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The Sclerostin-Bone Protein Interactome

Hemamalini Devarajan-Ketha^a, Theodore A. Craig^a, Benjamin J. Madden^c, H. Robert Bergen III^{b,c}, and Rajiv Kumar^{a,b}

^a Division of Nephrology and Hypertension, Department of Medicine, 200 First Street SW, Rochester, MN 55905, USA.

^b Department of Biochemistry and Molecular Biology, 200 First Street SW, Rochester, MN 55905, USA.

^c Mayo Proteomics Research Center, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

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 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 1M 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 $\alpha 1$, 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 protein 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.

Keywords

Sclerostin; protein interaction; protein affinity chromatography; bone formation; bone resorption

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

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Address inquiries to: Rajiv Kumar MS-120 Mayo Clinic 200 First St SW Rochester, MN 55905 rkumar@mayo.edu Telephone: 507-284-0020 Fax: 507-538-9536.

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the regulation of osteoblastic activity [1,2,3,4,5]. 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 [2,3,4,5]. 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 [8,9,10,11,12]. Sclerostin has been shown to influence the activity of known BMP and Wnt signaling pathways [1,13,14,15,16,17,18,19]. 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.

Materials and Methods

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 D₃: 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).

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 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.

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 Dalton 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 48h 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 μ L 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.

Protein Identification by Tandem Mass Spectrometry

Individual gel bands and the inter-band regions (IBR) were excised (**Figure 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 μ L (0.005 μ g/ μ L) 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 μ L 2% trifluoroacetic acid (TFA). Pooled extracts were concentrated to less than 5 μ L, 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 $\times$ 200 mm column (Michrom Magic C18, 3 μ m). 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 polydimethylcyclsiloxane (PCM) ion of m/z = 445.120024 or a common phthalate ion m/z = 391.284286 for real time internal calibration [22].

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.

Functional significance was assigned by searching the UniProt database (<http://www.uniprot.org/>).

Criteria for protein identification

Scaffold (version Scaffold_2_00_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [23]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony [24].

Bioactivity and interaction assays

Alkaline phosphatase activity was measured in the presence or absence of 0.1 μ M insect cell sclerostin [25], using increasing amounts of alkaline phosphatase (from 0.023 to 0.23 μ M), *p*-nitrophenyl phosphate as substrate and by measuring absorbance at 480 nm. Interactions

between carbonic anhydrase II and sclerostin were measured using bio-layer interferometry (Octet 96 instrument, ForteBio Inc., Menlo Park, CA) [26]. Insect cell sclerostin at concentrations decreasing (in three-fold dilutions) from 1 μ M in Octet assay buffer (1X PBS containing 0.1mg/mL BSA, 0.002% Tween-20) was used. The assay was run with SuperStreptavidin Biosensors and biotinylated carbonic anhydrase II bound to and immobilized on the sensors. Sensorgrams from the 6 lowest concentrations (1.3-333 nM) were used to calculate K_{DS} . Buffer without sclerostin was run as reference.

Statistical analysis

Differences between alkaline phosphatase activity in the presence or absence of sclerostin were analyzed using regression analysis and analysis of co-variance with the JMP statistical analysis program (SAS Institute, Cary, NC). Curves were fitted and kinetic parameter calculated using software supplied with the OctetRED96 instrument.

Results and Discussion

Bone decalcification and affinity chromatography

The bone-decalcification method yielded 184 mg soluble protein from 12.6 g of bone. From 92 mg of bone protein applied to the MBP-sclerostin column, 49 μ g of protein was eluted with the high salt buffer (**Figure 1**). Only 15 μ g protein eluted from the MBP column.

Captured proteins and their biological roles

Thirty nine full-length unique sclerostin-interacting proteins were identified (**Table 1**). Each protein had a M_r approximately equivalent to that of the full-length protein. Several peptides ($n = 2$ to 40 peptides per protein) with 6% to 62% sequence coverage were identified from each protein. Further characterization of the identified proteins on the basis of cellular location and function was performed using the UniProt database. Several protein fragments were found to interact with sclerostin. All the identified proteins (full-length and peptide fragments), the number of unique peptides found for each protein, their cellular location and molecular and biological functions are shown in **Supplemental Table 1**.

Several full-length sclerostin-interacting proteins with an established role in bone mineralization processes, including annexins 1 and 2, collagens, alkaline phosphatase, carbonic anhydrase, gremlin 1, fetuin A, secreted frizzled related protein 4 (sFRP4), and slit homolog 3 were identified [27,28,29]. Heparin binding proteins including neuropilin-1, platelet factor 4, biglycan, and thrombospondin 1, were identified. Calcium and metal (Zn, Cu and Fe) binding proteins such as annexins 1 and 2, neuropilin-1, eosinophil peroxidase, carbonic anhydrase-1 and alkaline phosphatase were represented. Several ATP binding, DNA binding, catalytic, structural, and motor proteins were found to interact with sclerostin. **Table 2** and **Supplemental Table 1** describe the role of each of the members involved in the bone mineralization processes.

Alteration of alkaline phosphatase bioactivity by sclerostin

To examine the biological relevance of these interactions, we tested the effect of added sclerostin on the bioactivity of one of the interacting proteins, namely, alkaline phosphatase. Added sclerostin inhibited the bioactivity of alkaline phosphatase over the range tested, with a statistical difference in the slope of activities compared to buffer alone ($p = 0.243$).

Affinity of sclerostin for carbonic anhydrase

We also examined the affinity of binding of sclerostin to carbonic anhydrase, by bio-layer interferometry using carbonic anhydrase as a capture molecule on the probe tip Octet96 interferometer. The $K_D = 1.40 \times 10^{-8} \pm 4.8 \times 10^{-9}$ M from steady state analysis.

Modulation of multiple signaling pathways in bone by sclerostin

Bone is a highly calcified matrix in which many bioactive proteins are embedded. Sclerostin is secreted by osteocytes and is likely to function by binding and regulating the activities of other protein targets present in the bone microenvironment. The mechanism of action of sclerostin has been proposed to involve the regulation of BMP and Wnt activity [1,13,14,15,16,17,18,19]. Additionally, data from our laboratory has shown that sclerostin interacts with Cyr61 and the ErbB3 receptor and regulates their function [20]. However, with the exception of the latter experiments, most previous experiments have addressed the regulation by sclerostin of pathways known to alter bone growth and differentiation. No experiments have taken an unbiased approach to examine the interactions of sclerostin from bone.

Sclerostin has previously been shown to interact with modulators of the BMP and Wnt pathways such as BMP4, BMP2 and LRP 5/6 [1,13,14,15,16,17,18,19]. We have now identified four new BMP/activin inhibitors (gremlin, biglycan, asporin and follistatin) that interact with sclerostin. The first three, were detected as full-length proteins. Peptide fragments derived from follistatin were identified. Gremlin 1, a cystine-knot protein, functions as a BMP antagonist, thereby suppressing osteoblast function [30]. Biglycan contains leucine-rich repeats, and inhibits bone formation [31]. Asporin modulates BMPRI activity [32]. Follistatin inhibits activin/BMP signaling by a direct interaction with activins and BMPs [28,33]. Thus, by interacting with gremlin, biglycan, asporin, and follistatin, sclerostin might potentiate the inhibitory effects of these proteins on BMP signaling. Sclerostin has been previously shown to interact with the LRP 5/6 receptor [15,34]. We show now that sclerostin also interacts with several other Wnt pathway regulatory molecules such as secreted frizzled related protein 4 (sFRP4), casein kinase II, and TRAF2 and NCK-interacting kinase [35,36,37,38]. Sclerostin was found to interact with SLIT 3 and SLIT2 that may act as molecular guidance cues in cellular migration, and whose functions are mediated by interactions with roundabout homolog receptors [39]. These proteins are expressed during limb development in mesenchymal cells and chondrocytes [39]. The annexins which are synthesized by chondrocytes and osteoblasts and are postulated to play a role in mineralization [40] bind sclerostin, and it is possible that sclerostin may regulate their activities. Extracellular-matrix associated proteins such as the collagens, which are vital for normal bone formation bind sclerostin [41]. They may act to sequester sclerostin in bone.

Several membrane-specific sclerostin-interacting proteins were identified, demonstrating that sclerostin influences the activity of several important cellular regulatory pathways. For example, interactions with cilia-associated proteins, such as ezrin and myocilin suggest that sclerostin may alter ciliary processes that influence biomineralization [42].

Sclerostin bound alkaline phosphatase which is present in osteoblasts and the absence of which is associated with hypophosphatasia [43]. The activity of alkaline phosphatase was inhibited by sclerostin. Additionally, sclerostin was bound with high affinity to carbonic anhydrase, an enzyme responsible for generating hydrogen ions needed for bone resorption mediated by osteoclasts [44,45]. The membrane bound endopeptidase, PHEX, mutations in which are present in X-linked hypophosphatemic rickets [46], is bound by sclerostin, and *sost* mRNA is expressed in greater amounts in bone from *Hyp* mice than in normal controls

[47]. These data suggest that sclerostin may be important in the etiology of the mineralization defect found in hypophosphatemic rickets [48].

Our report validates the role of sclerostin in multiple signaling pathways and also suggests a mutual regulatory relationship amongst the various proteins exhibiting inhibitory effects in the bone development process. Although functional studies are required to validate the physiological relevance of these interactions, this study has identified several new putative protein interaction partners of sclerostin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- An affinity capture-mass spectrometry method identified sclerostin-binding partners.
- Novel sclerostin-binding partners from bone matrix were found.
- Sclerostin interacts with proteins that influence bone formation and resorption.

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

Table 1

Full-length sclerostin interacting proteins in specific gel regions. Band 1 was maltose-binding protein. Biological function was assigned by searching the UniProt Database (<http://www.uniprot.org/>).

Band or Region	Proteins	MW (kDa)	# Unique Peptides	Percent Coverage	Biological Function
Band 2	Aspartate amino transferase	46	2	6	Enzyme
Band 3	Glia-derived nexin	44	5	19	Serine protease inhibitor with activity toward thrombin, trypsin, and urokinase.
Band 4	sFRP4	35	5	22	Wnt signaling.
Band 4	Annexin A1	39	2	6	Regulation of mineralization
Band 4	Annexin A2	39	2	6	As above.
Bands 5	Latexin	26	10	57	Tissue carboxypeptidase inhibitor.
Band 5	Granzyme M	26	8	35	Natural killer cell granular protease.
Band 6	Casein kinase 2 β	25	11	48	Wnt signaling
Bands 7	Histone H2B	14	13	62	DNA binding.
Band 8	Histone H2A	14	6	45	DNA binding.
Band 9	Midkine	15	3	23	Deficiency associated with higher bone mass
Band 10	Platelet Factor 4	11	6	37	Megakaryocyte differentiation, inhibits osteoblast function
Band 11	Platelet Factor 4	11	4	37	As above.
Band 11	Histone type 4	11	6	50	DNA binding.
Interband region 6	Myosin 4	223	40	23	Contraction.
	Collagen α 1	340	27	11	EC matrix, mineralization.
	α 1 inhibitor 3	164	23	19	Trypsin inhibitor.
	SLIT3	168	17	16	Regulation of osteoblast function. Interacts with gremlin 1.
	Glycogen phosphorylase	97	15	22	Enzyme.
	Serotransferrin	76	13	23	
	Eosinophil peroxidase	81	7	14	Enzyme.
	Myosin 7	223	7	14	Muscle contraction.
	Alkaline phosphatase	57	6	16	Osteoblast differentiation marker. Absence associated with hypophosphatasia.
	PRPF40	69	5	10	Binds to WASL/N-WASP. Plays a role in the regulation of cell morphology and cytoskeletal organization.

Band or Region	Proteins	MW (kDa)	# Unique Peptides	Percent Coverage	Biological Function
	Neuropilin	103	5	9	Co-receptor for semaphorin. Involved in angiogenesis.
	Podocan	69	5	10	Negatively regulates cell proliferation and cell migration.
	PLBL1	63	2	21	Phospholipase acting on various phospholipids.
	Glucose-6-phosphate isomerase	63	3	8	Enzyme.
Interband region 1	Glia-derived nexin	44	16	43	See above.
	Casein Kinase II Subunit α I	45	5	15	Wnt pathway regulation.
	Casein Kinase II Subunit α II	45	4	15	Wnt pathway regulation.
	Fetuin A	38	2	5	Regulation of endochondral ossification.
Interband region 2	Latexin	26	5	27	As above.
	Carbonic anhydrase 1	28	3	13	Enzyme.
Interband region 3	Gremlin 1	21	10	32	BMP antagonist. Conditional deletion of gremlin in mice results in increased bone mass. Interacts with SLIT2.
	Platelet factor 4	11	7	38	As above.
	Midkine	15	3	30	As above.
Interband region 4	Serine protease inhibitor Kazal-type 3	9	3	45	Trypsin inhibitor.
	Protein S100	10	2	32	EF hand protein.
Interband region 5	Platelet factor 4	11	3	37	As above.

Table 2

Sclerostin-interacting partners with an established role in the regulation of bone.

Protein (abbreviation)	# of Unique Peptides	Function
Alkaline Phosphatase	6	Osteoblast differentiation marker. Absence associated with hypophosphatasia [43].
Annexins A1 and A2	4, 3	Regulation of extra cellular matrix mineralization, Interacts with collagens [43].
Asporin	6	Negative regulation of TGF- β signaling via the LRR motif. Regulation of osteoblast function [32]
Biglycan	4	Regulates BMP4 induced osteoblast differentiation, Targeted disruption leads to an osteoporosis type phenotype [31].
Carbonic Anhydrase 1 and 2	3, 3	Osteoclast function [44,45]
Casein kinase II Subunit α	25	Wnt pathway regulation; alters BMP signaling in bone [49].
Collagens 2A1, 5A1, 1A1	6, 4, 3	Formation of extra-cellular matrix [41]
Fetuin A, Alpha-2-HS-glycoprotein	8	Regulation of endochondral ossification [50].
Follistatin	8	Regulates extracellular matrix mineralization [28].
Gremlin 1	12	BMP antagonist. Conditional deletion of Gremlin in mice results in increased bone mass phenotype. Interacts with SLIT2 [30,51]
Midkine	3	Midkine deficiency leads to higher bone mass [52]
Phosphate Regulating Enzyme with homology to Zn metallo-endopeptidase (PHEX)	9	Loss of function mutation results in hypophosphatemic rickets and impaired mineralization [46,48].
Slit Homologs (SLIT 3, SLIT2)	25, 11	Regulation of osteoblast function. Interact with GREM1 [39].
TRAF2 and NCK-Interaction Kinase	11	Wnt pathway regulation [38].