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Prokaryotic expression of bone sialoprotein and identification of casein kinase II phosphorylation sites

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Abstract

Bone sialoprotein is an extracellular noncollagenous acidic protein that plays a role in bone mineralization and remodeling. Its expression is restricted to mineralized tissues and is subjected to variety of posttranslational modifications including phosphorylation and glycosylation. We have expressed the full-length and half domains of bovine bone sialoprotein in a prokaryotic system and identified the phosphorylation sites of casein kinase II. The N-terminal automated solid-phase sequencing defined four phosphorylated peptides: residues 28–38 (LEDS^{P}EENGVFK), 51–86 (FYPELKRFAVQSSS^{P}DS^{P}S^{P}EENGNGDS^{P}S^{P}EEEEEEEETS^{P}), 151–165 (EDES^{P}DEEEEEEEEEE), and 295–305 (GRGYDS^{P}YDGQD). Nine phosphoserines were identified within the four peptides. Seven of them were in the N-terminus (S31, S64, S66, S67, S75, S76, and S86) and two were in the C-terminus (S154 and S300) of the protein.

Keywords: Bone sialoprotein; Phosphorylation; GST; Expression; CKII

Bone sialoprotein (BSP) is a noncollagenous phosphorylated glycoprotein. It contains two polyglutamic acid domains and the cell attachment recognition sequence, RGD. BSP expression is restricted to the mineralized tissues including bone, mineralizing cartilage, and dentin. The restricted expression of bone sialoprotein indicates that the transcription of the gene is suppressed in nonmineralized tissues [1]. The expression of bone sialoprotein in tumors has been coupled with the formation of mineral microcrystals and with the metastasis of tumor cells to bone [2]. There are evidences that noncollagenous extracellular matrix phosphoproteins contribute to many biological functions during the formation of bone tissue. Bone sialoprotein gene expression is upregulated during new bone formation and repair. These include bone calcification [3], bone resorption [4], bone remodeling [5], bone repair [6], and bone cell differentiation [7]. Early investigations indicated that bone glycosylated phosphoproteins play a role in the nucleation of the calcium-phosphate apatite crystals [8]. Recent hydroxyapatite nucleation studies concluded that the polyglutamic acid domains of bone sialoprotein are responsible for the calcium-phosphate crystal formation [9,10]. Furthermore, it was proposed that the glycosylated form of bone sialoprotein contributes to calcium-phosphate-binding procedure [11]. Bone sialoprotein is subjected to variety of posttranslational modifications including phosphorylation and glycosylation, and is primarily phosphorylated at serines [12]. The dephosphorylated form of bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase lacks the ability to promote RGD-dependent osteoclast attachment [13]. Previous works defined the phosphorylation regions of native bone sialoprotein and the protein kinases that are involved in their phosphorylation [14], and reported for the first time, the purification of casein kinase II from bone [15]. The precise roles of the

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glycosylation and phosphorylation status of bone sialoprotein remain to be clearly defined. Expression of bone sialoprotein in a prokaryotic system is essential to study the biological functions of the protein. In previous reports, the expression of full-length bone sialoprotein in a prokaryotic system was not achieved [16,10,11]. We report on the expression of bovine bone sialoprotein, full-length and half domains in a prokaryotic system and on the identification of the phosphorylated sites by casein kinase II.

Materials and methods

Generation of bovine bone sialoprotein constructs. A bovine bone sialoprotein cDNA clone was used as a PCR template to generate three fragments. The PCR cocktail was 50 pg cDNA in 10 μl of 1× high fidelity buffer (TripleMaster PCR System), 200 μM of each dNTP, 1 pmol of each primer, and 0.1 U TripleMaster enzyme mixture (Brinkman Biosystems). The PCR thermal profile was 30 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 60 s in an Eppendorf MasterCycler Gradient (Brinkman Biosystems). The bvBSP F1: 5′ cc gga tcc atg ctt tca atg aaa aat ttg aat c 3′ and bvBSP R3: 5′ cc gaa ttc tca ggg aag cca gat ggc agc tag 3′ primers were used to generate the N-terminus fragment. The bvBSP F2: 5′ cc gga tcc atg agg aag gct ggg gct aca g 3′ and bvBSP R4: 5′ cc gaa ttc cct tca ctg gtg gct gta gta atc 3′ primers were used to generate the C-terminus fragment. Meanwhile the bvBSP F1 and bvBSP R4 primers were used to generate the full-length fragment. Each forward primer contains a *Bam*HI restriction site and the translation initiation codon. Each reverse primer contains an *Eco*RI restriction site and a translation determination codon. The PCR product for each cDNA fragment was digested with *Bam*HI and *Eco*RI, gel purified, and ligated to pGEX-2T expression vector (previously digested with the same enzymes and gel purified). The N-terminus construct, which encompasses the amino acids sequence from L17 to P139, was termed pGEX-2T-NT-bvBSP. The C-terminus construct, which encompasses the amino acid sequence from R140 to Q310, was termed pGEX-2T-CT-bvBSP. The full-length construct, which encompasses the amino acid sequence from L17 to Q310, was termed pGEX-2T-bvBSP.

Selection of recombinant clones. A fraction of the ligation reaction for each construct was used to transform *Escherichia coli* DH5α bacterial competent cells. The plasmid DNA was extracted using a microextraction procedure described elsewhere [17]. Recombinant clones were selected by PCR, using primers originated from the vector sequences. The PCR cocktail was 50 pg plasmid DNA in 10 μl of 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25 °C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 pmol of each primer, and 0.1 U Taq DNA Polymerase (Promega). The PCR thermal profile was 30 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 60 s in an Eppendorf MasterCycler Gradient (Brinkman Biosystems). The PCR product was run onto 1% agarose gel containing 100 ng/ml Et-Br and documented by the AlphaImager 2000 system. A few of the recombinant clones for each construct were selected for DNA sequencing to assess the integrity and the correct reading frame of the cDNA sequences.

Expression of recombinant proteins. Clones displaying integrate cDNA sequence and correct reading frame were selected for protein expression in *E. coli* BL21 bacterial cells (Invitrogen). A few picograms of each construct were used to transform BL21 competent cells. A single colony for each construct was inoculated into LB medium supplied with 50 μg/ml ampicillin, grown overnight at 37 °C with shaking at 250 rpm. The bacterial growth was diluted 1:10 with fresh LB medium supplied with 50 μg/ml ampicillin, grown for additional 2 h at 37 °C with shaking, and then induced with IPTG to final concentration of 100 μM for 2 h at 30 °C. The bacterial pellet was suspended in ice-cold 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3), mildly sonicated, and supplied with Triton X-100 to final concentration of 1%. After centrifugation, the clear supernatant was rotated with glutathione agarose (beads). The GST fusion proteins bound to the beads were extensively washed with the binding buffer. A portion of each GST fusion protein was mixed with protein loading dye, fractionated onto 12% SDS–PAGE at 10 Vs/cm in 8 × 10 cm vertical mini polyacrylamide gel electrophoresis system (Bio-Rad). After electrophoresis, the gels were washed four times with 25% isopropanol, each for 30 min, then soaked in staining solution (25% isopropanol, 10% formamide, 15 mM Tris–HCl, pH 8.8, and 0.005% Stains-all) for 2 h in the dark on an orbital shaker at room temperature, rinsed with 25% isopropanol, and documented by the AlphaImager 2000 system.

Thrombin digestion. The bovine thrombin protease (Amersham) was mixed with aliquots of the beads-GST-fusion protein at ratio of 1 U/100 μg in 1× PBS [18]. The mixture was rotated at room temperature for 6 h, centrifuged, and the supernatant containing the recombinant protein was transferred to a new Eppendorf tube. The glutathione *S*-transferase and the thrombin remained bound to the beads. The resultant recombinant proteins were termed rNT-bvBSP, rCT-bvBSP, and rbvBSP, for the N-terminus, C-terminus, and the full length of the protein, respectively. The protein concentration for each recombinant protein was determined using Lowry's protein assay [19] and a portion of each was fractionated onto 12% SDS–PAGE at 10 Vs/cm in 8 × 10 cm vertical mini polyacrylamide gel electrophoresis system (Bio-Rad), and stained by Stains-all to assess the quality of the protein.

Phosphorylation by casein kinase II. One hundred micrograms of rbvBSP and 50 μg of each of the rNT-bvBSP and rCT-bvBSP were phosphorylated by [^{32}P]ATP using 100 ng of recombinant casein kinase II expressed in Sf9 insect cells (Upstate Biotechnology, Lake Placid, NY) in 0.5 ml of 0.1 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM EGTA for 1 h at room temperature. ^{32}P-labeled recombinant bovine BSP was dialyzed against 50 mM NH_4HCO_3 (pH 8.0) and digested with 2% (w/w) tosylphenylalanyl chloromethyl ketone-treated bovine trypsin (Sigma). The samples were speed vacuum dried, suspended in 0.2 ml of H_2O and 0.1% (v/v) trifluoroacetic acid, and individually subjected to RP-HPLC on a Vydac C18 column (25 × 0.46 cm). The peptides were eluted by a linear gradient from H_2O and 0.1% (v/v) trifluoroacetic acid to 60% CH_3CN and 0.55% (v/v) trifluoroacetic acid over 80 min at a flow rate of 1 ml/min. The absorbance at 219 nm was recorded continuously, and fractions of 1 ml were collected. Aliquots from each fraction were counted for ^{32}P radioactivity and speed vacuum dried.

Identification of the phosphorylation sites. N-terminal sequencing was carried out by Edman degradation [20] using an automated solid-phase amino acid sequenator Model 477A (Applied Biosystems, Foster City, CA) under the same conditions described previously [21]. Glass filters coated with Biobrene were used, and the proteins or peptides were adsorbed on these filters. This approach was used for N-terminal sequence analysis in which the prime interest was to identify the N-terminal sequence. The sequencing conditions for identification of the ^{32}P-labeled peptide regions and the specific sites of phosphorylation were as described previously [22].

Results

Expression of recombinant protein

The insertion of cDNA fragment in frame downstream the glutathione *S*-transferase (GST) permits for