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Technical Note

Runx2 regulates the expression of GNAS on SaOs-2 cells

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Abstract

Runx2 is a key regulator of osteoblast-specific gene expression and controls the expression of multiple target genes during osteoblast differentiation. Although some transcriptional targets for Runx2 are known, it is believed that the osteogenic action of Runx2 is mediated by additional target genes, and increasing studies are performed in order to identify such Runx2-responsive genes. To identify genes following the inhibition of Runx2 in osteoblastic cell line, SaOs-2 was stably transfected with a dominant negative mutant of Runx2 (Δcbfa1) under the control of a strong promoter. Comparison of gene expression patterns by differential display on selected SaOs-2 clones allowed us to observe that GNAS mRNA which encodes for the Gsα protein is overexpressed (5 to 8 fold) in cells presenting high levels of Δcbfa1. This overexpression was also observed at the protein level and seemed to be reflected by an increased basal cAMP level. Gel shift experiments performed in this study indicate that Runx2 is able to bind to the promoter of GNAS, suggesting a direct regulation at the transcriptional level. Well-described GNAS mutations like fibrous dysplasia or Albright hereditary osteodystrophy are linked to abnormality in osteoblast function, and numerous evidences showed that Gsα coupled adrenergic receptors increase the expression of osteotrophic factors and regulate bone mass. Regulation of Gsα protein by Runx2 seems to be of particular interest considering the increasing evidences on bone metabolism regulation by G proteins.

Keywords: Cbfa1/RUNX2; GNAS; Differential display; SaOs-2

Introduction

Bone remodeling requires tight control of gene activation and suppression in response to physiological cues. Runx2 (Cbfa1/AML3/PEBP2alphaA) plays a critical role in governing physiologically responsive control of skeletal genes. Abnormal expression of Runx proteins has been linked to perturbation in transcription associated with developmentally compromised skeletogenesis and skeletal disease. For example, Runx2-deficient transgenic mice had all their skeletal elements made either of cartilage or mesenchymal cells and died shortly after birth, without breathing [1,2]. These mice showed a complete lack of osteoblast, leading to a total absence of ossification. Some heterozygous Runx2-deficient mice present a phenotype identical to a human genetic dominant skeletal disorder called cleidocranial dysplasia (CCD), characterized by hypoplastic clavicles, patent fontanelles and sutures [1]. Further genetic analyses identified different mutations in Runx2 gene on CCD patients [3].

Runx2 was identified as a key regulator of osteoblast-specific gene expression by its ability to bind to the specific *cis*-acting elements present in osteocalcin promoter and other genes expressed in osteoblasts. The early and cell-specific expression of this gene together with its biological role in vivo indicates that Runx2 must control the expression of multiple target genes that are expressed in osteoblast differentiation earlier than osteocalcin. Although some transcriptional targets for Runx2 are known, it is believed that the osteogenic action of Runx2 is mediated by additional target genes, and increasing studies are performed in order to identify such Runx2-responsive genes [4,5].

Using as a global analytical method the differential display on stably transfected cells, we developed a screening procedure in order to identify genes affected by the inhibition of Runx2

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in osteoblastic cell line. The human osteosarcoma cell line SaOs-2 was stably transfected with a chimeric construct composed of the DNA-binding site of Runx2 (Δcbfa1 [6]) under the control of a strong promoter, this construct acts in a dominant negative manner. Previously, comparison of gene expression patterns by differential display on selected SaOs-2 clones allowed us to identify four genes that may be under the control of Runx2 [7]. In this present work, we demonstrated that GNAS was overexpressed in cells presenting high levels of Δcbfa1.

The human GNAS gene encodes for the α subunit of G protein (Gsα) which stimulates adenylyl cyclase. It has been identified as the underlying genetic cause of several clinical disorders, and some of these are clearly linked to abnormality in osteoblast function like fibrous dysplasia [8] or Albright hereditary osteodystrophy [9]. Moreover, several studies showed that Gsα coupled receptors increased the expression of osteotrophic factors and regulated bone mass. Among recent studies, Gsα coupled β-adrenergic receptors present on osteoblasts have been shown to regulate their proliferation and therefore to control the bone formation [10].

Gel shift experiments performed in our study indicate that Runx2 is able to bind to the promoter of GNAS in vitro, suggesting a direct regulation at the transcriptional level. Considering the increasing evidences of bone metabolism regulation by Gsα protein, this observation is of particular interest since regulation of GNAS by Runx2 has not been described before.

Materials and methods

Plasmid construction

By generation of transgenic mice overexpressing Runx2 binding domain, Ducy et al. showed that their construct acted in a dominant negative manner [6]. We fused the same DNA sequence corresponding to the human Runx2 binding domain (from *Nco*I to *Hin*dIII) to the CMV promoter and to a synthetic epitope in order to ensure a strong expression and an efficient detection of the chimeric protein in transfected cells [7]. Briefly, human bone marrow cDNA was used as a template to amplify by PCR the Runx2 DNA-binding domain. The PCR product was subcloned in pCMVTag4A (Stratagene Europe, The Netherlands). In order to confirm the correct orientation and sequence integrity, the construct (pΔcbfa1-tag) was sequenced using the ABI PRISM® Big Dye™ Terminator v 3.3 cycle sequencing Ready reaction kit (Applied Biosystems, Les Ulis, France) and the Abi Prism® 310 automated DNA sequencer (Applied Biosystems). The production of the flagged protein was checked by Western blot realized with flag antibody, thus confirming that the correct chimeric protein was produced [7].

Cell culture and stable transfection

The human osteosarcoma cell line SaOs-2 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum (FCS) (Eurobio), 2 mM glutamine, 50 UI/ml penicillin (Eurobio) and 50 μg/ml streptomycin (Eurobio) at 37°C in 5% CO_2. The day before transfection, cells were plated on 21 cm^2 dishes at a density of 4×10^5 cells per dish. Cells were transfected using 6 μl of the FuGene6 reagent (Roche Applied Science, Meylan, France) according to the manufacturer procedure and with 2 μg of plasmid: pCMVTag4A vector alone to generate control or pΔcbfa1-tag. Selection in supplemented Dulbecco's Modified Eagle's Medium (DMEM) (Eurobio) containing geneticin (G418) (Roche Applied Science) at a concentration of 1 mg/ml was started 48 h later. After 2 weeks of selection, positive clones were isolated using cloning cylinders (Sigma, Saint-Quentin Fallavier, France), and, after 3 months of expansion, a second round was engaged for 3 months of proliferation.

RNA extraction

Total RNA was extracted from confluent cultures of various cell subclones with Extract-all (Eurobio) according to the kit manufacturer protocol. Total RNA was quantified by spectrophotometer at 260 nm wave length, and the integrity of RNA was controlled by the 28S/18S rRNA ratio after agarose gel electrophoresis. Contaminating DNA was removed from RNA samples by a 30 min digestion at 37°C with DNase I (Roche Applied Science).

Differential display analysis

Differential display analysis was carried out following the Delta Differential Display kit protocol (Clontech). Two micrograms of treated RNA was reverse transcribed in a 15 μl reaction mixture with 200 units of Superscript reverse transcriptase II (Life technologies, Cergy Pontoise, France), 0.67 mM dNTPs and 0.067 μM oligo(dT) primer. The reaction was carried out for 60 min at 42°C and stopped with incubation 10 min at 75°C. Two dilutions (1/10 and 1/40) of each cDNA were made. Two microliters of diluted cDNAs was PCR-amplified using 1 unit of Taq-polymerase (Roche Applied Science), 50 μM dNTPs, 1 μM of an arbitrary primer and l μM of an oligo(dT) primer. As controls, cDNA was substituted by H_2O or DNAse-treated RNA without prior reverse transcription. Samples were incubated as follows: 94°C 5 min; 40°C 5 min; 68°C 5 min for 1 cycle, 94°C 30 s; 40°C 30 s; 68°C 5 min for 2 cycles, 94°C 20 s; 40°C 30 s; 68°C 5 min for 31 cycles; and 68°C 2 min. Five microliters of each PCR product was mixed with 5 μl of loading dye, denatured 5 min at 94°C and electrophoresed on a 50% urea–6% denaturing polyacrylamide gel in 1× TBE. Gels were stained by the silver nitrate staining method according to the manufacturer's protocol (Promega, Charbonnières, France). Differentially expressed bands were cut out from gel and incubated in 30 μl of sterilized water at 95°C for 20 min to elute cDNA fragments. The eluates were reamplified with the same primer pair and under the same PCR conditions by which they were generated. Reamplified DNA fragments were cloned according to the Advantage PCR Cloning Kit protocol (Clontech) and sequenced as described previously. Sequences were compared to the GenBank Database using the BLAST algorithm available at the National Center of Biotechnology Information home page (http://www.ncbi.nlm.nih.gov).

Real-time PCR

Quantitative PCR was performed using a LightCycler system (Roche Applied Science) according to the manufacturer's instructions. Reactions were performed in 10 μl with 1 μl cDNA, 0.5 μM primers, 4 mM $MgCl_2$ and 1 μl of LightCycler-FastStartDNA Master SYBR Green I mix (Roche Applied Science). Protocol consisted in a hot start step (8 min at 95°C) followed by 40 cycles including a 10 s denaturation step (95°C), a 10 s annealing step and an elongation step at 72°C varying from 15 s to 40 s. Efficiencies of PCR were optimized according to Roche Applied Science's recommendations on a standard sample expressing all studied genes. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and a subsequent gel electrophoresis. Quantification data represent the mean of two experiments. The sequences for the primers used for each of the genes analyzed are summarized in Table 1. Relative quantification analyses were performed by RelQuant 1.01 Software (Roche Applied Science).

Western blot analysis

Briefly, proteins of transfected cells were extracted with RIPA buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM $NaVO_4$/0.1% SDS, 1% NP40, 1% desoxycholic acid and 2 mM PMSF), and, after a centrifugation at 13,000 rpm, the supernatant was collected. Protein concentrations were measured by a BSA protein assay kit (Bio-Rad Life Science Group, Marnes-la-Coquette, France). Fifty micrograms of proteins and the prestained SDS-PAGE standards (low range, Bio-Rad Life Science Group) were separated by