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Lrp6 is a target of the PTH-activated αNAC transcriptional coregulator

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ABSTRACT

In the nucleus of differentiated osteoblasts, the alpha chain of nascent polypeptide associated complex (αNAC) interacts with cJUN transcription factors to regulate the expression of target genes, including *Osteocalcin* (*Bglap2*). PTH induces the phosphorylation of αNAC on serine 99 through a Gαs-PKA dependent pathway. This leads to activation of αNAC and expression of *Bglap2*. To identify additional target genes regulated by PTH-activated αNAC, we performed ChIP-Seq against αNAC in PTH-treated MC3T3-E1 cells. This identified *Low density lipoprotein receptor-Related Protein 6* (*Lrp6*) as a potential αNAC target. LRP6 acts as a co-receptor for the PTH receptor to allow optimal activation of PTH signaling. PTH increased *Lrp6* mRNA levels in primary osteoblasts. Conventional quantitative ChIP confirmed the ChIP-Seq results. To assess whether αNAC plays a critical role in PTH-stimulated *Lrp6* expression, we knocked-down *Naca* expression in MC3T3-E1 cells. Reduction of αNAC levels decreased basal expression of *Lrp6* by 30% and blocked the stimulation of *Lrp6* expression by PTH. We cloned the proximal mouse *Lrp6* promoter (−2523/+120 bp) upstream of the luciferase reporter. Deletion and point mutations analysis in electrophoretic mobility shift assays and transient transfections identified a functional αNAC binding site centered around −343 bp. ChIP and ChIP-reChIP against JUND and αNAC showed that they cohabit on the proximal *Lrp6* promoter. Luciferase assays confirmed that PTH-activated αNAC potentiated JUND-mediated *Lrp6* transcription and *Jund* knockdown abolished this response. This study identified a novel αNAC target gene induced downstream of PTH signaling and represents the first characterization of the regulation of *Lrp6* transcription in osteoblasts.

1. Introduction

Continuous infusion of Parathyroid Hormone (PTH) promotes calcium and phosphorus release by activating osteoclast-mediated resorption of bone [1,2]. However, intermittent administration of PTH at a low dosage promotes bone formation [3–5]. Intermittent PTH (iPTH) dosing leads to an increase in osteoblast number caused by exit of osteoprogenitors from the cell cycle, which promotes their differentiation into bone-forming cells [6–8]. Concomitantly, the hormone activates survival signaling in osteoblasts and delays apoptosis [9]. Thus iPTH treatment causes pleiotropic effects that all contribute to increase osteoblast number.

PTH signals in cells by activating the Parathyroid Hormone Receptor 1 (PTH1R), a seven-transmembrane domain G protein-coupled receptor [10]. Optimal activation of PTH1R by PTH involves its interaction with the Low density lipoprotein receptor-Related Protein 6 (LRP6) [11–14]. When *Lrp6* expression is disrupted in mice, the anabolic action of iPTH is blunted [12].

Following receptor engagement, the biological effect of PTH is mediated through signaling cascades and downstream targets controlling proliferation, maturation, and differentiation events. Because of its clinical relevance, extensive work has been performed to uncover the key signaling pathways modulated by PTH. Results from numerous studies suggest that multiple signal transduction pathways act in parallel or synergistically to achieve the full anabolic response to iPTH treatment (reviewed in [10,15]).

We have characterized one of these signaling cascade that initiates with PTH binding to its receptor, activation of $G\alpha_s$ to stimulate cAMP

Abbreviations: αNAC, α chain of nascent polypeptide associated complex; PTH, Parathyroid Hormone; PKA, protein kinase A; ChIP, chromatin immunoprecipitation; ChIP-Seq, ChIP-sequencing; LRP6, Low density lipoprotein receptor-Related Protein 6; iPTH, intermittent PTH; PTH1R, PTH receptor 1; 6Bnz-cAMP, N^6-benzoyladenosine cyclic adenosine monophosphate; pS99, phosphoserine 99-αNAC; PIC, protease inhibitor cocktail; RNA-Seq, RNA sequencing; FPKM, fragments per kilobase of exon per million fragments mapped; αNACbds, αNAC binding site; bZIP, basic domain-leucine zipper; CRE, cyclic AMP response element; CREB, CRE binding protein; JNK, JUN N-terminal Kinase

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accumulation and PKA activity, and phosphorylation of the transcriptional coregulator αNAC to send it to the nucleus where it affects target gene transcription to regulate bone mass [16]. αNAC (alpha chain of the nascent polypeptide associated complex, encoded by the *Naca* gene) shuttles to the nucleus where it can positively or negatively regulate gene transcription during mesenchymal cell differentiation through differential interaction with histones deacetylases (HDAC) corepressor molecules [17–19]. In the nucleus of differentiated osteoblasts, αNAC associates with transcription factors such as cJUN to regulate the expression of target genes, including *Osteocalcin* (*Bglap2*) [17,19,20]. αNAC can specifically bind DNA [21] and we have shown that the potentiation of cJUN-dependent transcription of the *Bglap2* gene by αNAC requires its binding to the *Bglap2* promoter [17]. The αNAC protein is extensively post-translationally modified by phosphorylation events that regulate its half-life, subcellular localization, interaction with partner molecules, and activity [16,22–25].

Treatment of osteoblasts with PTH(1–34) or a PKA-selective activator leads to translocation of αNAC to the nucleus [16]. αNAC is phosphorylated by PKA at residue serine 99 and phospho-S99-αNAC accumulates in the nucleus of osteoblasts exposed to PTH(1–34) but not in treated cells expressing dominant negative PKA. Nuclear accumulation is abrogated by an S99A mutation but enhanced by a phosphomimetic residue (S99D). Chromatin immunoprecipitation (ChIP) analysis showed that PTH(1–34) treatment leads to accumulation of αNAC at the *Bglap2* promoter. Altered gene dosage for $G\alpha_s$ and αNAC in compound heterozygous mice results in reduced bone mass, establishing the physiological relevance of this signaling cascade and confirming that $G\alpha_s$ and αNAC form part of a common genetic pathway [16].

To identify additional relevant target genes affected by PTH-mediated αNAC phosphorylation, we performed Chromatin Immunoprecipitation with deep sequencing (ChIP-Seq) against αNAC in MC3T3-E1 osteoblastic cells treated with vehicle or PTH. This strategy identified the *Lrp6* gene as a novel αNAC target. Our results define the first mechanism for the transcriptional control for *Lrp6*. They also suggest a model through which the iPTH anabolic effect can be maximized.

2. Materials and methods

2.1. Cells, antibodies, and reagents

The MC3T3-E1 (subclone 4) osteoblastic cell line [26] (generously provided by Dr. Renny T. Franceschi, University of Michigan, Ann Arbor, MI) was cultured in minimum essential medium alpha (αMEM; Invitrogen, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) from HyClone (Logan, UT). The HEK-293 cell line (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with the same supplement. Primary osteoblasts were isolated from 5-day-old C57BL/6 mice as previously described [16]. PTH(1–34) (catalog no. H4835) was purchased from Bachem (Torrance, CA) and N^6-benzoyladenosine cAMP (6Bnz-cAMP; catalog no. B009) was from Biolog (Bremen, Germany). The affinity-purified chicken anti-αNAC [27], the rabbit polyclonal anti-αNAC [21] and the rabbit polyclonal anti-αNAC-phospho-S99 (anti-pS99) [16] antibodies have been previously described. The mouse anti-αTubulin (catalog no. T6074) was purchased from Sigma-Aldrich (St. Louis, MO). The rabbit anti-JUND (catalog no. sc-74) and the naive rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX). The protease inhibitor cocktail (PIC; catalog no. P8340) was obtained from Sigma-Aldrich.

2.2. Chromatin immunoprecipitation (ChIP) and ChIP sequencing (ChIP-Seq)

Forty million MC3T3-E1 cells treated with vehicle or 100 nM PTH (1–34) were fixed for 10 min at room temperature by adding a concentrated formaldehyde solution directly to the culture media to a final concentration of 1%. For reChIP experiments, 2 mM disuccinimidyl glutarate (DSG) was added to the cells, 35 min before adding the formaldehyde. The cross-linking reaction was stopped by adding 0.125 M glycine for 5 min at room temperature. Fixed cells were washed twice, scraped in cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and centrifuged for 5 min at 500*g*. To isolate the nuclei, cells were successively resuspended and centrifuged in nucleus-chromatin preparation (NCP) buffer 1 (10 mM HEPES, pH 6.5; 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100 [vol/vol]) and NCP buffer 2 (10 mM HEPES, pH 6.5, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). The nuclear pellet was lysed by resuspension in 1 ml of cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [vol/vol] IGEPAL® CA-630, 0.5% [wt/vol] deoxycholate, 0.1% [wt/vol] SDS, 5 mM EDTA, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF and 1× PIC). The lysate was sonicated for 24 min using a 1 ml AFA fiber milliTUBE and a Focused-ultra sonicator M220 (Covaris, Woburn, MA) set at 10% duty cycle, 75 watts intensity and 200 cycles per burst. Sonicated lysates were cleaned of debris by centrifugation at 13000*g* for 10 min. Chromatin immunoprecipitation (ChIP) was performed during 16 h at 4 °C on a rotating wheel using the rabbit polyclonal anti-αNAC antibody (dilution 1:50), the rabbit polyclonal anti-pS99 antibody (dilution 1:10) or the anti-JUND (3 μg) bound to 1.5 mg of Dynabeads® protein G (Thermo Fisher Scientific). Protein-DNA-bead complexes were successively washed four times with RIPA buffer, four times with lithium chloride buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 1% [vol/vol] IGEPAL® CA-630, 1% [wt/vol] deoxycholate), and one time with 100 mM Tris-HCl, pH 8.0. For reChIP experiment, washed complexes were eluted from the beads by incubating in 50 μl of elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM DTT) for 30 min at 37 °C. Following elution, samples were diluted 20× in RIPA buffer and the second ChIP was performed as described above. Following final immunoprecipitation and washing steps, washed complexes were eluted from beads by heating in 150 μl of 1% SDS for 10 min, and cross-links were reversed by heating the samples for 5 h at 65 °C. DNA was purified by phenol-chloroform extraction and precipitated using ethanol and 20 μg of glycogen as a carrier. The chromatin from input and ChIP samples was resuspended in 50 μl of TE (10 mM Tris-HCl, PH 8.0, 1 mM EDTA) buffer. *Lrp6* promoter fragments were amplified using 5′-GAAAGCGTAAGCCGCAATG-3′ forward and 5′-GTGGTCGGGACTACTTTCTG-3′ reverse primers and *Bglap2* promoter fragments using previously described primers [17]. Reactions were performed using PowerUP SYBR green master mix following the manufacturer's instructions (Thermo Fisher Scientific) and carried out in a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific).

ChIP-Seq was performed by the Genome Quebec and McGill University Innovation Centre (Montreal, QC, Canada). Briefly, ChIP-Seq libraries from immunoprecipitated DNA were constructed using the TruSeq gDNA library kit from Illumina (San Diego, CA) following the manufacturer's instructions. Single reads of 50 base pairs were obtained using a HiSeq 2000 sequencing system (Illumina).

2.3. RNA isolation, real-time PCR and RNA sequencing (RNA-Seq)

Prior to RNA isolation, cells were washed 3 times with cold PBS. RNA was isolated using 1 ml of TRIzol reagent following the manufacturer's instructions (Thermo Fisher Scientific, Mississauga, ON). Using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific), 1 μg of RNA was reverse-transcribed into cDNA following the manufacturer's instructions. Gene expression was assessed using the TaqMan Universal PCR master mix (Thermo Fisher Scientific) and with the *Lrp6* (Mm00999795_m1) or *Jund* (Mm04208316_s1) TaqMan assay (Thermo Fisher Scientific). The *β2-microglobulin gene* (*B2m*) (Mm00437762_m1) and *Glyceraldehyde 3-phosphate dehydrogenase*