on both rDMP1F and rDMP1C, indicating that these proteins are capable of triggering the formation of focal adhesions. *b*, cells were treated with rDMP1C and then harvested. Western blot with anti-p-FAK antibody indicates that the presence of extracellular rDMP1 resulted in the activation of focal adhesion kinase. Loading was controlled by blotting for total FAK.

nal ends (data not shown) (21) (a gift from Dr. Chunlin Qin at Baylor School of Dentistry).

Focal Adhesion Formation—Because no osteocyte cell line expressing DMP1 was currently available,[3] we used three different cell types in the course of this study: hMSCs, preosteoblast cells (MC3T3-E1), and an odontoblast cell line known as MDPC23. To test the hypothesis that DMP1 signaling potential is mediated by integrin receptors, we first assessed the formation of focal adhesions, which can be visualized by staining for vinculin in hMSC cells. When hMSCs were plated on rDMP1-F/C-coated slides (Fig. 1*a*), FAs could be clearly visualized along actin filaments and specifically at the edges of cell processes. These data indicated that extracellular presence of rDMP1-F or rDMP1-C was sufficient to trigger the formation of focal adhesions. To confirm an integrin involvement in this formation, we assessed the activation of FAK by Western blot (Fig. 1*b*). Indeed, the rDMP1 group showed a clear and marked increase in the level of phosphorylated FAK when compared with the control group; similar results were also obtained in MDPC23 odontoblast-like cells (data not shown). Taken together, these data confirm that mechanisms known to be involved in the formation of FAs are set into action upon DMP1 exposure. This in turn confirms an integrin-mediated response to extracellular DMP1.

DMP1 Activates the MAPK Pathway—Based on the work of others who have shown the significance of MAPKs to bone development (18), we first turned our investigation to the possibility that DMP1 might be acting through similar machinery.

DMP1 is known to be processed into two fragments: a 37-kDa N-terminal protein and a 57-kDa C-terminal protein (as the role of the free N terminus is not yet clear, for this study

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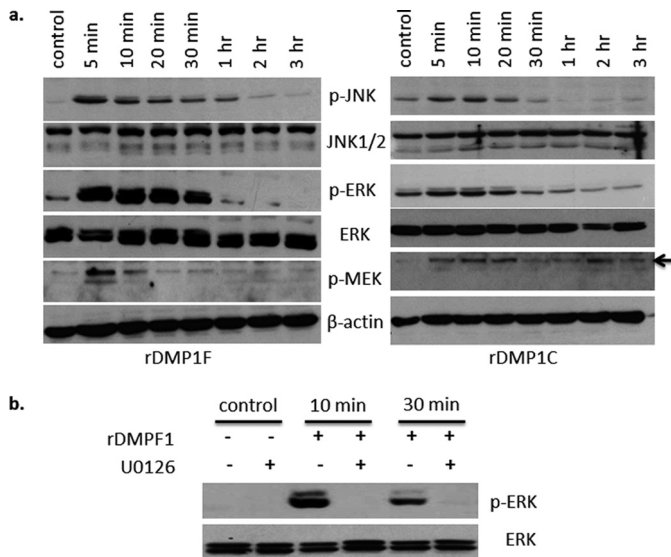[3] Dr. J. Q. Feng, personal communication.

FIGURE 2. **DMP1 activates signaling through the MAPK pathway in hMSC cells.** hMSC cells were first treated with DMP1, then analyzed by Western blot for the activation of known components of the MAPK signaling cascade. *a,* sharp increases in phosphorylated JNK, ERK, and MEK were detected in response to rDMP1F treatment. *b,* the activation of ERK by rDMP1 signaling can be effectively blocked with the ERK-specific inhibitor, U0126.

FIGURE 3. **DMP1 signals through** $\alpha v \beta 3$ **integrin.** Pretreatment with anti-$\alpha v \beta 3$ specific integrin antibody resulted in a lower level of activation of both JNK and ERK in response to rDMP1F (*a*) or rDMP1C (*b*), indicating that signal induction by DMP1 can be significantly reduced by blocking $\alpha v \beta 3$ availability. *c,* to confirm that this blocking effect was indeed specific to the anti-integrin antibody, an IgG1 isotype control was shown not to have such an inhibitory effect.

we have directed our attention to the full-length and C-terminal species). To determine whether intracellular MAPKs were activated by the presence of extracellular DMP1, hMSC cells were plated on recombinant DMP1 and then analyzed for Western blotting. As shown in Fig. 2*a*, MEK1/2, JNK, and ERK were activated in response to both DMP1-F and DMP1-C. The response was clearest at 5 min but was still detectable to varying degrees at 30 min after challenge. These results were also verified in MDPC23 and MC3T3-E1 cells, which showed similar patterns of MAPK activation upon DMP1 treatment (data not shown).

To confirm that the observed activation was specific, we tested the effect of the MEK inhibitor, U0126, on the observed response to DMP1. MEK activity is required for the phosphorylation of ERK1/2 and subsequent ERK-mediated signal transduction. Indeed, when cells were treated with 10 μM U0126 for 1 h before DMP1 challenge (Fig. 2*b*), we observed a complete absence of p-ERK at 10- and 30-min time intervals, confirming that the signaling potential of DMP1 was specifically conducted through MAPKs. It should finally be noted that DMP1 was observed to have no effect on activation of the MAPK family member, p38, or on Smad 1 or Smad 2, which are intracellular signaling effectors of the transforming growth factor-β family (data not shown).

DMP1 Activation of MAPK Is Mediated via Integrin $\alpha v \beta 3$— As our data show, exposure of cells to DMP1 induces the formation of focal adhesion clusters, indicating that integrins are involved in DMP1 signaling activity. In addition, Jain *et al.* (13) and Kulkarni *et al.* (22) have shown that DMP1 can bind to $\alpha v \beta 3$ integrin receptors (13, 22). To test the hypothesis that DMP1-C or -F signaling through the MAPK pathway is mediated by $\alpha v \beta 3$ integrin, we assessed the activation of ERK and JNK after the addition of an antibody directed against integrin $\alpha v \beta 3$. If the DMP1 signaling potential is mediated by $\alpha v \beta 3$
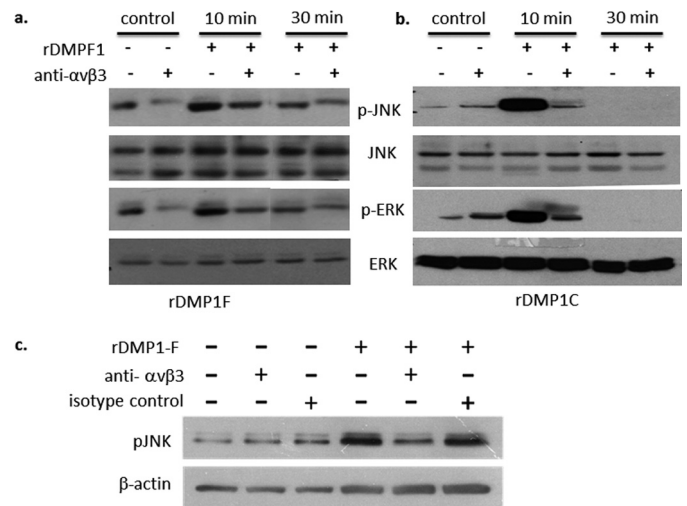
integrin, we should then observe a decline in phosphorylation of downstream targets when this integrin's ability to engage a ligand is blocked. Indeed, as shown in Fig. 3, *a* and *b*, the level of phosphorylated ERK and JNK decreased when anti-$\alpha v \beta 3$ integrin antibody was added before exposure of the cells to rDMP1-F or -C. We were able to confirm through use of an IgG control that this blocking was indeed specific to $\alpha v \beta 3$ (Fig. 3*c*). The results strongly suggested that DMP1 signaling is carried out via interaction with $\alpha v \beta 3$ integrin in hMSC cells.

*DMP1 Signals through $\alpha v \beta 3$ Integrin via Association—*It was next of interest to determine whether this signaling might be facilitated through the formation of a complex including DMP1 and $\alpha v \beta 3$. When cells were exposed to DMP1 and then subjected to immunoprecipitation with either α-$\alpha v \beta 3$ integrin or α-DMP1 antibodies and the precipitates were analyzed by Western blotting, it could be bidirectionally confirmed that these two proteins sustain an interaction, one that is strong enough to be maintained through radioimmune precipitation assay buffer (Fig. 4). Thus far our experiments together confirm that DMP1 signaling is via an integrin-mediated mechanism and, more specifically, involves interaction between DMP1 and $\alpha v \beta 3$ integrin.

*Extracellular Treatment with DMP1 Induces Translocation of JNK to the Nucleus in MC3T3-E1 Cells—*JNKs are normally located in the cytoplasm, but upon phosphorylation their translocation to the nucleus is observed (23). Once in the nucleus, these kinases target a known set of transcriptional activators responsible for the up-regulation of gene expression. To determine whether signals induced by the extracellular presence of DMP1-C could result in a nuclear translocation of JNK, we visualized the distribution of these proteins in MC3T3-E1 cells (Fig. 5, *a* and *b*). Before exposure to DMP1, JNK was confirmed to be diffusely present throughout the cytoplasm, but when cells were exposed to rDMP1-C, the distribution of JNK was specifically nuclear. Furthermore, when the activity of JNK was
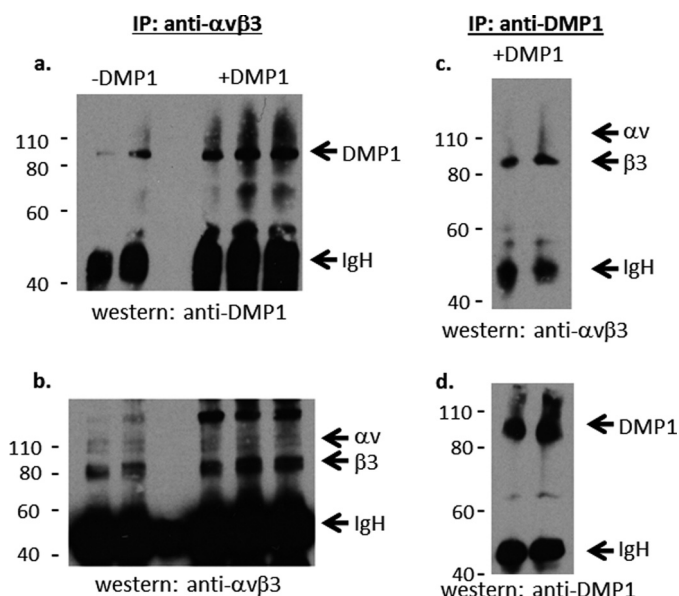
FIGURE 4. **DMP1 signaling via αvβ3 integrin occurs through the formation of a complex.** When cells were exposed to extracellular DMP1 and then subjected to immunoprecipitation, DMP1 could be detected in a complex with αvβ3 integrin. This interaction could be observed via immunoprecipitation (*IP*) with αvβ3 integrin (*a*) and blot-back for DMP1 as well as immunoprecipitation with anti-DMP1 (*c*) and blot-back for αvβ3 integrin. *Panels b* and *d* represent blot-backs with the immunoprecipitation antibody.
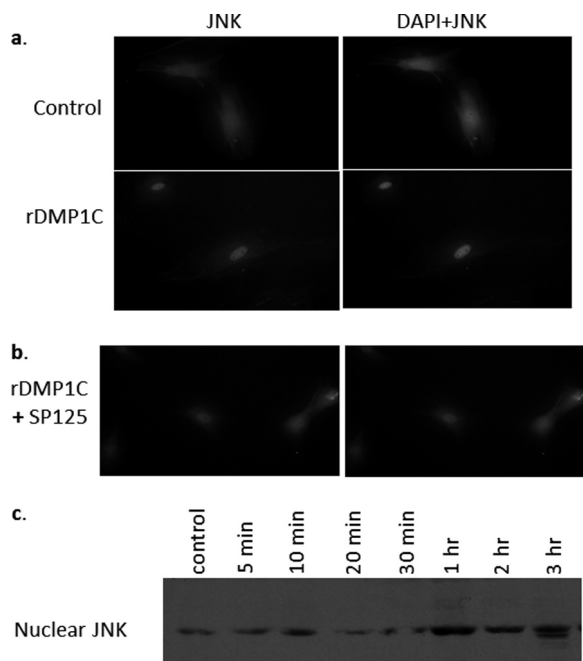

FIGURE 5. **JNK translocates to the nucleus of MC3T3-E1 cells in response to extracellular treatment with DMP1.** When cells were treated with rDMP1C, the distribution of JNK changed from a diffuse pattern to one of clear nuclear localization (*a*). This redistribution was efficiently inhibited by specifically blocking the activity of JNK with the inhibitor SP600125 (*b*). When cells were treated with DMP1, separated into nuclear and cytosolic fractions, and then subjected to α-JNK immunoblotting, an increase in nuclear JNK was observed at 1 h (*c*).

inhibited pharmacologically using the potent and selective SP600125 inhibitor, this nuclear translocation was blocked, as seen in Fig. 5b. To confirm this observed nuclear translocation, cells were treated with DMP1 for various lengths of time and separated into nuclear and cytosolic fractions, and the nuclear

proteins were immunoblotted for phospho-JNK. Indeed, an increase in JNK was seen in the nucleus, most notably at the 1-h time point (Fig. 5c).

Nuclear MAPK Targets Are Phosphorylated in Response to DMP1—The nuclear forms of JNK are known to phosphorylate ATF2 and c-Jun, and ERK is known to phosphorylate c-*fos* (24). These phosphorylated proteins subsequently potentiate the activation of transcription of target genes involved in osteoblast differentiation (18). To determine whether the presence of the DMP1 signaling effect could be traced all the way to the activation of transcription, we looked at the phosphorylation state of particular MAPK targets in response to extracellular DMP1. As shown in Fig. 6, phosphorylation levels of the transcription factors c-Jun and ATF-2 were both shown to increase as detected by Western blot.

To confirm this result in the other cell types as well as to verify the specificity of the effect, we treated hMSC, MC3T3-E1, and MDPC23 cells with DMP1 and examined the phosphorylation state of its putative nuclear targets. As shown in Fig. 7*a*, a strong increase in phosphorylated c-Jun and ATF2 was detected in all three cell types in response to DMP1. Furthermore, treatment with SP600125 to block the function of JNK had a clear and complete inhibitory effect on the phosphorylation state of these factors, indicating that this activation was in fact mediated through MAPKs.

Finally, we asked whether the DMP1 ability to elicit activation of transcriptional effectors resulted ultimately in an increase in transcriptional activity of a known responsive promoter. MC3T3-E1 cells were first transiently transfected with two plasmids; that is, the GAL4-c-Jun plasmid encoding the GAL4-DNA binding domain fused to the c-Jun activation domain and a reporter plasmid containing a luciferase gene driven by a 5×GAL4-responsive promoter). The activation of transcription of the 5×Gal4-containing reporter plasmid is dependent on c-Jun phosphorylation by JNK family members (25–27). The measured luciferase activity showed a dose-dependent activation of transcription in response to the presence of extracellular DMP1F or DMP1C for 24 h (Fig. 7*b*).

The data were analyzed to test for significant mean differences in luciferase activity among the three DMP1 concentration groups using a one-way analysis of variance between cases ($p \leq .0004$). Post hoc pairwise comparisons were done using the Tukey Honestly Significant Difference procedure. This found only the control compared with the 0.50 group to have a significant difference ($p = .020$), although the 0.15 group compared with the 0.50 approached significance ($p = .091$).

Taken together our data confirm that DMP1 elicits a signaling effect that can be traced from the extracellular matrix via integrins MAPK proteins (ERK and JNK) and down to the level of transcriptional activation of targets such as c-Jun and ATF2. These transcription factors were shown to be involved in osteoblastic gene expression (28).

DISCUSSION

In this study we examine the potential for the SIBLING family member, DMP1, to initiate intracellular signaling. We show that the extracellular presence of DMP1 is sufficient to initiate the formation of focal adhesion-like clusters and subsequent
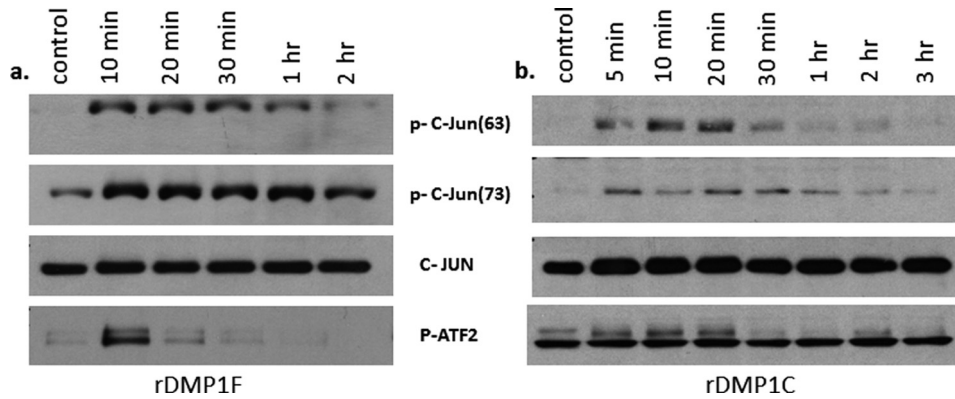
FIGURE 6. **DMP1 signaling regulates phosphorylation of c-Jun and ATF2.** *a*, the extracellular presence of rDMP1F is sufficient for induction of phosphorylation of the transcription factors c-Jun and ATF2 in hMSC cells. *b*, rDMP1C treatment resulted in c-Jun phosphorylation but not that of ATF2.
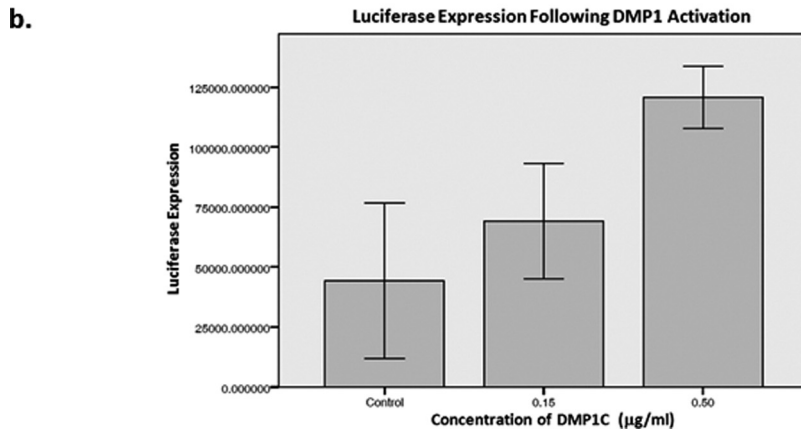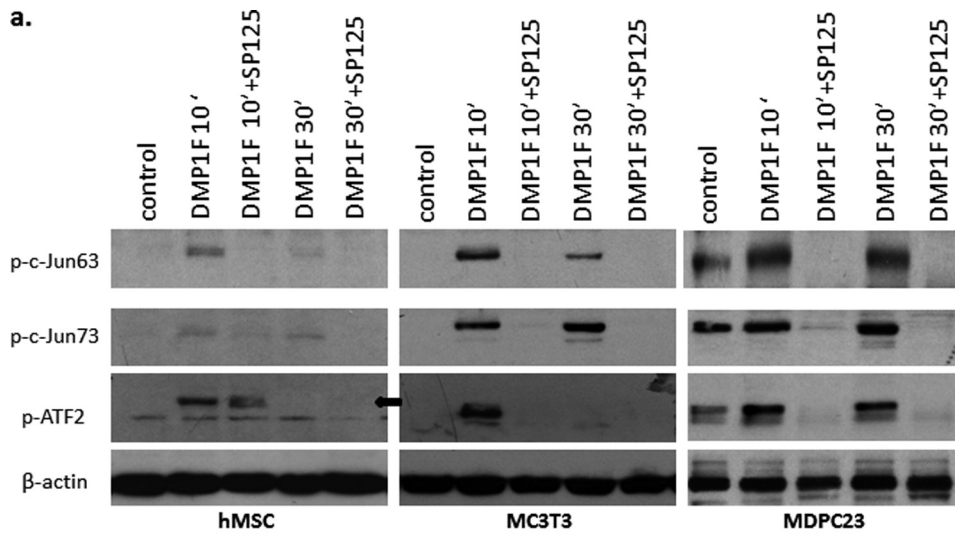

FIGURE 7. **Extracellular DMP1 activates transcriptional activation through MAPK signaling.** *a*, DMP1 treatment of hMSC, MC3T3-E1, and MDPC23 cells resulted in phosphorylation of the transcription factors c-Jun and ATF-2, and this activation was specifically blocked upon inhibition of the MAPK family member, JNK, using SP600125. *b*, MC3T3-E1 cells transfected with reporter plasmids bearing the c-Jun transactivation domain were then treated with rDMP1. Both rDMP1F and rDMP1C were capable of effecting downstream c-Jun activity.

activation of MAPK-ERK signaling via an $\alpha v\beta 3$ integrin-dependent mechanism. This MAPK signaling involves JNK and its translocation to the nucleus, where it appears to up-regulate the transcriptional activation of c-Jun.

Integrins are heterodimeric transmembrane receptors critical to a variety of processes including cell shape, mobility, growth and differentiation, and regulation of the cell cycle (14).

They are known conduits through which mechanical and chemical signals are conveyed from the extracellular aspect to the intracellular milieu and are the means by which cells achieve a physical connection to their microenvironment. The work of Feng *et al.* (1) has shown that DMP1-null mice exhibited a striking lack of osteocyte processes and that the null osteocytes do not seem able to attach to their lacunae. Coupled

with data confirming that DMP1 promotes cell attachment via its RGD sequence, that it can bind to MMP9 as a bridge to bind cell surface receptors, either integrin $\alpha v \beta 3$ or CD44 in colon cancer cells to improve cellular invasion (29), and that it may be involved in the initiation of osteoblast differentiation (30), there is compelling evidence for DMP1 ability to activate cellular signaling pathways (31). We hypothesized that the DMP1 null defect could in part be due to a loss of signal from DMP1 at the extracellular aspect and wondered whether this signal was integrin-mediated.

To this end we examined the ability of extracellular DMP1 to activate signaling machinery at the intracellular aspect and observed both a formation of focal adhesion-like structures and concomitant activation of FAK in response to rDMP1-F presence. It has been shown that *in vitro* cell attachment to DMP1-coated surfaces is specifically dependent on $\alpha v \beta 3$ integrin (32). Indeed, when we used an antibody to block $\alpha v \beta 3$ function, we observe a loss of FAK activation in the presence of DMP1. Furthermore, we were able to coimmunoprecipitate a DMP1-$\alpha v \beta 3$ integrin complex under stringent co-immunoprecipitation conditions, indicating that not only do the proteins exist in complex with one another but that their association within this complex is relatively robust. We conclude, thus, that DMP1 signals through $\alpha v \beta 3$ integrin and propose that this signaling role may be necessary to ensure physiological osteocyte homeostasis.

MAPK pathways transduce a wide array of extracellular signals, thereby regulating a host of vital cellular processes (33). In bone, MAPK signaling is critical for *in vivo* osteoblast activity and bone development (18), and Caverzasio and Manen (34) showed that MAPK may be involved in C3H10T1/2 cell differentiation. We asked, thus, whether the initiation of cellular signaling by DMP1 might involve a MAPK pathway. Our results indicate that known effectors of MAPK signaling, namely JNK, ERK, and MEK, are activated in response to DMP1 and that this activation is specifically inhibitable.

Our data are consistent with a study wherein bone morphogenetic protein, BMP, was shown to be involved in the differentiation of dental follicle cells to osteoblasts/cementoblasts via MAPK activity (35). Another ECM protein present in bone and dentin and known to be involved in the mineralization process in these tissues, phosphophoryn, was also found to be able to activate MAPK (36) and was shown to exert control over activation of Smad1 (37). In contrast, we observed neither Smad1 nor Smad 2 activation after DMP1 challenge. Our data also indicate that p38 is not activated in response to DMP1. Hence, we speculate that extracellular DMP1 may be a physiological regulator of osteoblast/odontoblast-specific genes via the MAPK-ERK-JNK pathways. Indeed, the DMP1 null mice revealed that DMP1 is not only required for the formation of both the organic and inorganic components of dentin, but its absence also results in changes in osteocyte morphology, reduced bone elasticity, and progressive morphological bone abnormalities concomitant with aging (38). In humans, a DMP1 gene defect has been shown to be the cause of a skeletal disorder known as autosomal-recessive hypophosphatemic rickets. The etiology of this disorder has been shown to lie in defective osteocyte maturation and inefficient regulation of phosphate homeostasis (1). We suggest that the absence of DMP1 function in the extracellular microenvironment results in changes in cell/matrix interactions and subsequent signaling pathways, which we have begun to describe in this manuscript. Furthermore, we hypothesize that these DMP1-mediated signals could play a role in the maintenance of the osteocyte phenotype.

At this juncture it is not known whether the signaling functions we have identified are specific to DMP1 or whether other SIBLING molecules may have similar functions in this context. As we have previously indicated, another SIBLING protein, phosphophoryn, has also been shown to activate the MAPK and the Smad pathways leading to bone/dentin specific gene activation (36, 37). It will be of future interest to extend this study to other family members as well as to look at the downstream effects of this signaling pathway on other transcriptionally active targets relevant to mineralized tissue biology.

In summary, we show that DMP1 can bind to the integrin receptor $\alpha v \beta 3$, thereby transducing a signal to the intracellular aspect via FAK activation. In the cytoplasm, the MAPK pathway mediates DMP1 activity via ERK and JNK phosphorylation and subsequent stimulation of specific transcriptional activators. It appears, as demonstrated in both the mouse and human defects, that DMP1 function in the extracellular space is necessary for the formation of morphologically competent osteocytes. It will be of future interest to determine whether this defect is indeed specifically due to an absence of MAPK signals normally sustained through DMP1-$\alpha v \beta 3$ interaction.

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