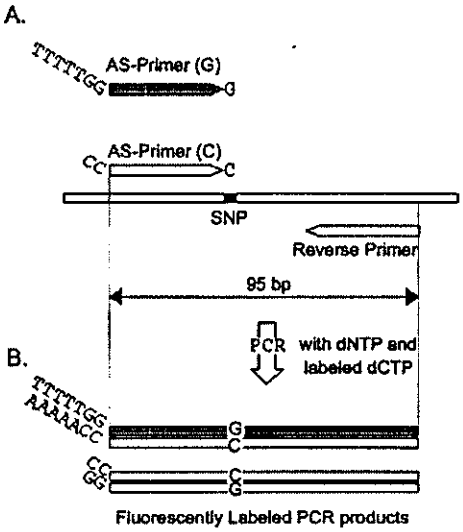

Fig. 1. Schematic representation of the Sd-PCR method. (A) Two allele-specific forward primers having a seven-base or two-base stretch of TTTT-TGG or CC at their 5′ ends amplify a 95-bp flanking sequence of the SNP with a specific reverse primer. (B) Allele-specific PCR products are labeled fluorescently by incorporation of dye-conjugated dCTP.

reverse primer for each PCR reaction. The C allele was designed to produce a 102-bp fluorescent DNA fragment and the G allele a 97-bp fragment.

Each genomic DNA sample (10 ng) was amplified with 250 nM each primer (two polymorphic forward and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris–HCl, 1.5 mM $MgCl^2$, 50 mM KCl, 1 U of *Taq* DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin–Elmer, Norwalk, CT). The Sd-PCR reaction was carried out on a thermal cycler (Gene-Amp System 9600, Perkin–Elmer) with initial denaturation at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s), and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system using GeneScan Analysis Software version 2.1 (Applied Biosystems, Foster City, CA). To confirm the accuracy of the Sd-PCR method, direct resequencing was carried out using the ABI Prism BigDye Terminator system.

Statistical analysis

Quantitative associations between genotypes and adjusted BMD values (g/cm^2) or between genotypes and Z scores were analyzed using Instat 3 software (GraphPad Software) via analysis of variance (ANOVA), with regression analysis as a post-hoc test. The three genotypic categories of GnRH-W16S (+47C/G; rs6185), C/C, C/G, and G/G, were converted into incremental values 0, 1, and 2 respectively. These values correspond to the number of chromosomes possessing a G allele of the SNP. Significant association was defined when the given P value of the ANOVA F test was less than 5% ($P < 0.05$). A χ^2 test was used to ascertain Hardy-Weinberg equilibrium among genotypes ($P > 0.05$).

Results

Genotyping by Sd-PCR

The Sd-PCR is a method that transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. It is based on the "mutagenically separated PCR" technique proposed by Rust [12] and has been refined by us into an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. A flow chart outlining the procedure is shown in Fig. 1. We first genotyped 24 samples of test genomic DNAs (48 alleles) using the Sd-PCR method, achieving a success rate of 100%. In parallel, the same 24 DNAs were examined at the SNP site by direct resequencing using the ABI Prism Big-Dye Terminator system, to confirm the accuracy of the Sd-PCR method. This experimental comparison proved the complete accuracy (48 correspondence/48 typings) of the Sd-PCR method. We used the validated system to genotype 384 individual DNAs for the W16S SNP (G/C at nt +47) of the GnRH gene. In those experiments the C allele was transformed into a fluorescent DNA fragment of 102 bp, and the G allele at the same nucleotide position was transformed into a fluorescent fragment 97 bp long. The two sizes were well separated and easily distinguishable by electrophoresis on a polyacrylamide gel (Fig. 2).

To confirm the reproducibility of the Sd-PCR assay we prepared two forward allele-specific primers harboring different artificial mismatches for each variant allele. A computerized algorithm was used to design these four primers, enabling us to test four different combinations of primer sets. We genotyped the 24 test samples (48 alleles) in quadruplicate and compared the results with each other and to the results from direct resequencing; all were completely concordant. Based on this satisfactory experience, we made it a rule to routinely carry out Sd-PCR with four different combinations of primer sets for 24 alleles as a preliminary test before choosing the most suitable pair of primers.

Fig. 2. Representative gel image of three genotypes for GnRH-W16S SNP using the Sd-PCR method. A 3-μl aliquot of denatured labeled PCR products was discriminated by a five-base mobility difference on electrophoresis using an automated ABI Prism 377 DNA sequencing system.

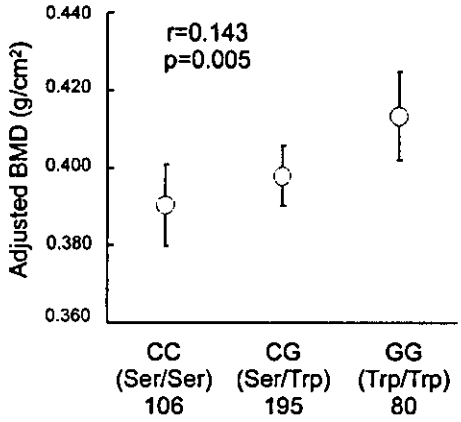

Fig. 3. Effect of GnRH alleles on quantitative adjusted BMD. Open circles indicate mean values; error bars indicate 95% confidence interval (95% C.I.); *r*, correlation coefficiency; p, *P* values, calculated by ANOVA *F* test.

Association of 16S alleles with low radial BMD in Japanese women

To test whether potentially functional amino acid variants of GnRH might be involved in the pathogenesis of postmenopausal osteoporosis, we selected for study an amino acid-substituting SNP in the GnRH gene (rs6185 in the NCBI dbSNP database). This SNP is located within the signal peptide domain and causes substitution of serine for tryptophan at residue 16 (W16S); the change is likely to affect processing of the precursor GnRH signal peptide on account of its location. As mentioned above, we applied the Sd-PCR method to determine genotypes at this site in DNA from 384 Japanese women, who were then classified into one of the three possible genotypic groups. Among our subjects, the C allele (corresponding to the second nucleotide of a serine codon) was a major variant, with a frequency of 54%; the proportions of different genotypes were in Hardy-Weinberg equilibrium.

Correlation between the genotypes and the adjusted values of BMD, examined by logistic regression analysis, was significant (Fig. 3). Homozygous C allele carriers (16S/S) had the lowest BMD values (0.390 ± 0.054 g/cm^2), homozygous G allele carriers (16W/W) had the highest (0.413 ± 0.052 g/cm^2), and heterozygous individuals (16W/S) had intermediate adjusted BMD levels (0.398 ± 0.055 g/cm^2), indicating a dosage effect ($r = 0.143$, $P = 0.005$).

Correlation of 16Ser alleles with low BMD in a case-control comparison

To examine the above correlation in a case-control study, we compared distributions of 16Ser alleles between 125 osteoporosis patients (low BMD; Z scores, −1.0) and 92 control individuals (sufficient BMD levels; Z scores, 1.0); χ^2 tests revealed a significant association between GnRH genotype and affected status, i.e., the 16S allele was more frequent among patients than among controls ($\chi^2 = 4.74$, $P = 0.041$; Table 1). A statistically significant association was ascertained by analyzing allele frequencies in both groups using Fisher's exact test (relative risk, 1.22, 95% confidence interval; 1.01–1.47, $P = 0.041$; Table 1). The results supported a consistent contribution of the SNP GnRH-16Ser to a lowering of bone mineral density not only in the general population but also in the comparison of cases with controls among Japanese women.

Discussion

We examined the effect of an amino acid-substituting SNP (rs6185), within the signal peptide-encoding domain of the GnRH gene, GnRH-W16S, on bone mineral density (BMD) and development of osteoporosis in a population-based quantitative analysis and in a case-control comparison involving osteoporosis patients. BMD levels and the frequency of osteoporosis were significantly correlated with the presence of 16Ser alleles encoded at nt +47, when we applied an Sd-PCR method of SNP genotyping.

Our SNP genotyping method can be summarized as follows: the Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. It is based on the "mutagenically separated PCR" proposed by Rust [12] and has been refined by us into an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. This procedure incorporates double-nucleotide mismatches at the 3′ end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other designed to allow distinct allelic discrimination through almost exclusive amplification of one allele over the other.

Innovations that were incorporated into the Sd-PCR method presented here are (1) addition of a five-thymidine stretch at the 5′ end of one of the two polymorphic (forward) primers, for discriminating the two alleles according to size; (2) incorporation of fluorescent dyes into the prod-

Table 1
Distribution of Trp and Ser alleles among cases (low Z scores) versus distribution among controls (high Z scores)

	Low Z score	High Z score	Total	*P* value
Genotype				
CC (Ser/Ser)	42 (33.6%)	24 (32.2%)	64	
CG (Ser/Trp)	60 (48.0%)	39 (32.3%)	103	
GG (Trp/Trp)	23 (18.4%)	29 (32.4%)	49	
Total	125 (100%)	92 (100%)	216	0.041[a]
Allele				
C Allele (Ser)	144 (57.6%)	87 (47.3%)	231	
G Allele (Trp)	106 (42.4%)	97 (52.7%)	201	
Total	250 (100%)	184 (100%)	432	0.041[b]

[a] *P* value was calculated by χ^2 test of trend ($P < 0.05$).
[b] *P* value was calculated by Fisher's exact test ($P < 0.05$).

ucts during DNA synthesis in the PCR reactions; (3) automated size separation on DNA sequencing (polyacrylamide) gels; (4) laser detection of the two fluorescent single-stranded DNA fragments that represent respective polymorphic alleles; and (5) multiplex processing (up to six SNPs) by gel electrophoresis and detection of fluorescent fragments simultaneously using multiple loading of fragments labeled with different fluorescent colors. These approaches successfully improved the accuracy and success rate of our genotyping assay.

Gonadotropin-releasing hormone is a key molecule in the hypothalamic–pituitary–gonadal axis that controls estrogen status. Dramatic changes in GnRH levels in serum during the perimenopausal period in women underscore the key role of GnRH in governing the entire process of hormonal regulation. Treatment of hypogonadism by GnRH agonists/antagonists sometimes causes estrogen deficiency due to increased negative feedback, with a resulting increase in biochemical markers of bone turnover and bone loss [16–18]. All of these facts indicate that normally regulated GnRH secretion is a requirement for maintenance of normal bone mass.

Estrogen status correlates not only with premenopausal peak bone mass [3] but also with increased bone turnover in postmenopausal women [1,5]. Thus, multiple factors involved in the estrogen regulatory system are assumed to contribute to acquisition of peak bone mass as well as postmenopausal bone loss. A longitudinal study of bone loss in a follow-up study of our cohort and/or an examination of peak bone mass among younger women would help to clarify these issues. In addition, reproducible association or linkage should be tested in a different ethnic group by means of both standard and alternative methods of association studies, including the transmission disequilibrium test (TDT).

Multiple reports have described correlations between variations in signal peptide sequences and altered function of those peptides [19–22]. In general, a signal peptide sequence contains a hydrophobic core region consisting of 6–15 amino acid residues [23]. In GnRH, the 11 hydrophobic amino acids from 7Leu to 18Val (LLAGLILLTWCV) are conserved among mammalian species and among gene families. The 16Trp (wild-type) residue lies at the C-terminal end of the hydrophobic core. Alteration of 16Trp to 16Ser would entail a loss of hydrophobicity from 0.50 to −0.55 units, suggesting that this alteration might cause a shift in translocation efficiency of the peptide from free ribosomes to endoplasmic reticulum or a change in some other aspect of intracellular transport of molecules. Those suggestions require further investigations, which might include, for example, an in vitro translated peptide-translocation assay that would measure the amount of peptide bind to microsomal membrane in vitro, and pulse–chase experiments on cultured cells.

In summary, we have developed the Sd-PCR genotyping method that can transform nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles, an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. This procedure was applied to identify a novel osteoporosis susceptibility gene, *GnRH*, whose polymorphic alleles encode an amino acid variation (W16S) that correlated significantly with bone mineral density among postmenopausal women in the general Japanese population as well as among osteoporosis patients. Structural inspection indicated that this amino acid sequence variation could affect processing of the signal peptide. In that case, variation in the *GnRH* gene might explain, at least in part, the pathogenesis of postmenopausal osteoporosis and this information could lead to development of new treatment designs and plans for prevention of the disease.

Acknowledgments

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CHAPTER 16

RING Finger-B Box-Coiled Coil (RBCC) Proteins as Ubiquitin Ligase in the Control of Protein Degradation and Gene Regulation

Kazuhiro Ikeda, Satoshi Inoue, Masami Muramatsu

Abstract

The protein family harboring the RING finger motif, defined as a linear array of conserved cysteines and histidines, has grown enormously in the last decade. The members of the family are involved in various biological processes including growth, differentiation, apoptosis, transcription and also in diseases and oncogenesis. It has been postulated that the RING finger domains have crucial roles in these phenomena themselves, in some cases, working with other domains in other proteins, although the precise mechanisms and common features of RING finger function have not been fully elucidated. However, most recently, an accumulating body of evidence has revealed that some of the RING finger proteins work as E3 ubiquitin ligases in ubiquitin-mediated specific protein degradation pathway. In this review, we focus on the RING finger protein with special reference to E3 ligase.

Structure of RING Finger

The RING finger protein sequence motif was first identified in the human gene RING1 – Really Interesting New Gene 1 – which is located proximal to the major histocompatibility region on chromosome 6.[1,2] The RING finger motif can be defined as a unique linear series of conserved cysteine and histidine residues: Cys-X_2-Cys-X_{11-16}-Cys-X-His-X_2-Cys -X_2-Cys-X_{7-74}-Cys-X_2-Cys (RING-CH or C_3HC_4 type), where X can be any amino acid (Fig. 1). So far, three-dimensional structures of RING domains from human PML (for promyelocytic leukemia protein),[3] immediate early equine herpes virus (IEEHV) protein,[4] human recombination-activating gene 1 protein (RAG1),[5] human MAT1 (for menage a trois-1 protein)[6] and human Cbl (for Casitas B-lineage lymphoma protein)[7] with a cognate ubiquitin-conjugating enzyme (E2) have been solved at atomic resolution. These studies have confirmed that the RING finger binds zinc ions in a similar manner as the classical zinc finger motif. Particularly, the RING finger is composed of a unique 'cross-brace' arrangement with two zinc ions and folds into a compact domain comprising a small central β sheet and an α helix. There are subfamilies of RING fingers which have Cys5 substituted with histidine (RING-H2) and a cysteine or histidine substituted with other metal binding residues such as aspartic acid and threonine.[8,9] Although the RING domain was initially found

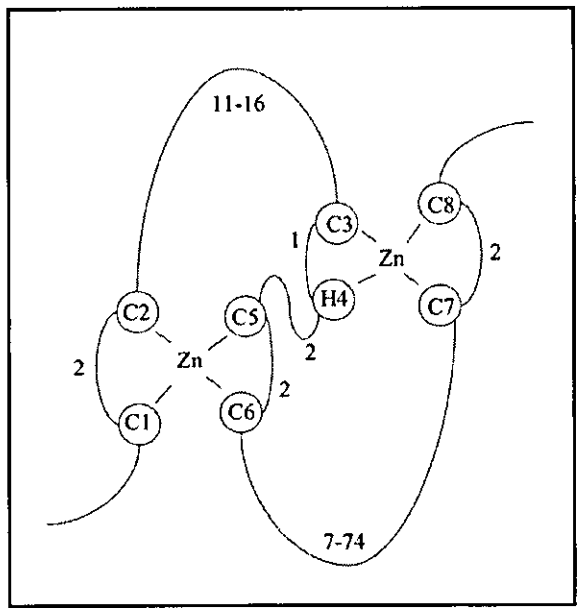

Figure 1. Schematic representation of the structure of RING finger domain. The metal-ligand residues, either cysteine (C) or histidine (H), are shown as numbered spheres. The numbers next to the loops connecting the metal-ligand residues indicate the minimum and maximum number of loop residues.

in only a few genes, more than 3000 proteins harboring the RING finger domain have been detected from diverse eukaryotes in the SMART database as of July 2003. Because of this evolutional conservation and variation in loop lengths, the RING domain appears to have a considerable flexibility within the rigid structure.

Family of RING Finger Protein

The RING fingers and their variants are generally located close to an amino or carboxyl terminus though there are no fixed rules. Most of the RING finger is associated with certain protein domains to form larger conserved motifs which may define the func-

Zinc Finger Proteins: From Atomic Contact to Cellular Function, edited by Shiro Iuchi and Natalie Kuldell.

tion of the protein, thus the family being divided into subfamilies along with the associated domains (Fig. 2). A similar domain architecture often corresponds with a similar function. For instance, TRAFs (for tumor necrosis factor (TNF) receptor-associated factors) 2-5 have an N-terminal RING domain followed by five zinc fingers, a coiled coil, and a C-terminal TRAF domain.[10] TRAF1 has all of these domains except for the RING. Members of TRAF family have been shown to be involved in TNF-related cytokine signal transduction through interactions between their TRAF domains and the intracytoplasmic parts of receptors of the TNF receptor family which are suicide receptors to transfer apoptotic signals into the cells.[11-13]

The inhibitors of apoptosis gene family, IAP1, IAP2 and XIAP, have a RING domain at their C termini and BIR (baculovirus IAP repeat) domain at their N termini. The BIR domains of the proteins bind and inhibit caspase.[14,15] Interestingly, the RING fingers of XIAP and IAP2 possess E3 ubiquitin ligase activity and are thought to be responsible for self-degradation when an apoptotic signal is transduced.[16] In addition, the anti-apoptotic activity of the protein is lost when the RING domain is mutated.[17]

There are interesting subfamilies uniquely possessing two RING fingers. Triad1 (for two RING fingers and DRIL1) and parkin have two RING finger domains separated by the double RING finger linked (DRIL) domain. Triad1 was identified as a nuclear RING finger protein, which is up-regulated during retinoic acid induced granulocytic differentiation of acute leukemia cells.[18] Parkin is a responsible gene for familial autosomal recessive Parkinson's disease.[19,20] Parkin binds to the E2 ubiquitin-conjugating enzymes through its C-terminal RING finger and has ubiquitin–protein ligase activity.[21] Parkin ubiquitinates and promotes the degradation of a putative G protein-coupled transmembrane polypeptide, Pael (parkin-associated endothelial-like) receptor, the insoluble form of which is accumulated in the brains of Parkinson's disease.[22] The insoluble parkin overexpressed in cells causes unfolded protein-induced cell death, whereas coexpression of Parkin suppresses the accumulation of Pael receptor and subsequent cell death.[21] Parkin also ubiquitinates and promotes the degradation of CDCrel-1 (for cell division cycle related-1) and itself.[23] Familial-linked mutations disrupt the ubiquitin–protein ligase function of Parkin and impair Parkin and CDCrel-1 degradation.

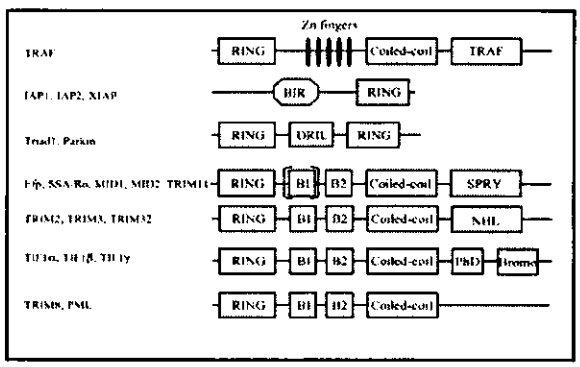

Figure 2. Structures of the RING finger protein family. Representative RING finger proteins with frequently associated domains are presented.

Function of RING Finger

It has been shown in early studies that the RING finger proteins have crucial roles in the growth, differentiation, transcription, signal transduction and oncogenesis.[24] For example, PML is fused to the retinoic acid receptor α (RARα) in acute promyelocytic leukemia (APL) translocation,[25-27] BRCA1 is mutated in early-onset breast cancer and ovarian cancer,[28] TIF1α is a positive cofactor of nuclear hormone receptors[29] and TRAF transduces signals from members of the TNF receptor superfamily to the transcription factor NF-κB.[14] Although those studies appear to show some essential roles played by the RING finger domains in the function of these proteins, the general function of the RING finger domain has not been resolved. However, recently, it was uncovered that the RING finger proteins are involved in the ubiquitin-mediated protein degradation pathway.

The ubiquitin-dependent protein degradation is a specific and sophisticated mechanism in which a target protein to be destroyed is tagged with the ubiquitin. Ubiquitination is accomplished by a complex process involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3).[30] Ubiquitin ligase mediates the transfer of ubiquitin from E2 to a substrate, marking it for degradation by the 26S proteosome. Therefore, E3 enzyme is thought to be important for the specific recognition of the substrate in the ubiquitination pathway. There is accumulating evidence that RING finger domains are identified in E3 complexes and proteins, suggesting the broad use of these domains for ubiquitination. As mentioned above, RING finger domain has the conserved cysteine and histidine residues. The C_3HC_4 type RING finger is found in several E3 proteins including Cbl,[31] BRCA1,[32] Efp (for estrogen-responsive finger protein)[33] and Mdm2 (for murine double minute 2).[34] The RING-H2 subtype is found in Rbx1 (for RING box protein 1) and Apc11 (for anaphase promoting complex (APC) subunit 11) in SCF (Skp1-Cullin-F-box) and APC E3 complexes,[35] respectively, and other ubiquitin ligases. Thus, evidence is accumulating that the RING finger proteins has crucial roles as an E3 ubiquitin ligase in diverse biological functions and diseases. Cbl is one of the initially identified E3 ligase which is involved in the regulation of various tyrosine kinase-linked receptors such as growth factor receptors (for example EGF and PDGF receptors), cytokine receptors and immuno-receptors (for example T-cell, B-cell and Fc-receptors).[36] Cbl recognizes activated protein tyrosine kinases and recruits E2 ubiquitin conjugating enzymes through its SH2 and RING finger domain, respectively. For EGF and PDGF receptors, increased recruitment of Cbl to the activated receptor complex leads to enhanced ubiquitination and degradation of the activated receptor. In contrast, oncogenic mutation in the Cbl RING finger which fails to bind E2 ubiquitin conjugating enzymes abrogates Cbl-mediated EGF receptor ubiquitination and degradation.[37] Thus, it appears that Cbl functions as an adapter to recruit the ubiquitination machinery to activated tyrosine kinase-linked receptors and stimulates receptor ubiquitination and degradation. This causes enhanced down-regulation of the receptor from the cell surface and attenuation of growth factor receptor signaling.

The RING finger protein Mdm2 is identified as an E3 ubiquitin ligase of the tumor-suppressor protein p53 which is a transcription factor and a potent inhibitor of the cell cycle. Mdm2 can bind to p53 and promote its ubiquitination and subsequent degradation by the proteasome.[38,39] It is also known that Mdm2 can ubiquitinate itself, suggesting that some of E3s self-regulate their own stability. The RING finger of Mdm2 is necessary for

both p53 ubiquitin and Mdm2 auto-ubiquitination. Substitution of the Mdm2 RING finger domain with the RING finger from another RING protein maintains the autoubiquination and degradation of Mdm2 but is not able to stimulate p53 ubiquitination. Moreover, mutations in the RING finger domain do not impair binding capacity between Mdm2 and p53. These observations suggest that the RING finger domain appears to be required for specific recognition of substrates in some degree, but is not generally involved in substrate binding.[40]

RBCC/TRIM Subfamily

Frequently, the RING is associated with cysteine-rich B-box domains followed by a predicted coiled coil domain. The B-box domain can be defined as a series of conserved cystein and histidine residues: B1 [Cys-X_2-Cys-X_{7-10}-Cys-X_2-Cys-X_{4-5}-Cys-X_2-Cys/His-X_{3-6}-His-X_{2-8}-His] and B2 [Cys-X_{2-4}-His/Cys-X_{4-9}-Cys-X_2-Cys/His-X_4-Cys/His-X_2-His/Cys] where X can be any amino acid. Structural analysis revealed that it consists of 15 or fewer β-strands.[41] The coiled coil is a common protein motif involving a number of α-helices wound around each other in a highly organized manner and is often used to control oligomerization.[42] These RING, one or two B-boxes and a coiled coil domain motifs are called RBCC or tripartite motif (TRIM)(Fig. 3).[43] This largest subfamily was first identified in a putative transcriptional regulator, Xenopus XNF7[24] and about 50 members have been identified since then. Though either one or two B-boxes are present, the spacing between the RING, B-boxes and the coiled coil is highly conserved with 38-40 residues between the RING and first B-box, and less than 10 amino acids between the second B-box and the coiled coil. There is no apparent homology among these separating sequences. According to the recent progress of genomic analysis, it was revealed that the chromosomal localization of the RBCC/TRIM subfamily genes has an intriguing feature. Although the genes encoding the RBCC/TRIM family members are dispersed throughout the genome, there are two distinct clusters on chromosomes 11p15 and 6p21-22 (Fig. 4). *TRIM22, SSA1/TRIM21, TRIM34, TRIM6, TRIM5, TRIM3* and *SS-56* are clustered in 11p15. *RFP/TRIM27, TRIM31, TRIM10, TRIM15, TRIM26, TRIM38, TRIM39, TRIM40* and *HZFW1* are clustered in 6p21-22. In particular, mRNAs for *TRIM 21, 22* and *34* are shown to be up-regulated by interferons.[44-46] These findings suggest that duplication of an ancestral *RBCC/TRIM* gene at these genomic loci may have occurred and, regulation and function may be conserved to some extents in these genes.[43]

The RBCC/TRIM proteins are associated with certain domains such as B30.2-like or SPRY, NHL, PHD and BROMO domains at their C-terminus. These additional domains may contribute to the function of the subfamily. The B30.2-like domain is a series of 160-170 amino acids containing three highly conserved motifs (LDP, WEVE and LDYE) which is named after the B30-2 exon within the human class I histocompatibility complex locus. The SPRY domain, which was originally named from SPla and the RYanodine Receptor, is composed of around 140 amino acids containing the latter two conserved motifs in B30.2-like domain. At present, the B30.2-like domain is considered as a subclass of the SPRY domain family. The SPRY domain is contained in many of the RBCC/TRIM subfamily including XNF-7, RPT-1, SSA-Ro and STAF50. The significantly conserved SPRY domains imply the biological importance of this gene, however the function of the SPRY domain is not known. The NHL domain name was derived from the three founding members: NCL-1, HT2A, and LIN-41.[47] NCL is involved in rRNA metabolism. HT2A was identified as an interacting partner of the

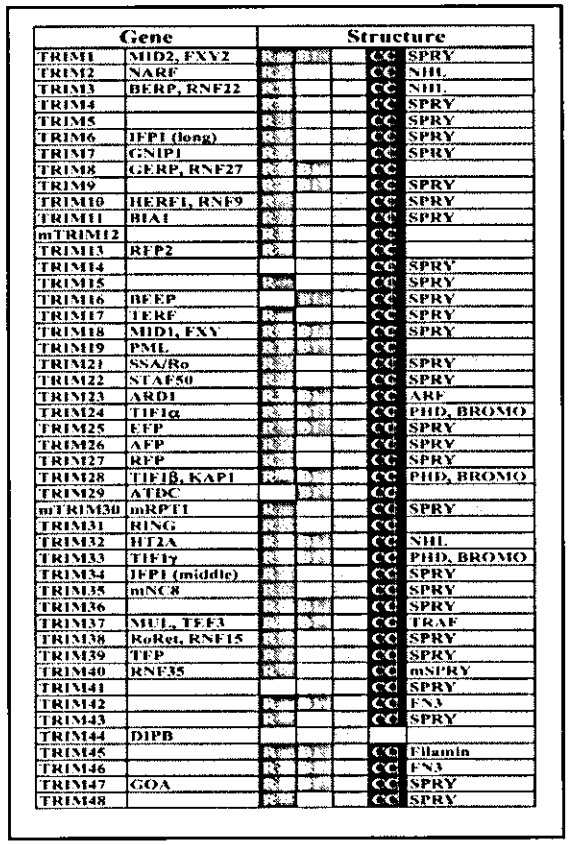

Figure 3. RBCC/TRIM family genes. The gene names are listed in numerical order of *TRIM* genes followed by commonly used names in the second column. Their domain structures are schematically shown to the right.

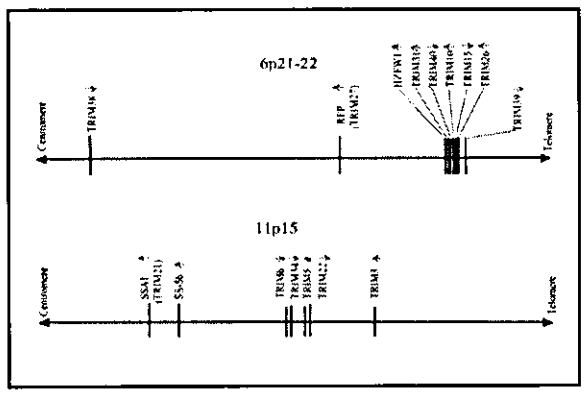

Figure 4. Clustered localization of the *RBCC/TRIM* genes in 6p21-22 and 11p15. *TRIM22, SSA1, TRIM34, TRIM6, TRIM5, TRIM3* and *SS-56* are clustered in 11p15, whereas *RFP, TRIM31, TRIM10, TRIM15, TRIM26, TRIM38, TRIM39, TRIM40* and *HZFW1* are clustered in 6p21-22. The sense strand orientations of each gene are indicated by the arrows.

HIV Tat protein and Lin41 is involved in posttranscriptional regulation of mRNA. The NHL motif has a slight homology with WD40 domain, suggesting a protein-protein interaction. The C-terminal PHD fingers and bromodomains are found in TIF1α and KAP1/TIF1β. The PHD domain is a motif characteristically defined by seven cysteines and a histidine that are highly homologous to the RING motif and is contained in some transcription factors. The PHD domain of MEKK1 (MEK kinase 1) exhibited E3 ubiquitin ligase activity toward ERK (extracellular signal-regulated protein kinase) 2, suggesting a negative regulatory mechanism for decreasing ERK1/2 activity.[48] The bromodomain is also found in transcription factors, can bind histones with acetylated lysines and appears to be involved in chromatin remodeling.[49] KAP1/TIF1β and TIF1α are involved in transcriptional regulation. Genes belonging to this RBCC/TRIM family are implicated in a variety of processes such as development and cell growth and are involved in several human diseases. PYRIN,[50] MID1 (Midline 1)[51] and MUL (for mulibrey nanism proteins)[52] are mutated in familial Mediterranean fever, X-linked Opitz/GBBB syndrome and mulibrey nanism, respectively, whereas PML, RFP (ret finger protein) and TIF1α acquire oncogenic activity when fused to RARα, RET or B-raf, respectively.

It has been shown that the RBCC/TRIM proteins can oligomerize through their coiled coil domains. In homodimerization of RFP proteins, the coiled coil region with the B-box but not the RING finger is required.[53] In this case, while the B-box is not an interacting interface itself, the mutation of conserved cysteine residues within the B-box affects the ability of RFP to multimerize, suggesting that its structural integrity is necessary for this interaction to occur.[53] The coiled coil domain of RFP is also necessary for interaction with Enhancer of polycomb protein (EPC) to repress gene transcription.[54] The homodimerization and binding with EPC occurs with the proximal coil in RFP protein. RFP also directly interacts and colocalizes with PML in a subset of the PML NBs (nuclear bodies).[55] This interaction is mediated by the RFP B-box and the distal two coils. The association of RFP with the PML NBs is altered by mutations that affect RFP/PML interaction and in APL patients-derived cells. These results indicate that RFP have an important role in regulating cellular growth and differentiation. MID1 protein, which is mutated in patients with Opitz GBBB syndrome, and the highly related gene MID2 also make both homo- and hetero-dimers mediated by the coiled coil motifs. The dimerization is a prerequisite for the association of MID and Alpha 4 (a regulatory subunit of PP2-type phosphatases) and the complex formation with microtubules which seems important for normal midline development.[56] In contrast, it has been shown that the entire RBCC/TRIM domain is required for hetero-oligomerization or binding natural ligands. The RBCC region of KAP1/TIF1β associates with the KRAB (Krüppel-associated box) transcriptional repressor domain of KOX-1.[57] From extensive studies of the interaction, it has been revealed that the interaction is specific for the KAP1 RBCC/TRIM domain. Namely, when each RBCC/TRIM motifs of KAP1 was swapped with other corresponding ones of MID1, KAP1 did not bind the KRAB domain any more. Therefore, each domain of the RBCC may function as an independent functional unit and have important roles in the specific recognition of interacting partners or oligomers formation. In other RBCC/TRIM proteins, only one copy of the B-box motif is present, but inspection of the whole family reveals a conserved residue spacing between the RING, B-box and coiled coil domains.[43] This strongly suggests that the overall architecture of the RBCC/TRIM motif is highly conserved, perhaps relating to the motif acting as a scaffold for higher-order protein-protein interactions.[57] Molecular modeling suggests that the position and orientation of the B-box (adjacent to the coiled coil) would be critical for the correct alignment of the α-helices that form the coiled coil. Interestingly, unlike the RING and coiled coil motifs, the B-box is only found in RBCC/TRIM family members suggesting that it is a critical determinant of the overall motif and its function.[43]

As mentioned above, the latest findings of RING fingers in E3 ubiquitin ligases imply that the members of this RBCC/TRIM subfamily are potential candidates for specific regulators/adopters in ubiquitin-dependent protein degradation. In fact, some genes belonging to the subfamily has been proven to act as E3 ligase. We next discuss such genes focusing on the recent findings.

Ring Fingers that Act As E3 Ligases

Efp

Estrogen-responsive finger protein (Efp) is a member of the RING-finger, B1 and B2-boxes, coiled coil and SPRY (RBCC-SPRY) subfamily in the RING finger family. Efp was isolated as an estrogen-responsive gene by genomic binding-site cloning using a recombinant estrogen receptor (ER) protein.[58] The estrogen-responsive element (ERE) to which ER can bind is found at the 3'-untranslated region (UTR) in the *Efp* gene and the gene's expression is predominantly detected in female reproductive organs including uterus, ovary and mammary gland[59] and in breast and ovarian cancers.[60] Estrogen-induced expression is found in the uterus, brain and mammary gland cells. *Efp* knockout mice have an underdeveloped uterus and estrogen responses of uterin cells from knockout mice are markedly attenuated, suggesting that Efp is necessary for estrogen-induced cell growth.[61] Moreover, tumor growth of breast cancer MCF7 cells implanted in female athymic mice has been demonstrated to be reduced by treatment with antisense Efp oligonucleotide. In contrast, Efp-overexpressing MCF7 cells in ovariectomized athymic mice generate tumors in the absence of estrogen.[33] These results indicate that Efp mediaes estrogen-dependent growth in breast cancer cells. We identified 14-3-3Σ which is responsible for reduced cell growth, as a binding factor to Efp and found an accumulation of 14-3-3Σ in *Efp* knockout mouse embryonic fibroblasts. Furthermore, it has been revealed that Efp is an ubiquitin ligase (E3) that targets proteolysis of 14-3-3Σ. Specifically, the RING which preferentially bound to ubiquitin-conjugating enzyme UbcH8 has been shown to be essential for the ubiquitination of 14-3-3Σ. Our findings provide an insight into the cell-cycle machinery and tumorigenesis of breast cancer by identifying 14-3-3Σ as a target for proteolysis by Efp, leading to cell proliferation. The degradation of 14-3-3Σ is subsequently followed by dissociation of the protein from cyclin–Cdk complexes, leading to cell cycle progression and tumor growth (Fig. 5).

MID1, MID2

Opitz GBBB syndrome (OS; Opitz syndrome) is a genetically and phenotypically complex disorder defined by characteristic facial anomalies, structural heart defects, as well as anal and genital anomalies.[62,63] A positional cloning approach has revealed a candidate gene designated *MID1*[51] which is a member of the RBCC-SPRY family. Most of the mutations identified so far in patients with Opitz syndrome cluster in the SPRY domain of

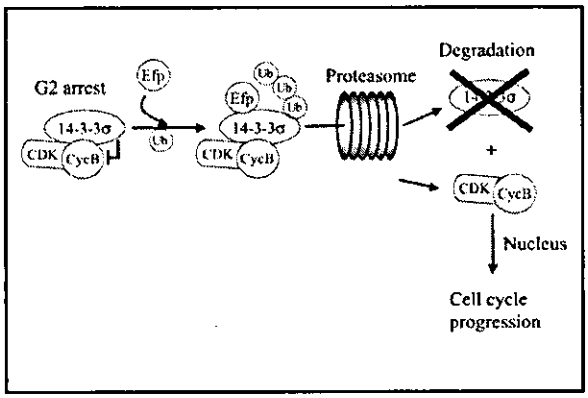

Figure 5. Models of Efp action as E3 ligase. Estrogen-induced RING finger protein Efp recognizes a cell cycle inhibitor 14-3-3Σ which keeps Cyclin B in cytoplasm. Efp modifies 14-3-3Σ with ubiquitin and the resulting ubiquitinated 14-3-3Σ is recruited to 26S proteosome to be destroyed. The dissociated cyclin B is now capable of entering the nucleus where it drives cell cycle.

MID1. It has been shown that MID1 associates with microtubules, whereas mutant forms of MID1 do not.[64] These results suggest that MIDI has a physiological role in microtubule dynamics.

Recently, the α4 protein, a regulatory subunit of protein phosphatase 2A (PP2A)[65] was isolated by yeast two-hybrid screening with MID1 as bait. It was demonstrated that the B-box 1 is sufficient for a strong interaction with α4. MID2,[66] which is highly similar to MID1, also binds α4. Cellular localizations of MID1 and α4 are coincident with cytoskeletal structures and MID1 with a mutation at the C terminus that mimics the mutant protein of some individuals with OS results in the formation of cytoplasmic clumps containing both proteins. The identified substrate for E3 ligase activity of MID1 is a cytosolic PP2A. In contrast, addition of a proteasome inhibitor to OS-derived fibroblasts expressing dysfunctional MID1 does not cause either enrichment of PP2A or accumulation of the enzyme's polyubiquitinated forms,[67] suggesting that MID1 mutations result in decreased proteolysis of the C subunit of PP2A in individuals with OS.

PML

PML also belongs to a subfamily of proteins containing a RBCC/TRIM motif.[43,68] PML has been implicated in the pathogenesis of acute promyelocytic leukemia that arises following a reciprocal chromosomal translocation that fuses the *PML* gene located on chromosome 15 with the retinoic acid receptor alpha (*RARα*) gene located on chromosome 17. The resulting PML-RARα fusion protein preserves most of the functional domains of both PML and RARα, but it lacks C-terminus of PML and N-terminus of RARα The fusion protein shows cell type- and promoter-specific differences from the wild type RARα,[25,26,69] while it maintains a responsiveness to retinoic acid. Overexpression of PML-RARα inhibits vitamin D3 and transforming growth factor β-induced differentiation and also reduces serum starvation-induced apoptosis in U937 cells.[70] In addition, dimerization of PML with PML-RARα is required to block differentiation.[71] Thus, PML-RARα is considered to function as a dominant negative protein by interfering with the function of PML and RARα.

In normal cells, cellular distribution of PML is found to form a discrete subnuclear compartment (nuclear body, NB)[72,73] or PML oncogenic domain.[74] Other proteins containing Sp100[75] and PLZF (for promyelocytic leukemia zinc finger)[76] have been reported to localize to the NBs. Interestingly, PLZF-RARα fusion protein is also found in a rare form of APL.[77] It is shown that the nuclear bodies were dispersed into a microspeckle pattern in APL cells but reformed with retinoic acid treatment by which APL cells differentiated into granulocytes. In addition, the NB is the preferred site where the early steps of transcription and replication of DNA virus occurs.[78] Therefore, the regulation of NB formation is thought to be involved in the pathogenesis of APL. Recently, PML is shown to be covalently modified by SUMO-1 (Small Ubiquitin-like Modifier-1) of ubiquitin-like proteins.[79] Mutations in the PML RING finger disrupt the nuclear body formation in vivo[3,69] and cause a failure of growth suppression,[80,81] apoptosis and anti-viral activities[82] of PML. The dependence on an intact RING finger for PML NBs formation implies specific protein interactions regulated by the RING structure. Recent studies have shown that PML RING interacting with the SUMO-1 E2 enzyme UBC9 is SUMO modified and the sumolylation of PML has an important role in regulating the formation of NBs.[83]

PML has two B-boxes (B1 and B2) adjacent to the RING domain. Mutations of conserved zinc-chelating residues in B1 and/or B2 boxes collapsed PML NB formation, whereas they did not affect PML oligomerization.[84] PML B-boxes are also involved in growth suppression.[80] It has been revealed that PML is sumoylated in B1 box which is responsible for binding of the 11S proteasomal subunit to PML NBs.[85]

The coiled coil region in PML is indispensable for multimerization or heterodimerization with PML-RARα,[69,71,86] formation of PML NB and growth suppression activity.[80] Notably, the important role of the coiled coil domain for the complex formation is also suggested from the studies of other RBCC/TRIM subfamily.[43,57]

TRIM8

TRIM8, a member of RBCC subfamily, is shown to interact with SOCS-1 (suppressor of cytokine signaling-1) which is induced by cytokines and inhibits cytokine signaling by binding to downstream signaling molecules such as JAK (Janus kinase) kinases.[87-89] The B-box coiled coil region of TRIM8 is sufficient for efficient interaction with SOCS-1, but the RING portion of the protein is not required for the binding. By contrast, both the SOCS box and the SH2 domain in SOCS-1 appear to be necessary for the interaction between SOCS-1 and TRIM8. It was found that exogenous coexpression of TRIM8/GERP with SOCS-1 decreased the stability of SOCS-1 protein and TRIM8 restored the IFN-γ-mediated transcription which was inhibited by the expression of SOCS-1.[90] These results suggest that TRIM8 is the putative E3 ligase for SOCS-1 and inhibits SOCS-1 function by targeting it for proteasomal degradation.

TRIM11

TRIM11 is a member of the protein family composed of a RING finger domain, which is a putative E3 ubiquitin ligase, a B-box domain, a coiled coil domain and a SPRY domain. A recent experiment with yeast two-hybrid screening has revealed that TRIM11 can interact with Humanin[91] which is a newly identified anti-apoptotic peptide that specifically suppresses Alzheimer's disease (AD)-related neurotoxicity. It is known that Bax

(Bcl2-associated X protein) has a crucial role in apoptosis. In response to death stimuli, Bax protein changes the conformation exposing membrane-targeting domains, translocates to mitochondrial membrane and releases the cytochrome c and other apoptogenic proteins. Indeed, Humanin is shown to bind with Bax and prevents the translocation of Bax from cytosol to mitochondria.[92] Moreover, Humanin blocks Bax association with isolated mitochondria and suppresses cytochrome c release. Therefore, Humanin seems to exert its anti-apoptotic effect by interfering the Bax function.

The coiled coil domain of TRIM11 is indispensable for the interaction with Humanin. The SPRY domain also contributes to the recognition of Humanin, whereas SPRY domain alone cannot. It was found that the intracellular level of Humanin was drastically reduced by the coexpression of TRIM11, and mutation of the RING finger domain or treatment with proteasome inhibitor attenuates the effect of TRIM11 on the intracellular level of Humanin.[91] These results suggest that the TRIM11 participates in the ubiquitin-mediated degradation of Humanin as an E3 ligase.

SSA/Ro (SSA1, TRIM21)

Sjögren syndrome is an autoimmune disease in which exocrine glands including salivary and lacrimal glands develop a chronic inflammation, and whose symptoms are dry eyes, dry mouth and fatigue. Autoantibodies to Ro recognize a ribonucleoprotein complex composed of small single-stranded RNAs and of one or more peptides. Although the Ro autoantigen is heterogeneous and found in most tissues and cells with differences in structure and quantity across tissues, it is detected in 35 to 50% of patients with systemic lupus erythematosus and in up to 97% of patients with Sjögren syndrome.[93] The 60-kD protein (Ro60) and the 52-kD protein (Ro52) were identified[94] and, another novel 56-kD protein (Ro56/SS-56) has been identified, recently.[95] Ro52 and Ro56 proteins belong to RBCC-SPRY subfamily. It is thought that the Ro autoantigen is involved in the regulation of transcription because it possesses functional domains associated with gene-regulation and binds to nucleic acids.[93] Its precise function is not understood, however. In a study, Ro52 was reported to be ubiquitinated in the cell.[96] The observation suggests that Ro52 may be downregulated by the ubiquitin–proteasome pathway in vivo. Interestingly, sera from patients with Sjögren syndrome showed heterogeneity in their reactivity to poly-ubiquitinated Ro52, probably because of their differing antigenic determinants. This heterogeneity of the reactivity may be associated with the varying clinical features found in Sjögren patients.

Conclusion

Here, we summarized the structural characteristics and functions of RING finger proteins specifically in terms of the E3 ligase activity. However, relatively few proteins have been really proven to function as E3 ligase. Thus, most RING finger proteins remain to be further investigated. Investigation of the RING finger proteins as a novel E3 ligase family will elucidate important mechanisms of cellular protein degradation and provide new insight into the physiological and pathophysiological roles of the pathway. Particularly, the molecular mechanisms of specific substrate recognition by E3 with the RING and other associated domains must be determined. Moreover, the RING finger proteins such as PML may possess unknown functions other than E3 ligase. Functional analysis of the RING finger proteins will help to understand biological roles of the family including the ubiquitin-mediated protein degradation pathway.

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Vitamin K_2 Regulation of Bone Homeostasis Is Mediated by the Steroid and Xenobiotic Receptor SXR*

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Vitamin K_2 is a critical nutrient required for blood clotting that also plays an important role in bone formation. Vitamin K_2 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_2 action in bone formation was thought to involve its normal role as an essential cofactor for γ-carboxylation of bone matrix proteins. However, there is evidence that suggests vitamin K_2 also has a transcriptional regulatory function. Vitamin K_2 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_2 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_2. Vitamin K_2 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_2 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, bisphosphonate, and selective estrogen receptor modulators (4). Vitamin K_2 was first reported to promote fracture healing in 1960 (5), and several studies showed that vitamin K_2 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_2 is used clinically in Japan to treat osteoporosis either alone or in conjunction with $1\alpha,25\text{-}(OH)_2$ vitamin D_3 (17–20).

Vitamin K_2 is a family of naphthoquinones, the most biologically important of which is menatetrenone (for a review, see Ref. 21). Vitamin K_2 was discovered as a critical nutrient required for blood clotting. It acts as a cofactor for the microsomal γ-carboxylase that facilitates the post-translational conversion of glutamic acid to γ-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[1] (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-

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[1] 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 16α-carbonitrile; PBP, 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.

dogenous 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_2 could also activate SXR.[2] This observation led us to consider the possibility that vitamin K_2 might act as a *bona fide* SXR ligand to mediate biological processes other than xenobiotic metabolism and clearance. Since vitamin K_2 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_2 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_2 induced the expression of the prototypical SXR target gene *CYP3A4* in these cells. Vitamin K_2 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_2 and bone development. The known SXR activators rifampicin and hyperforin induce the same panel of bone markers as does vitamin K_2, further confirming a role for SXR in the regulation of these genes. Finally we found that vitamin K_2 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_2 modulates the expression of osteoblastic bone markers through SXR and infer that vitamin K_2 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′-CAAGCGGAAGAAAAGTGAACG-3′; reverse primer, 5′-CTGGTCCTCGATGGGCAAGT-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)$_4$-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 $MgCl_2$ 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_2SO as 0.1 M stocks, diluted in Me_2SO 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-galactosidase 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_6-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. [^{3}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 $MgCl_2$. Aliquots of lysate were combined with reaction buffer (1 M diethanolamine, pH 9.8, 1 mM $MgCl_2$, 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_2, 1α,25-$(OH)_2$ vitamin D_3, 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′-CTAGCCCCAAAAAGAGTTGCAA-3′), osteopontin (OPN) (F, 5′-CAGACGAGGACATCACCTCA-3′; R, 5′-TGGCTGTGGGTTTCAGCA-3′), matrix Gla protein (MGP) (F, 5′-ATCGCTACTTCAGGAAGCGCC-3′; R, 5′-TGACTCTCCTTTGACCCTGACCCTCAC-3′), osteoprotegerin (OPG) (F, 5′-CCTCTCATCAGCTGTTGTGTG-3′; R, 5′-TATCTCAAGGTAGCGCCCTTC-3′), glyceraldehyde-3-phosphate dehydrogenase (F, 5′-TGGACCTCATGGCCCACA-3′; R, 5′-TCAAGGGGTCTACATGGCAA-3′), CYP3A4 (F, 5′-GGCTTCATCCAATGGACTGCATAAAT-3′; R, 5′-TCCCAAGTATAACACTCTACACAGACAA-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

[2] B. Blumberg, unpublished observations.

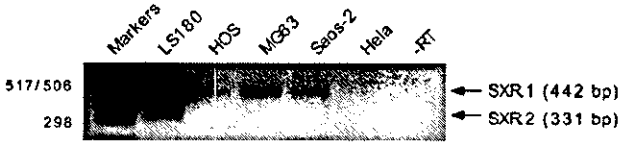

FIG. 1. ***SXR* is expressed in osteosarcoma cell lines.** The human cell lines HOS, MG-63, Saos-2, LS180, and HeLa were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was isolated using TriZol reagent. 1 μg of total RNA from each cell type was reverse transcribed 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 μM vitamin K_2 or pregnenolone 16α-carbonitrile (PCN). Total RNA was isolated, reverse transcribed, and analyzed using the following mouse primer sets: MGP (F, 5′-TCTCACGAAAGC-ATGGAGTC-3′; R, 5′-ATCTCGTAGGCAGGCTTGTT-3′), OPG (F, 5′-CTGCTGAAGCTGTGGAAACA-3′; R, 5′-AAGCTGCTCTGTGGTGAG-GT-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 ± 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).[3] It is not clear at present what role SXR is playing in other tissues. We were intrigued by the ability of vitamin K_2 to activate SXR in preliminary experiments. To ascertain whether SXR might be mediating the effects of vitamin K_2, 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_2 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_2. Accordingly we tested the ability of vitamin K_2 to activate SXR in dose-response experiments. As shown in Fig. 2*A*, vitamin K_2 activates CMX-GAL-SXR robustly with the highest levels of activation approximately equivalent to 1 μM 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_2 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_3 in cultured HepG2 and LS180 cells (49, 50), although the 1α,25-$(OH)_2$ vitamin D_3 induction of *CYP3A4* is mediated by the vitamin D_3 receptor rather than by SXR (51). RIF induced the expression of *CYP3A4* in all three lines (Fig. 2*B*). Vitamin K_2 was able to induce *CYP3A4* expression at both

[3] M. M. Tabb and B. Blumberg, unpublished data.

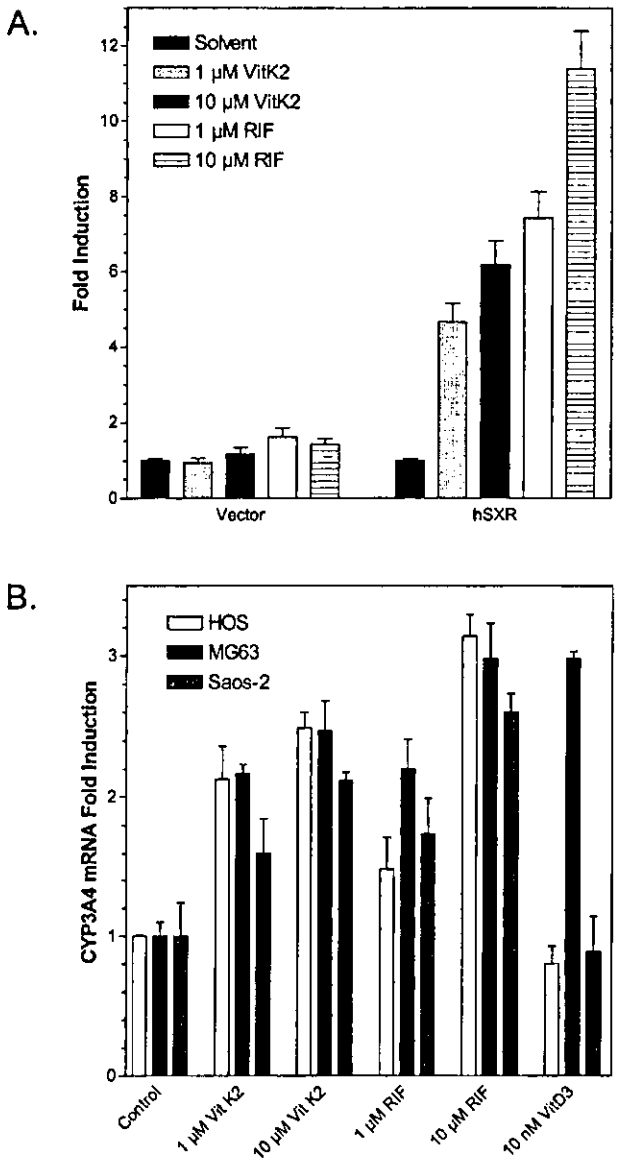

FIG. 2. **Vitamin K_2 activates SXR and induces SXR target genes.** *A*, activation of SXR by RIF and vitamin K_2. COS-7 cells were transiently transfected with either GAL-SXR or CMX-GAL4 (control) together with tk(MH100)4-luc reporter and CMX-β-galactosidase transfection control plasmids. After transfection, cells were treated with control medium or medium containing a dilution series of RIF, vitamin K_2, or 1α,25-$(OH)_2$ vitamin D_3. Only values for 1 and 10 μM rifampicin, 1 and 10 μM vitamin K_2, and 10 nM 1α,25-$(OH)_2$ D_3 are shown for clarity. *B*, *CYP3A4* induction by RIF and vitamin K_2. 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 μM in all three lines. 1α,25-$(OH)_2$ vitamin D_3 could not induce *CYP3A4* expression in Saos-2 or HOS cells (Fig. 2*B*).

Vitamin K_2 Specifically Binds to SXR in Vitro and in Vivo—Since vitamin K_2 activates SXR in transient transfections (Fig. 2*A*) and induces the expression of a prototypical SXR target gene in osteosarcoma cells (Fig. 2*B*), we next sought to determine whether vitamin K_2 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

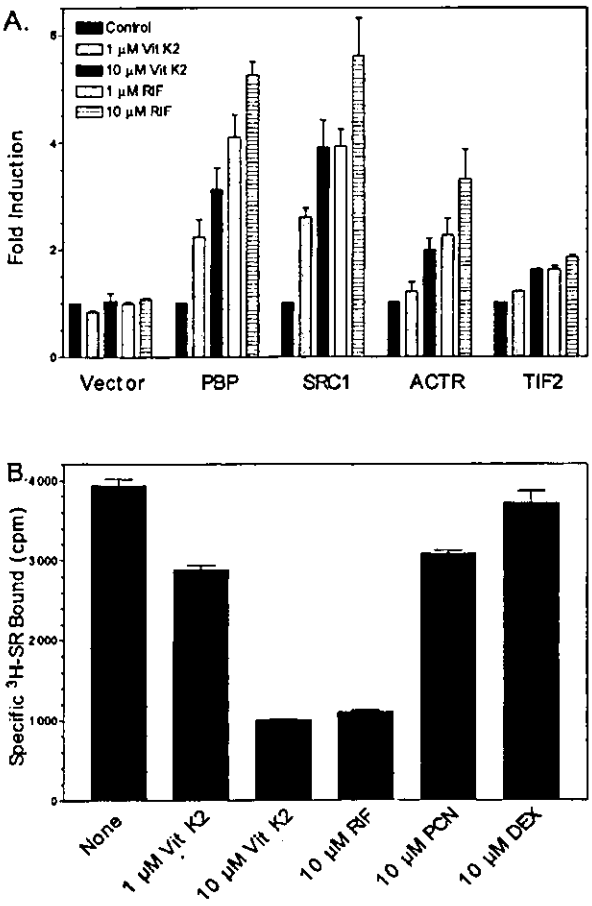

FIG. 3. **Vitamin K_2 specifically binds to SXR *in vitro* and *in vivo*.** *A*, vitamin K_2 induces SXR to recruit coactivators. COS-7 cell were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS and transiently transfected with a GAL reporter together with expression 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 μM RIF or vitamin K_2. Values represent the average of triplicates ± S.E. Experiments were repeated twice with similar results. *B*, vitamin K_2 specifically binds to the purified SXR ligand binding domain. His_6-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 [^{3}H]SR12813 (*^{3}H-SR*) in the presence of the indicated compounds or solvent control. Values represent the average of triplicates ±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. 3*A*, VP16-SXR was able to interact with PBP, SRC-1, and ACTR in the presence of vitamin K_2 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_2 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. 3*A*). We infer that vitamin K_2 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_2 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 [^{3}H]SR12813 and recombinant His_6-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. 3*B*, vitamin K_2 and RIF are able to displace [^{3}H]SR12813 from the SXR ligand binding domain, whereas the control compounds PCN and dexamethasone did not compete effectively for receptor binding. The K_i for vitamin K_2 binding to SXR was determined to be 5.1 μM, a value in the range of other known SXR ligands (28, 34). We infer from these results that vitamin K_2 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_2, Rifampicin, and Hyperforin on Bone Biomarker Genes in Osteosarcoma Cell Lines—Vitamin K_2 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_2 and 1α,25-$(OH)_2$ vitamin D_3, 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 1α,25-$(OH)_2$ vitamin D_3 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. 4*A*, 1α,25-$(OH)_2$ vitamin D_3 treatment increased ALP activity 1.7-fold in HOS and Saos-2 cells and 4.0-fold in MG-63 cells. Vitamin K_2 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. 4*A*). As will be seen with other markers below, there are notable differences in the response of particular cell lines to treatment with vitamin K_2 or 1α,25-$(OH)_2$ vitamin D_3. For example, 1α,25-$(OH)_2$ vitamin D_3 is particularly effective at inducing ALP enzyme activity in MG-63 cells (Fig. 4*A*). The effect of vitamin K_2 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_2 and 1α,25-$(OH)_2$ vitamin D_3 in a dose-dependent manner in all three cell lines with 1α,25-$(OH)_2$ vitamin D_3 being more potent (Fig. 4*B*). Statistically significant changes in ALP enzyme and mRNA levels were seen at vitamin K_2 concentrations of as little as 1 μM (Fig. 4, *A* and *B*), which is similar to therapeutic levels at which vitamin K_2 is used clinically. RIF and hyperforin produced effects very similar to vitamin K_2 (Fig. 4*B*). The concordance between ALP protein levels (Fig. 4*A*) and ALP mRNA levels (Fig. 4*B*) 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_2 elicited a dose-dependent increase in OPN

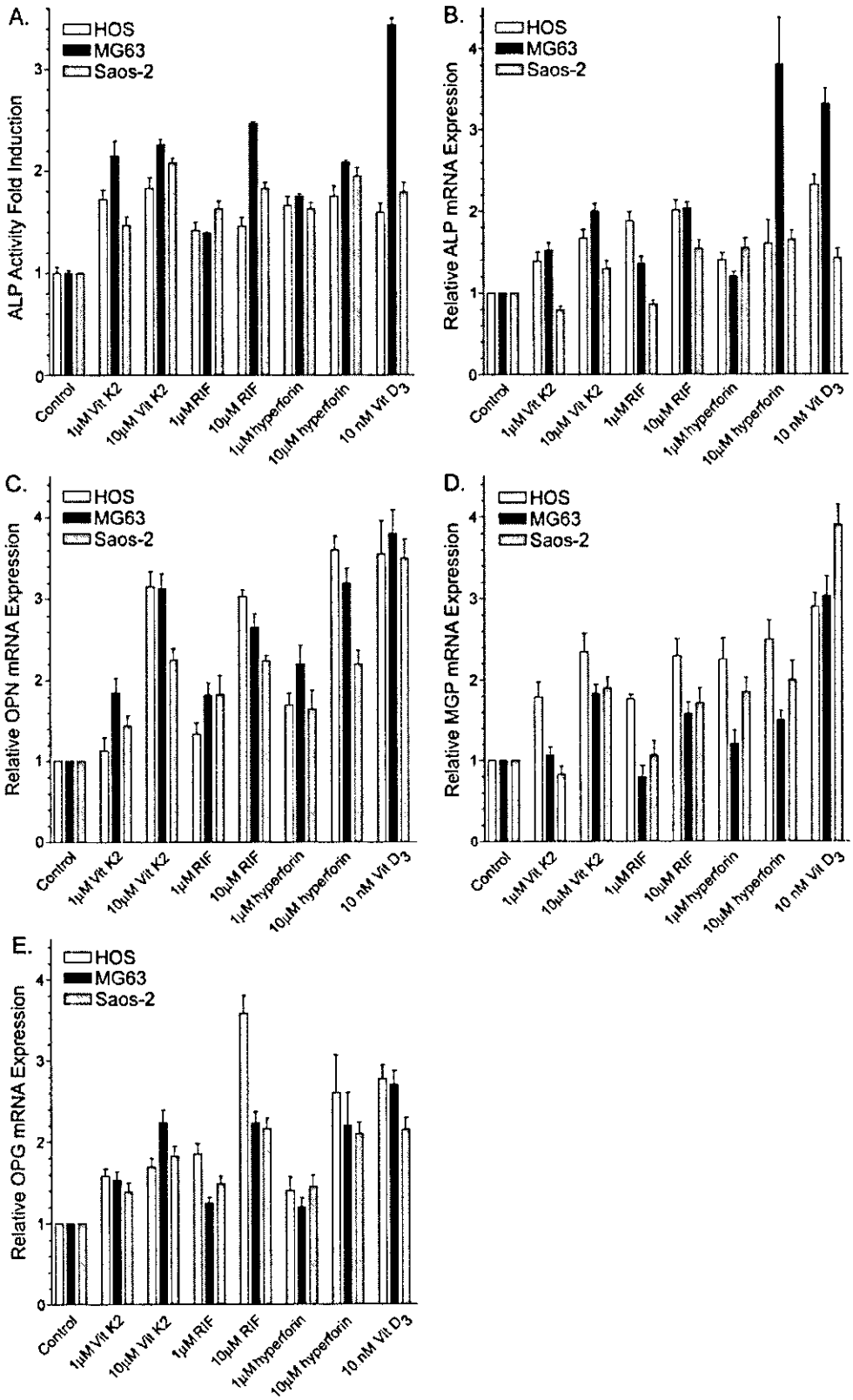

FIG. 4. **Effects of vitamin K_2 and rifampicin on osteoblastic marker genes in osteosarcoma cell lines.** Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS in the presence or absence of ligands for 48 h. ALP activity 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 μM dose approaching the response observed with 1α,25-(OH)$_2$ vitamin D$_3$ (Fig. 4*C*). RIF and hyperforin again elicited nearly identical results to those of vitamin K$_2$ treatment (Fig. 4*C*).

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). 1α,25-(OH)$_2$ vitamin D$_3$ 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$_2$, RIF, hyperforin, and 1α,25-(OH)$_2$ vitamin D$_3$ treatment (Fig. 4*D*). Unlike the other markers studied, MGP mRNA levels were only stimulated by the highest doses of vitamin K$_2$ and RIF used (10 μM) 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-κB ligand (RANKL). Recombinant OPG has been shown to block osteoclastogenesis *in vitro* and to increase bone density *in vivo* (68). 1α,25-(OH)$_2$ vitamin D$_3$ (10^{-7} 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$_2$ as well as by 1α,25-(OH)$_2$ vitamin D$_3$ and hyperforin (Fig. 4*E*). Rifampicin was an effective inducer of OPG mRNA in all three cell lines, particularly HOS cells (Fig. 4*E*), confirming and extending the published results. In summary, three structurally different human SXR activators, RIF, hyperforin, and vitamin K$_2$, 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$_2$ 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 μM 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$_2$, RIF, or vitamin D$_3$ 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$_2$ 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$_2$ 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$_2$ or RIF.

Vitamin K_2 Induction of Bone Marker Genes Requires SXR—The data presented above provide strong evidence that the stimulation of osteoblast bone markers by vitamin K$_2$ 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

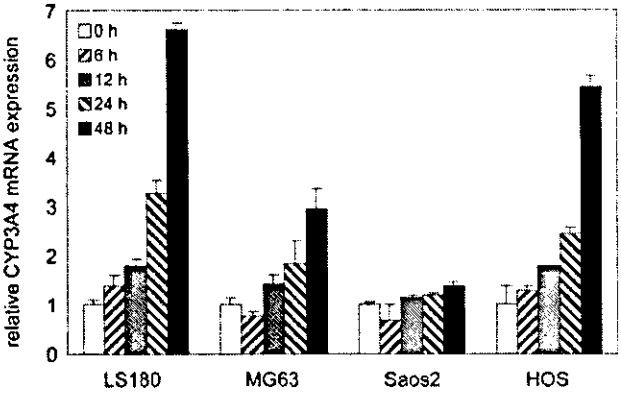

FIG. 5. **Time course of rifampicin induction of *CYP3A4*.** LS180 colon carcinoma cells or MG-63, Saos-2, or HOS osteosarcoma cells were treated for the indicated times with 10 μM rifampicin in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was reverse transcribed and analyzed by QRT-PCR using primers for human *CYP3A4*. Values represent the average of triplicates ± 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$_2$, 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$_2$ 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$_2$ 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$_2$ plays several important roles in bone metabolism. Similar to its role in promoting blood clotting, vitamin K$_2$ is an essential cofactor for γ-carboxylase, an enzyme that catalyzes the conversion of specific glutamic acid residues to Gla residues. Vitamin K$_2$ is required for γ-carboxylation of bone matrix Gla-containing proteins such as MGP and osteocalcin. Incomplete γ-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$_2$ 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$_2$ decreases bone resorption by inhibiting the formation of osteoclasts (10) as well as their bone resorptive activity (11, 12). Vitamin K$_2$ treatment also induces apoptosis of osteoclasts (13) while inhibiting apoptosis of osteoblasts (71) thereby shifting the balance toward bone formation. Vitamin K$_2$ was also shown to enhance the induction of osteocalcin mRNA levels mediated by co-administered 1α,25-(OH)$_2$ vitamin D$_3$ (7, 37). The function of vitamin K$_2$ 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-

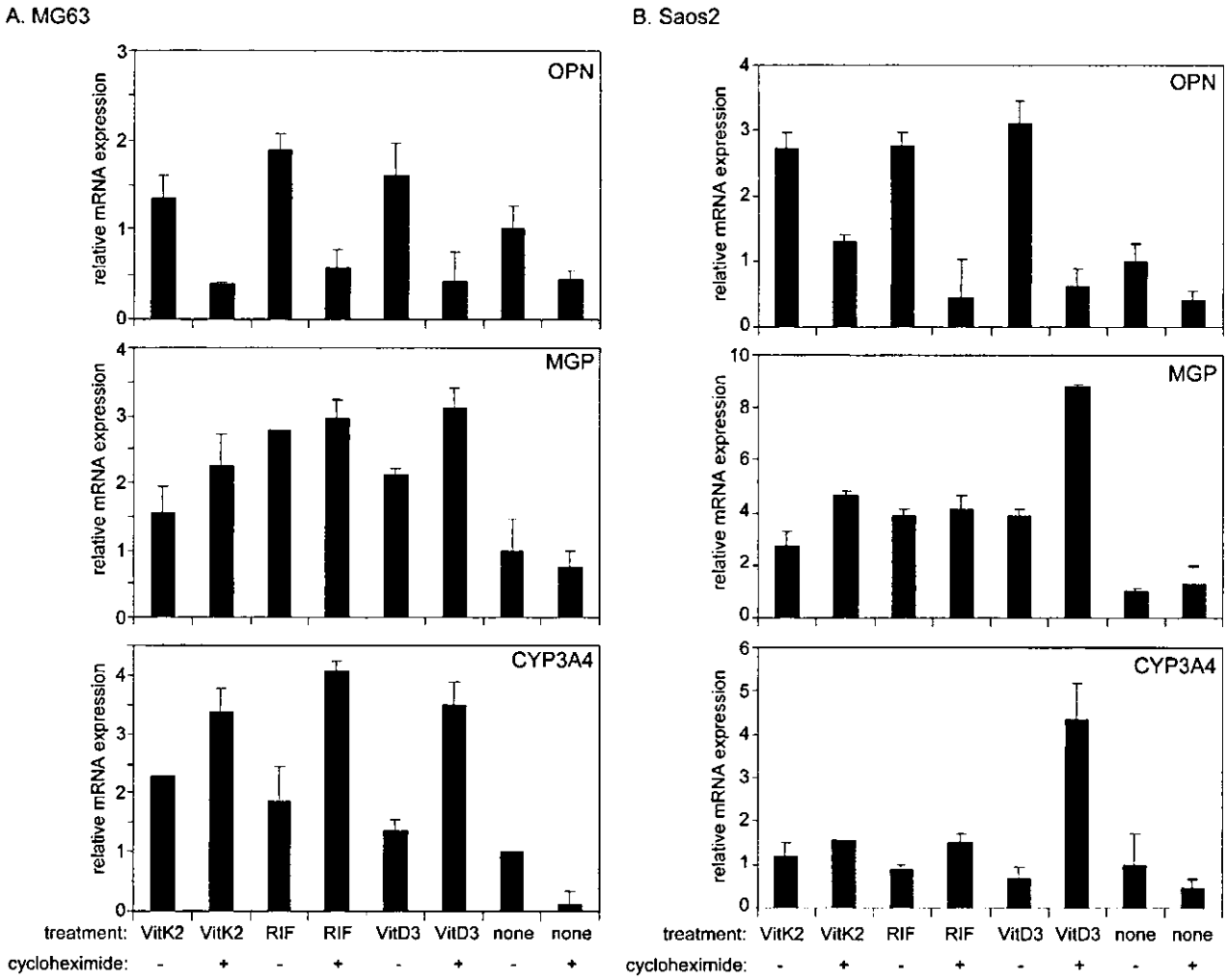

FIG. 6. **Vitamin K_2 has direct transcriptional effects on MGP and *CYP3A4* but not OPN.** Either MG-63 (*A*) or Saos-2 cells (*B*) were pretreated with 10 μg/ml cycloheximide for 30 min prior to addition of 10 μM vitamin K_2, 10 μM rifampicin, 10 nM vitamin D_3, or vehicle only. Total RNA was harvested 24 h later, reverse transcribed, and analyzed by QRT-PCR using primers for OPN, MGP, and *CYP3A4*. Values represent the average of triplicates ± 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_2 activated SXR-dependent reporter gene constructs led us to hypothesize that vitamin K_2 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_2 activated SXR in a dose-dependent manner (Fig. 2*A*) and up-regulated the known SXR target gene *CYP3A4* (Fig. 2*B*) as well as the osteoblast marker genes ALP, OPN, MGP, and OPG (Fig. 4). Vitamin K_2 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_2 are required for SXR binding (Fig. 3) and activation (Fig. 2), it should be noted that the therapeutic levels of vitamin K_2 in the plasma of patients average about 1 μM (74), and the levels in bone (2.5-fold) and liver (10-fold) are considerably higher (75). Therefore, the concentration of vitamin K_2 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_2. MGP and OPG were induced by vitamin K_2 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_2 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_2 on osteoblasts, vitamin K_2-mediated inhibition of osteoclast function, induction of osteoclast apoptosis, and inhibition of osteoblast apoptosis suggest a

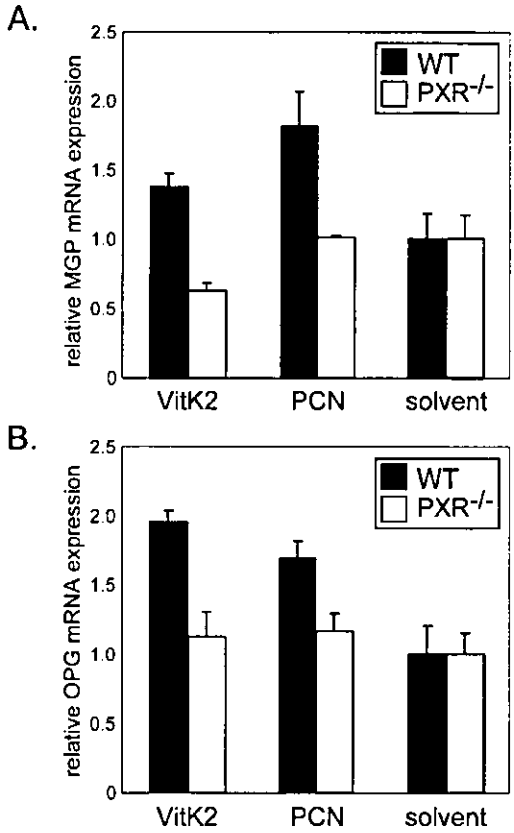

FIG. 7. **Vitamin K_2 induction of bone marker genes in murine primary osteocytes requires PXR.** Osteocyte cultures derived from calvaria isolated from WT (*black bars*) or PXR knockout mice (*white bars*) were treated with 10 μM vitamin K_2 or PCN or vehicle only for 48 h. Total RNA was isolated, reverse transcribed, and analyzed by QRT-PCR using primers specific for mouse MGP or OPG. Values represent the average of triplicates ± S.E. *Vit*, vitamin.

much broader role for vitamin K_2. 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_2 on bone marker genes. Therefore, our demonstration that vitamin K_2 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_2 on osteoblasts and underscores the clinical utility of vitamin K_2 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_2 (Fig. 4). Therefore, we conclude that vitamin K_2 exerts positive effects on the expression of osteoblastic marker genes via transcriptional activation of SXR. The loss of vitamin K_2 inducibility of osteoblast markers in primary bone cell cultures from *PXR* knockout mice confirms that SXR/PXR plays a central role in vitamin K_2 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.

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