Biochemical and Biophysical Research Communications 417 (2012) 830–835

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The sclerostin-bone protein interactome

Hemamalini Devarajan-Ketha [a], Theodore A. Craig [a], Benjamin J. Madden [c], H. Robert Bergen III [b,c], Rajiv Kumar [a,b,*]

[a] Division of Nephrology and Hypertension, Department of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
[b] Department of Biochemistry and Molecular Biology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
[c] Mayo Proteomics Research Center, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

ARTICLE INFO

Article history:
Received 9 December 2011
Available online 22 December 2011

Keywords:
Sclerostin
Protein interaction
Protein affinity chromatography
Bone formation
Bone resorption

ABSTRACT

The secreted glycoprotein, sclerostin alters bone formation. To gain insights into the mechanism of action of sclerostin, we examined the interactions of sclerostin with bone proteins using a sclerostin affinity capture technique. Proteins from decalcified rat bone were captured on a sclerostin-maltose binding protein (MBP) amylose column, or on a MBP amylose column. The columns were extensively washed with low ionic strength buffer, and bound proteins were eluted with buffer containing 1 M sodium chloride. Eluted proteins were separated by denaturing sodium-dodecyl sulfate gel electrophoresis and were identified by mass spectrometry. Several previously unidentified full-length sclerostin-interacting proteins such as alkaline phosphatase, carbonic anhydrase, gremlin-1, fetuin A, midkine, annexin A1 and A2, and collagen α1, which have established roles in bone formation or resorption processes, were bound to the sclerostin-MBP amylose resin but not to the MBP amylose resin. Other full-length sclerostin-interacting proteins such as casein kinase II and secreted frizzled related protein 4 that modulate Wnt signaling were identified. Several peptides derived from proteins such as Phex, asporin and follistatin that regulate bone metabolism also bound sclerostin. Sclerostin interacts with multiple proteins that alter bone formation and resorption and is likely to function by altering several biologically relevant pathways in bone.

1. Introduction

Understanding the mechanism of action of factors that regulate bone formation could result in information relevant to the pathogenesis and treatment of bone diseases such as osteoporosis. Sclerostin is an osteocyte-derived, secreted glycoprotein which is important in the regulation of osteoblastic activity [1–5]. In humans, a lack of expression, or reduced expression of sclerostin, results in the sclerosing bone dysplasias, sclerosteosis and van Buchem disease that are associated with osteoblastic hyperactivity, progressive skeletal overgrowth, a high bone mass, and cranio-facial abnormalities [2–5]. Transgene, gene knock-out and neutralizing antibody experiments support the role of sclerostin in the maintenance of bone mass [1,6,7].

Bone formation occurs by intra-membranous and endochondral mechanisms, both of which are influenced by numerous growth factors, including the bone morphogenetic proteins (BMPs) and Wnts [8–12]. Sclerostin has been shown to influence the activity of known BMP and Wnt signaling pathways [1,13–19]. Only limited, unbiased studies of sclerostin interactions with other proteins have been performed [20,21]. No sclerostin targets have been identified using bone-derived proteins and an unbiased approach for identifying binding partners. Therefore, we conducted studies to identify novel bone-derived protein partners of sclerostin using affinity capture followed by mass spectrometric identification of targets. The results demonstrate that sclerostin binds several previously unidentified factors important in bone function.

2. Materials and methods

2.1. Materials

Amylose resin was purchased from New England Biolabs (Ipswich, MA); Nu-Page BIS-TRIS 4–12% gels from Invitrogen (Carlsbad, CA); and bicinchoninic acid (BCA) protein assay reagents from Thermo Fisher Scientific (Waltham, MA). One-year old (400–500 g), Sprague Dawley rats (Harlan, Madison, WI), fed Lab Diet 5053, Pico Lab rodent diet (vitamin D_3: 2.2 IU/g, calcium: 0.81%; phosphorus: 0.63%) were used to obtain bone tissue. Alkaline phosphatase (EC 3.1.3.1) and carbonic anhydrase II (EC 4.2.1.1) were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of MBP and MBP-sclerostin columns

Human sclerostin (aa 24–213)-MBP, or MBP, were expressed using pMAL-C4E in *E. coli Origami 2* (*DE3*) cells [20]. Sclerostin-MBP

* Corresponding author. Address: MS-120, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA. Fax: +1 507 538 9536.
E-mail address: rkumar@mayo.edu (R. Kumar).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.048

fusion protein or MBP alone were separately bound to amylose resin columns. The columns were washed with 4 L wash buffer (WB, 20 mM tris(hydroxymethyl)aminomethane (TRIS), 200 mM NaCl, pH 7.4) and 1 L of high salt buffer (HSB, 20 mM Tris, 600 mM NaCl, pH 7.4). Immediately prior to use, the columns were exchanged into WB.

2.3. Bone decalcification, protein extraction and affinity chromatography

Femurs, tibiae and fibulae from four rats were washed in ice cold water and 70% ethanol. Cartilage was removed, the bones were flash frozen in liquid nitrogen and ground to a fine powder. Fifty mL of 1 M EDTA, pH 7.5, at 4 °C were added to 12.5 g of powdered tissue. The suspension was dialyzed against 4 L of decalcification buffer (1 M EDTA, pH 7.5; 1000 Da MW cut-off membrane) at 4 °C. After 24 h, the dialysate was homogenized with three, 60 s pulses of a Polytron (Brinkman Instruments, Ontario, Canada). Decalcification was continued with 1 M EDTA, pH 7.5, for another 24 h. The sample was homogenized a second time. Decalcification was continued for a total of 72 h. The dialysate was centrifuged at 15,000*g* for 2 h, exchanged into 20 mM Tris, 200 mM NaCl, pH 7.4, over 48 h and finally centrifuged at 15,000*g* for 1 h. Protein content of the sample was determined.

MBP and MBP-sclerostin columns were equilibrated with equal amounts of the protein extract over 2 h. The columns were washed with 2 L of WB at 4 °C. Proteins bound to each column were eluted with 10 × 10 ml of HSB at 4 °C. Eluants from MBP and MBP-sclerostin columns were concentrated to 30 μL each. Protein yield was measured. Equal amounts of protein were electrophoresed on a 4–12% BIS-Tris polyacrylamide gel. The gel was stained with Coomassie Blue.

2.4. Protein identification by tandem mass spectrometry

Individual gel bands and the inter-band regions (IBR) were excised (Fig. 1). Gel bands were destained in 50% acetonitrile, 50 mM Tris, pH 8.1. Proteins within the bands were reduced with 50 mM tris(2-carboxyethyl)phosphine, 50 mM Tris, pH 8.1, at 55 °C for 40 m, and alkylated with 40 mM iodoacetamide, 50 mM Tris, pH 8.1, at 22 °C for 40 m. Proteins were digested *in situ* with 30 μL (0.005 μg/μL) trypsin (Promega Corporation, Madison, WI) in 20 mM Tris pH 8.1, 0.0002% Zwittergent 3–16, at 37 °C for 4–16 h, followed by peptide extraction with 20 μL 2% trifluoroacetic acid (TFA). Pooled extracts were concentrated to less than 5 μL, brought up in 0.2% TFA for protein identification by nano-flow liquid chromatography electrospray tandem mass spectrometry using an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA) and a Thermo Finnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). The digested peptide mixtures were loaded onto a 250 nL OPTI-PAK trap (Optimize Technologies, Oregon City, OR) packed with a C8 solid phase (Michrom Bioresources, Auburn, CA). Chromatography was performed using 0.2% formic acid in both the A solvent (98% water, 2% acetonitrile) and B solvent (80% acetonitrile, 10% isopropanol/10% water), and a 5% B to 50% B gradient over 60 m at flow rate of 325 nL/m through a PicoFrit (New Objective, Woburn, MA) 75 mm × 200 mm column (Michrom Magic C18, 3 μm). The LTQ Orbitrap mass spectrometer was set to perform a FT full scan from 360–1400 m/z with resolution set to 60,000 (at 400 m/z), followed by linear ion trap MS/MS scans on the top five ions. Dynamic exclusion was set to 1 and selected ions were placed on an exclusion list for 30 s. The lock-mass option was enabled for the FT full scans using the ambient air polydimethyl cyclosiloxane (PCM) ion of m/z = 445.120024 or a common phthalate ion m/z = 391.284286 for real time internal calibration [22].

2.5. Database searching

Tandem mass spectra were extracted by BioWorks version 3.2. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.04), Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12) and X! Tandem (www.thegpm.org; version 2006.09.15.3) searched against the Swiss-Prot database release 2011-01. The search was left open to all species.

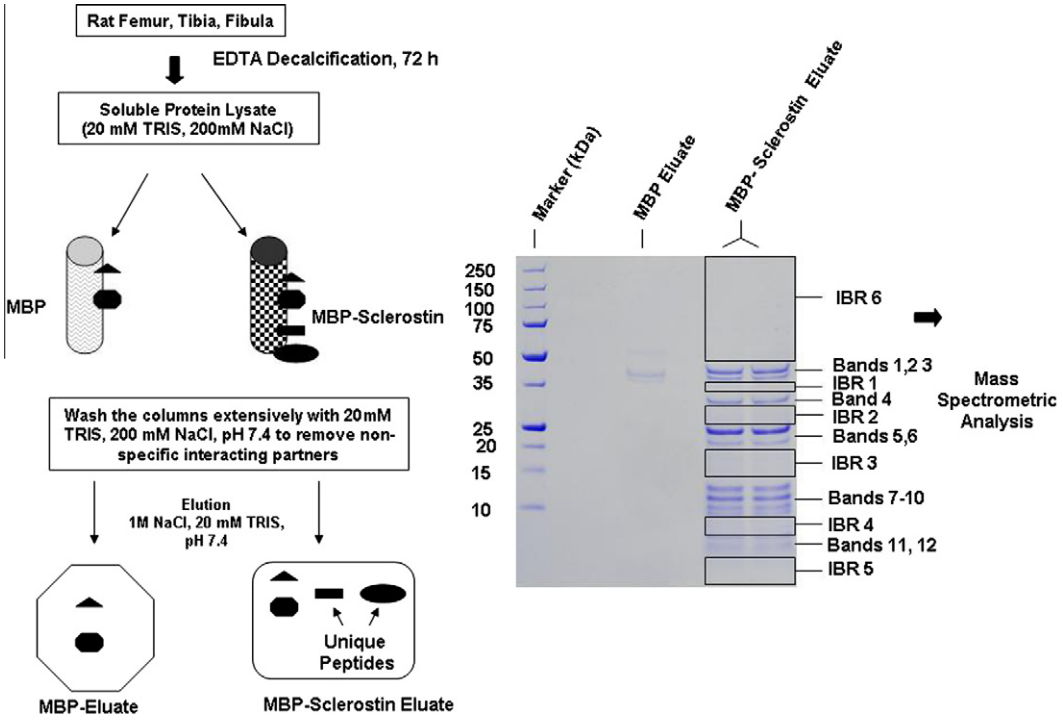

Fig. 1. Procedure for identifying bone derived proteins that interact with sclerostin.