Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Glycosylation status of bone sialoprotein and its role in mineralization

Lan Xu[a,*], Zhenqing Zhang[b,**], Xue Sun[b], Jingjing Wang[a], Wei Xu[c], Lv Shi[d], Jiaojiao Lu[b], Juan Tang[a], Jingjing Liu[a], Xiong Su[a]

[a] Department of Biochemistry and Molecular Biology, Soochow University Medical College, Suzhou 215123, China
[b] College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China
[c] Department of Orthopaedic Surgery, The Second Affiliated Hospital of Soochow University, Suzhou 215004, China
[d] Shanghai Green-Valley Pharmaceutical Co. Ltd., Shanghai 201200, China

ARTICLE INFO

Keywords:
Bone sialoprotein
Mineralization
Sialic acids
Glycans
Mass spectrometry

ABSTRACT

The highly glycosylated bone sialoprotein (BSP) is an abundant non-collagenous phosphoprotein in bone which enhances osteoblast differentiation and new bone deposition in vitro and in vivo. However, the structural details of its different glycosylation linkages have not been well studied and their functions in bone homeostasis are not clear. Previous studies suggested that the *O*-glycans, but not the *N*-glycans on BSP, are highly sialylated. Herein, we employed tandem mass spectrometry (MS/MS) to demonstrate that the *N*-glycanson the recombinant human integrin binding sialoprotein (rhiBSP) are also enriched in sialic acids (SAs) at their termini. We also identified multiple novel sites of *N*-glycan modification. Treatment of rhiBSP enhances osteoblast differentiation and mineralization of MC3T3-E1 cells and this effect could be partially reversed by efficient enzymatic removal of its *N*-glycans. Removal of all terminal SAs has a greater effect in reversing the effect of rhiBSP on osteogenesis, especially on mineralization, suggesting that sialylation at the termini of both *N*-glycans and *O*-glycans plays an important role in this regulation. Moreover, BSP-conjugated SAs may affect mineralization via ERK activation of VDR expression. Collectively, our results identified novel *N*-glycans enriched in SAs on the rhiBSP and demonstrated that SAs at both *N*- and *O*-glycans are important for BSP regulation of osteoblast differentiation and mineralization in vitro.

1. Introduction

Bone sialoprotein (BSP) is one of the major extracellular matrix (ECM) proteins of the bone and belongs to the small integrin-binding ligand *N*-linked glycoprotein (SIBLING) family. There are five known members of the SIBLING family: BSP, osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) [1,2]. BSP was proposed to be physiologically important for hydroxyapatite (HA) nucleation, cell attachment and collagen binding [3,4]. Our previous study demonstrated that BSP was able to enhance osteoblastic differentiation and new bone deposition in vivo by promoting the early bone mineralization [5]. No obvious cartilage formation was observed and molecular mechanisms for the BSP regulation of osteogenesis are not clear. Osteogenesis is regulated by activation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and protein kinase B/Akt [2,6,7].

BSP is a highly phoshorylated and glycosylated secretory protein that is enriched in sialic acids (SAs). Although the molecular mass of the core BSP protein is 33.6 kDa, the protein is usually identified as a 75 kDa band on SDS-PAGE [1,8]. The high apparent molecular weight is due to the extensive post-translational modifications (PTMs), including glycosylation. However, structural details of BSP glycosylation have not been determined and functions of different glycosylation residues in bone homeostasis are still not clear.

Early studies showed that there were about four *N*-glycosylation

Abbreviations: ALP, alkaline phosphatase; AKT, protein kinase B; BSP, bone sialoprotein; DSPP, dentin sialophosphoprotein; DMP, dentin matrix protein-1; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; ETD, electron transfer dissociation; JNK, c-Jun N-terminal kinase; HA, hydroxyapatite; HCD, high-energy collision dissociation; HPAEC, high performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; MAPK, mitogen-activated protein kinase; MEPE, matrix extracellular phosphoglycoprotein; Neu5Ac, N-acetyl neuraminic acid; Neu5Gc, N-glycolylneuraminic acid; OC, osteocalcin; OPG, osteoprotegerin; OPN, osteopontin; PAD, pulsed amperometric detector; PTMs, post-translational modifications; qPCR, quantitative real-time PCR; Runx2, runt-related transcription factor 2; RANK, receptor activator for nuclear factor-κB; RANKL, receptor activator for nuclear factor-κB ligand; rhiBSP, recombinant human integrin binding sialoprotein; SAs, sialic acids; VDR, vitamin D receptor

* Corresponding author.
** Correspondence to: College of Pharmaceutical Sciences, Soochow University Medical College, Suzhou 215123, China.
E-mail addresses: xulan@suda.edu.cn (L. Xu), z_zhang@suda.edu.cn (Z. Zhang).

http://dx.doi.org/10.1016/j.yexcr.2017.09.034
Received 9 May 2017; Received in revised form 19 September 2017; Accepted 24 September 2017

sites and eight *O*-glycosylation sites on BSP, and its *O*-glycans, rather than *N*-glycans are highly sialylated [8,9]. Sialic acids, also known as *N*-acetylneuraminic acid, is a monosaccharide found on glycoproteins or glycolipid chains. It can be attached to the ultimate ends of galactose, *N*-acetylgalactosamine, or to another SA. SAs play an important role in regulating interactions of glycosylated molecules in tumor metastasis [10,11]. However, the function of SAs in bone development is still not clear. BSP purified from human bone contains more SA modifications than the recombinant BSP protein in a human cell line and has higher affinity for HA, suggesting that BSP sialylation may play a role in bone homeostasis [8]. In the present study, we employed a nano-flow liquid chromatography-mass spectrometry (LC-MS) [12] method to analyze the structural details of the BSP *N*-glycans and to determine whether SAs are enriched at their termini. The glycopeptides were analyzed in both high-energy collision dissociation (HCD) and HCD product-dependent electron transferred dissociation (HCD-pd-ETD) modes in parallel to improve specificity and accuracy. The total sialylation of intact BSP and BSP with *N*-glycans removed were further quantitated by high performance anion-exchange chromatography analysis coupled with pulsed amperometric detection (HPAEC-PAD) following complete enzymatic removal of SAs, and we examined whether complete removal of all terminal SAs or removal of *N*-glycans are involved in the regulation of mineralization by BSP in cultured MC3T3-E1 cells. The roles of different glycosylation status of BSP in this regulation are compared and evaluated.

2. Materials and methods

2.1. Materials

The pre-osteoblast cell line MC3T3-E1 Subclone 14 cells from mouse calvaria were obtained from the Cell Bank of the Chinese Academy of Sciences. α-Minimal Essential Medium (α-MEM) and TRIzol® reagent were purchased from Life Technologies. Fetal bovine serum (FBS), L-ascorbic acid, β-glycerophosphate and dexamethasone were ordered from Sigma. Alkaline phosphatase Kit (ALP kit, N1891) ordered from Sigma. The ERK inhibitor, PD0325901, was purchased from Cayman Chemical. Enzymatic DeglycoMx Kit was ordered from QA-Bio, Inc. CHO-derived recombinant human integrin binding sialoprotein (rhiBSP, AAC95490) and goat anti-BSP (AF4014, 1:1000) antibody were purchased from R & D Systems. Rabbit anti-vitamin D receptor (VDR, 3277-1, 1:1000), anti-osteopontin (OPN, 2671-1, 1:1000) antibodies were purchased from Epitomics. Goat anti-osteoprotegerin (OPG, SC-390518, 1:1000), rabbit anti-OC (OC, SC-365797, 1:1000), rabbit anti-runt-related transcription factor 2 (Runx2, YT5356, 1:1000), and mouse anti-receptor activator NFκB ligand (RANKL, SC-59982, 1:1000) antibodies were purchased from Santa Cruz Biotechnology, Inc. Mouse anti-receptor activator NFκB (RANK, 64C1385, 1:1000) was purchased from Abcam. Rabbit anti-phospho-AKT (4058, 1:1000), anti-total AKT (9272, 1:1000), anti-phospho-ERK1/2 (9101, 1:1000), and anti-total ERK1/2 (9102, 1:1000) antibodies were obtained from Cell Signaling Technology.

2.2. Methods

2.2.1. Nano LC-MS/MS-Orbitrap analysis and data processing

The solution of rhiBSP (25 μg in 0.1 mL PBS) was digested by protease E (~ 0.2 U, Sigma) at 37 °C overnight and analyzed by nanospray LC-MS/MS on an Orbitrap Fusion Tribrid (Thermo Scientific) coupled to an EASY-nanoLC System (Thermo Scientific) without trap column. Glyco-peptide mixtures were loaded onto a C18 column (15 cm × 50 μmi.d.) and separated at a flow rate of 300 nL/min using a gradient of 5–22% solvent B (100% acetonitrile with 0.1% formic acid) in 40 min, followed by an increase to 90% B in 10 min and held for another 5 min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile.

The parameters used for MS and MS/MS data acquisition under the HCD-pd-ETD mode were: positive mode; top speed mode with 3 s cycle time; FTMS: scan range (m/z) = 350–2000; resolution = 120 K; AGC target = 2×10^5; maximum injection time (ms) = 50; Filter: precursor selection range = 350–2000; include charge state = 2–8; dynamic exclude after n times = 1; Decision: precursor priority = highest charge state then most intense; FTMSn (HCD): isolation mode = quadrupole; isolation window = 1.6; collision energy (%) = 30; resolution = 30 K; AGC target = 5×10^4; maximum injection time (ms) = 35; microscan = 1; Product ion trigger: at least n product ions detected = 1; top N product ions = 10; Product ion table = 138.0545, 204.0867, 366.1396; ITMSn (ETD): isolation mode = quadrupole; isolation window = 2; use calibrated charge dependent ETD parameters = true; AGC target = 5×10^4; maximum injection time (ms) = 100; microscan = 1.

For direct glycopeptide identification, the HCD and ETD MS^2 data were searched separately using Byonic (version 2.6.46) with the following search parameters: peptide tolerance = 20 ppm; fragment tolerance = 0.02 Da (HCD) and 0.6 Da (ETD); missed cleavages = 2; modifications: methionine oxidation (common2), *N*-glycan search (*N*-glycan 57 human plasma). Peptide-spectrum match (psm) with score ≥ 100 were accepted.

2.2.2. Removal of N-glycans or SAs from rhiBSP

rhiBSP solutions (100 μg in 1 mL PBS) were incubated with 5 mU PNGase F (E. meningosepticum, QA-Bio, Inc) or 5 mU sialidase (recombinant from *A. ureafaciens* in *E. Coli*, E-S001, QA-Bio, Inc) at 37 °C for 24 h, respectively. The de-sialylated rhiBSP or rhiBSP with *N*-glycans removed was collected by centrifugal ultrafiltration (Amicon Ultra-0.5 mL, Ultracel-10 K, Millipore) at 4500*g* for 10 min. The protein was washed twice with 0.3 mL PBS using the same method.

2.2.3. Quantitation of terminal SAs of rhiBSP

The solution (100 μg/mL) of intact rhiBSP, the *N*-glycan removed rhiBSP and de-sialyated rhiBSP were incubated with 5 mU sialidase at 37 °C for 24 h, respectively. The protein was collected by centrifugal ultrafiltration (Amicon Ultra-0.5 mL, Ultracel-3 K, Millipore, Billerica, MA) at 4500*g* for 10 min. The filter was washed twice with 0.3 mL PBS. The filtrates were combined with wash solution and were further diluted in 2 mL water prior to HPAEC-PAD analysis [13,14].

Two types of SAs, *N*-Acetyl neuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) were used as standards in this work. Neu5Ac and Neu5Gc were dissolved in ultrapure water to make a stock solution of 2 mg/mL. The standard curve of Neu5Ac was prepared at five different concentrations (0.05, 0.25, 0.50, 1, 5 μg/mL). An equally mixed solution (Neu5Ac/Neu5Gc) was prepared and diluted to 5 μg/mL for each ingredient.

The analysis was performed on a Metrohm 850 Professional System with a 919 IC auto-sampler plus, dual pumps and coupled with a pulsed amperometric detector (PAD, Herisau, Switzerland). SAs are separated in a Dionex Amino Trap trap column (4 × 50 mm) in line with a Dionex Carbopac PA10 analytical column (4 × 250 mm); using an isocratic mobile phase consisted of 50 mM NaOH and 150 mM NaOAc as described [14]. Data was acquired and analyzed using MagIC Net 2.4 (Herisau, Switzerland) software. All analysis was performed in duplicate.

2.2.4. Cell culture

MC3T3-E1 Subclone 14 cells were cultured in α-MEM containing 10% FBS, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate at 37 °C with 5% CO_2. Media was replaced every 3 days. Cells at around 90% confluency were replated at a concentration of 1.5×10^5 cells/well in 6 well cell culture plates. Differentiation was stimulated by incubation in conditioned media containing L-ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM), and dexamethasone (10^{-8} M) for up to 14 days as described [15].