

## Vitamin K<sub>2</sub> Regulation of Bone Homeostasis Is Mediated by the Steroid and Xenobiotic Receptor SXR\*

Received for publication, March 26, 2003, and in revised form, July 27, 2003  
Published, JBC Papers in Press, August 14, 2003, DOI 10.1074/jbc.M303136200

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Vitamin K<sub>2</sub> is a critical nutrient required for blood clotting that also plays an important role in bone formation. Vitamin K<sub>2</sub> supplementation up-regulates the expression of bone markers, increases bone density *in vivo*, and is used clinically in the management of osteoporosis. The mechanism of vitamin K<sub>2</sub> action in bone formation was thought to involve its normal role as an essential cofactor for  $\gamma$ -carboxylation of bone matrix proteins. However, there is evidence that suggests vitamin K<sub>2</sub> also has a transcriptional regulatory function. Vitamin K<sub>2</sub> bound to and activated the orphan nuclear receptor SXR and induced expression of the SXR target gene, *CYP3A4*, identifying it as a *bona fide* SXR ligand. Vitamin K<sub>2</sub> treatment of osteosarcoma cells increased mRNA levels for the osteoblast markers bone alkaline phosphatase, osteoprotegerin, osteopontin, and matrix Gla protein. The known SXR activators rifampicin and hyperforin induced this panel of bone markers to an extent similar to vitamin K<sub>2</sub>. Vitamin K<sub>2</sub> was able to induce bone markers in primary osteocytes isolated from wild-type murine calvaria but not in cells isolated from mice deficient in the SXR ortholog *PXR*. We infer that vitamin K<sub>2</sub> is a transcriptional regulator of bone-specific genes that acts through SXR to favor the expression of osteoblastic markers. Thus, SXR has a novel role as a mediator of bone homeostasis in addition to its role as a xenobiotic sensor. An important implication of this work is that a subset of SXR activators may function as effective therapeutic agents for the management of osteoporosis.

Osteoporosis is a common disease affecting the elderly, particularly postmenopausal women, although a significant minority of older men is also affected. It is defined as the gradual reduction in bone strength with advancing age that is manifested by such observations as bone fracture following minimal trauma (1–3). Several types of agents are used clinically in the United States to prevent or treat osteoporosis. These include estrogen/progestin replacement therapy, calcitonin, bisphos-

phonate, and selective estrogen receptor modulators (4). Vitamin K<sub>2</sub> was first reported to promote fracture healing in 1960 (5), and several studies showed that vitamin K<sub>2</sub> is closely associated with increased bone formation (6, 7) and decreased bone resorption (8–13). Low levels of dietary vitamin K are associated with increased risk of fractures (14–16). Accordingly vitamin K<sub>2</sub> is used clinically in Japan to treat osteoporosis either alone or in conjunction with 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (17–20).

Vitamin K<sub>2</sub> is a family of naphthoquinones, the most biologically important of which is menatetrenone (for a review, see Ref. 21). Vitamin K<sub>2</sub> was discovered as a critical nutrient required for blood clotting. It acts as a cofactor for the microsomal  $\gamma$ -carboxylase that facilitates the post-translational conversion of glutamic acid to  $\gamma$ -carboxyglutamyl (Gla) residues (for a review, see Ref. 14). Post-translational conversion of 9–12 Glu to Gla residues is required for the function of proteins such as prothrombin and Factors VII, IX, and X in the blood clotting cascade (for reviews, see Refs. 21 and 22). In addition, Gla-containing proteins such as osteocalcin and matrix Gla protein are abundant in bone tissues where they are thought to play important roles in regulating mineralization (for reviews, see Refs. 23 and 24).

Recent studies have demonstrated that the orphan nuclear receptor SXR<sup>1</sup> (25) (also known as PXR (26), PAR (27), and NR1I2) plays a central role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as *CYP3A4* and *MDR1* (28–32). SXR is activated by a diverse array of pharmaceutical agents including Taxol, rifampicin, SR12813, clotrimazole, phenobarbital, hyperforin (33–35), the herbal antidepressant St. John's wort (36), and peptide mimetic human immunodeficiency virus protease inhibitors such as ritonavir (28). These studies indicate that SXR functions as a xenobiotic sensor to coordinately regulate drug clearance in the liver and intestine. Indeed gene knockout studies have confirmed a role for SXR in regulating the metabolism of en-

<sup>1</sup> The abbreviations used are: SXR, steroid and xenobiotic receptor; PXR, pregnane X receptor; CYP, cytochrome P-450; RT-PCR, reverse transcriptase PCR; QRT-PCR, quantitative real time RT-PCR; RIF, rifampicin; ALP, alkaline phosphatase; OPN, osteopontin; MGP, matrix Gla protein; OPG, osteoprotegerin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SRC-1, steroid receptor coactivator-1; ACTR, activator for thyroid hormone and retinoid receptors; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; F, forward; R, reverse; PCN, pregnenolone 16 $\alpha$ -carbonitrile; BPB, peroxisome proliferator-activated receptor (PPAR)-binding protein; GRIP, glucocorticoid receptor-interacting protein; SMRT, silencing mediator of retinoid and thyroid receptors; NCoR, nuclear receptor corepressor; CHX, cycloheximide; WT, wild-type.

\* This work was supported by National Cancer Institute Grant CA-87222, National Institutes of Health Grant GM-60572, and a gift from Eisai Co., Ltd. Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ogenous steroids and dietary and xenobiotic compounds (29, 32).

During our original screening experiments that led to the discovery of SXR as a steroid and xenobiotic sensor, we noted that vitamin K<sub>2</sub> could also activate SXR.<sup>2</sup> This observation led us to consider the possibility that vitamin K<sub>2</sub> might act as a *bona fide* SXR ligand to mediate biological processes other than xenobiotic metabolism and clearance. Since vitamin K<sub>2</sub> was previously suspected to have a transcriptional regulatory function in addition to its role as an enzyme cofactor (37), we hypothesized that SXR might be the mediator of this activity. In this report, we demonstrate that vitamin K<sub>2</sub> transcriptionally activates SXR in a dose-dependent manner and binds directly to SXR *in vitro* and *in vivo*. SXR mRNA is expressed in osteosarcoma cell lines, and vitamin K<sub>2</sub> induced the expression of the prototypical SXR target gene *CYP3A4* in these cells. Vitamin K<sub>2</sub> up-regulates the steady state mRNA levels for a panel of osteoblastic bone markers in the osteosarcoma cell lines HOS, MG-63, and Saos-2, demonstrating a mechanistic connection between vitamin K<sub>2</sub> and bone development. The known SXR activators rifampicin and hyperforin induce the same panel of bone markers as does vitamin K<sub>2</sub>, further confirming a role for SXR in the regulation of these genes. Finally we found that vitamin K<sub>2</sub> was able to induce bone markers in primary osteocytes isolated from wild-type murine calvaria but not in cells isolated from *PXR* knockout mice. From these data, we conclude that vitamin K<sub>2</sub> modulates the expression of osteoblastic bone markers through SXR and infer that vitamin K<sub>2</sub> activation of SXR could be an important factor favoring the deposition of bone over its resorption. Therefore, SXR is likely to be involved in the maintenance of bone homeostasis in addition to its known role in hormonal homeostasis. This reveals a novel biological function for SXR and suggests that a subset of SXR activators may function as effective therapeutic agents for the management of osteoporosis.

#### EXPERIMENTAL PROCEDURES

**SXR Detection by RT-PCR**—HOS, MG-63, Saos-2, LS180, and HeLa cells were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was isolated using TriZol reagent (Invitrogen). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (Invitrogen). SXR was detected with the following primer set: forward primer, 5'-CAAGCGGAAGAAAGT-GAACG-3'; reverse primer, 5'-CTGGTCTCGATGGGCAAGT-3'. PCR was carried out at 37 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

**Cell Culture and Transfection**—COS-7 cells were cultured and transfected as described previously (38). Typically COS-7 cells were cultured in phenol red-free DMEM supplemented with 10% FBS. For transient transfection experiments, COS-7 cells were seeded into 96-well plates at a density of 5000 cells/well. The next day cells were transfected with either CMX-GAL-SXR or CMX-GAL4 (control) together with tk(MH100)<sub>4</sub>-luc reporter (39) and CMX-β-galactosidase transfection control plasmids using standard calcium phosphate precipitation methodology. 22–24 h after transfection, the cells were washed twice with phosphate-buffered saline supplemented with 1 mM MgCl<sub>2</sub> or DMEM-ITLB (DMEM containing 5 μg/ml insulin, 5 μg/ml holotransferrin, 5 μg/ml selenium, 0.5% defined lipid mix (Invitrogen), 0.12% (w/v) delipidated bovine serum albumin (Sigma)) (40). Ligands were typically purchased from Sigma and BIOMOL Research Laboratories Inc., made freshly from powder in Me<sub>2</sub>SO as 0.1 M stocks, diluted in Me<sub>2</sub>SO to appropriate concentrations, and added to media with vigorous vortex mixing. Ligands were added in DMEM-ITLB, and the cells were incubated for an additional 24–48 h. The cells were lysed *in situ*, and extracts were prepared and assayed for β-galactosidase and luciferase activity as described previously (41). Reporter gene activity was normalized to the β-galactosidase transfection controls, and the results were expressed as normalized relative luciferase units per OD β-galac-

tosidase per minute to facilitate comparisons between plates. Fold induction was calculated relative to solvent controls. Each data point represents the average of triplicates ± S.E. The experiments were repeated three times with similar results.

For coactivator recruitment experiments, GAL4-coactivator plasmids were generated by cloning the receptor interaction domains of human *TIF2* (GenBank™ accession number NM\_006540, amino acids 563–790), human *SRC-1* (GenBank™ accession number U59302, amino acids 600–800), or human *ACTR* (GenBank™ accession number AF036892, amino acids 600–788) into pCMX-GAL4. The GAL4-PBP construct was described previously (30). To construct herpesvirus VP16 activation domain fusion proteins, full-length SXR was PCR-amplified and ligated in-frame into pCDG-VP16 vector (25). All constructs were sequenced to verify that no errors were introduced during the PCR.

**Ligand Binding Assays**—N-terminal His<sub>6</sub>-tagged human SXR ligand binding domain was expressed in *Escherichia coli* together with the SRC-1 receptor interaction domain essentially as described previously (28). Active protein was refolded from inclusion bodies solubilized in denaturation buffer (6 M guanidinium-HCl, 50 mM HEPES, pH 7.4, 0.2 M NaCl, 25 mM dithiothreitol, 1% (w/v) Triton X-100) by rapid 10-fold dilution into binding buffer (50 mM HEPES, pH 7.4, 1 M sucrose, 0.2 M NaCl, 0.1 mM dithiothreitol, 0.1% (w/v) CHAPS) followed by dialysis overnight at 4 °C against binding buffer. Binding assays were performed by coating 96-well nickel chelate FlashPlates (PerkinElmer Life Sciences) with a 10-fold molar excess of protein for 1 h at 22 °C in binding buffer (50 mM HEPES, pH 7.4, 200 mM NaCl, 1 M sucrose, 0.1% CHAPS). Unbound protein was removed from the wells by washing four times with binding buffer. [<sup>3</sup>H]SR12813 (33) (Amersham Biosciences) was added to a final concentration of 50 nM in each well either alone or together with competitor ligands in binding buffer as indicated. Incubation was continued for 3 h at room temperature. Total counts were measured using a Topcount scintillation counter (Packard Instrument Co.). Counts remaining after the addition of 10 μM clotrimazole were taken as nonspecific background and subtracted from all wells (33). All assays were performed in triplicate and reproduced in independent experiments.

**Alkaline Phosphatase (ALP) Activity Assay**—ALP activity was measured as described previously (42). Briefly, cells were harvested by washing twice with phosphate-buffered saline, then collected with a cell scraper, and transferred to 1.5-ml microcentrifuge tubes. Cell pellets were obtained by centrifugation at 14,000 rpm at 4 °C, and lysates were prepared with a solution containing 0.2% (v/v) Nonidet P-40 and 1 mM MgCl<sub>2</sub>. Aliquots of lysate were combined with reaction buffer (1 M diethanolamine, pH 9.8, 1 mM MgCl<sub>2</sub>, and 10 mM *p*-nitrophenyl phosphate) and incubated at 37 °C for 30 min. Absorbance at 405 nm was measured using a Spectra MAX Plus spectrophotometer (Amersham Biosciences), and the enzyme activity was calculated as described previously (6). ALP activity was corrected for protein content, which was determined using the Bio-Rad protein assay kit.

**Quantitative Real Time RT-PCR Analysis of Bone Biomarker Genes in Osteosarcoma Cell Lines**—Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were obtained from American Type Culture Collection (Manassas, VA) and cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Cells were treated with vitamin K<sub>2</sub>, 1α,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>, rifampicin, or solvent controls for 48 h. Total RNA was isolated and reverse transcribed as described above. Quantitative real time RT-PCR (QRT-PCR) was performed using the following primer sets: ALP (F, 5'-CATGGCTTTGGGCAGAAGGA-3'; R, 5'-CTAGCCCCAAAAGAGTTGCAA-3'), osteopontin (OPN) (F, 5'-CAGACGAGGACATCACCTCA-3'; R, 5'-TGGCTGTGGGTTTCAGCA-3'), matrix Gla protein (MGP) (F, 5'-ATCGCTACTTCAGGAAGCGCC-3'; R, 5'-TGACTCTCTTTGACCCTGACCCTCAC-3'), osteoprotegerin (OPG) (F, 5'-CCTCTCATCAGCTGTTGTGTG-3'; R, 5'-TATCTCAAGGTAGC-GCCCTTC-3'), glyceraldehyde-3-phosphate dehydrogenase (F, 5'-TGG-ACCTCATGGCCCCACA-3'; R, 5'-TCAAGGGTCTACATGGCAA-3'), *CYP3A4* (F, 5'-GGCTTCATCCAATGGACTGCATAAAT-3'; R, 5'-TCC-CAAGTATAACACTACACAGACAA-3'), and the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection system (MJ Research). All samples were quantitated by the comparative cycle threshold (Ct) method for relative quantitation of gene expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (43).

**Isolation of Calvaria, Culture of Primary Bone Cells, and QRT-PCR**—Calvaria were isolated from newborn wild-type and *PXR* knockout mice (postnatal day 1–5) and were digested sequentially with 0.1% collagenase, 0.05% trypsin, 4 mM EDTA in 1× phosphate-buffered saline essentially as described in Refs. 44 and 45. Bone cells released upon digestion were cultured in phenol-red free DMEM, 10% FBS and

<sup>2</sup> B. Blumberg, unpublished observations.

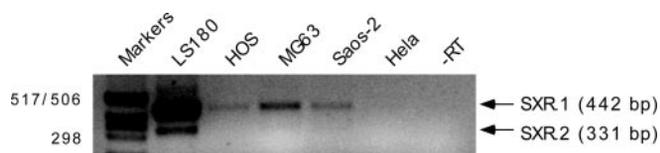


FIG. 1. **SXR is expressed in osteosarcoma cell lines.** The human cell lines HOS, MG-63, Saos-2, LS180, and HeLa were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was isolated using TriZol reagent. 1  $\mu$ g of total RNA from each cell type was reverse transcribed using Superscript II reverse transcriptase and subjected to RT-PCR analysis. RT-PCR products were resolved on a 1% agarose gel. The *hSXR.1* product is 442 bp. The *hSXR.2* product is 331 bp.

treated for 48 h with 10  $\mu$ M vitamin K<sub>2</sub> or pregnenolone 16 $\alpha$ -carbonitrile (PCN). Total RNA was isolated, reverse transcribed, and analyzed using the following mouse primer sets: MGP (F, 5'-TCTCAGAAAGC-ATGGAGTC-3'; R, 5'-ATCTCGTAGGCAGGCTTGT-3'), OPG (F, 5'-CTGCTGAAGCTGTGGAACA-3'; R, 5'-AAGCTGCTCTGTGGTGTGAGT-3'), and glyceraldehyde-3-phosphate dehydrogenase (F, 5'-AACTT-TGGCATTGTGGAAGG-3'; R, 5'-GGATGCAGGGATGATGTTCT-3').

**Statistical Analysis**—Differences between two groups were analyzed using two-sample, two-tailed Student's *t* test. A *p* value less than 0.05 was considered to be significant. All data are presented in the text and figures as the mean  $\pm$  S.E.

## RESULTS

**SXR Is Expressed in Osteosarcoma Cell Lines**—SXR functions as a xenobiotic sensor and is expressed at high levels in the liver and intestine where it modulates the levels of CYP enzymes and ATP-binding cassette family transporters (31, 46). SXR is expressed at lower levels in normal and neoplastic breast tissues (47) and breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and MDA-MB-435) (47).<sup>3</sup> It is not clear at present what role SXR is playing in other tissues. We were intrigued by the ability of vitamin K<sub>2</sub> to activate SXR in preliminary experiments. To ascertain whether SXR might be mediating the effects of vitamin K<sub>2</sub>, we first determined whether SXR was expressed in a panel of osteosarcoma cell lines using RT-PCR. SXR expression was observed in the LS180 human colon adenocarcinoma cells and in the osteosarcoma cell lines HOS, MG-63, and Saos-2. SXR mRNA was not detected in HeLa cells or in negative controls (Fig. 1). It has been previously reported that SXR is expressed in LS180 cells (28, 30), whereas it is not expressed in HeLa cells (48). SXR is expressed at highest levels in LS180 cells with lower levels in the osteosarcoma cell lines (Fig. 1).

**Vitamin K<sub>2</sub> Activates SXR**—SXR is activated by a diverse array of pharmaceutical agents including Taxol, rifampicin, SR12813, clotrimazole, phenobarbital, and hyperforin. As noted above, our early screening efforts aimed at identifying SXR ligands also demonstrated SXR activation by vitamin K<sub>2</sub>. Accordingly we tested the ability of vitamin K<sub>2</sub> to activate SXR in dose-response experiments. As shown in Fig. 2A, vitamin K<sub>2</sub> activates CMX-GAL-SXR robustly with the highest levels of activation approximately equivalent to 1  $\mu$ M rifampicin (RIF). In contrast, no activation was observed using CMX-GAL4 alone, demonstrating that the activation results from a specific interaction with the SXR ligand binding domain.

Next we tested the ability of vitamin K<sub>2</sub> to induce the SXR target gene *CYP3A4* in cultured osteosarcoma cells. It has been reported previously that *CYP3A4* expression is induced by RIF and vitamin D<sub>3</sub> in cultured HepG2 and LS180 cells (49, 50), although the 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> induction of *CYP3A4* is mediated by the vitamin D<sub>3</sub> receptor rather than by SXR (51). RIF induced the expression of *CYP3A4* in all three lines (Fig. 2B). Vitamin K<sub>2</sub> was able to induce *CYP3A4* expression at both

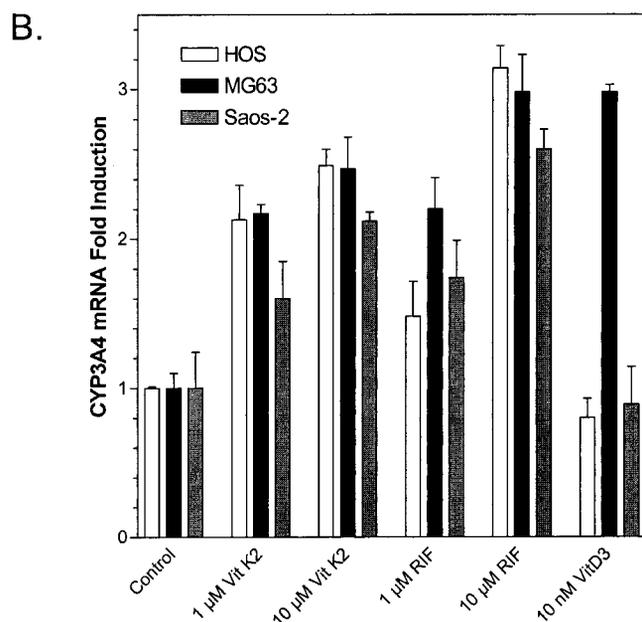
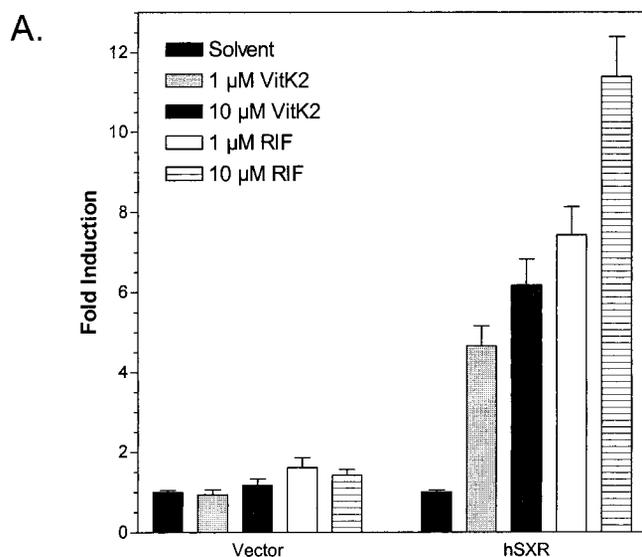
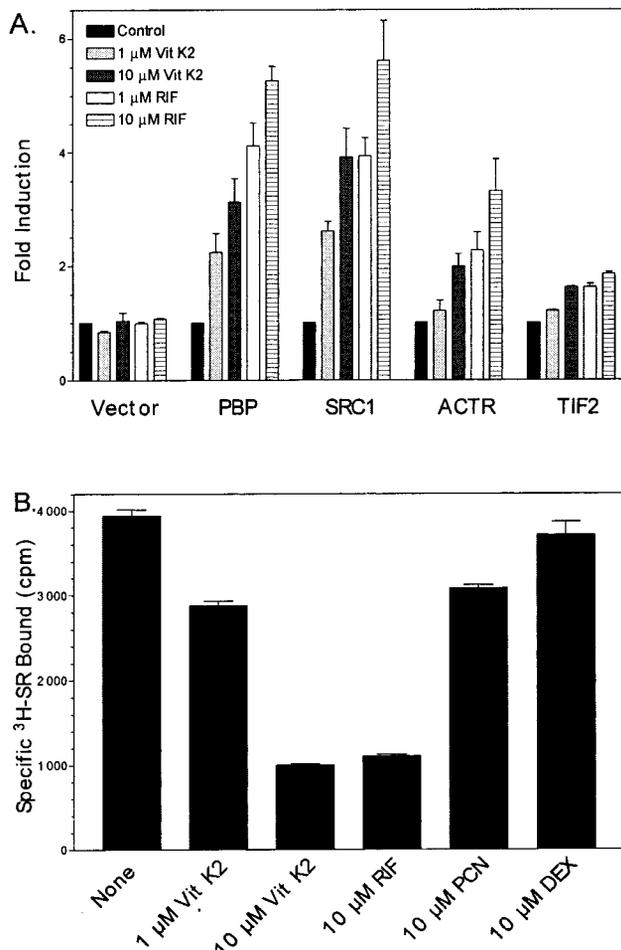


FIG. 2. **Vitamin K<sub>2</sub> activates SXR and induces SXR target genes.** A, activation of SXR by RIF and vitamin K<sub>2</sub>. COS-7 cells were transiently transfected with either GAL-SXR or CMX-GAL4 (control) together with tk(MH100)4-luc reporter and CMX- $\beta$ -galactosidase transfection control plasmids. After transfection, cells were treated with control medium or medium containing a dilution series of RIF, vitamin K<sub>2</sub>, or 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>. Only values for 1 and 10  $\mu$ M rifampicin, 1 and 10  $\mu$ M vitamin K<sub>2</sub>, and 10 nM 1 $\alpha$ ,25-(OH)<sub>2</sub> D<sub>3</sub> are shown for clarity. B, *CYP3A4* induction by RIF and vitamin K<sub>2</sub>. Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were cultured in the presence or absence of ligands for 48 h. Total RNA was isolated and analyzed by QRT-PCR with primers for human *CYP3A4*. Vit, vitamin.

1 and 10  $\mu$ M in all three lines. 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> could not induce *CYP3A4* expression in Saos-2 or HOS cells (Fig. 2B).

**Vitamin K<sub>2</sub> Specifically Binds to SXR in Vitro and in Vivo**—Since vitamin K<sub>2</sub> activates SXR in transient transfections (Fig. 2A) and induces the expression of a prototypical SXR target gene in osteosarcoma cells (Fig. 2B), we next sought to determine whether vitamin K<sub>2</sub> binds to SXR. One important measure of ligand binding is the ability of a compound to induce a nuclear receptor to interact with coactivator proteins. Accordingly we conducted coactivator recruitment experiments that utilized VP16-SXR together with fusions between the GAL4 DNA binding domain and the receptor-interacting domains of

<sup>3</sup> M. M. Tabb and B. Blumberg, unpublished data.



**FIG. 3. Vitamin K<sub>2</sub> specifically binds to SXR *in vitro* and *in vivo*.** A, vitamin K<sub>2</sub> induces SXR to recruit coactivators. COS-7 cells were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS and transiently transfected with a GAL reporter together with expression vectors for the GAL4 DNA binding domain (Vector) or the GAL4 DNA binding domain linked to the receptor interaction domains of the indicated nuclear receptor coactivators. Cells were treated with 1 and 10  $\mu$ M RIF or vitamin K<sub>2</sub>. Values represent the average of triplicates  $\pm$  S.E. Experiments were repeated twice with similar results. B, vitamin K<sub>2</sub> specifically binds to the purified SXR ligand binding domain. His<sub>6</sub>-SXR ligand binding domain was coexpressed with the SRC-1 receptor interaction domain and purified. The receptor complex was bound to nickel chelate FlashPlates and incubated with 50 nM [<sup>3</sup>H]SR12813 (<sup>3</sup>H-SR) in the presence of the indicated compounds or solvent control. Values represent the average of triplicates  $\pm$  S.E. and were replicated in independent experiments. *Vit*, vitamin; *DEX*, dexamethasone.

the nuclear hormone receptor coactivators *SRC-1*, *TIF2*, *ACTR*, and *PBP* (30). As shown in Fig. 3A, VP16-SXR was able to interact with PBP, SRC-1, and ACTR in the presence of vitamin K<sub>2</sub> or the known SXR ligand RIF. The results from the coactivator recruitment experiments paralleled those of the activation assays. As is the case for other SXR ligands (30), vitamin K<sub>2</sub> and RIF preferentially induced interaction between SXR and PBP or SRC-1 while producing weaker but detectable interactions between SXR and TIF2 or ACTR (Fig. 3A). We infer that vitamin K<sub>2</sub> promotes association between SXR and nuclear receptor coactivators SRC-1 and PBP as would be expected from an authentic SXR ligand.

We next tested whether vitamin K<sub>2</sub> binds to purified SXR protein *in vitro* using a sensitive scintillation proximity ligand binding assay similar to that used by other investigators (28, 33). This assay used [<sup>3</sup>H]SR12813 and recombinant His<sub>6</sub>-tagged SXR coexpressed with the SRC-1 receptor-interacting

domain (28) and nickel chelate FlashPlates (PerkinElmer Life Sciences). SR12813 interacts specifically with SXR with a dissociation constant of 40 nM (33). As seen in Fig. 3B, vitamin K<sub>2</sub> and RIF are able to displace [<sup>3</sup>H]SR12813 from the SXR ligand binding domain, whereas the control compounds PCN and dexamethasone did not compete effectively for receptor binding. The *K<sub>i</sub>* for vitamin K<sub>2</sub> binding to SXR was determined to be 5.1  $\mu$ M, a value in the range of other known SXR ligands (28, 34). We infer from these results that vitamin K<sub>2</sub> specifically binds to SXR *in vitro* and *in vivo* and conclude that it acts as a *bona fide* ligand for this receptor.

**Effects of Vitamin K<sub>2</sub>, Rifampicin, and Hyperforin on Bone Biomarker Genes in Osteosarcoma Cell Lines**—Vitamin K<sub>2</sub> is used as a therapeutic agent to treat osteoporosis in Japan and is thought to act by stimulating the deposition of bone (6, 7) and decreasing bone resorption (8–13), although the mechanism of action remained unclear before our experiments. We tested the effect of vitamin K<sub>2</sub> and 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>, two known therapeutic agents, on the expression of a panel of osteoblast marker genes in the human osteosarcoma cell lines HOS, MG-63, and Saos-2 and compared these with the effects of the SXR activators RIF and hyperforin in the same cells. We chose 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> as a positive control compound in this study because it is also used therapeutically to treat osteoporosis and is known to transcriptionally regulate a range of biological processes, including bone growth, bone remodeling, and the expression of osteoblastic markers such as ALP (52–54), osteocalcin (55), and OPN (56, 57).

ALP is a widely distributed glycosylated membrane-bound ectoenzyme. Bone ALP is located on the surface of osteoblasts and is thought to play a major role in bone formation and mineralization (58, 59). ALP levels are considered to reflect osteoblastic activity and can therefore be used as a biochemical marker for assessing metabolic bone disease, including bone metastasis (60). As seen in Fig. 4A, 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> treatment increased ALP activity 1.7-fold in HOS and Saos-2 cells and 4.0-fold in MG-63 cells. Vitamin K<sub>2</sub> treatment led to a dose-dependent 1.4–2.0-fold increase in ALP levels in these cells, and RIF completely paralleled this effect (Fig. 4A). As will be seen with other markers below, there are notable differences in the response of particular cell lines to treatment with vitamin K<sub>2</sub> or 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>. For example, 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> is particularly effective at inducing ALP enzyme activity in MG-63 cells (Fig. 4A). The effect of vitamin K<sub>2</sub> treatment on ALP enzyme in HOS cells is in agreement with other reports (6).

Consistent with the effects on ALP enzyme, QRT-PCR analysis showed that ALP mRNA expression levels were significantly increased by vitamin K<sub>2</sub> and 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> in a dose-dependent manner in all three cell lines with 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> being more potent (Fig. 4B). Statistically significant changes in ALP enzyme and mRNA levels were seen at vitamin K<sub>2</sub> concentrations of as little as 1  $\mu$ M (Fig. 4, A and B), which is similar to therapeutic levels at which vitamin K<sub>2</sub> is used clinically. RIF and hyperforin produced effects very similar to vitamin K<sub>2</sub> (Fig. 4B). The concordance between ALP protein levels (Fig. 4A) and ALP mRNA levels (Fig. 4B) suggests that QRT-PCR analysis of marker gene expression will be predictive of osteoblastic activity in cultured osteosarcoma cell lines.

OPN is one of the major non-collagenous bone matrix proteins produced by osteoblasts (61, 62). It is an early marker of osteoblast differentiation and a prominent component of the mineralized bone matrix that has been implicated in tissue mineralization and in the attachment of osteoclasts to the bone matrix. Vitamin K<sub>2</sub> elicited a dose-dependent increase in OPN

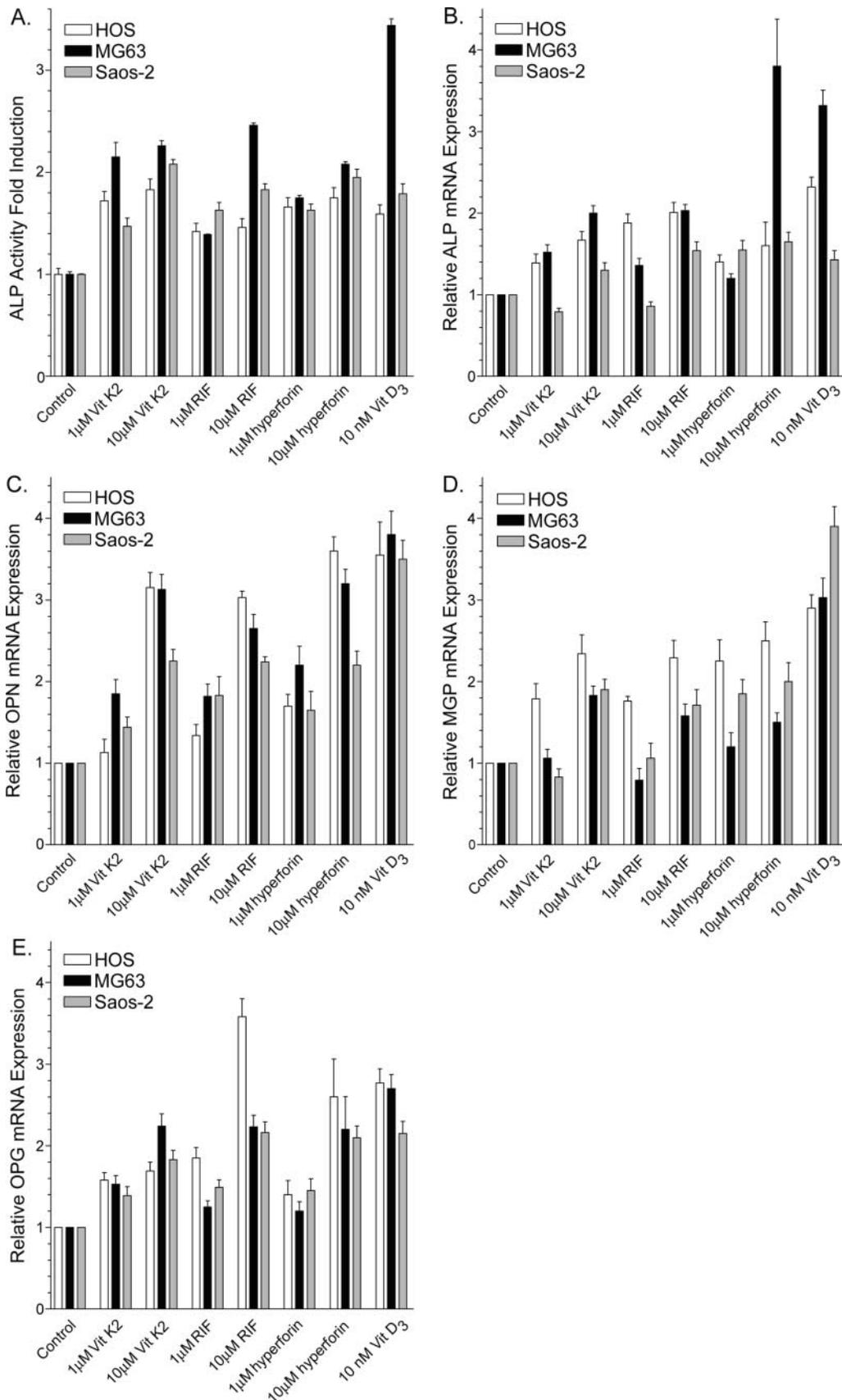


FIG. 4. **Effects of vitamin K<sub>2</sub> and rifampicin on osteoblastic marker genes in osteosarcoma cell lines.** Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS in the presence or absence of ligands for 48 h. ALP activity and bone marker gene expression were determined by ALP activity assay and quantitative real time RT-PCR. Data from quantitative real time RT-PCR are shown as the mRNA expression levels of marker genes normalized to glyceraldehyde-3-phosphate dehydrogenase. *A*, ALP activity assay. *B*, ALP mRNA expression. *C*, OPN mRNA expression. *D*, MGP mRNA expression. *E*, OPG mRNA expression. Data from HOS (white bars), MG-63 (black bars), and Saos-2 cells (gray bars) is shown. Values represent the average of triplicates ± S.E. The experiments were repeated twice with similar results. *Vit*, vitamin.

expression with the 10  $\mu\text{M}$  dose approaching the response observed with 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (Fig. 4C). RIF and hyperforin again elicited nearly identical results to those of vitamin K<sub>2</sub> treatment (Fig. 4C).

MGP is a vitamin K-dependent extracellular matrix protein with a wide tissue distribution that is particularly abundant in bone and cartilage. MGP is thought to play a key role in the inhibition of tissue calcification (63, 64). 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> treatment was previously reported to increase both the level of MGP mRNA expression and the rate of MGP secretion into culture medium in osteosarcoma cells (65). We found that MGP mRNA levels were up-regulated by vitamin K<sub>2</sub>, RIF, hyperforin, and 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> treatment (Fig. 4D). Unlike the other markers studied, MGP mRNA levels were only stimulated by the highest doses of vitamin K<sub>2</sub> and RIF used (10  $\mu\text{M}$ ) in MG-63 and Saos-2 cells.

OPG (also known as osteoclastogenesis-inhibitory factor) is a member of the tumor necrosis factor receptor family (66, 67). It is thought to function as a soluble decoy receptor activator of nuclear factor- $\kappa$ B ligand (RANKL). Recombinant OPG has been shown to block osteoclastogenesis *in vitro* and to increase bone density *in vivo* (68). 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (10<sup>-7</sup> M) increased OPG mRNA levels by 90 and 50% in a fetal osteoblastic cell line (hFOB) and normal trabecular osteoblastic cells (hOB), respectively (69). OPG mRNA levels were up-regulated by both low and high doses of vitamin K<sub>2</sub> as well as by 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> and hyperforin (Fig. 4E). Rifampicin was an effective inducer of OPG mRNA in all three cell lines, particularly HOS cells (Fig. 4E), confirming and extending the published results. In summary, three structurally different human SXR activators, RIF, hyperforin, and vitamin K<sub>2</sub>, induce osteoblast bone markers in three different osteosarcoma cell lines supporting a role for SXR in regulation of some osteoblastic bone markers.

We next asked whether the effects of vitamin K<sub>2</sub> or RIF on the expression of the bone markers above were direct transcriptional effects. We first tested induction of the known SXR target gene *CYP3A4* with 10  $\mu\text{M}$  rifampicin at time points from 6 to 48 h. There was very little induction of *CYP3A4* detected before 24 h after treatment in the positive control LS180 cells or in any of the osteosarcoma cells (Fig. 5). Therefore, the osteosarcoma cell lines were treated with vitamin K<sub>2</sub>, RIF, or vitamin D<sub>3</sub> in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) for 24 h, and RNA was analyzed by QRT-PCR using primers for *OPN*, *MGP*, and *CYP3A4*. As shown in Fig. 6, the expression of both *MGP* and *CYP3A4* induced by vitamin K<sub>2</sub> and RIF was not decreased by the presence of CHX in either the MG-63 or Saos-2 cell lines. CHX has been demonstrated to increase the stability of a number of mRNAs, explaining the increased expression of *MGP* and *CYP3A4* seen in the presence of CHX. In contrast, the induction of *OPN* expression by vitamin K<sub>2</sub> and RIF was eliminated in the presence of CHX. No induction of osteoblast marker genes was seen at 6 or 12 h of treatment (data not shown), and no induction of ALP or OPG could be detected even after 24 h of treatment. These results indicate that *CYP3A4* and *MGP* are direct transcriptional targets of activated SXR but that *OPN* requires ongoing protein synthesis for induction by vitamin K<sub>2</sub> or RIF.

**Vitamin K<sub>2</sub> Induction of Bone Marker Genes Requires SXR**—The data presented above provide strong evidence that the stimulation of osteoblast bone markers by vitamin K<sub>2</sub> occurs via direct transcriptional activation of SXR. To confirm that these effects were indeed mediated by SXR, we utilized primary bone cell cultures derived from collagenase/trypsin-digested calvaria isolated from wild-type (WT) and *PXR* knockout

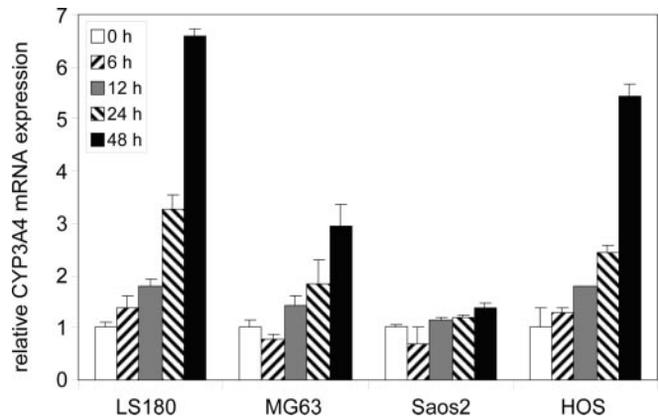


FIG. 5. Time course of rifampicin induction of *CYP3A4*. LS180 colon carcinoma cells or MG-63, Saos-2, or HOS osteosarcoma cells were treated for the indicated times with 10  $\mu\text{M}$  rifampicin in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was reverse transcribed and analyzed by QRT-PCR using primers for human *CYP3A4*. Values represent the average of triplicates  $\pm$  S.E.

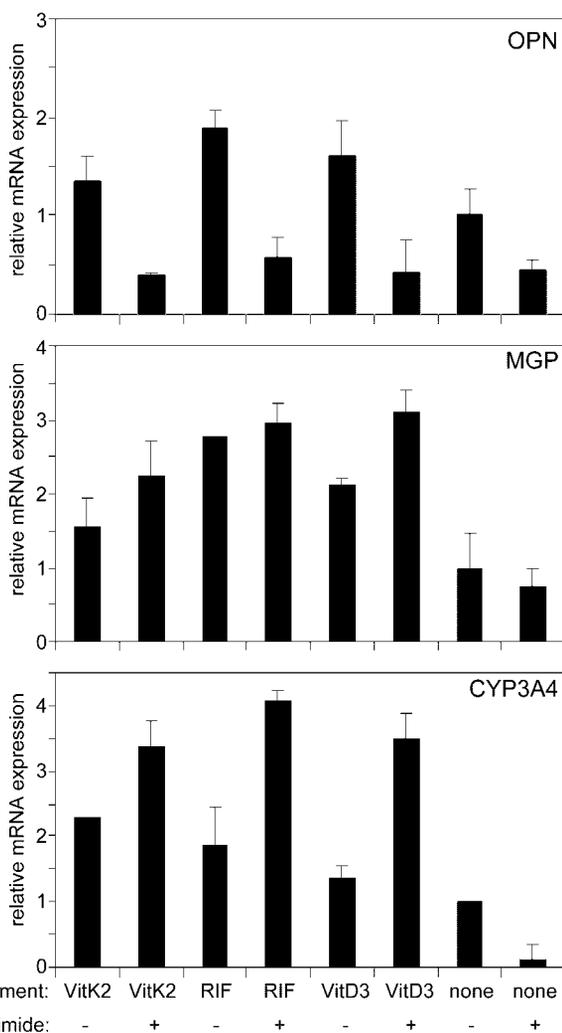
mice. Previous studies have shown that the cells isolated from calvaria by this method are primarily osteoblasts (45). *PXR* is the rodent homolog of human SXR, and the *PXR* knockout mouse has been used to demonstrate the key role SXR/*PXR* plays in the control of xenobiotic metabolism (29, 32). WT or *PXR* knockout cells were treated for 48 h with vitamin K<sub>2</sub>, the rodent *PXR* activator PCN, or vehicle only. Total RNA was then isolated and analyzed using QRT-PCR with primers specific for mouse *MGP* and *OPG*. As shown in Fig. 7, vitamin K<sub>2</sub> and PCN were able to induce *MGP* and *OPG* expression in WT cells; however, the ability to induce either *MGP* or *OPG* was lost in the *PXR* knockout cells. Therefore, vitamin K<sub>2</sub> induction of osteoblast marker genes requires the expression of *PXR*/*SXR* in bone cells.

## DISCUSSION

The regulation of bone mass in mammals is a complex process that requires a balance between the bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) (70). Vitamin K<sub>2</sub> plays several important roles in bone metabolism. Similar to its role in promoting blood clotting, vitamin K<sub>2</sub> is an essential cofactor for  $\gamma$ -carboxylase, an enzyme that catalyzes the conversion of specific glutamic acid residues to Gla residues. Vitamin K<sub>2</sub> is required for  $\gamma$ -carboxylation of bone matrix Gla-containing proteins such as *MGP* and osteocalcin. Incomplete  $\gamma$ -carboxylation of osteocalcin and *MGP* resulting from vitamin K deficiency is associated with osteoporosis and increased risk of fracture (15, 16). In addition to its function as an enzymatic cofactor, vitamin K<sub>2</sub> has a dual role in mediating bone homeostasis. It acts in an anabolic manner to stimulate the synthesis of osteoblastic markers and deposition of bone (6, 7). Vitamin K<sub>2</sub> decreases bone resorption by inhibiting the formation of osteoclasts (10) as well as their bone resorptive activity (11, 12). Vitamin K<sub>2</sub> treatment also induces apoptosis of osteoclasts (13) while inhibiting apoptosis of osteoblasts (71) thereby shifting the balance toward bone formation. Vitamin K<sub>2</sub> was also shown to enhance the induction of osteocalcin mRNA levels mediated by co-administered 1 $\alpha$ ,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (7, 37). The function of vitamin K<sub>2</sub> was previously only well understood as an enzymatic cofactor.

SXR is expressed at high levels in the liver and intestine where it acts as a xenobiotic sensor that regulates the expression of cytochrome P-450 enzymes such as *CYP3A4* and *CYP2C8* and ATP-binding cassette family transporters such as *MDR1* and *MRP2* (28, 30). SXR is thus a master regulator of xenobiotic clearance, coordinately controlling steroid and xeno-

## A. MG63



## B. Saos2

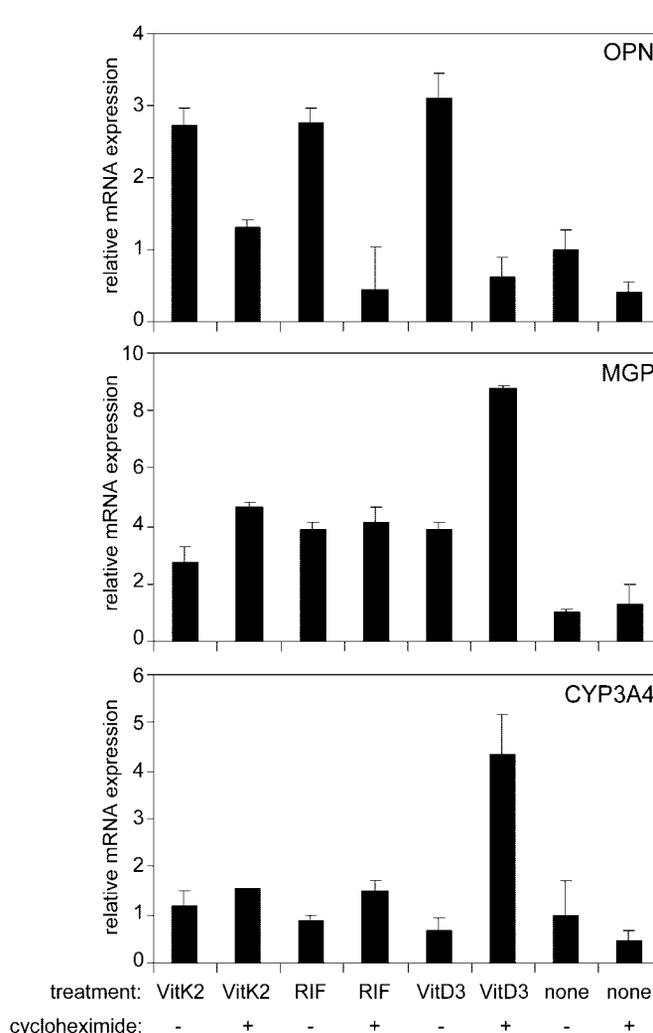


FIG. 6. **Vitamin K<sub>2</sub> has direct transcriptional effects on MGP and CYP3A4 but not OPN.** Either MG-63 (A) or Saos-2 cells (B) were pretreated with 10  $\mu$ g/ml cycloheximide for 30 min prior to addition of 10  $\mu$ M vitamin K<sub>2</sub>, 10  $\mu$ M rifampicin, 10 nM vitamin D<sub>3</sub>, or vehicle only. Total RNA was harvested 24 h later, reverse transcribed, and analyzed by QRT-PCR using primers for OPN, MGP, and CYP3A4. Values represent the average of triplicates  $\pm$  S.E. *Vit*, vitamin.

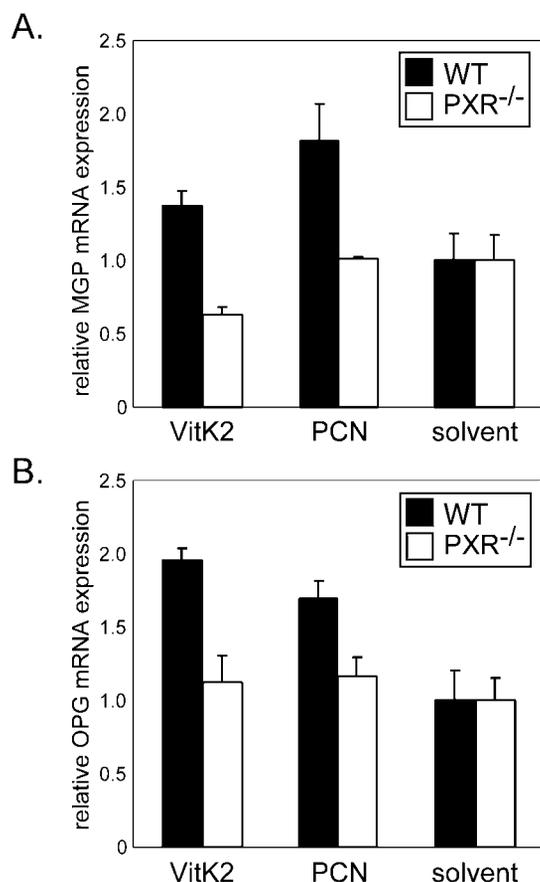
biotic metabolism (31, 46). *SXR* is also expressed at lower levels in other tissues such as normal and neoplastic breast tissue (47), although no function has yet been described for *SXR* in these tissues. There are no data available about the expression of *SXR* in normal human bone; however, its mouse ortholog *PXR* is abundantly expressed in bone as measured by *in situ* hybridization (26). Our observation that vitamin K<sub>2</sub> activated *SXR*-dependent reporter gene constructs led us to hypothesize that vitamin K<sub>2</sub> could regulate the transcription of bone markers through activation of *SXR*.

In support of this hypothesis, we demonstrated that *SXR* is expressed in three well studied osteosarcoma cell lines, HOS, MG-63, and Saos-2 (Fig. 1). These cell lines are considered to be osteoblastic in nature and are commonly used as models for osteoblast formation (6, 72, 73). Vitamin K<sub>2</sub> activated *SXR* in a dose-dependent manner (Fig. 2A) and up-regulated the known *SXR* target gene *CYP3A4* (Fig. 2B) as well as the osteoblast marker genes *ALP*, *OPN*, *MGP*, and *OPG* (Fig. 4). Vitamin K<sub>2</sub> stimulated the recruitment of coactivator proteins by *SXR* and bound directly to the *SXR* ligand binding domain *in vitro*, identifying it as an *SXR* ligand (Fig. 3). Although micromolar levels of vitamin K<sub>2</sub> are required for *SXR* binding (Fig. 3) and activation (Fig. 2), it should be noted that the therapeutic levels of vitamin K<sub>2</sub> in the plasma of patients average about 1  $\mu$ M (74),

and the levels in bone (2.5-fold) and liver (10-fold) are considerably higher (75). Therefore, the concentration of vitamin K<sub>2</sub> required to activate *SXR* is achieved in clinical use.

Cultured cells derived from sequential collagenase-digested calvaria are routinely used to study bone physiology. Utilizing primary osteocytes isolated from the calvaria of *PXR* knockout mice, we were able to demonstrate a requirement for *PXR* in the induction of *MGP* and *OPG* by vitamin K<sub>2</sub>. *MGP* and *OPG* were induced by vitamin K<sub>2</sub> in the WT cells but not in the *PXR* knockout cells. The basal levels of both *MGP* and *OPG* expression were increased in the *PXR* knockout cells (4- and 3-fold higher expression compared with WT, respectively; data not shown). A similar increase in basal expression was previously noted for *CYP3A11* in the *PXR* knockout mouse (29), further supporting a role for *SXR/PXR* in the regulation of these bone marker genes.

As described above, prior to this study a growing body of evidence existed linking vitamin K<sub>2</sub> to positive effects on the formation of bone and protection against fractures. Although its role as an enzymatic cofactor that favors the formation of Gla residues is undoubtedly critical for bone development, the anabolic effects of vitamin K<sub>2</sub> on osteoblasts, vitamin K<sub>2</sub>-mediated inhibition of osteoclast function, induction of osteoclast apoptosis, and inhibition of osteoblast apoptosis suggest a



**FIG. 7. Vitamin K<sub>2</sub> induction of bone marker genes in murine primary osteocytes requires PXR.** Osteocyte cultures derived from calvaria isolated from WT (black bars) or PXR knockout mice (white bars) were treated with 10  $\mu$ M vitamin K<sub>2</sub> or PCN or vehicle only for 48 h. Total RNA was isolated, reverse transcribed, and analyzed by QRT-PCR using primers specific for mouse MGP or OPG. Values represent the average of triplicates  $\pm$  S.E. Vit, vitamin.

much broader role for vitamin K<sub>2</sub>. Aside from its reported ability to increase the production of bone-specific ALP (6), there were previously no data that indicated a direct effect of vitamin K<sub>2</sub> on bone marker genes. Therefore, our demonstration that vitamin K<sub>2</sub> up-regulates the steady state mRNA levels for a broad panel of osteoblastic markers including ALP, MGP, OPN, and OPG (Fig. 4) provides a mechanistic basis for the anabolic effects of vitamin K<sub>2</sub> on osteoblasts and underscores the clinical utility of vitamin K<sub>2</sub> in the treatment of osteoporosis. The SXR activators RIF and hyperforin produce effects on the expression of bone markers very similar to those elicited by vitamin K<sub>2</sub> (Fig. 4). Therefore, we conclude that vitamin K<sub>2</sub> exerts positive effects on the expression of osteoblastic marker genes via transcriptional activation of SXR. The loss of vitamin K<sub>2</sub> inducibility of osteoblast markers in primary bone cell cultures from PXR knockout mice confirms that SXR/PXR plays a central role in vitamin K<sub>2</sub> induction of osteoblast marker genes. These results widen the pharmacological implications of SXR action beyond drug interactions and the xenobiotic response and may provide new insight into bone formation and the treatment of osteoporosis.

**Acknowledgments**—We thank Dr. C. Zilinski and members of the Blumberg laboratory for comments on the manuscript, Hiroshi Umesaka (Eisai Co., Ltd.) for gifts of vitamin K<sub>2</sub> and related compounds, and Dr. Ronald M. Evans at the Salk Institute in whose laboratory this work was initiated.

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