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AP1 binding site is another target of FGF2 regulation of bone sialoprotein gene transcription

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Abstract

Bone sialoprotein (BSP) is an early marker of osteoblast differentiation. We previously reported that fibroblast growth factor 2 (FGF2) regulates BSP gene transcription via FGF2 response element (FRE) in the proximal promoter of rat BSP gene. We here report that activator protein 1 (AP1) binding site overlapping with glucocorticoid response element (GRE) AP1/GRE in the rat BSP gene promoter is another target of FGF2. Using the osteoblastic cell line ROS17/2.8, we determined that BSP mRNA levels increased by 10 ng/ml FGF2 at 6 and 12 h. Runx2 protein levels increased by FGF2 (10 ng/ml) at 3 h. Treatment of ROS17/2.8 cells with FGF2 (10 ng/ml, 12 h) increased luciferase activities of constructs including −116 to +60 and −938 to +60 of the rat BSP gene promoter. Effects of FGF2 abrogated in constructs included 2 bp mutations in the FRE and AP1/GRE elements. Luciferase activities induced by FGF2 were blocked by tyrosine kinase inhibitor herbimycin A, src-tyrosine kinase inhibitor PP1 and MAP kinase kinase inhibitor U0126. Gel shift analyses showed that FGF2 increased binding of FRE and AP1/GRE elements. Notably, the AP1/GRE-protein complexes were supershifted by Smad1 and c-Fos antibodies, c-Jun and Dlx5 antibodies disrupted the complexes formation, on the other hand AP1/GRE-protein complexes did not change by Runx2 antibody. These studies demonstrate that FGF2 stimulates BSP gene transcription by targeting the FRE and AP1/GRE elements in the rat BSP gene promoter.

Keywords: Activator protein 1; Bone sialoprotein; Fibroblast growth factor 2; FGF2 response element; Glucocorticoid response element; Transcription

1. Introduction

Fibroblast growth factor 2 (FGF2), a potent mitogen in many cell types including osteoblasts, is a member of the heparin-binding growth factor family. It has been implicated in a range of normal physiological processes from embryonic mesoderm induction and pattern formation to angiogenesis and wound repair (Mason, 1994; Yamasaki et al., 1996). FGF2 has been reported to act as a local regulator of bone formation, since FGF2 is synthesized by osteoblasts and is stored in a bioactive form in the extracellular matrix (Mayahara et al., 1993; Nakamura et al., 1995; Globus et al., 1989; Liang et al., 1999). Over-expression of the FGF2 gene in transgenic mice causes premature mineralization, flattening and shortening of

Abbreviations: BSP, bone sialoprotein; FGF2, fibroblast growth factor 2; FRE, FGF response element; bp, base pair(s); nts, nucleotides; PKC, protein kinase C; PKA, cAMP dependent protein kinase; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase kinase; Pit-1, pituitary-specific transcription factor-1; LUC, luciferase; AP1, activator protein 1; AP2, activator protein 2; GRE, glucocorticoid response element; NFκB, nuclear factor-kappaB; CRE, cAMP response element; α-MEM, α-minimal essential medium; TAE, transforming growth factor-β activation element; Runx2, runt homeodomain protein 2; Dlx5, distalless 5.

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long bones (Coffin et al., 1995), whereas disruption of the FGF2 gene leads to decreased bone mass and bone formation (Montero et al., 2000). FGF2 is an immediate-early gene induced by mechanical stress in osteogenic cells (Li and Hughes-Fulford, 2006). FGF2 inhibits the expression of alkaline phosphatase, type I collagen and osteocalcin in ROS17/2.8 cells (Rodan et al., 1989). FGF2 also inhibits type I collagen transcription in MC3T3-E1 cells (Hurley et al., 1993). However, the combination of FGF2 and forskolin markedly up-regulates osteocalcin mRNA levels in MC3T3-E1 cells (Boudreaux and Towler, 1996). An osteocalcin FGF2 response element (GCAGTCA motif) has been identified in the rat osteocalcin gene promoter as a target of FGF2 and cAMP stimulation (Boudreaux and Towler, 1996). The induction of human osteocalcin transcription by FGF2, requires the interaction of CCAAT motif that overlaps with three tandem repeats of a NF-1 half-site (TTGGC) (Schedlich et al., 1994). In the previous study, we reported that FGF2 stimulates bone sialoprotein (BSP) transcription (Shimizu-Sasaki et al., 2001), and FGF2 and cAMP synergistically induce BSP gene expression in ROS17/2.8 osteoblast-like cells (Shimizu et al., 2006).

BSP is a prominent component of the mineralized bone matrix that has been implicated in tissue mineralization. Studies on the developmental expression, tissue localization and structural properties of BSP in newly forming bone have shown that the expression of BSP is essentially restricted to differentiated cells in mineralizing tissues and that it might initiate hydroxyapatite formation during bone formation *de novo* (Oldberg et al., 1988; Ganss et al., 1999; Chen et al., 1992; Hunter and Goldberg, 1993). And also BSP is expressed by breast, prostate and lung cancers and to be associated with the formation of ectopic hydroxyapatite microcrystals in the tumor tissues (Waltregny et al., 2000). The rat, mouse and human BSP genes have been cloned and partially characterized (Li and Sodek, 1993; Benson et al., 1999; Kerr et al., 1993; Kim et al., 1994). BSP gene promoters have an inverted TATA box (nt −24 to −19) (Li et al., 1995), which overlaps a vitamin D response element (Kim et al., 1996), and an inverted CCAAT box (−50 to −46), which required for basal transcription (Kim and Sodek, 1999; Shimizu and Ogata, 2002). In addition, a FGF2 response element (FRE; −92 to −85) (Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Shimizu et al., 2006, Nakayama et al., 2006), a cAMP response element (CRE; −75 to −68) (Samoto et al., 2002, 2003; Takai et al., 2007), a pituitary-specific transcription factor-1 motif (Pit-1; −111 to −105) (Ogata et al., 2000), a transforming growth factor-β activation element (TAE; −499 to −485) (Ogata et al., 1997), and a homeodomain protein-binding site (HOX; −199 to −192) (Benson et al., 2000; Nakayama et al., 2006) have been characterized. Further upstream, a glucocorticoid response element (GRE) overlapping an AP1 site (Ogata et al., 1995; Yamauchi et al., 1996; Takai et al., 2007) has also been identified. FGF2 effect on BSP is mediated through FRE in the proximal promoter of the rat BSP gene. FGF2 also increased AP1 binding activities (Shimizu-Sasaki et al., 2001).

To determine the molecular mechanism of FGF2 regulation of the BSP gene, we have analyzed the effects of the FGF2 on the expression of BSP in ROS17/2.8 cells. These studies have revealed that FGF2-induced BSP transcription is mediated through both FRE and AP1/GRE elements in the rat BSP gene promoter.

2. Materials and methods

2.1. Materials

Alpha minimal essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin and streptomycin and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA). pGL3-basic, pSV-ß-galactosidase control vector, MAP kinase kinase inhibitor U0126 were purchased from Promega (Madison, WI). The protein kinase inhibitors H89 and H7 were from Seikagaku Corporation (Tokyo, Japan), and the tyrosine kinase inhibitor herbimycin A was purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). PP1 was from Biomol Research Laboratories, Inc (Plymouth Meeting, PA). EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from Takara-bio (Tokyo, Japan). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). ECL Plus Western Blotting Detection Reagents were from GE Healthcare UK Ltd. (Buckinghamshire, England). All chemicals used were of analytical grade.

2.2. Cell culture

The rat clonal osteoblastic cell line ROS17/2.8 was used in these studies (Ogata et al., 1995; Majeska et al., 1980). Cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% FCS then cultured in α-MEM without serum and incubated with FGF2 (10 ng/ml) for time periods 3–12 h. Nuclear proteins were extracted by the method of Dignam et al. (1983) with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MaCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9).

2.3. Real-time PCR

ROS17/2.8 cells were incubated with FGF2 (10 ng/ml) for time periods 3–12 h. Total RNA was extracted with guanidium thiocyanate and, following purification. Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using EXScript RT reagent Kit. Quantitative real-time PCR was performed using the following primer sets: BSP forward, 5′-AGACCACAGCTGACGCTGGA-3′; BSP reverse, 5′-CCGTTGACGACCTGCTCATT-3′; GAPDH forward, 5′-GACAACTTTGGCATCGTGGA-3′; GAPDH reverse, 5′-ATGCAGGGATGATGTTCTGG-3′; using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara-bio, Tokyo, Japan). The amplification reactions were performed in 25 μl of final volume containing 2× SYBR Premix EX Taq (12.5 μl), 0.2 μM forward and reverse primers (0.5 μl) and 25 ng cDNA (2.5 μl). To reduce variability