Analytical Biochemistry 499 (2016) 85–89

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A strategy to quantitate global phosphorylation of bone matrix proteins

Grażyna E. Sroga, Deepak Vashishth*

Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form
7 January 2016
Accepted 25 January 2016
Available online 3 February 2016

Keywords:
Global phosphorylation
Human bone
Mouse bone
Extracellular matrix proteins
Osteopontin

ABSTRACT

Current studies of protein phosphorylation focus primarily on the importance of specific phosphoproteins and their landscapes of phosphorylation in the regulation of different cellular functions. However, global changes in phosphorylation of extracellular matrix phosphoproteins measured “in bulk” are equally important. For example, correct global phosphorylation of different bone matrix proteins is critical to healthy tissue biomineralization. To study changes of bone matrix global phosphorylation, we developed a strategy that combines a procedure for in vitro phosphorylation/dephosphorylation of fully mineralized bone in addition to quantitation of the global phosphorylation levels of bone matrix proteins. For the first time, we show that it is possible to enzymatically phosphorylate/dephosphorylate fully mineralized bone originating from either cadaveric human donors or laboratory animals (mice). Using our strategy, we detected the difference in the global phosphorylation levels of matrix proteins isolated from wild-type and osteopontin knockout mice. We also observed that the global phosphorylation levels of matrix proteins isolated from human cortical bone were lower than those isolated from trabecular bone. The developed strategy has the potential to open new avenues for studies on the global phosphorylation of bone matrix proteins and their role in biomineralization as well for other tissues/cells and protein-based materials.

Current studies of protein phosphorylation focus primarily on the importance of specific phosphoproteins and the landscapes of their phosphorylation in regulation of different cellular functions. Mass spectrometry (MS)[1] has become the technique of choice to identify phosphorylated residues within proteins, whereas fragmentation analysis by tandem mass spectrometry (MS/MS) is used to confirm the peptide sequence and to assign the site of modification [1–4]. It is worth noting that the reliable localization of phosphorylation sites in proteins represents one of the most challenging tasks in phosphoproteomic studies. Although powerful, MS tends to be biased toward certain phosphorylated sites, and in general it is not quantitative. Thus, routine analyses of, for example, age-related changes in global phosphorylation levels of proteins using MS equipment are impractical for daily tests performed on patient specimens in a present-day clinical setting.

The quantitative measurement of global protein phosphorylation in comparison with the total amount of protein is essential to clinical and pharmacological research and to biomedical applications. From the perspective of bone tissue research, such studies are particularly important for, for example, the understanding of biomineralization regulation occurring in vivo [5] and/or biomineralization performed in vitro for various purposes of bone tissue engineering [6].

Thus, for the needs of bone research and biomedical engineering, we developed a strategy that combines in vitro phosphorylation and dephosphorylation of mineralized bone with an assay for the quantification of global phosphorylation of bone matrix proteins. The phosphorylation levels were measured “in bulk” using a pIMAGO-based system recently developed by Iliuk and coworkers [7]. In the current study, we have, for the first time, quantified global phosphorylation levels of extracellular bone matrix proteins isolated from bone tissues. The samples originated from male and female human cadaveric donors of different ages as well as from wild-type (WT) and osteopontin knockout [OPN (−/−)] C57BL/6 mice. To this end, we were able to capture a

* Corresponding author.
E-mail address: vashid@rpi.edu (D. Vashishth).

[1] *Abbreviations used*: MS, mass spectrometry; MS/MS, tandem mass spectrometry; WT, wild-type; OPN (−/−), osteopontin knockout; CK2, casein kinase II; rhOPN, recombinant human osteopontin; MT plate, microtiter plate; avidin–HRP, avidin-linked horseradish peroxidase; PTM, post-translational modification.

http://dx.doi.org/10.1016/j.ab.2016.01.017
0003-2697/

difference in the global phosphorylation levels between matrix proteins originating from WT and OPN (−/−) mice. We also demonstrated that there is a difference in the levels of global phosphorylation between bone matrix proteins isolated from human cortical and trabecular bone. We expect that the developed strategy has potential to open new avenues for studies on global phosphorylation of bone matrix proteins and their role in bone biomineralization and fragility fractures as well as for other tissues/cells and protein-based materials.

Materials and methods

Human and mouse bone samples

Tibias (posterior area) from a total of 8 human female (4) and male (4) cadaveric donors (young [25.0 ± 2.0 years], middle age [61.0 ± 2.0 years], and elderly [85.0 ± 2.0 years] donors as well as 1 female donor of 77 years) served as the source of cortical and/or trabecular (spatially matched) bone tissue samples. The specimens were obtained from the centralized National Disease Research Interchange (NDRI) biobank and were known to be free of osteoarthritis, diabetes, and other metabolic bone diseases as well as HIV and HVB.

Tibias from a total of 3 WT and 3 OPN (−/−) C57BL/6 mice were harvested, cleaned, and cut using a slow-speed diamond blade (Buehler, Lake Bluff, IL, USA). The mice bones served as the source of cortical bone.

Both human and mice bone pieces were repeatedly washed in cold distilled water until the washings were free of blood and then were defatted using isopropyl ether. After freeze-drying, the specimens were stored at −80 °C until their use.

Dephosphorylation and phosphorylation of mineralized bone samples and osteopontin

Three types of experiments were performed on bone samples and osteopontin (recombinant human osteopontin, R&D Systems, Minneapolis, MN, USA): phosphorylation, dephosphorylation, and non-enzyme-treated control reactions.

Phosphorylation was conducted by incubating the samples overnight at 30 °C with casein kinase II (CK2) and the reaction buffer from New England Biolabs (Ipswich, MA, USA). Adenosine triphosphate was added to the buffer as the phosphoryl donor for CK2. The incubation solution also contained 50 mM $CaCl_2$, protease inhibitors (100× Protease Inhibitor Cocktail Kit, final concentration 1×, Pierce Biotechnology, Rockford, IL, USA) and antibiotics (ampicillin and kanamycin).

Dephosphorylation was performed by incubating the samples overnight at 37 °C with calf intestinal alkaline phosphatase and the reaction buffer from New England Biolabs. The incubation solution also contained 50 mM $CaCl_2$, protease inhibitors (100× Protease Inhibitor Cocktail Kit, final concentration 1×) and antibiotics (ampicillin and kanamycin).

All control reactions contained only the enzyme incubation buffers supplemented with 50 mM $CaCl_2$, protease inhibitors (100× Protease Inhibitor Cocktail Kit, final concentration 1×), and antibiotics (ampicillin and kanamycin).

Isolation of bone matrix proteins for pIMAGO-based assay

Extracellular bone matrix proteins were isolated as described by Sroga and coworkers [8,9] with some modifications to the published procedures. Specifically, in addition to protease inhibitors (100× Protease Inhibitor Cocktail Kit, final concentration 1×), a mixture of phosphatase inhibitors (100× Halt Phosphatase Inhibitor Cocktail, final concentration 1×, Pierce Biotechnology) was added to the protein extraction buffer.

Quantitation of global phosphorylation levels for extracellular bone matrix proteins and osteopontin using pIMAGO-based assay

Quantitative measurement of global phosphorylation levels in protein extracts isolated from each bone sample and all control reactions was performed as described by Iliuk and coworkers [7] using a newly developed pIMAGO phosphoprotein detection system. Briefly, the extracted proteins, recombinant human osteopontin (rhOPN), and phosphorylated casein (additional phosphoprotein control [7]) were bound to the wells of the polystyrene microtiter plates (MT plates). The PBS buffer alone (0.5× and 1×) and plasmid DNA alone (0, 10, 20, 40, 80, and 100 ng DNA/well) were also included in the assay as controls. After a series of blocking (Tris-buffered saline with 5% nonfat dried milk, pH 7.4) and washings (Tris-buffered saline with 0.1% Tween 20, pH 7.4) steps, the pIMAGO reagent (Tymora Analytical, West Lafayette, IN, USA) was added to the MT plate wells to allow the attachment of pIMAGO to the phosphate groups on proteins. The pIMAGO is a water-soluble nanopolymer that is functionalized with titanium(IV) ions that specifically bind to phosphoproteins. The nanopolymer also contains biotin groups that allow the enzyme-linked spectrophotometric detection.

In the next step, pIMAGO excess was removed by a minimum of three washings (Tris-buffered saline with 0.1% Tween 20, pH 7.4), and the avidin-linked horseradish peroxidase (avidin−HRP) was added to the wells in order to proceed with the colorimetric detection. The absorbance of the solutions in each well was determined at 415 nm using a microtiter plate reader (Infinite 200, Tecan).

Results and discussion

In vitro dephosphorylation and phosphorylation of mineralized bone samples

It was necessary to modify the composition of the buffers supplied with enzymes from New England Biolabs for the needs of the reactions performed on mineralized bone tissues. Thus, to maintain bone's mineral content, 50 mM $CaCl_2$ was used. Preservation of the natural mineral content in bone samples is important if, for example, bone fracture tests were going to follow the phosphorylation/dephosphorylation procedures. To block the activity of proteases, which are naturally present in the extracellular bone matrix, a cocktail of protease inhibitors was added to the reaction buffers. In addition to sterilization of the used solutions (i.e., depending on the compounds, autoclaving or sterile filtration), antibiotics (ampicillin and kanamycin) were also added to prevent the potential growth of bacteria.

Isolation of bone matrix proteins for pIMAGO-based assay

For the analysis of global protein phosphorylation, protein extracts should reflect as accurately as possible the natural phosphorylation levels of all proteins isolated, for example, at a certain age of a donor or at a particular time point from implanting a graft. Thus, to prevent undesired dephosphorylation of bone matrix proteins after the completion of all the reactions, the cocktail of phosphatase inhibitors was also introduced to the protein extraction buffer in order to block the activity of alkaline phosphatases naturally present in bone matrix.