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Identification and characterization of Runx2 phosphorylation sites involved in matrix metalloproteinase-13 promoter activation

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ABSTRACT

Matrix metalloproteinase-13 (MMP-13) plays a critical role in parathyroid hormone (PTH)-induced bone resorption. PTH acts via protein kinase A (PKA) to phosphorylate and stimulate the transactivation of Runx2 for MMP-13 promoter activation. We show here that PTH stimulated Runx2 phosphorylation in rat osteoblastic cells. Runx2 was phosphorylated on serine 28 and threonine 340 after 8-bromo cyclic adenosine mono phosphate (8-Br-cAMP) treatment. We further demonstrate that in the presence of 8-Br-cAMP, the wild-type Runx2 construct stimulated MMP-13 promoter activity, while the Runx2 construct having mutations at three phosphorylation sites (S28, S347 and T340) was unable to stimulate MMP-13 promoter activity. Thus, we have identified the Runx2 phosphorylation sites necessary for PKA stimulated MMP-13 promoter activation and this event may be critical for bone remodeling.

1. Introduction

Parathyroid hormone (PTH) is an essential regulator of calcium homeostasis. The hormone has multiple actions, including indirect activation of the osteoclast through osteoblastic production of RANKL [1] resulting in increased bone resorption, as well as many direct changes in the functions of the osteoblast. Besides the stimulation of the production of RANKL, bone resorbing hormones such as PTH increase the production of proteases such as matrix metalloproteinase-13 (MMP-13; collagenase-3) which appear to be required for bone and cartilage matrix degradation and the later action of osteoclasts [2–4]. MMP-13 appears to play a critical role in PTH-induced bone resorption and calcemic responses and endochondral bone formation [5,6].

In the rat osteoblastic cell line, UMR 106-01, PTH induces MMP-13 gene transcription through a protein kinase A (PKA)-dependent pathway requiring de novo protein synthesis [7,8]. Investigation of the regulatory region of the MMP-13 gene identified the PTH-response elements as being the runt domain (RD at −132/−126) and the activator protein-1 (AP-1 at −48/−42) binding sites in the MMP-13 promoter. We have demonstrated a PTH-dependent cooperative interaction between the sites and the proteins (Runx2/Fos/Jun) binding to them [9]. However, there was no significant change in the abundance of Runx2 protein binding to the RD site and there was also no change in the levels of Runx2 mRNA or protein in UMR cells after PTH treatment [8]. Using a Gal4 fusion construct of the activation domain 3 (AD3) of Runx2, and in vitro reactions with PKA, we have shown that PTH acts via PKA to phosphorylate and stimulate the transactivation of Runx2 through a PKA consensus site in AD3 [8].

Runx2 has been found to be an essential transcription factor for inducing osteoblast differentiation [9–13]. Runx2 regulates expression of several genes including alkaline phosphatase, types I and II collagen, RANKL, TGF-β type I receptor, C/EBPdelta, nuclear matrix associated proteins, and TWIST, a basic helix–loop–helix transcription factor [14]. Runx2 seems to be involved in the balance between bone formation and bone resorption and participates in several bone and bone related diseases [14–18]. Runx2 activity can be modulated in several ways, including direct regulation of its gene expression, post-translational modifications, and protein–protein interactions. In this study we investigated how Runx2

Abbreviations: PTH, parathyroid hormone; PKA, protein kinase A; 8-Br-cAMP, 8-bromo cyclic adenosine mono phosphate; MMP-13, matrix metalloproteinase-13; MALDI, matrix-assisted laser desorption/ionization; TLC, thin layer chromatography; CAT, chloramphenicol acetyl transferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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is modified in response to PTH treatment, and how this modification is biologically significant for MMP-13 promoter activation. We identified Runx2 phosphorylation sites in vivo and their requirement for PKA stimulation of MMP-13 promoter activity in osteoblastic and non-osteoblastic cells.

2. Materials and methods

2.1. Reagents and cells

Tissue culture media and reagents were obtained from Invitrogen. The Runx2, c-myc tag and phospho tyrosine antibodies were purchased from Santa Cruz Biotechnology, CA. The phospho threonine antibody was obtained from Calbiochem, CA. Other chemicals were obtained from Sigma, St. Louis. UMR 106-01 cells were maintained in culture in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. COS-7 and C3H10T1/2 cells were maintained in DMEM medium plus 10% FBS.

2.2. Immunoprecipitation

Cells were lysed in immunoprecipitation buffer containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). Protein A/G beads were added to the extracts and allowed to pre-absorb for 1 h, after which primary antibody and protein A/G bead mixture were added. Incubation was continued at 4 °C with agitation for 2 h or overnight. After washing, the beads were suspended in 2× SDS sample buffer, boiled for 5 min and centrifuged. The proteins in the resulting supernatant were separated by SDS–PAGE and subjected to Western blotting (8, 9).

2.3. Western blot analysis

The proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking in Tris-buffered saline containing 5% (w/v) non-fat dry milk, the membrane was exposed to primary antibody overnight at 4 °C. The membrane was washed and exposed to horseradish peroxidase-conjugated secondary antibody. The immunoreactive signals were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences).

2.4. Metabolic labeling of Runx2 protein

COS-7 cells were transiently transfected with pCMV–c-myc–Runx2 construct [15] for 24 h. The cells were then incubated with 100 μCi of [^{32}P]phosphate (Phosphorus-32; Amersham BioSciences) in phosphate free DMEM medium containing 0.1% FBS for 2 h, followed by control or 8-Br-cAMP treatment. Whole cell lysates were prepared and immunoprecipitated with antibody to the c-myc tag. The immunoprecipitated proteins were fractionated by 12% SDS–PAGE, followed by autoradiography.

2.5. Phosphoamino acid analysis

The radioactive band (Runx2) as visualized by autoradiography was cut out and incubated for 45 min at 95 °C in the presence of 6 N HCl. The resulting samples were dried and redissolved in a solution containing unlabeled phosphorylated serine, threonine, and tyrosine standards (Sigma Company, St. Louis). Separation of the amino acids by thin layer chromatography (TLC) electrophoresis and staining of the amino acid standards with ninhydrin were done as described previously [19]. Visualization of Runx2-derived ^{32}P-phosphorylated amino acid(s) was accomplished by autoradiography.

2.6. Identification of phosphorylation sites by mass spectrometry

COS-7 cells were transiently transfected with pCMV–c-myc–Runx2 construct [15] for 24 h. The cells were then treated with control or 8-Br-cAMP containing media. Whole cell lysates were prepared and immunoprecipitated with antibody to the c-myc tag. The immunoprecipitated proteins were fractionated by 12% SDS–PAGE, followed by silver staining (Amersham Biosciences). The gel bands corresponding to the Runx2 proteins based on their sizes were excised and washed with 30% ACN in 50 mM ammonium bicarbonate prior to 7 μg DTT reduction and 35 μg iodoacetamide alkylation. Trypsin (0.2 μg) was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 μl of 1% trifluoracetic acid (TFA) followed by C_{18} Ziptip (Millpore Corporation, Billerica, MA) desalting according to manufacturer's protocol. The peptides were dried in a Speedvac, and re-suspended in 10 μl of solvent A (2% acetonitrile (ACN), 0.1% formic acid (FA)) for LC–MS/MS analysis. In brief, the peptides were first separated by Reversed Phase Liquid Chromatography (RPLC, capillary PepMap100 column (75 μm × 150 mm, 3 μm, 100 Å, C_{18}), Dionex, Sunnyvale, CA, USA) in a 60 min linear gradient from 10% solvent A to 40% solvent B (95% ACN, 0.1% FA). The RPLC eluent was directly introduced into a nano-ESI source on an API-US QTOF tandem MS system (Waters Corporation, Milford, MA). The ESI capillary voltage was set at 3000 V. The MS spectra (*m/z* 400–1900) were acquired in the positive ion mode. Argon was used as the collision gas and the collision energy was set within a range between 17 and 55 V, depending on the charge states and the *m/z* values of the ions analyzed. MS/MS spectra were acquired in data-dependent mode, in which the top five most abundant precursors with two to five charges from each MS survey scan were selected for fragmentation. Protein identification was performed by searching the MS/MS spectra against mammalian NCBI database using a local MASCOT search engine (V. 1.9). Oxidized methionine, carbamidomethyl labeled-cysteine and serine/threonine phosphorylation were set as variable modifications as the search parameters. The MS/MS spectra of phosphopeptides were manually confirmed.

2.7. Site-directed mutagenesis and transient transfections

The mutations in the Runx2 constructs were carried using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The serine and threonine amino acids were converted to alanine. The constructs were verified by sequencing at the UMDNJ-RWJMS-core DNA automatic sequencing facility. The plasmid DNAs were transiently transfected into cells using GeneJammer (Stratagene). Briefly, cells were plated at 2–4 × 10^5/well in six-well plates in 10% FBS-containing medium. The following day, the cells were transfected with 1 μg DNA and 5 μl GeneJammer per well in 1 ml of serum-free medium. After 3 h, 1 ml of 10% FBS-containing medium were added. After 24 h, the cells were treated with either control or 8-Br-cAMP (10^{-3} M)-containing media for 24 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of final concentrations of 250 μM *n*-butyryl-coenzyme A and 23 mM [^{14}C]-chloramphenicol (0.125 μCi/assay). The values were normalized to protein as determined by the Bradford dye binding (BioRad, Hercules, CA) method. A standard curve using purified CAT was performed every experiment to determine the linear range of the enzyme assay. The Renilla luciferase construct was co-transfected to normalize the transfection efficiency. The Renilla luciferase assay was carried out using the Renilla luciferase assay kit from Promega [8,9].