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Protamine stimulates bone sialoprotein gene expression

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ABSTRACT

Protamine is a small, arginine-rich, nuclear protein that replaces histone late in the haploid phase of spermatogenesis and is believed to be essential for sperm head condensation and DNA stabilization. Protamine has many biological activities and has roles in hematopoiesis, immune responses, the nervous system and bone metabolism. Bone sialoprotein (BSP) is a mineralized connective tissue-specific protein expressed in differentiated osteoblasts that appears to function in the initial mineralization of bone. Protamine (71.35 ng/ml) increased BSP mRNA levels by 6 h in osteoblast-like ROS 17/2.8 cells. In a transient transfection assay, protamine (71.35 ng/ml) increased luciferase activity of the construct (−116 to +60) in ROS 17/2.8 cells and rat bone marrow stromal cells. Luciferase activities induced by protamine were blocked by protein kinase A, tyrosine kinase and ERK1/2 inhibitors. Introduction of 2 bp mutations to the luciferase constructs showed that the effects of protamine were mediated by a cAMP response element (CRE), a fibroblast growth factor 2 response element (FRE) and a homeodomain protein-binding site (HOX). Gel shift analyses showed that protamine (71.35 ng/ml) increased the nuclear protein binding to CRE, FRE and HOX. CREB, phospho-CREB, c-Fos, c-Jun, JunD and Fra2 antibodies disrupted the formation of CRE-protein complexes. Dlx5, Msx2, Runx2 and Smad1 antibodies disrupted FRE– and HOX–protein complex formations. These studies demonstrate that protamine induces BSP transcription by targeting CRE, FRE and HOX sites in the proximal promoter of the rat BSP gene. Moreover, phospho-CREB, c-Fos, c-Jun, JunD, Fra2, Dlx5, Msx2, Runx2 and Smadl transcription factors appear to be key regulators of protamine effects on BSP transcription.
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1. Introduction

Protamine was originally isolated from the sperm of salmon and other species of fish (Balhorn, 2007; Carr and Silverman, 1999). It is a highly cationic peptide and, shows a PI of 10–11 due to a high arginine content. Protamine sulfate is used as an antidote to heparin and as a carrier of insulin, and also has antibacterial properties (Fukushima et al., 2010; Horrow, 1985; Johansen et al., 1995). The molecular mass of protamine is usually ~4 kDa, and more than one-half of its amino acid sequence consists of arginine. Protamine enhances the hepatocyte growth factor (HGF) induced liver regeneration (Liu et al., 1998), and is a popular polycation for the electrostatic coating of nanoparticles and multilayer films used in drug delivery. Protamine based polyelectrolyte multilayer thin films support the long-term proliferation and differentiation of pre-osteoblast cells on non-cross-linked film-coated surfaces (Samuel et al., 2011). However, little is known about the role of protamine in osteogenesis, osteoblast differentiation and bone formation.

Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that is characterized by the ability to bind to hydroxyapatite, through polyglutamic acid sequences, and to mediate cell attachment, through an RGD sequence (Ganss et al., 1999; Ogata, 2008; Oldberg et al., 1988). The expression of BSP is essentially restricted in the mineralized connective tissues. Studies on the developmental expression of BSP have shown that BSP mRNA is produced at high levels at the onset of bone, dentin and cementum formation (Chen et al., 1991; Chen et al., 1992). Furthermore, the temporo-spatial deposition of BSP into the extracellular matrix (Bianco et al., 1991; Chen et al., 1992) and the ability of BSP to nucleate hydroxyapatite crystal formation (Hunter and Goldberg, 1993) indicate a role for this protein in the initial mineralization of bone, dentin and cementum (Ganss et al., 1999; Ogata, 2008). BSP is also expressed in breast, lung, thyroid and prostate

Abbreviations: AP1, activator protein 1; α-MEM, α-minimum essential medium; bp, base pair(s); BSP, bone sialoprotein; CRE, cAMP response element; CREB, CRE binding protein; Dlx5, distal-less homeobox 5; FCS, fetal calf serum; FGF2, fibroblast growth factor 2; FRE, FGF response element; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; LUC, luciferase; nts, nucleotides; PKA, cAMP dependent protein kinase; PKC, protein kinase C; PTH, Parathyroid hormone; Runx2, runt homeodomain protein 2.

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cancers, suggesting that BSP might play a role in the pathogenesis of bone metastasis (Bellahcène et al., 1994; Ibrahim et al., 2000; Waltregny et al., 2000). The human, rat, and mouse BSP genes have been cloned and characterized (Benson et al., 1999; Kerr et al., 1993; Kim et al., 1994; Kiyoshima et al., 2002; Li and Sodek, 1993). These promoters include a highly conserved region that extends upstream from the transcription start site to nt −370 (Ogata, 2008). This region includes a functional inverted TATA element (−24 to −19) (Li et al., 1995) and an inverted CCAAT box (−50 to −46), which is required for basal transcription (Kim and Sodek, 1999). In addition, a cAMP response element (CRE; −75 to −68) (Araki et al., 2009; Li et al., 2010; Mezawa et al., 2009; Samoto et al., 2003; Yang et al., 2010), a fibroblast growth factor 2 (FGF2) response element (FRE; −92 to −85) (Li et al., 2011; Nakayama et al., 2006; Wang et al., 2010; Wang et al., 2011b; Samoto et al., 2003; Shimizu-Sasaki et al., 2001; Wang et al., 2010), a runt-related transcription factor 2 (Runx2) binding site (Shimizu et al., 2006), a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) (Ogata et al., 2000), a homeodomain protein binding site (HOX; −199 to −192) (Benson et al., 2000; Nakayama et al., 2006; Wang et al., 2010), a transforming growth factor-β (TGF-β) activation element (−499 to −485) (Ogata et al., 1997) and a glucocorticoid response element (−920 to −906) overlapping an AP-1 site (−921 to −915) (Ogata et al., 1995; Yamauchi et al., 1996) have also been characterized.

To determine the mechanism of BSP gene regulation by protamine, we have analyzed the effects of the protamine sulfate on the expression of BSP gene in osteoblast-like cells. These studies have revealed that protamine induced the BSP gene transcription that was mediated through CRE, FRE and HOX elements in the rat BSP gene promoter.

2. Materials and methods

2.1. Materials

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin and streptomycin and TrypLE™ Express were obtained from Invitrogen (Carlsbad, CA). The PGL3-basic vector, pSV-β-galactosidase (β-Gal) control vector and ERK1/2 inhibitor U0126 were purchased from Promega (Madison, WI). The protein kinase C (PKC) inhibitor H7 was from Seikagaku Corporation (Tokyo, Japan). The cAMP-dependent protein kinase (PKA) inhibitor KT5720 was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The tyrosine kinase inhibitor herbimycin A (HA) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). The Quikchange Site-directed Mutagenesis Kit was from Stratagene (La Jolla, CA). The EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). The ChIP-IT™ Express Enzymatic kit was purchased from Active Motif (Carlsbad, CA). Anti-rabbit IgG conjugated with HRP and ELC plus Western Blotting Detection Reagents were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). The salmon derived protamine sulfate was kindly provided by Maruha Nichiro Holdings, Inc. (Tokyo, Japan). All chemicals used were of analytical grade.

2.2. Cell culture

Rat osteoblast-like ROS 17/2.8 cells (Ogata et al., 1995) and rat stromal bone marrow cells (RBMC) (Jheon et al., 2009; Nakayama et al., 2006) were cultured in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm cell culture dishes and then cultured in α-MEM without serum and incubated with or without protamine sulfate for the dose response (7.135, 71.35 and 713.5 ng/ml, 12 h) or for time periods extending over 3–12 h. The total RNA was extracted with guanidium thiocyanate, as previously described (Ogata et al., 1997). RNA was isolated from triplicate cultures and analyzed for the expression of BSP mRNA and Runx2 mRNA by Northern hybridization and real-time PCR as described below.

2.3. Northern hybridization

Aliquots (20 μg) of total RNA were fractionated in a 1.2% agarose gel and transferred onto a Hybond-N+ membrane. Hybridizations were performed at 42 °C with ^{32}P-labeled rat BSP cDNA probe. Following hybridization, membranes were washed four times for 5 min each at 21 °C in 300 mM sodium chloride, 30 mM trisodium citrate, pH 7.0 containing 0.1% SDS. This was followed by two 20-min washes at 55 °C in 15 mM sodium chloride, 1.5 mM trisodium citrate, pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned using a Bio-imaging analyzer (Fuji BAS 2500, Tokyo; Japan).

2.4. Western blot

For Western blot analyses, total proteins from ROS17/2.8 cells were separated on 10% SDS-PAGE and transferred onto a membrane, which was then incubated for 3 h with anti-BSP (LF-100, provided by Dr. Larry W. Fisher) and α tubulin (B-7 sc-5286) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Anti-rabbit and anti-mouse IgG conjugated with HRP was used as the secondary antibodies. Immunoreactivities were detected by ELC plus Western Blotting Detection Reagents.

2.5. Real-time PCR

ROS 17/2.8 cells were cultured in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm cell culture dishes and then cultured in α-MEM without serum for 12 h and incubated with or without protamine for time periods extending over 3–24 h. Total RNA (1 μg) was used as a template for cDNA, which was prepared using an EXScript RT reagent Kit. Quantitative real-time PCR was performed using the following primer sets: Runx2 forward, (5′-CAAGTGGCCAGGTTCAACGA-3′); Runx2 reverse (5′-TGTGAAGACCGTTATGGTCAAAGTG-3′); GAPDH forward (5′-GACAACTTTGGCATCGTGGA-3′); GAPDH reverse (5′-ATGCA GGGATGATGTTCTGG-3′), using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara, Tokyo, Japan). The amplification reactions were performed using 25 μl of the final volume containing 2x SYBR Premix EX Taq (12.5 μl), 0.2 M forward and reverse primers (0.1 μl) and 50 ng cDNA (5 μl) for Runx2 and 10 ng cDNA (1 μl) for GAPDH. To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes (Takara, Tokyo, Japan). Post-PCR melting curves confirmed the specificity of single-target amplification and the expression of Runx2 relative to GAPDH was determined in triplicate.

2.6. The construction of plasmids

To determine regions of the promoter that respond to protamine, chimeric constructs of various sized promoter sequences were ligated to a luciferase (LUC) gene in the plasmid pLUCB (kindly provided by Dr. V. Giguerre). The constructs pLUC 1–5 were produced by cutting the rat BSP promoter inserts from the pCAT 1–5 plasmids (Li and Sodek, 1993) with Hind III and Sal I and re-ligating the inserts into the pLUCB at the corresponding restriction sites (pLUC1, −18~+60; pLUC2, −43~+60; pLUC3, −116~+60; pLUC4, −425~+60; pLUC5, −801~+60). The pLUC 6 (−938~+60) construct was made by ligating the rat promoter segment −2992 (Xba I) to −801 (Hind III) to the 5′-end of pLUC5. This ~3-kb insert was then deleted unidirectionally with exonuclease III, and the 5′-ends of each deletion were sequenced to determine the 5′-cleavage site. Mutation LUC constructs were made by PCR using the Quikchange Site-directed Mutagenesis Kit within the context of the homologous −116 to +60 (pLUC3) and −425 to

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