Journal of Cellular Biochemistry 101:466–476 (2007)

A Proteomic Analysis of Adult Rat Bone Reveals the Presence of Cartilage/Chondrocyte Markers

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Abstract The non-mineral component of bone matrix consists of 90% collagenous, 10% non-collagenous proteins. These proteins regulate mineralization, growth, cell signaling and differentiation, and provide bone with its tensile strength. Expression of bone matrix proteins have historically been studied individually or in small numbers owing to limitations in analytical technologies. Current mass-spectrometric and separations technologies allow a global view of protein expression patterns in complex samples. To our knowledge, no proteome profile of bone matrix has yet been reported. Therefore, we have used mass spectrometry as a tool to generate a profile of proteins present in the extracellular matrix of adult rat bone. Overall, 108 and 25 proteins were identified with high confidence in the metaphysis and diaphysis, respectively, using a bottom up proteomic technique. Twenty-one of these proteins were present in both the metaphysis and diaphysis including the bone specific proteins, osteocalcin, type I collagen, osteopontin, osteoregulin, and bone sialoprotein. Interestingly, type II collagen, a protein thought to be exclusively expressed in cartilage, was identified in both the metaphysis and diaphysis. This observation was validated by Western blot. Additionally, the presence of aggrecan, another protein expressed in cartilage was identified in the bone matrix extracts by Western blot. The proteome profile generated using this technology represents an initial survey of the acid soluble proteins of bone matrix which provides a reference for the analysis of deviations from the normal composition due to perturbations or disease states. J. Cell. Biochem. 101: 466–476, 2007.

Key words: proteomics; bone matrix proteins; LC/MS; endochondral ossification

Bone strength is determined by a number of factors including bone size and geometry, levels and quality of mineralization, tissue turnover, and microarchitecture. Diagnostic tools including bone densitometry are currently used to predict fracture risk, but are not able to capture all aspects of bone strength including flexibility and resistance against fracture of bone. Recent reviews indicate the need to identify diagnostic measurements to supplement bone densitometry in predicting fracture risk [Recker and Barger-Lux, 2004; Felsenberg and Boonen, 2005; Rubin, 2005, Srivastava et al., 2005]. Changes in material properties of bone, including the orientation and mineralization of the collagen fibrils can affect its strength [Oxlund et al., 1996]. A recent study by Garnero et al. [2006] concluded that changes in collagen post-translational modification contribute to the flexibility of the bone and its ability to resist fracture. These changes in mechanical properties of the bones were independent of bone mineral density, a commonly used predictor of bone strength. In addition to collagens (which contribute 90% of the protein mass), the non-mineral component of bone contains a variety of non-collagenous proteins. These proteins regulate mineralization, growth, mediate cell signaling, and control cellular differentiation. Cumulatively, these processes provide bone with its tensile strength. Therefore, comprehensive information and knowledge of the

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

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Received 30 August 2006; Accepted 11 October 2006
DOI 10.1002/jcb.21196

proteome of bone matrix is essential for understanding the physiology and metabolic activity of bone.

Mass spectrometry has already been used in bone research to study protein expression in various systems. Proteins extracted from human demineralized bone matrix have been separated using 2D-gel electrophoresis and individual proteins identified by MALDI-TOF. From this analysis, alpha B-crystallin was found to be present in bone matrix [Behnam et al., 2002]. Separation of proteins by 2D-gel electrophoresis followed by MALDI-TOF analyses have also been used to identify proteins involved in the mechanism of action of glucocorticoids on osteoblast cell lines [Olkku et al., 2004] as well as identifying proteins associated with TRAF6 regulating osteoclast differentiation in vitro [Ryu et al., 2005]. Global differential expression of proteins in cell culture have been monitored in the investigations of the differentiation of osteoblasts [Kim et al., 2005; Salasznyk et al., 2005] and osteoclasts [Kubota et al., 2003; Czupalla et al., 2005] as well as in the responses of osteoblasts [Conrads et al., 2004, 2005] and bone marrow mesenchymal stem cells [Wang et al., 2004] to treatment. All but one of these studies used 2D-gel electrophoresis followed by mass spectral analysis of excised spots to identify differentially expressed proteins. The exception was Conrads et al. [2004, 2005] who observed global changes in total cell lysates using isotope-coded affinity tag (ICAT) technology. Two-DE followed by mass spectrometry has been utilized to study differential expression of proteins in bones between sham and ovariectomized (OVX) rats. Proteins exhibiting differential expression include peroxiredoxin 2, myosin light polypepetide 2, and ubiquitin-conjugating enzyme E2-16 kD [Fan et al., 2005]. Large-scale comprehensive identification of proteins was not attempted in any of these studies.

Therefore, the objective of the current study was to use mass spectrometry as a tool to generate a profile of proteins in the extracellular matrix of normal adult metaphyseal and diaphyseal bone. Defining most of the proteins in the metaphysis and diaphysis of bone will facilitate a better understanding of bone metabolism and provide a method to investigate specific protein expression patterns. The proteome profile generated using this technology represents the first pass at a comprehensive survey of acid soluble bone matrix proteins and shows differences in the population of proteins in metaphyseal and diaphyseal regions of bone. An unexpected result was the observation of cartilage matrix proteins in bone in regions well removed from any potential mineralized cartilage contamination. These data provide a reference for the analysis of deviations from normal physiology due to perturbations, age, or disease states.

MATERIALS AND METHODS

Bone Protein Extraction

Femurs from 6-month-old female Sprague–Dawley rats were excised, cleaned of all muscle and connective tissue, and the growth plate was removed. The bones were immediately frozen in liquid nitrogen and stored at −80°C until sample processing. Approximately 3 mm of the proximal and distal metaphyseal ends were separated from the diaphysis and pooled. The diaphysis (6 mm of the femoral midregion) was collected by removing an additional 1–2 mm of the proximal and distal metaphyseal ends, removing any contamination with the secondary spongiosa. The metaphyseal ends and the diaphysis were rinsed with PBS and then with acetone until the rinsing solutions were clear to remove bone marrow and lipids. The bone fragments were lyophilized, crushed between two aluminum plates under liquid nitrogen, and subsequently ground to a powder with a mortar and pestle under liquid nitrogen. The proteins were extracted from the powdered metaphysis and diaphysis using a 10-fold excess of 10% formic acid (v/w) for 2 h at 4°C. The extracts were dialyzed against water overnight followed by 0.02 M ammonium bicarbonate (pH 8) for 2 h at 4°C to neutralize the formic acid.

Articular cartilage was scraped from the growth plate of the distal femur, pooled with cartilage scraped from the proximal tibial metaphysis and ground to a fine powder under liquid nitrogen with a mortar and pestle. Ten milligrams of the ground articular cartilage and the collagenous residue that remained following formic acid extraction of bone were rinsed with water and extracted overnight at 4°C in a 50-fold excess of buffer containing 4 M guanidine hydrochloride, 0.01 M Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, and protease inhibitors (Complete EDTA, Roche Applied Science, Indianapolis, IN) as well as pepstatin (Roche Applied

Science). The supernatant was dialyzed against 0.1 M sodium acetate in 0.1 M Tris-HCl pH 7.3 for 1 h at 4°C using a 3500 Da MWCO dialysis membrane (Spectrum Labs, Rancho Dominguez, CA). The dialyzed samples were dried in a speedvac and resuspended in a solution containing 50 mM sodium acetate, 50 mM Tris-HCl pH 7.5, and 10 mM EDTA. The protein concentration of the formic acid and guanidine hydrochloride extracts of bone as well as the guanidine hydrochloride extracts of articular cartilage was measured using the Bradford assay.

Western Blot

Ten micrograms of formic acid extract from the metaphysis and diaphysis were incubated in SDS sample reducing buffer for 5 min at 95°C. Residues (10 μg) from the insoluble fractions were incubated in SDS sample reducing buffer for 10 min at 95°C. Articular cartilage from 3-week- and 6-month-old Sprague–Dawley rats were extracted using a 10-fold excess of reducing SDS sample buffer (v/w). The reduced samples were separated on 4–20% gradient Tris-Glycine gel, and transferred to nitrocellulose membranes, and blocked for 1 h using reagents included in LumiGLO Western Blotting Kit (KPL, Inc., Gaithersburg, MD). Immunoblotting was conducted by incubating the membrane with 2 μg/ml of primary mouse monoclonal antibody against type II collagen at room temperature for 2 h (Collagen II Ab-2 Clone 2B1.5, Lab Vision Corp., Fremont, CA) or 1 μg/ml primary rabbit polyclonal antibody against aggrecan at room temperature for 1 h (Abcam, Cambridge, MA). The membranes were washed four times using the wash solution included with the LumiGLO Western Blotting Kit and incubated with 1:10,000 dilution of secondary antibodies against mouse or rabbit for 1 h at room temperature followed by four additional washes. Bands were visualized by the horseradish peroxidase reaction and exposure of the membrane to Biomax X-Omat AR-2 film (Eastman Kodak, Rochester, NY).

Protein Fractionation and Mass Spectral Analysis

The formic acid soluble extracellular matrix extracts were fractionated by reversed phase chromatography using an Agilent 1100 Series HPLC (Palto Alto, CA) by injecting 100 μg of protein from each extract onto a 1 mm × 25 cm protein C4 Vydac column (Hesperia, CA) equilibrated with 1.0% CH_3CN, 0.1% TFA. Proteins were eluted from the column with a gradient from 1 to 70% CH_3CN, 0.1% TFA, developed over 60 min at a flow rate of 100 μl/min, and 2-min fractions were collected. A 50 μl volume of each fraction was added to an equal volume of reduction/alkylation cocktail (50% 0.050 M ammonium carbonate, 48.75% acetonitrile, 1% iodoethanol, and 0.25% triethylphosphine; final pH 10). The tubes were capped and incubated at 37°C for 60 min. The samples were then uncapped and evaporated on a speedvac until the sample was dry (approximately 2 h). The dried pellet was reconstituted directly in a 20 μl solution of 25 μg/ml of Worthington TPCK treated in 0.1 M ammonium bicarbonate (pH 8) and digested overnight at 37°C.

Mass Spectrometry Conditions and Protein Identification

Tryptic digests (10 μl) were desalted with C-18 Ziptips™ (Waters) and were injected onto a 75 μm × 5 cm New Objectives PicoFrit Aquasil C18 capillary reversed phase column. The Thermofinnigan Surveyor HPLC system was run at 50 μl/min with solvent A as 0.1% formic acid in water and solvent B as 0.1% formic acid in acetonitrile. The flow was split with an LC Packings flow splitter to approximately 1.5–2 μl/min and directed into a LC Packings FAMOS autosampler. Sample was loaded on the column at this flow rate for 8 min. A splitting tee was then opened between the autosampler and the column via a valve reducing the flow across the column to 200–300 nl/min for the duration of the separation. Bound peptides were eluted with a gradient from 2% B to 45% B developed over 35 min then increasing to 80% B for an additional 4 min. The outlet of the column was placed in a tube with a 15 psi flow of nitrogen directed at the mass spectrometer's inlet. An LTQ-FTICR mass spectrometer (Thermofinnagan) was used for analysis. The electrospray voltage was set to 2,500 V and was applied to a metal union in the flowpath. The mass spectrometer was operated in a triple play mode where a parent mass scan was collected in the LTQ, ions were selected in a data dependant manner and the most intense ion was selected for a FT SIM scan and a MS/MS scan on the LTQ. Analyzed ions were placed on the rejection list for 2 min and the cycle was repeated through the LC gradient.

Protein identification was accomplished by searching MS/MS acquired during the LC/MS analysis using the database searching programs *Sequest* (ThermoFinnigan) and *X! Tandem* (Beavis Informatics) [Eng et al., 1994; Craig and Beavis, 2003]. *X! Tandem* searches were performed against the International Protein Index (IPI) database (version 3.02) [Kersey et al., 2004; International Protein Index, European Bioinformatics Institute, 2005]. *Sequest* searches were performed against the nonredundant protein database (RefSeq database Jan 4, 2005) provided by NCBI [National Center for Biotechnology Information, 2005]. *Sequest* searches were run separately with trypsin specificity and again with no enzyme specificity. All searches were executed with the following potential mass modifications: oxidized methionine, de-amidated asparagine and glutamine, hydroxylated proline, and γ-carboxyglutamic acid. The output parameters from *Sequest* and *X! Tandem* were combined and used as input to a classification model that produces an overall score for correct identification [Higgs et al., submitted]. False positive identifications were controlled by running the searches against reversed versions of the protein databases and estimating *P*-values: the probability of observing a model score from the reversed database search that exceeds the observed score from the correct database. False discovery rates (q-values) for peptide identifications were estimated from the *P*-values using the method described by Benjamini and Hochberg [1995]. Peptides with q-values less than 0.10 and mass accuracy less than 0.03 Da were retained for further biological interpretation.

RESULTS AND DISCUSSION

This study was conducted in order to generate a profile of proteins in the extracellular matrix of normal adult rat bone. Knowledge of this profile will facilitate examination of the effects of perturbations, age, or disease state on the protein expression profile. Mass spectrometry also provides methods to investigate specific protein expression patterns independent of antibody availability and specificity. In the present study, we generated a protein profile of the metaphyseal and diaphyseal regions of bone to identify populations of proteins that reflect the differences in the bone turnover rate in trabecular as compared to cortical bone. The outcome of this work was the identification of 108 and 25 proteins with high confidence in the metaphyseal and diaphyseal regions of bone, respectively. Twenty-one of these proteins were observed in both of the regions of bone we examined (Fig. 1). The complete list of proteins that were observed are listed in Table I, with supporting information and spectra from the top matched peptide for each protein available in Supplemental Tables I and II. The profile of proteins identified in the acid-soluble matrix of metaphyseal and diaphyseal bone includes proteins associated with the bone matrix (e.g., Type I collagen, osteopontin, osteocalcin, matrix gla protein), or bone cell function such as proteases and phosphatases (e.g., cathepsin K and cathepsin D, tartrate resistant acid phosphatase 5, alkaline phosphatase), antioxidants (e.g., superoxide dismutase, thioredoxin), nuclear proteins (e.g., sp120, far upstream binding protein, histones) as well as secreted proteins (e.g., vitamin D-binding protein), and proteins potentially captured from circulation (e.g., alpha-2-HS-glycoprotein, hemopexin, transthyretin).

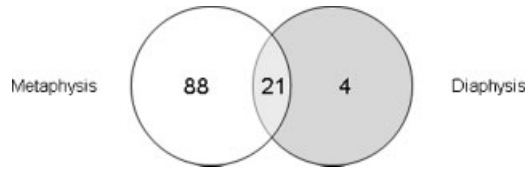

Fig. 1. Analysis of proteins in the formic acid soluble fraction of bone identified proteins common as well as unique to metaphyseal and diaphyseal bone matrix.

A greater number of proteins were identified in the metaphysis as compared to the diaphysis (Table I; Fig. 1). The proteins that were exclusively identified in the metaphysis represent a population of proteins consistent with a higher rate of bone turnover in the metaphyseal region of bone as compared to the diaphyseal region. Some of these proteins include ribosomal proteins involved in protein synthesis, plasma transport proteins (e.g., hemopexin, and vitamin D-binding protein precursor), enzymes associated with bone turnover (tartrate resistant acid phosphatase 5, cathepsins D and K, and matrix metalloproteinase 13), and proteins involved in bone mineralization (bone sialoprotein and osteonectin). The identification of non-secreted proteins such as the ribosomal proteins in the extracellular matrix of the metaphyseal region may be due to cell lysis

TABLE I. Unique and Common Acid Soluble Proteins in Metaphyseal as Compared to Diaphyseal Bone

International protein index	Protein annotation	# Distinct peptide in metaphyseal bone	# Distinct peptide in diaphyseal bone
IPI00327469.1	Alpha-2-HS-glycoprotein precursor	31	23
IPI00204991.1	Collagen alpha1	29	17
IPI00327895.3	Osteopontin precursor	25	13
IPI00188921.1	Collagen alpha 2(I) chain precursor	24	16
IPI00188909.1	Collagen alpha 1	16	11
IPI00205809.3	Collagen alpha 1 type II	10	2
IPI00205467.1	Dentin matrix acidic phosphoprotein 1 precursor	9	7
IPI00191327.1	Osteoregulin	9	3
IPI00417757.1	Procollagen, type XI, alpha 2	8	5
IPI00231643.3	Superoxide diSmutaSe 1	8	2
IPI00188804.1	60S acidic ribosomal protein P2	8	—
IPI00324019.1	Alpha-1-antiproteinase precursor	7	—
IPI00324380.3	Transthyretin precursor	6	1
IPI00200920.1	Far upstream element binding protein 2	6	—
IPI00230925.3	Thymosin beTa-4	6	—
IPI00197709.1	Osteocalcin precursor	5	6
50675	S20638 desmoyokin—mouse (fragment)	5	—
IPI00190240.1	Ribosomal protein S27a	5	—
IPI00195516.1	Hemopexin precursor	5	—
IPI00194097.1	Vitamin D-binding protein precursor	4	—
IPI00194148.1	Similar to 40S RIBOSOMAL PROTEIN S19	4	—
IPI00195160.1	Sulfated glycoprotein 1 precursor	4	—
IPI00201561.1	Peroxiredoxin 2	4	—
IPI00203523.1	60S ribosomal protein L23a	4	—
IPI00207668.1	Afamin precursor	4	—
IPI00210090.3	SP120	4	—
IPI00211779.1	Peroxiredoxin 1	4	—
IPI00212314.3	Moesin	4	—
IPI00231701.4	High mobility group protein 2	4	—
IPI00195241.1	Beta-2-glycoprotein I precursor	3	1
17981389	AHNAK-related protein [*Rattus norvegicus*]	3	—
IPI00200593.1	Contrapsin-like protease inhibitor 1 precursor	3	—
IPI00202717.1	Tartrate-resistant acid phosphatase type 5 precursor	3	—
IPI00205448.1	Bone sialoprotein II precursor	3	—
IPI00230916.5	Ribosomal pRotein L13	3	—
IPI00231368.3	Thioredoxin	3	
IPI00231632.3	HigH mobility group box 1	3	—
IPI00231697.3	Stathmin 1	3	—
IPI00326433.4	10 kDa heat shock protein, mitochondrial	3	—
IPI00365861.2	Angiogenin	3	—
IPI00231099.3	Spp-24 precurSor	2	3
2499391	Bone sialoprotein	2	2
IPI00208051.1	Neutrophil antibiotic peptide NP-4 precursor	2	2
27688933	Similar to Collagen alpha1 [*Rattus norvegicus*]	2	1
15928578	FUB1 MOUSE Far upstream element binding protein 1 (FUSE binding protein 1) (FBP)	2	—
6754994	PCB1 MOUSE Poly(rC)-binding protein 1 (Alpha-CP1) (hnRNP-E1)	2	—
IPI00189627.1	Chromogranin A precursor	2	—
IPI00191794.1	Non-histone chromosomal protein HMG-17	2	—
IPI00194974.1	Heterogeneous nuclear ribonucleoprotein K	2	—
IPI00200114.1	Similar to erythroid differentiation-related factor	2	—
IPI00204359.1	Beta-2-microglobulin precursor	2	—
IPI00206378.1	Cathepsin K precursor	2	—
IPI00208209.1	PRx IV	2	—
IPI00212731.1	Cathepsin D precursor	2	—
IPI00231346.3	Ribosomal pRotein L30	2	—
IPI00231864.3	CytoChrome C, somatic	2	—
IPI00231955.4	Calmodulin 3	2	—
IPI00326596.2	Stathmin 2	2	—
IPI00327143.1	Alkaline phosphatase, tissue-non-specific isozyme precursor	2	—
IPI00359085.1	Similar to hypothetical protein DKFZp434L0117	2	—
IPI00367840.1	Similar to riboSomal protein S27a	2	—
IPI00373416.1	Procollagen tyPe XI alPha 1	2	—
IPI00464535.1	Splice Isoform 2 Of Plasminogen activator inhibitor 1 RNA-binding protein	2	—
IPI00470260.2	GM2 ganglioside activator protein	2	—
IPI00215485.1	Insulin-like growth factor binding protein 5 precursor	1	7

(*Continued*)

TABLE I. (*Continued*)

International protein index	Protein annotation	# Distinct peptide in metaphyseal bone	# Distinct peptide in diaphyseal bone
IPI00211401.1	Matrix Gla-protein precursor	1	5
IPI00207174.1	Splice Isoform 1 Of Insulin-like growth factor I precursor	1	3
IPI00231262.3	S100 calcium binding protein A9 (calgranulin B)	1	2
45598372	22 kDa neuronal tissue-enriched acidic protein [*Mus musculus*]	1	—
6754750	Moesin	1	—
IPI00188079.2	Similar to RiboSome-binding protein 1 (RiboSome receptor protein) (mRRp)	1	—
IPI00189424.1	SPARC precursor	1	—
IPI00189631.1	Cytochrome P450-like protein	1	—
IPI00189813.1	Actin, alpha skeletal muscle	1	—
IPI00192749.1	Ubiquitin-like protein SMT3B	1	—
IPI00194683.1	Telomerase protein component 1	1	—
IPI00197553.1	Splice Isoform 1 Of Nucleophosmin	1	—
IPI00197703.1	Apolipoprotein A-I precursor	1	—
IPI00200591.1	Contrapsin-like protease inhibitor 3 precursor	1	—
IPI00204703.1	47 kDa heat shock protein precursor	1	—
IPI00207980.1	60S ribosomal protein L23	1	—
IPI00208053.1	Neutrophil antibiotic peptide NP-2 precursor	1	—
IPI00208271.1	Paired mesoderm homeobox protein 2A	1	—
IPI00209976.1	ATPase inhibitor, mitochondrial precursor	1	—
IPI00210238.1	Ribosomal pRotein S23	1	—
IPI00211336.1	Similar to SH3 domain binding glutamic acid-rich protein-like 3	1	—
IPI00212708.2	Fetub protein	1	—
IPI00212776.1	40S ribosomal protein S3	1	—
IPI00213405.1	BART-1 protein	1	—
IPI00213638.3	Lens epithelium-derived growth factor a	1	—
IPI00213988.1	Similar to riboSomal protein L31	1	—
IPI00231107.3	Parathymosin	1	—
IPI00231275.3	Beta-galactoside-Binding lectin	1	—
IPI00231317.3	Matrix Metalloproteinase 13	1	—
IPI00231370.3	S100 calcium binding protein A8 (calgranulin A)	1	—
IPI00231692.3	Ribosomal pRotein S15	1	—
IPI00358804.1	Similar to Muf1-pending protein	1	—
IPI00359024.1	Similar to Extra cellular link domain-containing 1	1	—
IPI00359402.1	Similar to heterogeneouS nuclear ribonucleoprotein G—human	1	—
IPI00360717.1	Similar to phoSphoriboSylaminoimidazole carboxylaSe	1	—
IPI00361513.1	Similar to ATP-binding caSSette tranSporter Sub-family A member 9	1	—
IPI00364741.1	Similar to CG9638-PA	1	—
IPI00365352.1	Similar to interleukin 17 receptor	1	—
IPI00365868.1	Similar to AcylphoSphataSe, organ-common type iSozyme (AcylphoSphate phoSphohydrolaSe)	1	—
IPI00366060.1	Similar to Enhancer of filmentation 1 (MEF1) (CRK-aSSociated SubStrate-related protein) (CAS-L) (PP105) (Neural precurSor cell expreSSed developmentally downregulated 9)	1	—
IPI00370705.1	Similar to Mpo protein	1	—
IPI00371946.1	Similar to MyriStoylated alanine-rich C-kinaSe SubStrate	1	—
IPI00421490.1	(MARCKS) Nicotinate phosphoribosyltransferase-like protein	1	—
IPI00199670.4	Alpha-2 antiplasmin	—	2
IPI00200398.1	Sclerostin precursor	—	2
IPI00204373.1	Pleiotrophin_precursor	—	2
IPI00422076.1	Thrombospondin_1	—	2

associated with a high rate of turnover in the trabecular bone. While some of these proteins may be present in the diaphysis, they are present at levels below the limit of detection of the mass spectrometer under these conditions.

The profile of proteins identified in both the metaphysis and the diaphysis includes growth factors such as insulin-like growth factor binding protein 5 (IGFBP-5), insulin-like growth factor 1 (IGF-1) precursors. Insulin-like growth factors in general are well known for their roles in regulating cell proliferation and function (for a review, see Kubota et al., 2003). IGFBP-5 accumulates in the extracellular matrix with decreasing levels incorporated in the cortical bone with age [Nicolas et al., 1995; Rutter et al., 2005]. Overexpression of IGFBP-5 results in the inhibition of collagen matrix maturation and osteopenia due to a decrease in osteoblast function [Devlin et al., 2002; Durant et al., 2004; Atti et al., 2005]. Alternatively, systemic administration of IGFBP-5 in OVX mice resulted in enhanced bone formation due to an increase in osteoblast number [Andress, 2001] and when complexed with IGF-1 resulted in an increase in cortical thickness and bone mineral density in femoral mid-shaft and tibial metaphysic [Bauss et al., 2001]. This apparent effect of IGFBP-5 and IGF-1 on cortical bone is reflected in the greater abundance of peptides identified in the diaphysis as compared to the metaphyseal region of bone (Table I).

Matrix Gla-protein precursor was also identified in both metaphyseal and diaphyseal regions of bone, with more peptides identified in the diaphyseal region of bone. Matrix Gla-protein is expressed in areas that undergo endochondral ossification as well as cartilaginous tissues. The protein is expressed by vascular smooth muscle cells and resting proliferative and late hypertrophic chondrocytes, but not in early hypertrophic chondrocytes or osteoblasts [Luo et al., 1995]. Matrix Gla-protein functions in the extracellular matrix as an inhibitor of mineralization while transgenic expression of matrix Gla-protein in osteoblasts of mice prevented bone mineralization [Murshed et al., 2004]. Matrix-Gla protein may function to prevent over-mineralization of the bone especially in the diaphyseal region of bone known to have a slower turnover.

Proteins that were exclusively identified in the diaphyseal region consisting of predominately cortical bone include thrombospondin-1, pleiotrophin, and sclerostin. Thrombospondin-1 is a glycoprotein that interacts with collagens, proteoglycans, and fibrinogen in the extracellular matrix and has anti-angiogenic properties, possibly regulating blood vessel formation in bone [Armstrong and Bornstein, 2003; Rajani et al., 2005; Alford and Hankenson, 2006]. Pleiotrophin has been identified as a product of the osteoblast and is localized at sites of new periosteal and endochondral bone formation [Tare et al., 2002; Li et al., 2005] possibly due to its ability to recruit and promote attachment of osteoprogenitor cells to sites of new bone formation [Gieffers et al., 1993; Rauvala et al., 2000]. Sclerostin is a protein expressed by mature osteocytes surrounded by a mineralized matrix, hypertrophic chondrocytes, and osteoblasts, and acts as a negative regulator of bone formation [Winkler et al., 2003; Sutherland et al., 2004a,b; Van Bezooijen et al., 2004; Poole et al., 2005]. The presence of these proteins exclusively in the diaphyseal region of bone may be due to the enriched number of osteocytes in cortical bone and its interaction with bone periosteum.

An unexpected protein identified in both the metaphyseal and diaphyseal regions of bone was type II collagen $\alpha 1$ (Table I, Supplemental Tables I and II). This was confirmed by Western blot analysis of both the acid soluble and the insoluble residue with anti Type II collagen antibody. Low molecular weight immunoreactive bands to type II collagen were observed in the acid extract of bone (Fig. 2A, lanes 2 and 3). These bands migrated below the molecular weight range of intact type II collagen extracted from cartilage (lane 1). This suggests that formic acid extracted only fragments of type II collagen. We subsequently extracted the acid insoluble residue with SDS and also analyzed this extract by Western blot. This analysis revealed the presence of intact type II collagen (Fig. 2B, lanes 4 and 5). The relative level was much higher in the metaphysis than the diaphysis. The diaphyseal segments of the bone were well removed from any potential contamination with growth plate cartilage. This suggests that either type II collagen is not completely removed during bone remodeling, or that some is actually produced in the bone environment. Several lines of evidence (published or unpublished) support this thesis as the source of type II collagen in our analysis. (1) Increases in the expression of type II collagen as

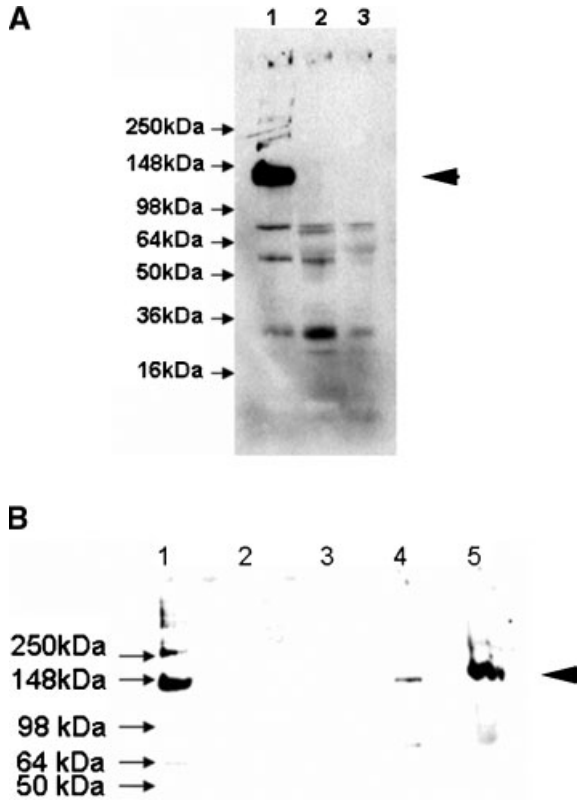

Fig. 2. Identification of Type II Collagen in rat metaphyseal and diaphyseal bone. Samples were prepared, separated on 4–20% Tris-Glycine gel and blotted to nitrocellulose membranes as described in Methods. The blots were probed with anti Type II Collagen antibody. **Panel A**: Overexposed blot of SDS extract from articular cartilage of a 6-month-old rat (**lane 1**), acid-soluble fraction of 6-month-old rat metaphyseal and diaphyseal bone (**lanes 2 and 3**, respectively). **Panel B**: SDS extract from articular cartilage of a 6-month-old rat (lane 1), acid-soluble fraction of 6-month-old rat diaphyseal and metaphyseal bone (lanes 2 and 3, respectively), and SDS extract of the formic acid insoluble residue from 6-month-old rat diaphyseal and metaphyseal bone (**lanes 4 and 5**). Intact type II collagen indicated by arrowheads.

well as other cartilage-associated genes have been reported in iliac crest biopsies from primary hyperparathyroidism patients 1 year after successful parathyroidectomy [Reppe et al., 2006]. (2) This bone expression and robust regulation of cartilage-associated genes has been confirmed by us in the rat OVX model of osteopenia. The expression of these chondrogenic markers decrease with OVX and are restored by treatment with bone anabolic agents, PTH or GSK-3 inhibitor (Kulkarni et al. under review JCB, Kulkarni et al., 2006). (3) Interestingly, we detect the expression of these chondrogenic genes in both metaphyseal and diaphyseal bone in vivo and primary osteoblasts derived from these regions. The diaphyseal RNA or primary cells in these studies are dissected from the midshaft of tibiae or femora. If this were a result of tissue contamination, it would require the presence of living chondrocytes in this region of the bone. (4) We have found Type II collagen protein to be present in cortical bone extracts from the diaphyseal femoral midshaft of a 7-month-old cow (data not shown). Given the size of the bones, this bone section is anatomically well removed from any potential contamination with cartilage. (5) The presence of cartilage markers in intramembranous bone during embryonic development has also been described. Transient expression of the type IIA procollagen, the $\alpha 1$ subunit of type XI collagen, and aggrecan genes in the frontal bone of normally developing embryonic calvaria and the genes for type II and X collagen were found to be further translated by the chick embryonic calvarial bone cells. Additionally, cells expressing Type IIB and type X collagen, characteristic of hypertrophic chondrocytes were identified in the chick embryonic calvaria [Nah et al., 2000]. Because of the presence of type II collagen in the bone extract, we decided to look for other chondrocyte-associated proteins. Type XI collagen is a fibrillar collagen thought to be specific to cartilage, interacting with type II collagen to regulate fibril thickness to give cartilage its tensile strength [Mendler et al., 1989; Van der rest and Garrone, 1991; Wu and Eyre, 1995; Chambers et al., 2002]. Type XI procollagen $\alpha 2$ was identified in the protein profile of metaphyseal and diaphyseal regions of bone (Table I, Supplemental Tables I and II) indicating type XI collagen is present in mature rat bone at regions well removed from cartilage. Although aggrecan was not detected with high confidence in the proteomic analysis of the bone matrix formic acid extracts, we had identified a couple of aggrecan peptides in preliminary analysis of bone extracts with low confidence (data not shown). In order to investigate this further, we subjected guanidine extracts of the formic acid insoluble residue to Western blot analysis with an anti-aggrecan antibody. Figure 3 shows that low molecular weight immunoreactive fragments of aggrecan were present in the extracts from metaphysis and diaphysis (compared to articular cartilage extracts). As with type II collagen, much more of the aggrecan fragment

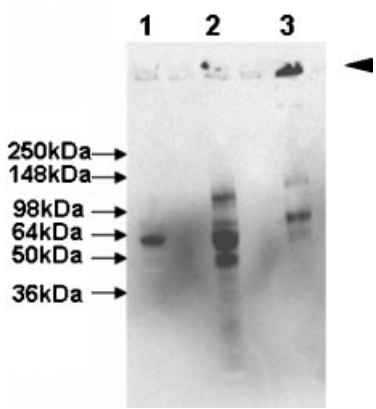

Fig. 3. Identification of aggrecan in rat metaphyseal and diaphyseal bone. Samples were prepared and separated on 4–20% Tris-Glycine gel and blotted to nitrocellulose membranes as described in Methods. The blots were probed with anti aggrecan antibody. Formic acid-soluble fraction of diaphyseal (**lane 1**) metaphyseal (**lane 2**) bone from a 6-month-old rat and SDS extract of articular cartilage from a 3-week-old rat (**lane 3**). Intact aggrecan denoted by arrowhead.

was present in the metaphysis than in the diaphysis. Our data is the first to identify the presence of cartilage-specific proteins, type II collagen, and aggrecan (Table I, Figs. 2 and 3), in mature rat bone. Additional confirmation of this observation by immunohistochemistry will help strengthen these findings and define precisely the cell type(s) in bone elaborating these markers and their role in bone remodeling.

CONCLUSIONS

These data represent the construction of a reference database of proteins in the diaphyseal and metaphyseal regions of bone. The proteins identified in this study indicate increased metabolism in the metaphyseal region of bone as compared to the diaphyseal region of bone. Of these proteins, the presence of cartilage markers in the bone matrix indicate either that these markers are not necessarily cartilage specific, cartilage tissue can persist well into mature sections of bone or that cartilage may play a role in the turnover of bone. This work describes the basis for future work in investigating changes in protein expression and may be used to design comparative studies at the protein level.

SUPPORTING INFORMATION AVAILABLE

The complete list of proteins with supporting information and spectra from the top matched peptide for each protein that was observed for the metaphysis and diaphysis are available in Supplemental Tables I and II, respectively.

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