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Journal of Cellular Biochemistry 107:284–292 (2009)

Journal of Cellular Biochemistry

Sp Proteins and Runx2 Mediate Regulation of Matrix Gla Protein (MGP) Expression by Parathyroid Hormone

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ABSTRACT

As part of its catabolic action in bone, parathyroid hormone (PTH) inhibits extracellular matrix mineralization. We previously showed that PTH dose-dependently induces matrix gla protein (MGP) expression in osteoblasts and this induction is at least partially responsible for PTH-mediated inhibition of mineralization. Recently, we identified PKA and ERK/MAPK as the key signaling pathways involved in PTH regulation of MGP expression. The goal of this study was to further characterize the mechanism by which PTH stimulates expression of MGP. Deletion analysis of the murine *Mgp* gene promoter identified a PTH-responsive region between −173 bp and−49 bp. Using gel-mobility shift assays we found that Sp1/Sp3, and Runx2 bind to distinct sites within this region. Mutation of either the Sp or the Runx2 site reduced MGP induction by PTH, while mutation of both sites completely abolished PTH responsiveness. Overexpression of Runx2 or Sp1 activated the *Mgp* reporter, while Sp3 was a dose-dependent repressor of Sp1 and PTH-induced MGP expression. Collectively, these data show that PTH regulates MGP gene transcription in osteoblasts through altered activities of Sp and Runx2 transcription factors. J. Cell. Biochem. 107: 284–292, 2009.

KEY WORDS: PARATHYROID HORMONE; MATRIX GLA PROTEIN; Sp TRANSCRIPTION FACTORS; RUNX2; OSTEOBLASTS

Parathyroid hormone (PTH) action on the skeletal system is complex, with differential effects produced depending upon the mode of treatment [Tam et al., 1982]. Continuous PTH treatment induces net bone loss due to inhibition of osteoblast activity and increased bone resorption, while intermittent PTH administration causes net gain in bone due to increased bone formation [Hock and Gera, 1992; Stewart et al., 2000; Burr et al., 2001]. PTH exerts its action through the PTH1 receptor (PTH1R), a seven transmembrane domain G-protein coupled receptor expressed on the cell surface. Activation of this receptor generates multiple second messengers, which in turn activate various signaling pathways including protein kinase A (PKA), protein kinase C (PKC) and MAPK pathways [Civitelli et al., 1988; Partridge et al., 1994; Swarthout et al., 2001]. The cAMP response element binding protein (CREB), AP-1 transcription factors and Cbfa1/Runx2 are well studied PTH-responsive transcription factors in osteoblasts [Pearman et al., 1996; Koe et al., 1997; Selvamurugan et al., 2000].

MGP is a 15-kDa secreted protein initially extracted and purified from demineralized bone matrix [Price et al., 1983]. Along with osteocalcin (OCN), MGP belongs to the mineral-binding γ-carboxyglutamic acid (Gla) containing protein family, which also includes numerous coagulation factors [Price and Williamson, 1985]. MGP is a potent inhibitor of extracellular matrix calcification, as mice deficient for the *Mgp* gene show severe vascular calcification and premature mineralization of long bones [Luo et al., 1997]. Transgenic mice overexpressing MGP in osteoblasts under the control of the *α1(I)-collagen* promoter exhibit a reduction in bone mineral levels, indicating that MGP can grossly disrupt bone formation [Murshed et al., 2004]. Similarly, it has been reported that tooth root dentin and cementum from *α1(I)-Mgp* transgenic mice are hypomineralized [Kaipatur et al., 2008] and retrovirus-induced overexpression of MGP in developing growth plates blocked endochondral ossification by delaying the normal process of chondrocyte differentiation [Yagami et al., 1999]. Murine MGP contains four Gla

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Grant sponsor: NIH; Grant number: DE016093; Grant sponsor: University of Minnesota.

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Received 6 February 2009; Accepted 10 February 2009 • DOI 10.1002/jcb.22124 • 2009 Wiley-Liss, Inc.
Published online 20 March 2009 in Wiley InterScience (www.interscience.wiley.com).

residues, through which it binds to calcium and hydroxyapatite (HA), but the exact mechanism by which MGP inhibits mineralization is not known [Hauschka et al., 1989; Roy and Nishimoto, 2002].

We recently reported that PTH induces expression of MGP in MC3T3 osteoblasts and that MGP is a key mediator of PTH's inhibition of matrix mineralization [Gopalakrishnan et al., 2001]. Further, we showed that PTH induction of MGP expression involves both PKA and ERK/MAPK signaling pathways, and that the 748 bp murine *Mgp* promoter region is sufficient for transcriptional activation by PTH in MC3T3-E1 cell [Suttamanatwong et al., 2007]. Several consensus transcription factor-binding sites are present in this region of the murine *Mgp* promoter including AP-1, AP-2, and *runt* domain binding sequences [Stheneur et al., 2003]. The purpose of this study was to identify specific DNA elements in the *Mgp* promoter necessary for regulation by PTH and to characterize the transcription factors that elicit this regulation. We find that regulation of MGP expression by PTH is mediated through Sp and Runx2 transcription factors bound to distinct sites within the −173 to −49 region of the murine *Mgp* gene promoter.

TABLE I. Sequence of Wild-Type and Mutant Probes Used for Gel-Shift Analysis of *Mgp* Promoter Binding Elements and Mutant Reporter Plasmids

Probe 6 wild-type	CCC CCT CCC GCC GGT TCC TCC CCC TTT CAG
Probe 6 M1	aaa CCT CCC GCC GGT TCC TCC CCC TTT CAG
Probe 6 M2	CCC aag CCC GCC GGT TCC TCC CCC TTT CAG
Probe 6 M3	CCC CCT aaa GCC GGT TCC TCC CCC TTT CAG
Probe 6 M4	CCC CCT CCC taa GGT TCC TCC CCC TTT CAG
Probe 6 M5	CCC CCT CCC GCC ttg TCC TCC CCC TTT CAG
Probe 6 M6	CCC CCT CCC GCC GGT gaa TCC CCC TTT CAG
Probe 6 M7	CCC CCT CCC GCC GGT TCC gaa CCC TTT CAG
Probe 6 M8	CCC CCT CCC GCC GGT TCC TCC aaa TTT CAG
Probe 6 M9	CCC CCT CCC GCC GGT TCC TCC CCC ggg CAG
Probe 6 M10	CCC CCT CCC GCC GGT TCC TCC CCC TTT act
Probe 1 wild-type	CTG CCC ACG CTG TGT AGA TAC TAT CTG G
Probe 1 M1	agt CCC ACG CTG TGT AGA TAC TAT CTG G
Probe 1 M2	CTG ggg ACG CTG TGT AGA TAC TAT CTG G
Probe 1 M3	CTG CCC cat CTG TGT AGA TAC TAT CTG G
Probe 1 M4	CTG CCC ACG agt TGT AGA TAC TAT CTG G
Probe 1 M5	CTG CCC ACG CTG gtg AGA TAC TAT CTG G
Probe 1 M6	CTG CCC ACG CTG TGT ctc TAC TAT CTG G
Probe 1 M7	CTG CCC ACG CTG TGT AGA gca TAT CTG G
Probe 1 M8	CTG CCC ACG CTG TGT AGA TAC gcg CTG G
Probe 1 M9	CTG CCC ACG CTG TGT AGA TAC TAT agt t

Wild-type residues are given in uppercase, mutated bases in lowercase.

MATERIALS AND METHODS

CELL CULTURE

A highly differentiating subclone of MC3T3-E1 cells (MC-14) was used for the studies [Wang et al., 1999; Petryk et al., 2005]. MC3T3-E1 cells express most of the known osteoblast markers and form a mineralized ECM following differentiation in ascorbic acid-containing medium. MC-14 cells were maintained in MEM media containing 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids and were not used beyond passage 20. PTH treatments were performed in 0.1% FBS containing MEM. For transfection experiments, MC-14 cells were plated at a density of 0.25×10^5 cells/cm^2 in 35-mm dishes. For nuclear extract isolation and overexpression experiments, cells were plated at 0.50×10^5 cells/cm^2.

DNA CONSTRUCTS

The murine *Mgp* promoter construct, pMGP-748-luc was previously described [Suttamanatwong et al., 2007]. This plasmid contains bases −748 to +1 of the murine *Mgp* gene inserted into the *HindIII* site of pGL3Basic (Promega) upstream of the firefly luciferase reporter gene. 5′-deletion constructs were created by exonuclease III digestion of the pMGP-748-luc linearized with *SacI* and *XhoI*, using the Erase-a-Base kit (Promega) as recommended by the manufacturer. Point mutations in the pMGP-luc reporter plasmids were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The relevant sequences of the mutant reporter plasmids are provided in Table I. Plasmids pCMV-Sp1 and its empty vector, p119, were kindly provided by Dr. Robert Tjian (University of California, Berkeley, CA). Plasmids pN3-Sp3 and pN3 vector were obtained from Dr. Guntram Suske (Philipps-University, Marburg, Germany). pCDNA3.1-HA-Runx2 and p6OSE2-luc reporter plasmids were obtained from Dr. Jennifer Westendorf (Mayo Clinic, Rochester, MN).

TRANSFECTION AND LUCIFERASE ACTIVITY ASSAYS

MC-14 cells were transfected by Lipofectamine (Invitrogen) with 1 μg of pMGP-luc reporters plus 0.05 μg/dish of pRL-SV40 Renilla luciferase vector (Promega) as well as Sp1, Sp3, and Runx2 expression vectors or the corresponding empty vectors as indicated. Following transfection, cells were grown overnight in MEM containing 10% FBS. The following day, cells were washed with Hank's Buffered Saline Solution, then treated for 6 h with PTH (10^{-7} M) or vehicle in MEM containing 0.1% FBS. After 6 h, cell lysates were harvested and the luciferase activity in the cell lysates was assayed using the Dual Luciferase Reporter Kit (Promega). Transfections were performed in triplicate and normalized either to *Renilla* luciferase or to total protein content, due to inductive effects of Sp1 and Sp3 on the *Renilla* luciferase reporter.

GEL MOBILITY SHIFT ASSAYS

Double-stranded oligonucleotide probes containing DNA sequence spanning from −173 to −49 of the *Mgp* promoter (Fig. 2A and Table I) were end-labeled with [α-^{32}P]dATP using Klenow DNA polymerase (Roche) in a reaction containing 100 ng of oligonucleotides, 5 mM each of cold G,C, and T nucleotides and 50 μCi of [α-^{32}P]dATP. The labeled oligonucleotide probe was purified using ProbeQuant G-50 Micro Columns (Amersham Biosciences) according to the manufacturer's instructions. Nuclear extracts (10 μg) from vehicle- or PTH-treated cells were prepared as previously published [Dignam et al., 1983] and incubated with an equal volume of 2× probe mix (100 mM HEPES, pH 7.8, 5 mM $MgCl_2$ 0.5 mM EGTA, pH 8.0, 2 mM dithiothreitol, 200 mM KCl, 8.25% Ficoll, 12.5 μg/ml salmon sperm DNA, and 41.25 pg/ml labeled probe) at room temperature for 30 min. For competition assays, nuclear extracts were pre-incubated with 25- to 75-fold excess of unlabeled probe. For supershift experiment, binding complexes were incubated with antibodies against Sp1, Sp3 or Runx2 (Santa Cruz Biotechnology), anti-Sp3 (Santa Cruz Biotechnology) or Runx2 (Santa Cruz Biotechnology). Complexes were resolved by 5% non-denaturing

polyacrylamide gel electrophoresis in 1× TBE for 90 min at 270 V at 4°C, dried, and visualized by autoradiography.

STATISTICAL ANALYSIS

All transfection experiments were performed in triplicate and repeated at least three times. Data are represented as mean ± Standard Deviation (SD). Statistical significance was determined using Student's *t*-test.

RESULTS

PTH-RESPONSIVE ELEMENT(S) ARE PRESENT BETWEEN BASES −173 TO −49 BP OF THE *MGP* PROMOTER

We previously showed that PTH treatment increased *Mgp* promoter activity in a time-dependent manner, with a maximal ~4-fold induction observed after 6 h [Suttamanatwong et al., 2007]. To identify the region(s) responsible for PTH-mediated transcriptional activation of MGP, we generated a series of 5' deletions of the murine *Mgp* promoter in the pGL3-basic luciferase reporter plasmid. As shown in Figure 1, the −748 bp *Mgp* promoter fragment was activated approximately fourfold by PTH treatment. Like the −748 bp fragment, the activity of −634, −545, and −173 bp fragments were stimulated upon PTH treatment approximately fourfold above their basal activities. In contrast, induction of the −49 bp fragment by PTH was reduced to approximately 1.5-fold above the reporter plus vehicle, representing an approximately 60% decrease in PTH-responsiveness compared with the −748 bp and −173 bp reporters. These observations suggest that element(s) necessary for PTH-responsiveness are contained within the −173 to −49 region of the *Mgp* promoter.

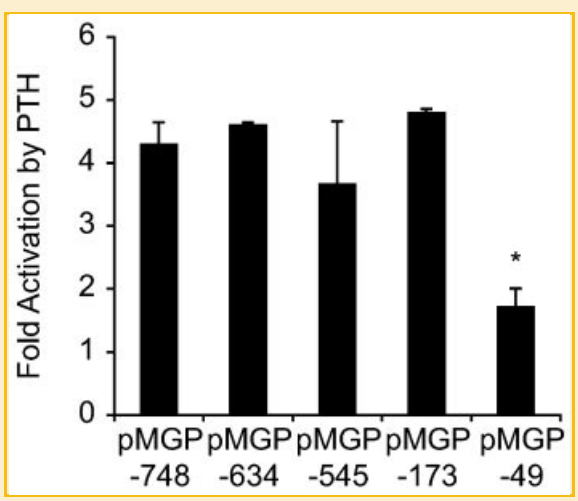

Fig. 1. Identification of PTH-responsive region(s) of the *Mgp* promoter. MC14 cells were transfected with the indicated 5′ deletions of the pMGP-luc reporter and treated with 10^{-7} M PTH or vehicle for 6 h. Luciferase values are graphed as the fold activation of each reporter by PTH relative to the activity of that treated with vehicle. $^{*}P \leq 0.02$ versus pMGP-748 reporter.

SP1 AND SP3 TRANSCRIPTION FACTORS BIND TO −63 TO −55 BP IN THE *MGP* PROMOTER

To identify potential transcription factor binding sites by which PTH regulates *Mgp* gene expression, we performed electrophoretic mobility shift assays (EMSA) using a set of overlapping double-stranded oligonucleotide probes that span the −173 to −49 bp region of the proximal *Mgp* promoter (Fig. 2A). As shown in Figure 2B, MC14 nuclear extracts contained a strong binding activity to probe 8, which spans the proximal half of the −173 to −49 region. In some experiments, treating the cells with PTH reduced binding (Fig. 2B and data not shown), but this effect was inconsistent and its significance is unclear. To further localize the position of the binding site, we tested probes 4, 5, and 6 (Fig. 2A), finding that the binding site was present in probe 6 but not probes 4 or 5, indicating that the binding site is contained within the 30 nucleotides between −78 and −49. To further refine the location of the binding site, we generated a series of mutant versions of probe 6 in which three adjacent nucleotides were mutated (Table I). Pre-incubation of nuclear extracts with excess unlabeled wild-type probe reduced binding to ^{32}P-labeled probe 6. Pre-incubation with unlabeled mutant probes M7, M7, M8 did not affect binding to labeled wild-type probe 6, while probes M5 and M9 only weakly reduced binding (Fig. 2C,D). Further experiments confirmed that the binding activity towards ^{32}P-labeled mutant probes M6, M7, and M8 is highly compromised (data not shown). Sequence analysis of the region mutated in probes M6, M7, and M8 indicated that it comprises a putative Sp-transcription factor core sequence (5′ TCCTCCCCC 3′) at −63/−55 bp (underlined in Fig. 2A).

To determine whether the binding activity in MC14 nuclear extracts reflects activity of an Sp transcription factor family member, we performed antibody supershift experiments in which we added antibodies against Sp1, Sp3 or Sp1 and Sp3 together to EMSA binding reactions with MC14 cells (Fig. 2E). Addition of anti-Sp1 antibody produced a supershift in the upper portion of the initial mobility-shifted band. Similarly, incubation with anti-Sp3 reduced the lower portion of the original band. Incubation with both antibodies shifted or eliminated the majority of the original band. These observations led us to conclude that Sp1 and Sp3 proteins are the major proteins bound to this site in the *Mgp* promoter in vivo. This result was further confirmed by pre-incubation with unlabeled Sp1 consensus oligonucleotide, which efficiently blocked MC14 cell extract binding to the *Mgp* probes (data not shown). These data indicate that both Sp1 and Sp3 transcription factors bind to the region near −60 bp of the MGP promoter.

REGULATION OF THE MGP PROMOTER BY Sp PROTEINS AND PTH

To determine the ability of Sp1 and Sp3 to regulate the *Mgp* promoter, we examined the effects of overexpressed Sp1 and Sp3 on the activity of the *Mgp* reporters. Figure 3A shows that transfection of increasing levels of Sp1 gave a dose-dependent activation of the −173 bp *Mgp* reporter, while Sp3 activated the reporter less than twofold above vector alone at all doses tested. We next asked whether Sp1 and PTH could cooperate to activate the pMGP-luc reporters (Fig. 3B). The −748 bp *Mgp* reporter was activated 3.3-fold above control by PTH, 2-fold by Sp1 and 12.5-fold by Sp1 and PTH together. Similarly, the −173-bp *Mgp* reporter was activated

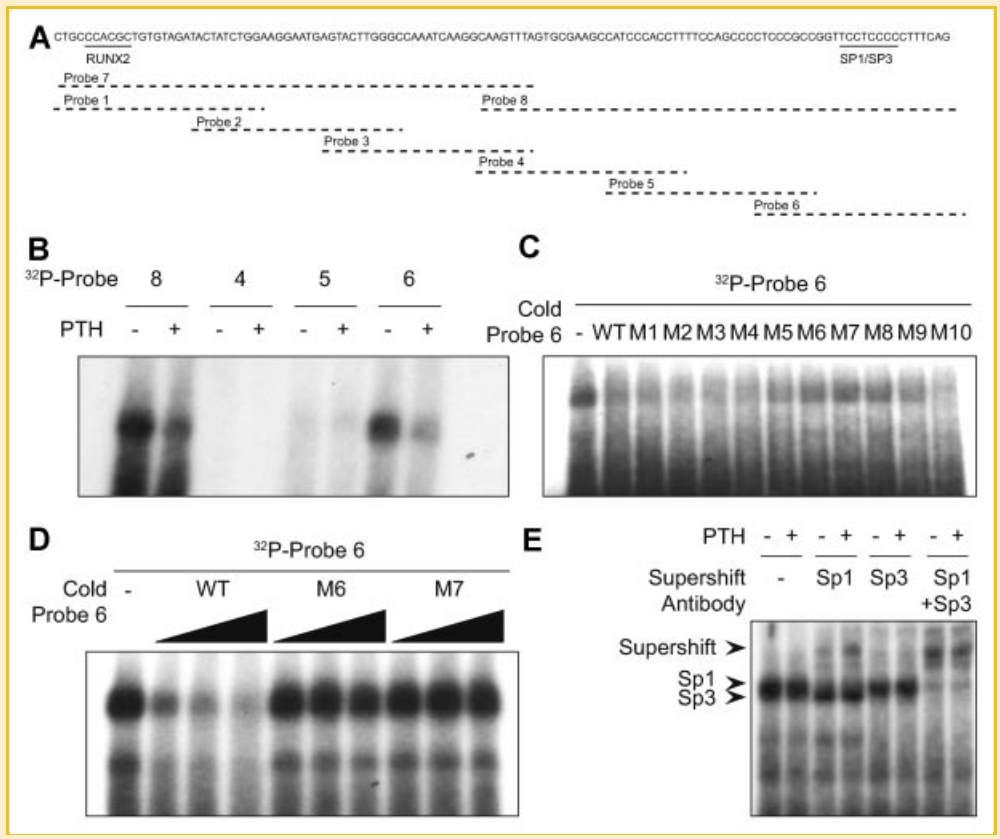

Fig. 2. Sp proteins bind to the PTH-responsive region of the *Mgp* promoter. A: Schematic of the −173 to −49 bp region of the *Mgp* promoter showing the position of the EMSA probes and the putative Sp and Runx2 binding sites. B: EMSA showing binding of nuclear extracts prepared from vehicle or PTH-treated MC14 cells. C,D: Competition assay in which binding to radiolabeled probe 6 was competed by pre-incubation with increasing wild-type "WT" or mutant variants of probe 6 "M1 through M10". E: Antibody supershift experiment showing that addition of anti-Sp1 or Sp3 antibodies further retards the mobility of binding complexes (Sp1) or impairs complex formation (Sp3).

3.3-fold above control by PTH, 2-fold by Sp1 and 15.8-fold by Sp1 and PTH together. These data indicate that PTH and Sp1 synergistically activate MGP expression. As Sp3 showed little ability to activate the *Mgp* reporters by itself (Fig. 3A), we next determined whether Sp3 modulated activation of the −173 bp *Mgp* promoter by Sp1 (Fig. 3C) or PTH (Fig. 3D). Low doses of Sp3 slightly increased promoter activity above that of Sp1 or PTH alone. However, as the amount of Sp3 increased we observed a dose-dependent repression of the reporter by Sp3.

Having found that Sp1 by itself activates the *Mgp* promoter and synergizes with PTH to strongly activate the reporter, we next asked whether the Sp binding site is necessary for activation by PTH. To this end, we introduced mutations corresponding to the Sp site M6 and M7 mutant EMSA probes (Table I) into the −748 and −173 bp MGP reporters and tested their activity and induction by PTH. Prior to treatment with PTH, the basal activity of the M6 and M7 mutant reporters was decreased by 40–70% compared to the wild-type reporters (Fig. 3E). Likewise, the ability of PTH to activate the Sp-site mutant reporters was reduced by approximately 50% of the wild-type reporters (Fig. 3F). Taken together, these data indicate that Sp1 contributes to basal activity and PTH-induced transcription of the *Mgp* promoter, while Sp3 acts as an inhibitor of MGP expression.

Runx2 BINDS TO THE PTH-RESPONSIVE REGION OF THE *MGP* PROMOTER

In addition to the Sp binding element in the proximal portion of the −173 to −49 bp PTH-responsive region, EMSA analysis also revealed a DNA binding activity that associated with the distal part of this region. As shown in Figure 4, MC14 nuclear extracts contain a protein that binds to probe 7 (Fig. 2A), which spans the distal portion of the region, from −173 to −108 (Fig. 4A). Gel shift analysis with probes 1, 2, and 3 localized the binding to the region from −173 to −144 contained in probe 1. Binding to these probes was not reproducibly affected by PTH treatment of the cells (Fig. 4A,C). We next tested a set of mutant versions of probe 1 (Table I), each with substitutions at three adjacent bases to further refine the location of the binding site. Binding to mutant probes M1, M2, M3, and M4 was strongly decreased relative to the wild-type probe, while binding to M5, M7, M8, and M9 was similar to the wild-type probe. We noted the presence of a sequence motif similar to the core OSE2 Runx2-binding element (5′-AACCAC) centered at nt −167 (underlined in Fig. 2A) that was either mutated or directly adjacent to the mutated residues in probes M1 through M4. To determine whether Runx2 binds to this site, we added anti-Runx2 antibody to the binding complexes with the wild-type probes 1 or 7.

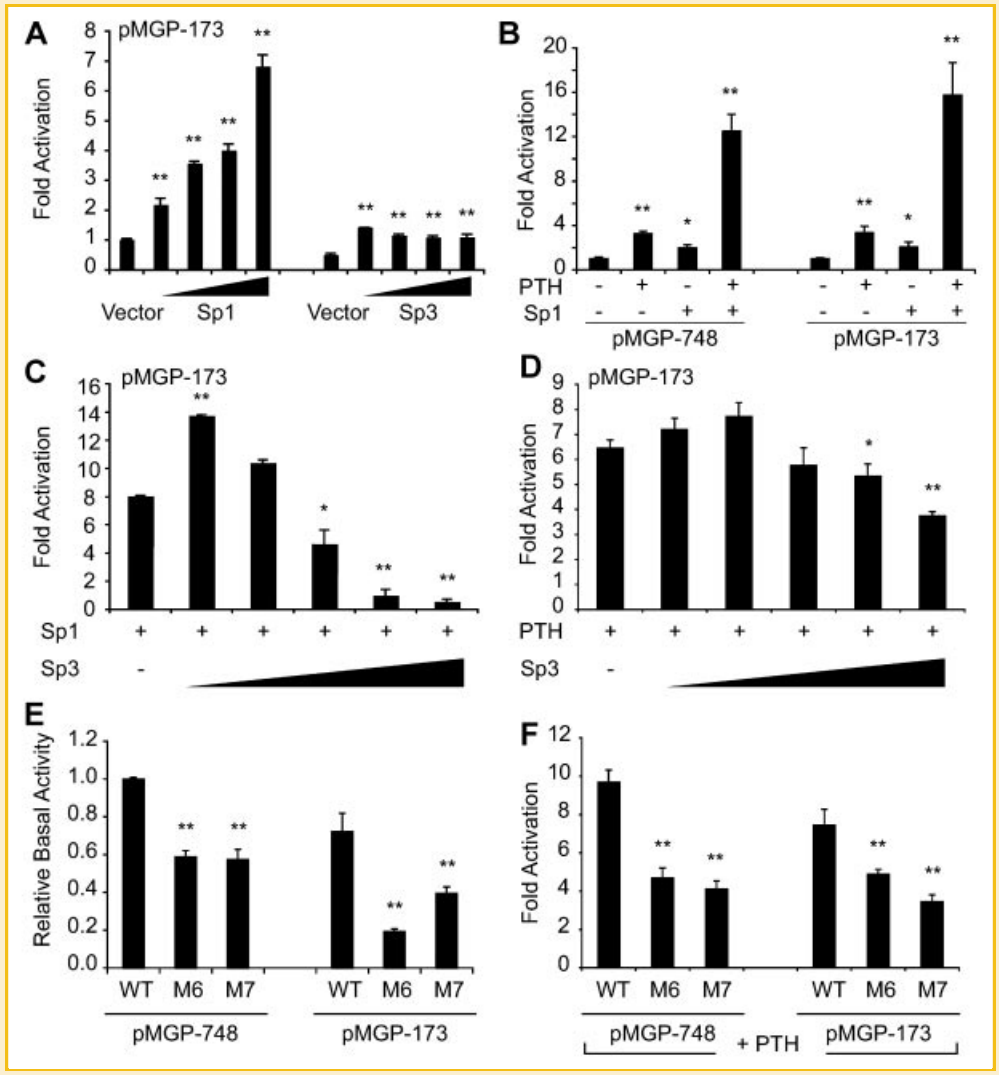

Fig. 3. Sp proteins regulate *Mgp* promoter activity. A: Relative activity of the pMGP-173 reporter with increasing doses of Sp1 or Sp3. B: Sp1 and PTH cooperatively activate the *Mgp* reporter. C,D: Sp3 modulates activation of the pMGP-173 reporter by Sp1 (C) and PTH (D). E,F: Activation by PTH of wild-type "WT" and Sp site mutant (M6 and M7, Table I) forms of the pMGP-173 reporter. E: Basal activity of Sp site M6 and M7 mutant forms of pMGP-748 and pMGP-173. F: Fold activation of wild-type and Sp1 site mutant *Mgp* reporters treated with PTH. $^{*}P \leq 0.02$; $^{**}P \leq 0.001$.

Figure 4C shows that addition of anti-Runx2 antibody strongly reduced binding, indicating that Runx2 is the protein responsible for this binding activity. These data establish that Runx2 binds to a site in the distal part of PTH-responsive region of the MGP promoter.

Runx2 AND PTH COOPERATIVELY ACTIVATE MGP EXPRESSION

To determine the functional significance of Runx2 on regulation of MGP expression we compared the ability of Runx2 and PTH to activate −173 bp *Mgp* reporter, using the p6OSE2-luc reporter as a positive control for Runx2 transcriptional activation (Fig. 5). As expected, Runx2 efficiently activated the p6OSE2 reporter, and although PTH had little effect on p6OSE2-luc by itself, it strongly synergized with Runx2 to activate the p6OSE2-luc reporter. The −173 bp *Mgp* reporter was activated between 5- and 8-fold by either Runx2 or PTH individually and approximately 15-fold by Runx2 and PTH together. To further test the significance of the Runx2 binding site on MGP expression, we introduced mutations corresponding to the mutated EMSA probes 1 M2 and M3 (Fig. 4 and Table I) into the −173 bp MGP reporter. In each case, the ability of Runx2 or PTH to stimulate the mutant reporters was significantly diminished compared to their effect on the wild-type reporter. These data indicate that Runx2 activates the *Mgp* promoter and cooperates with PTH to regulate MGP expression.

LOSS OF BOTH Sp AND Runx2 SITES ABOLISHES PTH RESPONSIVENESS

Mutation of either the Sp or Runx2 binding site compromised activation of the *Mgp* reporters by PTH. We next examined the effect of mutating both sites together. Like the Sp-site single mutation, basal activity of the double mutant reporter was significantly

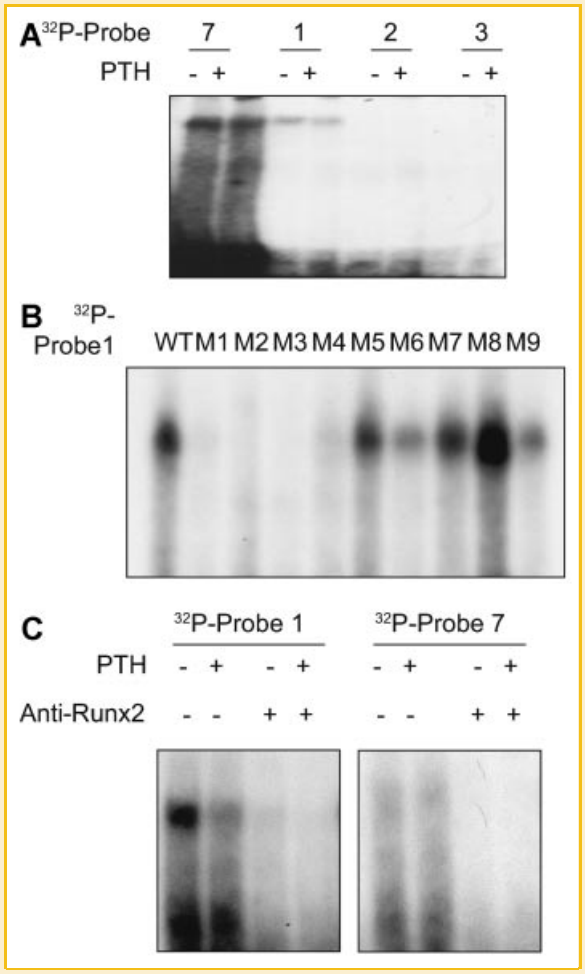

Fig. 4. Runx2 binds to the PTH-responsive region of the *Mgp* promoter. A: EMSA showing binding to probes from the distal portion of the *Mgp* promoter. B: Binding to mutant versions of Probe 1. C: Antibody supershift experiment showing that addition of anti-Runx2 antibody impairs formation of the mobility-shifted DNA-protein complex. See Figure 2 and Table I for details of these probes.

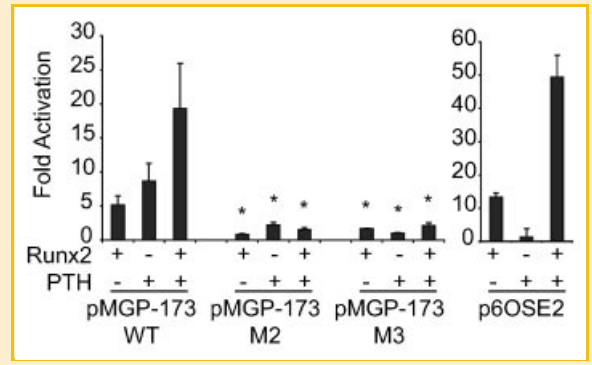

Fig. 5. Activation of the pMGP reporters by Runx2 and PTH. MC14 cells were transfected with wild-type (WT) and Runx2 site mutant reporters (Table I) together with Runx2 as indicated, and treated with vehicle or PTH. In parallel, cells were transfected with the p6OSE2-luc reporter as a positive control for Runx2 transcriptional activation. Luciferase activity is plotted as fold activation relative to each reporter alone. $^{*}P \leq 0.02$ versus wild-type reporter, same treatment.

reduced compared to the wild-type −173 bp reporter (Fig. 6). The double mutant reporter was completely unresponsive to stimulation by PTH. This observation suggests that the combined activities at both the Sp and Runx2 sites are necessary for most or all of the regulation of MGP expression by PTH.

DISCUSSION

It has been reported that MGP is an important contributor to the ability of PTH to inhibit extracellular matrix mineralization in MC3T3-E1 osteoblast-like cells [Gopalakrishnan et al., 2001]. Previously, we showed that PTH treatment induces MGP mRNA and that the proximal 748 bp of the murine *Mgp* promoter are sufficient for transcriptional activation by PTH [Suttamanatwong et al., 2007]. We also showed that PTH induction of MGP expression involves PKA and ERK/MAPK signaling pathways [Suttamanatwong et al., 2007]. The goal of this study was to further characterize the mechanism by which PTH regulates MGP expression. To this end, we made use of 5' deletion analysis to show that the PTH-responsive region lies between −173 and −49 bp relative to the *Mgp* transcriptional start site. Sequence analysis of this region revealed a number of potential transcription factor binding sites. We verified protein binding to the putative Sp and Runx2 binding sites, demonstrated that Sp1 and Runx2 activate the *Mgp* reporters and showed that mutation of the Sp and Runx2 binding sites impaired *Mgp* activation.

Our antibody supershift EMSA studies indicate that Sp1 and Sp3 both bind to the *Mgp* promoter. The Sp (Specificity Protein) family of transcription factors, which consists of Sp1–Sp8 and the Krüppel-like factors KLF1 to KLF16 [Suske, 1999; Ganss and Jheon, 2004], is characterized by three conserved Cys_2His_2 zinc fingers that form the DNA-binding domain. Sp1 and Sp3 are ubiquitously expressed in

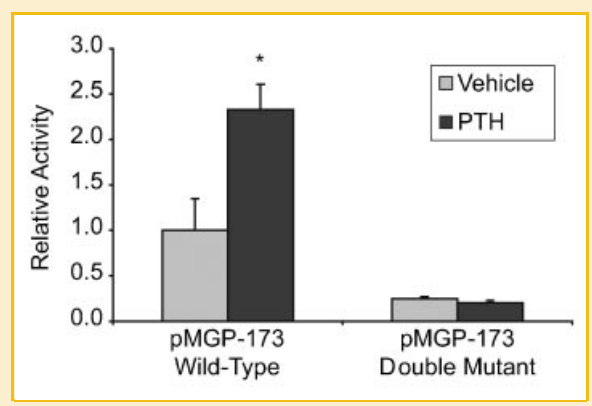

Fig. 6. Mutation of both Sp and Runx2 sites abolishes PTH-responsiveness. MC14 cells were transfected with the wild-type or Sp site/Runx2 site double mutant reporter and treated with vehicle or PTH for 6 h. Luciferase activities are graphed relative to the vehicle treated wild-type reporter. $^{*}P \leq 0.05$.

mammalian cells and Sp-binding sites are present on the promoters of genes associated with almost all cellular processes [Suske, 1999; Li et al., 2004]. Despite their ubiquity, numerous reports have indicated the importance of Sp1 and Sp3 as modulators of inducible and tissue-specific gene expression [Suske, 1999; Feng et al., 2000; Ghayor et al., 2001; Chadjichristos et al., 2002; Stains et al., 2003; Li et al., 2004], and specific mineralized tissue defects have been noted in mice deficient in Sp proteins. The function of Sp1 in bone has not been examined as *Sp1*$^{-/-}$ mice die around day 11 of gestation [Marin et al., 1997]. *Sp3*$^{-/-}$ mice develop in utero without any gross abnormalities but die within 10 min of birth due to respiratory failure [Bouwman et al., 2000]. Detailed analysis of the *Sp3*$^{-/-}$ mice revealed specific defects in tooth and bone formation. In a separate study, several skeletal malformations including mineralization defects in the vertebral bodies, sternum, skull and the craniofacial skeleton were noted in *Sp3*$^{-/-}$ mice [Gollner et al., 2001]. The authors also noted that lack of Sp3 resulted in reduced OCN mRNA levels, while Runx2 levels were not affected. It is important to note that Sp7 (osterix), which is expressed in skeletal tissues and is important for osteoblast differentiation, is the only tissue-specific Sp protein known [Nakashima et al., 2002]. Complete absence of mineralization of bones formed by intramembranous ossification and a general deficiency of osteoblast differentiation were noted in Sp7-deficient mice [Nakashima et al., 2002].

We found that Sp1 overexpression enhanced *Mgp* reporter activity, whereas the effects of Sp3 overexpression were more complex, with low doses of Sp3 being activating but higher doses inhibiting transcription. Due to the extensive similarity in their zinc finger DNA-binding domains, Sp1 and Sp3 recognize and bind with similar affinity to similar GC-rich Sp-binding sites, hence numerous reports have shown that Sp3 can antagonize Sp1-mediated transcriptional activation by competing for the same binding sites [Feng et al., 2000; Ghayor et al., 2001; Chadjichristos et al., 2002; Stains et al., 2003]. Similarly, the requirement for an optimal Sp1/Sp3 ratio in transcriptional regulation has been noted in osteoblast-related genes such as OCN and COL2A1. Stains and coworkers showed that a Sp1/Sp3 binding element at −70 to −57 in the rat OCN promoter is necessary and sufficient to confer gap junction sensitivity [Stains et al., 2003]. The authors showed a repression of OCN transcription as a result of displacement of the stimulatory Sp1 by the inhibitory effect of Sp3 on the promoter when gap junctional communication is perturbed. Thus, alterations in the relative expression levels or DNA binding activities of Sp1 and Sp3 represent potential mechanisms for influencing their activities by PTH and other signals. The transcriptional activities of Sp1 and Sp3 are subject to regulation by modifications such as phosphorylation, glycosylation, and acetylation. Phosphorylated Sp1 and Sp3 have been reported to up- or down-regulate gene transcription. Sp1 can be phosphorylated by numerous kinases including PKA [Rohlff et al., 1997; Zheng et al., 2000], PKC [Pal et al., 1998; Zheng et al., 2000], and ERK/MAPK [Pal et al., 1998; Merchant et al., 1999], the three pathways that are the main PTH responsive pathways. In addition, Sp1 can also be phosphorylated by DNA-dependent protein kinase [Jackson et al., 1990] and casein kinase II [Armstrong et al., 1997] but the sites targeted by these kinases in vivo are still unknown. Finally, the activities of Sp1 and Sp3 can be influenced by interactions with other transcription factors [reviewed by Bouwman and Philipsen, 2002]. The precise mechanism(s) by which PTH regulates Sp1 and Sp3 activities with respect to the *Mgp* promoter remain an area for future investigation.

In addition to Sp proteins, our data indicate that Runx2 participates in regulation of MGP expression. Previous evidence concerning a role of Runx2 in regulation of MGP expression in bone is limited. MGP was significantly upregulated in osteoblastic cells overexpressing Runx2 [Stock et al., 2004], suggesting a potential positive role for Runx2. However, MGP was highly expressed in chondrocytes of Runx2 knockout animals [Komori et al., 1997], suggesting that Runx2 is not required for MGP expression in cartilage. In normal vasculature, MGP is strongly expressed in endothelial and smooth muscle cells, while Runx2 is present at very low levels [Engelse et al., 2001]. During progression of vascular calcification, MGP expression is lost from calcified regions while Runx2 expression increases in atherosclerotic lesions [Engelse et al., 2001]. These data suggest antagonistic functions for Runx2 and MGP expression in the cardiovascular system.

Runx2 has been widely studied as a downstream target of PTH signaling. Our previous studies indicate that the effects of PTH on MGP expression require PKA and MEK activities, suggesting that these kinases may modulate Runx2's ability to regulate MGP. PTH has been shown to increase Runx2 transactivation of the collagenase-3 promoter through phosphorylation of the Runx2 activation domain 3 by PKA [Selvamurugan et al., 2000]. Similarly, Runx2 activity can be enhanced through phosphorylation by MEK [Xiao et al., 2002]. Another mechanism by which PTH stimulates Runx2 activity involves cooperative interactions with Runx2 and AP-1 transcription factors [D'Alonzo et al., 2002; Selvamurugan et al., 2006]. Polymorphisms at AP-1 sites in the MGP promoter are associated with altered levels of serum MGP in human subjects [Farzaneh-Far et al., 2001]. In our studies, we did not find evidence supporting AP-1 transcription factors in mediating PTH responsiveness, as mutation of potential AP-1 sites within the PTH-responsive region did not significantly alter MGP expression (data not shown). Finally, PTH has been reported to enhance Runx2-dependent transcription by stimulating Runx2 expression [Krishnan et al., 2003]. In our EMSA studies, we did not detect a change in Runx2 binding to the MGP promoter following PTH treatment (Fig. 4A and data not shown), suggesting that the increased MGP transcription from Runx2 does not arise from overt changes in Runx2 expression or its DNA binding activity.

In summary, our data establish Sp and Runx2 transcription factors as essential mediators of PTH-stimulated MGP expression. These results provide further insight into the regulation of MGP. However, the functional significance of Sp and Runx2 regulation of MGP is not known. Determining how these transcription factors interact and regulate MGP expression is essential for understanding of how these proteins control osteoblast behavior not only during normal bone growth but also in various pathological conditions.

ACKNOWLEDGMENTS

We would like to express our gratitude to Dr. Jennifer J Westendorf and Dr. Rachel Kahler for insightful discussions and technical assistance with gel-shift analyses.

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