Parathyroid hormone (1–34) and its analogs differentially modulate osteoblastic Rankl expression via PKA/SIK2/SIK3 and PP1/PP2A–CRTC3 signaling

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Osteoporosis can result from the loss of sex hormones and/or aging. Abaloparatide (ABL), an analog of parathyroid hormone–related protein (PTHrP(1–36)), is the second osteoanabolic therapy approved by the United States Food and Drug Administration after teriparatide (PTH(1–34)). All three peptides bind PTH/PTHrP receptor type 1 (PTHR1), but the effects of PTHrP(1–36) or ABL in the osteoblast remain unclear. We show that, in primary calvarial osteoblasts, PTH(1–34) promotes a more robust cAMP response than PTHrP(1–36) and ABL and causes a greater activation of protein kinase A (PKA) and cAMP response element–binding protein (CREB). All three peptides similarly inhibited sclerostin (Sost). Interestingly, the three peptides differentially modulated two other PKA target genes, c-Fos and receptor activator of NF-κB ligand (Rankl), and the latter both in vitro and in vivo. Knockdown of salt-inducible kinases (SIKs) 2 and 3 and CREB-regulated transcription coactivator 3 (CRTC3), indicated that all three are part of the pathway that regulates osteoblastic Rankl expression. We also show that the peptides differentially regulate the nuclear localization of CRTC2 and CRTC3, and that this correlates with PKA activation. Moreover, inhibition of protein phosphatases 1 and 2A (PP1/PP2A) activity revealed that they play a major role in both PTH-induced Rankl expression and the effects of PTH(1–34) on CRTC3 localization. In summary, in the osteoblast, the effects of PTH(1–34), PTHrP(1–36), and ABL on Rankl are mediated by differential stimulation of cAMP/PKA signaling and by their downstream effects on SIK2 and -3, PP1/PP2A, and CRTC3.

Osteoporosis is a prevalent disease in our aging population. Annually, more than 8.9 million osteoporotic fractures are reported worldwide, with a total cost of $25 billion (1). Standard treatment with bisphosphonates curtails bone resorption, but does not aid in forming new bone. Teriparatide, or PTH(1–34), was the first FDA-approved osteoanabolic therapy (2, 3) and studies aimed at delineating its effects on the skeleton remain under close investigation (4–6). The second and only other FDA-approved osteoanabolic treatment, abaloparatide (ABL),2 is an analog of parathyroid hormone–related protein (PTHrP(1–34)), and phase III clinical trials reported that ABL was similar, if not superior, to teriparatide in increasing bone mineral density in osteoporotic, postmenopausal women (7). Interestingly, it was also reported that serum CTX levels, the marker for bone resorption, were significantly lower than in patients treated with teriparatide. Additionally, bone resorption markers were unperturbed in ovariectomized rats (8) and monkeys (9) treated with ABL compared with the ovariectomized control groups.

PTH(1–34), PTHrP(1–36), and ABL bind the same G protein–coupled receptor, parathyroid hormone receptor, type 1 (PTHR1), which is expressed in the cells of the osteoblast lineage, chondrocytes, and kidneys (10). Although a variety of signaling cascades are activated upon ligand-receptor binding (reviewed in Ref. 11), the Gαq/cAMP pathway is known to mediate the bone anabolic response of PTH (12). Studies have described PTH signaling in the osteoblast/osteocyte (reviewed in Ref. 13) and its effects on their respective transcriptionomes (14, 15), but the specific effects of PTH on the regulation of the pro-osteoclastogenic cytokine, receptor activator of NF-κB ligand (RANKL), are of great interest due to its central role in the cross-talk between osteoblasts/osteocytes and the bone resorbing cells of the skeleton, the osteoclast (16–22).

Recently, a study showed that in the osteocyte, PTH regulates Rankl expression through the inhibition of salt-inducible kinases (SIKs) and nuclear translocation of cAMP-regulated transcriptional coactivator, CRTC2 (23), which is a known substrate of SIKs (24). This study also reported that PTH-induced SIK inhibition allows for nuclear translocation of histone

2 The abbreviations used are: ABL, abaloparatide; PTHrP, parathyroid hormone–related protein; RANKL, receptor activator of NF-κB ligand; SIK, salt-inducible kinase; HDAC, histone deacetylase; PP2A, protein phosphatase 2A; PKA, protein kinase A; CREB, cAMP response element–binding protein; SOST, sclerostin; MMP, matrix metalloproteinase; qRT, quantitative RT; myr-PKI, myristoylated PKA inhibitor; OA, okadaic acid; IBMX, isobutylmethylxanthine; FBS, fetal bovine serum; HRP, horseradish peroxidase; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole.
deacetylases, HDACs 4 and 5, which inhibit the transcription factor, MEF2c, and therefore decrease Sost expression (23). Previous studies have also reported differences in downstream PTHR1 effects between PTH(1–34), PTHrP(1–36), and ABL (25). Based on this, we hypothesized that in the osteoblast, PTH(1–34), PTHrP(1–36), and ABL would differentially modulate this particular signaling axis and ultimately result in differing effects on the regulation of Rankl mRNA. Additionally, because this cascade was reported to involve an unknown serine/threonine phosphatase and PTHrP is able to activate protein phosphatase 2A (PP2A) in the chondrocyte (26), we hypothesized that this activation would also be important in the osteoblast.

Here, we report that PTH(1–34), PTHrP(1–36), and ABL differentially induce cAMP/PKA signaling. Accordingly, different levels of PKA activation lead to differences in the up-regulation of Rankl mRNA by all three peptides, and we found that this phenomenon is a direct result of PKA activation. We also found that osteoblastic Rankl expression requires SIKs 2 and 3, CRTCs, and PP1/PP2A and that the three peptides, through PP1/PP2A, differentially regulate the nuclear translocation of CRTCs 2 and 3. Interestingly, PTH(1–34), PTHrP(1–36), and ABL equally inhibit osteoblastic Sost expression, which illustrates a demarcation between SIK/HDAC/MEF2c and SIK/CRTC signaling. Taken together, our data show that these peptides differentially regulate a particular arm of the cAMP/PKA/SIK signaling axis and ultimately result in lower expression of Rankl by ABL, which may provide an explanation for the decreased resorptive effects of ABL observed in vivo.

**Results**

**PTH(1–34), PTHrP(1–36), and ABL similarly bind PTHR1**

Previous studies aimed at comparing the binding efficacies between PTH(1–34) or PTHrP(1–36) with their cognate receptor, PTHR1, have shown that although both are able to bind with similar affinity, PTH(1–34) binds PTHR1 in both high affinity receptor conformations, R0 (G-protein uncoupled) and R5 (G protein-coupled), whereas PTHrP(1–36) favors the latter (27). These differences were also reflected in the ability of PTH(1–34) to elicit a more sustained cAMP response upon receptor internalization, whereas PTHrP(1–36) could not (28). More recently, comparative studies between PTH(1–34), PTHrP(1–36), and ABL in GP-2.3 cells have shown that ABL binds more selectively to the R5 conformation of PTHR1, thus resulting in a more transient cAMP response compared with PTH(1–34) (25). To determine whether these differences were observable in osteoblastic cells, we measured the capacities of PTH(1–34), PTHrP(1–36), and ABL to compete for binding to PTHR1 in the osteosarcoma cell line, UMR 106, using [125I]Nle8,21,Tyr34 rPTH(1–34) as a tracer radioligand (Table 1). We found no significant difference in pIC50 between the three peptides under these conditions.

**PTH(1–34) elicits a stronger cAMP response and downstream effects on PKA/CREB compared with PTHrP(1–36) and ABL**

Because we did not observe differences in PTHR1 binding, we hypothesized that in primary murine calvarial osteoblasts, a system that more closely resembles the physiological setting, more pronounced differences that will integrate downstream signaling would be observed. Upon treating primary osteoblasts with PTH(1–34), PTHrP(1–36), or ABL, we found that all three peptides achieved maximal cAMP responses at 30 min, but PTH(1–34) resulted in higher cAMP concentrations (240 ± 23 pmol/µl), compared with PTHrP(1–36) (175 ± 4 pmol/µl) or ABL (45 ± 5 pmol/µl; Fig. 1a). Because cAMP levels are the rate-limiting step to PKA activation, we therefore sought to observe how these differing cAMP responses affected PKA activity. Indeed, we found that whereas all three peptides achieve peak PKA activation between 1 and 2 min, PTH(1–34) reached 72% PKA activation, whereas PTHrP(1–36) and ABL resulted in 50 and 30%, respectively (Fig. 1b). The differences in magnitude of each peptide’s cAMP response were proportional to their effect on PKA activation. To assess the dose-dependent effects of these peptides, we performed a dose-response at 1 min and found that at 0.75 nM, PTH(1–34) resulted in 40% PKA activation, whereas PTHrP(1–36) and ABL elicited less than half of that at that dose (16 and 18%, respectively). Additionally, the relative differences in PKA activation between all three peptides grew smaller with larger doses, but ABL did not achieve the same levels of PKA activation (53%) as PTH(1–34) (73%) or PTHrP(1–36) (68%) (Fig. 1c). Previously, we reported that cAMP/PKA signaling leads to activation of the cAMP response element–binding protein (CREB) and the stimulation of c-Fos expression in osteoblastic cells (29, 30). We found that all three peptides resulted in maximal CREB activation (assayed by phosphorylation of CREB at Ser-133) at 5 min, and again, in proportions similar to their effects on cAMP/PKA (PTH(1–34) = 77%, PTHrP(1–36) = 55%, ABL = 20%; Fig. 1d). Dose-response of CREB activation at 5 min shows a similar curve as seen with PKA activation (Fig. 1e), thus confirming that the initial differences in cAMP response exerted by PTH(1–34), PTHrP(1–36), and ABL on cAMP are directly reflected in their downstream effectors, PKA and CREB.

**PTH(1–34), PTHrP(1–36), and ABL differentially regulate osteoblastic genes c-Fos and Rankl**

To determine the consequences, if any, of the initial signaling differences between PTH(1–34), PTHrP(1–36), and ABL on the regulation of osteoblastic genes, we performed time course and dose-response analyses, followed by qRT-PCR on a set of osteoblastic genes. After treating primary osteoblasts and/or osteoblastic UMR 106-01 cells with peptide concentrations ranging from 0.001 to 100 nM at 1, 2, and 4 h, we found no significant differences with almost all of the genes assayed (Table 2). PTH is a known regulator of sclerostin (Sost) (31) and...
Regulation of Rankl by PTH(1–34), PTHrP(1–36), and ABL

a) cAMP levels over time (min): PTH (1-34), PTHrP (1-36), ABL

b) Relative PKA Activation over time (min): PTH (1-34), PTHrP (1-36), ABL

c) Relative PKA Activation over dose (nM): PTH (1-34), PTHrP (1-36), ABL

d) pCREB/CREB ratio over time (min): PTH (1-34), PTHrP (1-36), ABL

e) pCREB/CREB ratio over dose (nM): PTH (1-34), PTHrP (1-36), ABL
Regulation of Rankl by PTH(1–34), PTHrP(1–36), and ABL

Differential regulation of Rankl relies on the PKA/PP1/PP2A signaling axis in vitro

Rankl gene expression has been shown to be regulated by cAMP/PKA activity in vascular cells (37), mesenchymal stem cells (38), and osteocytes (21, 23), and due to the differential effects of PTH(1–34), PTHrP(1–36), and ABL on this signaling axis, we hypothesized that modulating PKA activity would abolish any differences they elicit in the osteoblast with respect to Rankl mRNA expression. Indeed, pre-treatment with the PKA agonist, 8-bromo-cAMP (8-Br-cAMP) alone increased Rankl mRNA levels by 38-fold and abolished the effects of all three peptides on RANKL, suggesting Rankl mRNA cannot be enhanced above a maximal level (Fig. 3a). Conversely, pre-treatment with the PKA-specific antagonist, myristoylated PKA inhibitor (myr-PKI), did not affect basal Rankl mRNA levels, but completely abolished the effects of all three peptides (Fig. 3a). To confirm these findings in a different system, we performed similar experiments with primary osteoblasts derived from bone chips of adult C57Bl/6 WT femurs and obtained identical results (Fig. S1).

Protein phosphatase 1 (PP1) and PP2A are two major Ser/Thr phosphatases that account for the majority of phosphatase activity in eukaryotes. PP1 activity is modulated through the formation of heterotrimeric complexes with numerous regulatory subunits. Targeting to specific substrates involves interaction of PP1 catalytic subunit C with targeting subunits, of which 30 have been identified (39). In skeletal muscle, PP1 activity has been shown to be indirectly regulated by PKA, because it directly phosphorylates a PP1 targeting subunit, G<sub>32</sub> (40). PP2A has been implicated in the regulation of Rankl in MC3T3-E1 cells (41), in addition to its involvement in PTHrP signaling in the chondrocyte (26). PP2A holoenzymes consist of a dimer formed by a catalytic C subunit (PP2A-C) and scaffolding A subunit (PP2A-A), which is targeted to the substrate by a regulatory B subunit (42). We found that MC3T3-E1 cells are not PTH-responsive (data not shown), and therefore sought to determine phosphatase involvement in primary calvarial osteoblasts. Okadaic acid is a commonly used inhibitor of PP1/PP2A, and inhibition of either or both phosphatases can be determined by using two different concentrations (PP2A: IC<sub>50</sub> = 0.02–0.5 nm; PP1: IC<sub>50</sub> = 10–200 nm). Inhibition of PP2A by 50 nm okadaic acid (OA) decreased basal levels of Rankl by 75% and attenuated the effects of PTH(1–34), PTHrP(1–36), and ABL by 50, 55, and 80%, respectively. Inhibition of both Ser/Thr phosphatases, PP1 and PP2A by 200 nm OA decreased basal osteoclasts, we sought to investigate the differential effects of PTH(1–34), PTHrP(1–36), and ABL on osteoblastic Rankl expression.

Table 2

| Gene ID | Gene name                     | PTH | PTHrP | ABL |
|---------|-------------------------------|-----|-------|-----|
| Ihsp    | Bone Sialoprotein             | −   | −     | −   |
| Sost    | Sclerostin                    | −   | −     | −   |
| Opg     | Osteoprotegerin               | NC  | NC    | NC  |
| Alp     | Alkaline phosphatase          | +   | +     | +   |
| Coll1a1 | Collagen type 1               | +++ | +++   | +++ |
| Runx2   | Runx-related transcription factor 2 | + | + | + |
| Mmp13   | Matrix metalloproteinase 13   | +   | +     | +   |
| Mep1    | Monocyte chemotaxattractant protein-1 | + | + | + |
| c-Fos   | c-Fos                         | +++ | +++   | +++ |
| Rankl   | Receptor activator of nuclear factor kB ligand | +++ | +++ | +++ |

−, decrease; +, increase; and NC, no change.

matrix metalloprotease (MMP) 13 (32) expression, and all three peptides led to a 2-fold increase in Mmp13 mRNA in primary osteoblasts (Fig. 2a) and a complete suppression of Sost mRNA in UMR 106-01 cells (Fig. 2b), with no significant differences in the dose-response between the three peptides. Interestingly, both genes have been reported to be regulated by PTH in a cAMP/PKA/HDAC4/5-dependent mechanism (33, 34), suggesting that this arm of the cAMP/PKA signaling axis is similarly regulated by all three peptides, and likely is sensitive to low PKA activation. Because the inhibition of Sost represents one of the major anabolic effects of PTH, its identical inhibition by PTH(1–34), PTHrP(1–36), and ABL may account for the similar osteoanabolic effects observed in vivo.

Importantly, our analyses revealed two genes that were differentially regulated by the peptides: the transcription factor, c-Fos, and the pro-osteoclastogenic factor, Rankl. We have shown that c-Fos is an immediate-early downstream effector of PTH by way of cAMP/PKA signaling (29, 30), and here, we confirm peak c-Fos mRNA abundance at 1 h, where PTH(1–34) resulted in a 10-fold increase, whereas PTHrP(1–36) and ABL resulted in 5- and 4-fold increases, respectively, and all returned to basal levels by 4 h (Fig. 2c). Dose-response analysis of c-Fos mRNA abundance (Fig. 2d) mirrored the differential effects of all peptides on cAMP/PKA/CREB signaling and confirmed that in osteoblasts, PTHrP(1–36) and ABL are weaker effectors of this arm of cAMP/PKA signaling compared with PTH(1–34).

Rankl is a well-studied PTH-responsive gene in osteoblasts and osteocytes (16–18, 21, 35, 36), and here, we found peak fold-induction by all three peptides at 4 h and a significant difference in the effect of the peptides at 1 h (PTH(1–34) = 8-fold, PTHrP(1–36) = 3-fold, ABL = 3-fold; Fig. 2e and f). Due to the central role of RANKL as the driver of osteoclastogenesis and a mediator between osteoblasts/osteocytes and osteoclasts, we sought to investigate the differential effects of PTH(1–34), PTHrP(1–36), and ABL on osteoblastic Rankl expression.

Figure 1. PTHrP(1–36) and ABL are weaker activators of cAMP/PKA/CREB signaling compared with PTH(1–34). a, cAMP stimulation. Primary calvarial osteoblasts were treated with 750 nM peptides at the indicated times. Cells were lysed in buffer containing 2 mM IBMX. cAMP detection was performed by ELISA and readings were calculated against a cAMP standard curve as described under "Experimental procedures." Groups with dissimilar letters signify p < 0.05 compared with all the other groups. Area-under-the-curve values for PTH(1–34) were 11,971 (S.D. = 710.4); PTHrP(1–36), 9,253 (S.D. = 816.8); and ABL, 2,036 (S.D. = 309.9). All groups are p < 0.05 compared with each other. b and c, PKA activation. Primary calvarial osteoblasts were treated with (b) 10 nM peptides for up to 5 min or (c) with the indicated concentrations for 1 min prior to lysis. Relative PKA activity is expressed as a ratio of phosphorylated PKA substrate over total substrate. d and e, CREB phosphorylation. Primary calvarial osteoblasts were treated with (d) 10 nM peptides from 1 to 60 min or (e) with the indicated doses for 5 min prior to whole cell lysis and immunoblotting. Samples were quantified using Bio-Rad ImageLab software and calculated against the total detectable protein per lane. All data represent the mean ± S.D. of n = 3 independent experiments and images are representative of mean results.
levels of Rankl by 64% and decreased the effects of PTH(1–34), PTHrP(1–36), and ABL by 93, 59, and 75%, respectively (Fig. 3b). Adenoviral-mediated expression of the PP2A-specific inhibitor, SV40 small T-antigen, attenuated the effects of all peptides by 60–70% (Fig. 3c), and abolished any differential effects between the peptides, implicating PP2A as a phosphatase that plays a major role in the regulation of Rankl mRNA expression. Nevertheless, PP1 may also be involved. These data show that cAMP/PKA and PP1/PP2A signaling is required for maximal osteoblastic Rankl expression.

**Differential regulation of Rankl is observed in vivo and in a PKA-dependent fashion**

To corroborate our data in vivo, we injected 4-month-old male C57Bl/6 WT mice with 80 μg/kg/day of vehicle or peptides for 6 weeks and harvested tibial shafts to enrich for RNA derived from osteoblast/osteocyte-rich cortical bone. We found that PTH(1–34) injections led to a 3-fold increase in Rankl, whereas PTHrP(1–36) and ABL only resulted in a 1.5-fold increase (Fig. 3d). To examine the effects of PKA up-regulation in vivo, we deleted the regulatory subunit of PKA (PRKAR1a) in osteoblasts by crossing Col1-Cre-ERT mice with Prkar1afl/fl mice and induced deletion by tamoxifen injections. Four-week-old male and female mice were injected for 3 weeks and 5-month-old mice were injected once a week for 4 weeks prior to tissue harvest. We observed an increase in Rankl mRNA of 2.8-fold in 7-week-old female mice and 4-fold in male mice (Fig. 3e). We also observed a 5-fold increase of Rankl mRNA in 6-month-old mice (Fig. 3f). Taken together, the in vivo findings corroborate the results seen in primary cultured...
Regulation of Rankl by PTH(1–34), PTHrP(1–36), and ABL

Figure 3. Differential regulation of Rankl in vivo and in vitro utilizes the cAMP/PKA and R1P2A signaling axis. a, primary calvarial osteoblasts were pre-treated for 30 min with 1 mM 8-Br-cAMP or for 4 h with 15 μM myr-PKI prior to 1 nM peptides for 4 h. Data are expressed relative to the housekeeping gene β-actin. b, primary calvarial osteoblasts were pre-treated with 50 or 200 nM OA for 2 h prior to 4 h treatment with 1 nM peptides (1–34). Data are expressed relative to the housekeeping gene β-actin. c, primary calvarial osteoblasts were transfected overnight with adenoviral SV40 small T-antigen or GFP empty vector. Subsequently, cells were treated with 10 nM peptides for 4 h. Data are expressed relative to the housekeeping genes β-actin and Gapdh, normalized to each control, and represent the mean ± S.E. of n = 3 independent experiments. d, 16-week-old C57Bl/6 WT male mice were given 80 μg/kg/day of vehicle or peptides for 6 weeks (n = 10/group). Eighteen h after the final injections, tibial shaft RNA was harvested and processed for qRT-PCR. Data are expressed relative to the housekeeping genes β-actin and Gapdh and represent the mean ± S.E. e, 18-week-old C57Bl/6 WT male mice were given 80 μg/kg/day of vehicle or peptides for 6 weeks (n = 10/group). Eighteen h after the final injections, tibial shaft RNA was harvested and processed for qRT-PCR. Data are expressed relative to the housekeeping genes β-actin and Gapdh and represent the mean ± S.E. Groups with dissimilar letters signify p < 0.05.

Osteoblastic Rankl expression depends on SIK2/3 and CRTC3

Previous studies had reported that PTH-induced osteoblastic Rankl expression depended on the PKA-dependent phosphorylation of HDAC4 and its dissociation from the transcription factor, MEF2c (43). Surprisingly, we found that siRNA-mediated knockdown of either HDAC4 or MEF2c did not abrogate the effect of PTH(1–34) on Rankl mRNA expression (Fig. S2). Recently, SIKs have been shown to be prominent effectors of osteocytic Rankl and Sost expression. PTH has been shown to promote the PKA-dependent inhibitory phosphorylation of SIK2 and SIK3, which in turn, decreases the tonic phosphorylation of CREB-regulated transcription coactivator, CRTC2. CRTC2 dephosphorylation leads to its dissociation from 14-3-3 cytoplasmic proteins and allows its nuclear translocation and subsequent association with a bZIP transcription factor at the −75 kb enhancer region of the Rankl promoter (23). In primary osteoblasts, knockdown of SIK2 and SIK3 results in a 5-fold increase in basal Rankl mRNA levels, and a 2–3-fold increase in the effects of all peptides (Fig. 4a). Interestingly, knockdown of CRTC2 or CREB did not significantly alter the basal levels of Rankl mRNA or the effects of PTH(1–34), PTHrP(1–36), or ABL (Fig. 4b), implying that PKA signaling modulates osteoblastic Rankl expression through other effectors. However, knockdown of CRTC3 decreased basal Rankl mRNA levels by 50%, and reduced the effects of all peptides by 30% (Fig. 4c). This suggests that SIK2, SIK3, and CRTC3 play an important role in osteoblastic Rankl expression, and that CRTC2 and/or CRTC3 do not require CREB, but presumably bind other transcription factors containing bZIP domains on the Rankl promoter.

Compared with PTH(1–34), PTHrP(1–36), and ABL lead to less nuclear translocation of CRTC2 and CRTC3

SIKs have been shown to be directly inhibited by cAMP/PKA activity (44) and their direct targets (CRTC family members) are regulated by phosphorylation-dependent nuclear-cytoplasmic shuttling (24). We and others have shown that CRTC2 and CRTC3 are involved in osteocytic/osteoblastic Rankl expression. Therefore, we hypothesized that PTH(1–34), PTHrP(1–36), and ABL would differentially regulate nuclear/cytoplasmic CRTC2/CRTC3 distribution in osteoblasts. After 60 min of treatment, PTH(1–34) resulted in the highest proportion of nuclear-localized CRTC2 (70%) and CRTC3 (79%), whereas PTHrP(1–36) resulted in lower CRTC2 (30%) and CRTC3 (20%) nuclear localization, and also ABL (CRTC2 = 40%, CRTC3 = 46%, Fig. 5, a and b). These results mirror the relative effects of PTH(1–34), PTHrP(1–36), and ABL on cAMP/PKA signaling and suggest that compared with PTH(1–34), the...
decreased effects of PTHrP(1–36) and ABL on this particular signaling axis lead to a weaker inhibition of SIK2 and SIK3 and consequently, lesser nuclear localization of CRTC2 and CRTC3, which ultimately results in relatively lower levels of Rankl mRNA.

**PTH-induced translocation of CRTC2 and CRTC3 requires SIK2 and PP1/PP2A**

Because we found that PTH(1–34) and its analogs require SIKs 2 and 3, CRTC3, and PP1/PP2A to modulate Rankl mRNA expression, and all three peptides affect the nuclear translocation of CRTC2/CRTC3, we assessed the potential of PTH(1–34), the strongest of the three cAMP/PKA agonists, to regulate the localization of CRTC2 and CRTC3 in the absence of SIK2 and PP1/PP2A activity. siRNA-mediated SIK2 knockdown resulted in a 36% increase in basal levels of nuclear CRTC2, and enhanced the PTH(1–34)-induced nuclear translocation by 10% (Fig. 6a), thus confirming its inhibitory effect on CRTC2. Surprisingly, knockdown of SIK2 did not have any significant effects on basal levels of nuclear CRTC3, nor did it alter the...
Inhibition of PP1/PP2A by 200 nM OA decreased basal levels of nuclear CRTC2 by 17%, and decreased the effects of PTH(1–34) by 32% (Fig. 6c). Addition-
ally, PP1/PP2A inhibition did not alter basal levels of nuclear CRTC3, but dramatically decreased the effects of PTH(1–34) on CRTC3 localization by 76% (Fig. 6d). These data sug-
gest that whereas both PP1/PP2A and SIK2 are responsible for CRTC2 localization, PP1/PP2A seem to play a more
prominent role in controlling nuclear CRTC3 localization in PTH signaling.

Discussion

We have shown that whereas PTH(1–34), PTHrP(1–36), and ABL exert similar effects on a subset of genes (Table 2), PTHrP(1–36) and ABL appear to be weaker agonists of the cAMP/PKA/SIK2–SIK3/CRTC signaling axis, and this is par-

Figure 6. PTH(1–34) regulates CRTC2 and CRTC3 subcellular localization through SIK2 and PP1/PP2A, respectively. a and b, primary calvarial osteoblasts were
transfected with 1 μM si-control or si-SIK2 for 72 h prior to 1 h treatment with 50 nM PTH(1–34). c and d, primary calvarial osteoblasts were pre-treated with 200 nM OA
for 2 h prior to a 1 h treatment with 50 nM PTH(1–34). Cells were incubated with primary antibodies to detect (a and c) CRTC2 and (b and d) CRTC3 localization. Images
were captured and analyzed as described in the legend to Fig. 5. For each condition, n = 500 ± 50 cells were counted and data represent the mean ± S.D. of n = 3
independent experiments. Images shown are representative of mean results. Groups with dissimilar letters signify p < 0.05.
particularly clear in the differential effects on Rankl mRNA, as well as in CRTC2/CRTC3 localization. Our in vitro binding data suggest that in osteosarcoma cells, ABL binding to PTHR1 is similar to PTH(1–34). Because the assay was performed on intact cells and without GTPγS, which, in assays performed in membranes, enriches for the G protein-uncoupled conformation (R°) through inducing receptor-G protein dissociation (45), we were unable to distinguish between R° and R⁵ and further studies must be conducted to delineate this. Nevertheless, the binding of ABL was slightly weaker (~2-fold) than that of human PTH(1–34) and significantly weaker (~10-fold) than that of a rat PTH(1–34) analog, which suggests a relatively weaker overall affinity of ABL as compared with PTH ligands for the rat PTHR1 in the intact cell line. Although the differences we report with respect to the cAMP responses elicited by the peptides vary slightly from other reports (25), we believe that this is due to the nature of the cell types being studied. Our system employs primary calvarial osteoblasts, which express native levels of PTHR1. Nonetheless, we observed time and dose-dependent differences between all three peptides in their ability to activate PKA (Fig. 1, b and c), in addition to downstream activation of CREB (Fig. 1, c and d), which are both sequentially downstream of cAMP stimulation. Our data show that this pathway is titratable, as observed with PKA agonists and antagonists (Fig. 3a), and with the deletion of the PKA regulatory subunit in vivo (Fig. 3, d and e).

Because we observed weaker cAMP/PKA signaling by PTHrP(1–36) and ABL compared with PTH(1–34), and others have reported that this signaling accounts for the inhibitory effects of PTH on osteocyte Sost expression through SIK2/HDAC4/5/MEF2c (23), we expected to find differential inhibition of osteoblast Sost expression by all three peptides. Surprisingly, their inhibitory effects were identical and relatively sensitive in UMR 106-01 cells (Fig. 2b). We attempted to replicate this in primary calvarial osteoblasts, but basal Sost levels were much lower compared with UMR 106-01 cells and we were unable to observe any effects with PTH(1–34) (data not shown). Nonetheless, this finding suggests clear differences in the effects of all three peptides on the SIK/HDAC4/5/MEF2c and SIK/CRTC signaling axes, and that the former is more sensitive to changes in cAMP/PKA activity. Also, the equipotent effects of PTH(1–34), PTHrP(1–36), and ABL on Sost suppression may account for the equipotent effects observed in vivo (7).

It is also interesting to note the differences between the effects of these peptides on Sost mRNA versus Rankl mRNA. The minimum dose required for an observable change in Sost mRNA was 0.1 nM (1/2 max = 0.085 nM), whereas the effects of all three peptides on RANKL mRNA were observable at 1 nM, and both effects are maximal at 4 h. Because the regulation of each gene requires transcriptional termination and activation for Sost and Rankl, respectively, it is conceivable that this indicates that transcriptional termination may require a weaker stimulus compared with transcriptional activation.

SIKs are direct downstream effectors of cAMP/PKA signaling and respond to metabolic changes in different systems (47–49). There appears to be redundancy between SIK2 and SIK3 (50), and we observed this in our system, because knockdown of either protein results in the modulation of Rankl mRNA (Fig. 4). Although knockdown of SIK2 yielded greater effects on Rankl mRNA compared with SIK3, SIK3 knockdown was still effective in increasing osteoblastic Rankl mRNA. Although SIK3 is not a reported downstream target of PTH signaling, SIK3 deficiency phenocopies PTHrP overexpression in chondrocytes, suggesting that it is somehow involved in the pathway (51, 52).

Our results show that the effects of PTH(1–34) and its analogs differ in the primary osteoblast versus the osteocyte with respect to the regulation of Rankl (Fig. 7). Although others have reported that CRTC2 is the main cofactor involved in osteocytic Rankl expression (23), we report that PTH(1–34) and its analogs indeed affect CRTC2 localization, but CRTC3 appears to be the main effector in the osteoblast. Kim et al. (53) observed redundancy between CRTC2 and CRTC3 in bone marrow, because CRTC2/CRTC3 double knockout mice exhibited embryonic lethality, but only one allele of either CRTC2 or -3 was sufficient for viability. It is possible that there is redundancy between CRTC2 and CRTC3 in the osteoblast, and perhaps there may even be events in late osteoblast differentiation/maturation that generate a switch from CRTC3 to CRTC2 with respect to Rankl signaling. Simultaneous knockdown of CRTC2 and CRTC3 in the osteoblast would shed light on these open issues. However, this was attempted and the knockdown was not effective in primary calvarial osteoblasts (data not shown).

Several groups have implicated CREB in Rankl regulation through its upstream enhancers (17, 36, 54, 55). Interestingly, we have shown that the CREB coactivator, CRTC3, plays a role...
in enhancing Rankl up-regulation, but knockdown of CREB did not hinder the effects of PTH(1–34) or the other peptides on Rankl mRNA (Fig. 4, b and c). We postulate that other transcription factors with bZIP domains are able to bind the CRTC localization at the −75 kb enhancer region, but further studies must be conducted to determine this.

Our data implicate PP1 and PP2A as important mediators of PKA signaling in PTH-stimulated osteoblasts. Indeed, inhibition of PP1/PP2A by okadaic acid significantly altered the effects of PTH(1–34) on both Rankl mRNA (Fig. 3b) and CRTC localization (Fig. 3c), but inhibition of PP2A by SV40 small T-antigen was sufficient to attenuate the effects of all peptides on Rankl mRNA (Fig. 3d). This suggests that the PKA agonist, FTY720, was able to increase Rankl mRNA by stimulating PP2A phosphorylation of PP2A substrate, elongation factor-2 and phosphorylation of PP2A substrate (or protein phosphatase 3) was implicated in CRTC dephosphorylation, which require further investigation. Although calcineurin (calcium/calmodulin-dependent Protein Kinase I) its activation with okadaic acid. However, inhibition of PKA with H-89 did not block cAMP-dependent dephosphorylation, suggesting that the pathway of PPA activation in these cells is cAMP-dependent, but PKA-independent. The complexity of its regulation leaves many open questions about the mechanism of osteoblastic effects of all peptides on Rankl mRNA (Fig. 3d). Thus, we hypothesize that PP2A is the main Ser/Thr phosphatase involved in PTH signaling. Additionally, pre-treatment with the PKA agonist, FTY720, was able to increase Rankl mRNA 2-fold (data not shown). It has previously been shown that PKA phosphorylates three Ser residues on the PP2A subunit, B56δ, and this leads to the up-regulation of PP2A activity. In another study using NRK-52E and L6 cells, cAMP up-regulation by forskolin and isobutylmethylxanthine (IBMX) led to decreased phosphorylation of PP2A substrate, elongation factor-2 and these dephosphorylation events were blocked by PP2A inhibition with okadaic acid. However, inhibition of PKA with H-89 did not block cAMP-dependent dephosphorylation, suggesting that the pathway of PPA activation in these cells is cAMP-dependent, but PKA-independent. The complexity of its regulation leaves many open questions about the mechanism of osteoblastic effects of all peptides on Rankl mRNA (Fig. 3d). Thus, we hypothesize that PP2A is the main Ser/Thr phosphatase involved in PTH signaling. Additionally, pre-treatment with the PKA agonist, FTY720, was able to increase Rankl mRNA 2-fold (data not shown). It has previously been shown that PKA phosphorylates three Ser residues on the PP2A subunit, B56δ, and this leads to the up-regulation of PP2A activity. In another study using NRK-52E and L6 cells, cAMP up-regulation by forskolin and isobutylmethylxanthine (IBMX) led to decreased phosphorylation of PP2A substrate, elongation factor-2 and these dephosphorylation events were blocked by PP2A inhibition with okadaic acid. However, inhibition of PKA with H-89 did not block cAMP-dependent dephosphorylation, suggesting that the pathway of PPA activation in these cells is cAMP-dependent, but PKA-independent. The complexity of its regulation leaves many open questions about the mechanism of osteoblastic effects of all peptides on Rankl mRNA (Fig. 3d). Thus, we hypothesize that PP2A is the main Ser/Thr phosphatase involved in PTH signaling. Additionally, pre-treatment with the PKA agonist, FTY720, was able to increase Rankl mRNA 2-fold (data not shown). It has previously been shown that PKA phosphorylates three Ser residues on the PP2A subunit, B56δ, and this leads to the up-regulation of PP2A activity. In another study using NRK-52E and L6 cells, cAMP up-regulation by forskolin and isobutylmethylxanthine (IBMX) led to decreased phosphorylation of PP2A substrate, elongation factor-2 and these dephosphorylation events were blocked by PP2A inhibition with okadaic acid. However, inhibition of PKA with H-89 did not block cAMP-dependent dephosphorylation, suggesting that the pathway of PPA activation in these cells is cAMP-dependent, but PKA-independent. The complexity of its regulation leaves many open questions about the mechanism of osteoblastic effects of all peptides on Rankl mRNA (Fig. 3d). Thus, we hypothesize that PP2A is the main Ser/Thr phosphatase involved in PTH signaling. Additionally, pre-treatment with the PKA agonist, FTY720, was able to increase Rankl mRNA 2-fold (data not shown). It has previously been shown that PKA phosphorylates three Ser residues on the PP2A subunit, B56δ, and this leads to the up-regulation of PP2A activity.
Regulation of Rankl by PTH(1–34), PTHrP(1–36), and ABL

collagenase A at 37 °C by five sequential digestions, and cells from digest 3–5 were collected and plated at a density of 6.4 × 10^5 cells/cm² in αMEM supplemented with 10% FBS and 100 units/ml of penicillin and 100 μg/ml of streptomycin. After reaching confluence, osteogenic medium (50 μg/ml of ascorbic acid) was added for 3 days to allow osteoblastic differentiation. Primary bone cells were harvested using femoral chips from 3-month-old male C57BL/6 WT mice. Cells were cultured to confluence and after the first plating, were treated similarly to primary calvarial osteoblasts. The osteoblastic phenotype of all primary cells was confirmed by measuring the relative mRNA levels of osteoblastic genes, Col1α1 and Alp. For all experiments, cells were serum-starved with 0.1% FBS 24 h prior to peptide treatments.

In vivo study design

All experiments using mice were performed following protocols approved by the New York University Institutional Animal Care and Use Committee (IACUC). Intermittent subcutaneous peptide injections of 80 μg/kg/day were administered to 4-month-old C57BL/6 male mice for 6 weeks (n = 10/group). Eighteen h after the final injection, tibial shafts were harvested and processed for qRT-PCR analyses. Additionally, 4-week-old Col1-Cre-ER^T2/Pkraiα^fl/fl mice (male: n = 9, female: n = 8) were injected with 1 mg of tamoxifen/mouse once a week for 3 weeks and 5-month-old Col1-Cre-ER^T2/Pkraiα^fl/fl mice (n = 7) were injected with 2.5 mg of tamoxifen once a week for 4 weeks prior to tibial mRNA harvest. Pkraiα^fl/fl mice injected with tamoxifen were used as controls.

In vitro binding assays

Ligand binding to the rat PTHR1 in intact UMR 106 cells was assessed by competition methods using [125I]-Nle^8,21,Tyr^34]rat PTH(1–34) as a tracer radioligand (2.2 Ci/mmoll). Assays were performed in 96-well plates containing confluent cell monolayers in a buffer of Hanks’ balanced salts solution supplemented with 10 mM HEPES, pH 7.4, and 0.1% BSA. Reactions contained radioligand (30,000 cpm/well) and varying concentrations of an unlabeled ligand, and were incubated at 4 °C for 17 h. The wells were then rinsed three times with cold buffer, lysed with 1 M NaOH, and the lysate was counted for γ-irradiation. Data from 4 independent experiments, each with either 3 replicates per dose-point (1 plate, n = 2) or 6 replicates per dose-point (2 plates, n = 2) were combined and plotted by fitting to a sigmoidal dose-response model with variable slope, such that each data point is an average (±S.E.) of values from 4 independent experiments. Reported pIC_{50} values are the averages (±S.E.) of the values obtained for each peptide in each of the 4 experiments.

In vitro cAMP and PKA assays

cAMP concentrations were measured using a cAMP XP assay kit and performed as described by the manufacturer (Cell Signaling). Briefly, cells were plated in triplicate in 96-well plates and cultured as described above. Following peptide treatments using αMEM supplemented with 0.1% FBS, the cells were washed in warm 1× PBS and lysed with 100 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_4VO_4, 1 μg/ml of leupeptin) supplemented with 2 mM IBMX. Fifty μl were transferred into microtiter plates coated with anti-cAMP rabbit mAb. HRP substrate TMB was added to develop color, spectrophotometry measurements were taken at 405 nm, and readings were calculated against a cAMP standard curve. For PKA assays, cells were plated in triplicate in 6-well dishes and cultured as described above. PKA activity was measured using a PepTag nonradioactive protein kinase A assay and performed as described by the manufacturer (Promega). Following peptide treatments using αMEM supplemented with 0.1% FBS, the cells were washed in warm 1× PBS, scraped, and lysed in a Dounce homogenizer with 200 μl of PKA extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin) and 10 μl were incubated with PKA reaction buffer and Peptag A1 Peptide PKA substrate (P-L-S-R-T-L-S-V-A-A-K). Samples were processed in 1% agarose gels and imaged with Bio-Rad gel documentation system for densitometric detection of PKA substrates. Samples were analyzed by measuring the ratio between phosphorylated PKA substrates over total PKA substrates.

siRNA knockdown

si-control, si-SIK2, si-SIK3, si-CRTC2, si-CRTC3, si-CREB, si-HDAC4, and si-MEF2c Accell siRNA reagents were purchased from Dharmacon and transfection was performed as described by the manufacturer. Briefly, siRNA was diluted to a final concentration of 1 μM with αMEM supplemented with 2.5% fetal bovine serum (FBS) and incubated at 37 °C for 1 h. siRNA media were then added to cells and incubated for 72 h prior to peptide treatments. Confirmation of knockdowns was confirmed by Western blotting or RNA (Fig. S3).

Adenoviral infection

For adenoviral infection, primary calvarial osteoblasts were plated as described above. Cells were exposed to 10 pfu/cell of Ad-SV40 small T antigen overnight at 37 °C. The following morning, cells were serum-starved in αMEM supplemented with 0.1% FBS for 4 h prior to peptide treatments.

qRT-PCR

Total RNA was extracted using TRizol (Sigma). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a TaqMan reverse transcription kit (Applied Biosystems) with hexamer primers following the protocol described by the manufacturer. Gene expression levels were measured using SYBR Green PCR reagents (Applied Biosystems). Primer pairs used for quantitative detection of gene expression are listed in Table S1. The quantity of mRNA was calculated by normalizing the threshold cycle value (Ct) of specific genes to the Ct of the housekeeping gene β-actin and/or glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

Western blotting

Whole cell lysates were prepared using RIPA buffer (150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma) supplemented with 1× HALT protease and phosphatase inhibitor cocktails (Thermo). Total protein
concentration was determined by Bradford reagents (Bio-Rad). Twenty µg of lysates were resolved on TGX Stain-free SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes using the Trans-Blot Turbo Transfer system (Bio-Rad). Prior to immunoblotting, membranes were imaged to detect total protein content as described by the manufacturer. Membranes were blocked with 5% nonfat milk in TBST, and incubated overnight with primary antibodies. Subsequently, membranes were washed, incubated with HRP-linked secondary antibodies, and signals were detected by chemiluminescence (Pierce). Blots were imaged and quantified using Bio-Rad ImageLab software and calculated against total detectable protein per lane.

**Immunofluorescence**

Cells were fixed with 3.7% paraformaldehyde and 0.002% Triton X-100 for 30 min and blocked with 1% BSA in 1× PBS for 1 h. Cells were incubated with 1:250 dilution of primary antibodies for 1 h, followed by washes and incubation with 1:450 dilution of Alexa Fluor 488-conjugated secondary antibodies. Cells were incubated with 1:10,000 DAPI (Life Technologies) for nuclear staining and mounted using Fluoromount-G (Southern Biotechnology Associates, Inc.). Cells were incubated with 1:250 dilution of primary antibodies for 1 h, followed by washes and incubation with 1:400 dilution of Alexa Fluor 488-conjugated secondary antibodies. Images were captured with a Zeiss LSM 700 laser scanning confocal microscope and automated quantitation of nuclear localization was performed on ImageJ and measured by comparing intensities of GFP signal overlaid onto nuclear 4′,6-diamidino-2-phenylindole (DAPI) signal. For each condition, n = 500 ± 50 cells were counted for statistical analysis.

**Statistics**

All experiments were performed at least three times and in triplicate per experiment. Statistical differences were analyzed either by Student’s t test, or one-way or two-way analysis of variance using IBM SPSS (v24). Results are expressed as mean ± S.D. or S.E. and a p < 0.05 was considered significant comparing treatment groups.

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