Journal of Structural Biology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Microstructure dependent binding of pigment epithelium derived factor (PEDF) to type I collagen fibrils

Meagan Cauble [a], Phillip Yang [a], Ulrich Baumann [b], Jan M. Gebauer [b], Bradford G. Orr [c], Le T. Duong [d], Mark M. Banaszak Holl [a,e,f,*]

[a] Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, United States
[b] Institute of Biochemistry, University of Cologne, Otto-Fischer-Strasse 12-14, 50674 Cologne, Germany
[c] Department of Physics, University of Michigan, Ann Arbor, MI 48109, United States
[d] Bone Biology Group, Merck Research Laboratories, West Point, PA, United States
[e] Program in Macromolecular Science and Engineering, University of Michigan, Ann Arbor, MI 48109, United States
[f] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, United States

ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form 15 May 2017
Accepted 3 June 2017
Available online xxxx

Keywords:
PEDF
Collagen
AFM

ABSTRACT

Pigment epithelium derived factor (PEDF) is a multifunctional extracellular protein. In addition to its known anti-angiogenic and neurotrophic roles in collagen rich tissues, PEDF is thought to be involved in collagen fibril assembly due to its sequence specific binding to the collagen fibril and high expression in regions of active bone formation. In order to image the presence of the protein on the fibrils, PEDF was recombinantly made with a strep tag (*strep*-PEDF) and then gold nanoparticles conjugated to streptavidin (Au_{NP}) were used as a secondary tag. The gold nanoparticles were detected using phase imaging in tapping mode AFM to image where exogenous PEDF bound in rabbit femur. These findings demonstrate that PEDF binds heterogeneously in cortical rabbit femur. Exogenous PEDF binding was concentrated at areas between microstructures with highly aligned collagen fibrils. Binding was not observed on or within the collagen fibrils themselves.

1. Introduction

Pigment epithelium derived factor (PEDF) is a 50 kDa non-inhibitory member of the serpin class of proteins with various biological functions including anti-angiogenesis, anti-vasopermeability, anti-tumor, and neurotrophic activities (Kawaguchi et al., 2010). In bone, PEDF is primarily expressed and secreted by osteoblasts and is highly expressed at areas of active bone formation (Broadhead et al., 2010). Direct binding of PEDF to type I collagen occurs via ionic interactions between a negatively charged area on the surface of PEDF derived from aspartic acid and glutamic acid residues and a series of positively charged residues, KGXRGFXGL, in the collagen triple helix (Fig. 1) (Meyer et al., 2002). There are two high affinity binding sites on the human α1(I) chain for PEDF: KGHRGFSGL at residues 87–95 and IKGHRGFSGL at residues 929–938 (Sekiya et al., 2011).

The role of PEDF in bone formation, including mineralization and remodeling, is an area of intense current interest (Broadhead et al., 2010; Glorieux and Moffatt, 2013; Marini et al., 2014). PEDF appears to suppress factors that inhibit bone mineralization and enhance factors that promote mineralization. In particular, treatment of human osteoblasts with PEDF reduced expression of sclerostin and other markers of osteocytes including MEPE and DMP1 (Li et al., 2015). Mutation of the *SERPINF1* gene encoding for PEDF has been tied to poor matrix mineralization and patients with null mutations in *SERPINF1*, have a distinct form of *Osteogenesis Imperfecta* (OI) type VI, characterized by undermineralized bone and high fragility (Becker et al., 2011; Homan et al., 2011; Venturi et al., 2012). Both micro- and nano-scale mineralization defects have been observed in the bone tissue (Fratzl-Zelman et al., 2015). Thus, the roles of PEDF in bone formation and homeostasis are of great interest for both normal and diseased tissue. Studies to date have typically focused on the role of PEDF in the biochemical cascades associated with bone formation and mineralization (Li et al., 2015) and the level of expression in *OI* patients (Farber et al., 2014; Venturi et al., 2012). Although PEDF is known to bind to the Type I collagen triple helix (Meyer et al., 2002; Sekiya et al., 2011), the spatial distribution of PEDF in bone tissue has not been characterized.

* Corresponding author at: Department of Chemistry, University of Michigan, 930 N. University Avenue, Ann Arbor, MI 48109-1055, United States.
E-mail address: mbanasza@umich.edu (M.M. Banaszak Holl).

http://dx.doi.org/10.1016/j.jsb.2017.06.001
1047-8477/

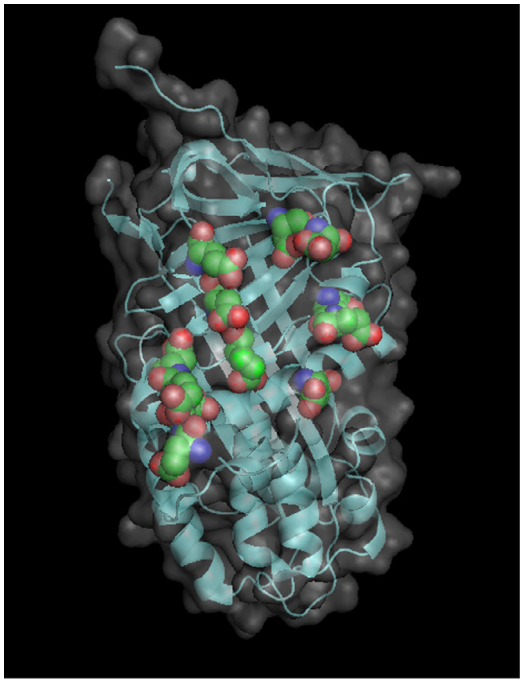

Fig. 1. Structure of human pigment epithelium derived factor (PEDF) with collagen binding site (PDB ID 1IMV). Data taken from (Meyer et al., 2002). The secondary structures of the protein are shown in blue and the surface map of the protein is shown in gray. The amino acids involved in the binding site for type I collagen are shown with green spheres representing carbon, blue spheres nitrogen, and red spheres oxygen atoms.

In this study, we used tapping-mode atomic force microscopy (AFM) combined with gold nanoparticle labeling to characterize binding patterns of PEDF in cortical rabbit femur. *Strep*-tagged PEDF was incubated with the bone followed by treatment with 10 nm gold nanoparticles (Au_{NP}) conjugated to streptavidin. Using phase-lag (phase) imaging, the location of the PEDF-Au_{NP} on the surface can be readily identified due to the substantial difference in phase lag for tapping on the hard gold particles as compared to the softer collagen fibrils. We observed that the exogenous PEDF does not bind to the top surface of the collagen fibrils or in regions of high collagen fibril alignment. Rather, we observed exogenous PEDF binding in regions showing reduced fibril alignment such as regions where bundles of aligned fibrils intersect. The observed binding density was heterogeneous on a ~0.2–2 μm length scale across the bone surface.

2. Materials and methods

2.1. Sample preparation and initial AFM imaging

Nine-month old New Zealand white rabbit bones were obtained from Merck Research laboratories and stored in 95% ethanol (Cauble et al., 2015; Pennypacker et al., 2011). Cortical bone was prepared for imaging with atomic force microscopy (AFM) using previously published procedures (Cauble et al., 2015; Erickson et al., 2013). Sections of the mid-diaphysis of cortical femur were polished using a 3 μm diamond suspension. Demineralization was achieved by suspending the samples in 0.5 M EDTA (pH 8.0) for 90 min while shaking at room temperature. The sample was sonicated for 5 min in nanopure water both before and after demineralization. After the sample was allowed to air dry, AFM imaging was performed at room temperature in tapping mode (Nanoscience Instruments; Aspire conical tapping mode AFM probes; 300 kHz, 40 N/m, radius 8 nm). A series of 50 μm × 50 μm scans were obtained with varying offset values to assist in locating the same $(3.5\ \mu m)^2$ imaging area for before/after incubation PEDF binding. In all cases, images are oriented with the long bone axis running horizontally on the page.

Protein sequence of the human PEDF, lacking the first 40 amino acids (V_{40}-P_{418}), were back translated and codon optimized for expression in *E. coli*. A single N-terminal strepII-tag was introduced and the gene synthesized as an insert in the pJExpression 411 vector (DNA 2.0). Expression was performed in *E. coli* BL21 (DE3) cells. LB media inoculated with an overnight culture were initially allowed to grow at 37 °C and subsequently cooled down to 20 °C. At an OD_{600} of approx. 1.2 protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside and the cultures incubated overnight at 20 °C. Cells were harvested, washed and stored frozen at −20 °C. For protein purification, cells were ruptured in the presence of 10 μg/ml DNAse in a cell disruptor (Constant Systems, UK), the lysis solution cleared by ultracentrifugation and the supernatant loaded on a streptactin column (IBA). After washing with at least 10 column volumes, the bound protein was eluted with 2 mM desthiobiotin in Tris buffered saline (TBS). The PEDF containing eluates were pooled, concentrated and polished twice using an HiLoad Superdex 200 pg (GE Healthcare) size exclusion chromatography column equilibrated with 20 mM Tris, 150 mM NaCl and 2 mM DTT at pH 7.5. In the last run the protein appeared as a monomodal peak and run as a single band on SDS-PAGE (results not shown). The secondary tag utilized in this study was an Alexa Fluor 488 Streptavidin 10 nm colloidal gold conjugate purchased from Thermo Fisher Scientific (A32361).

2.2. Preparation of solutions for labeling with Au_{NP} secondary tag

Stock solutions of 25 and 50 nM PEDF were prepared in the following buffer: 20 mM Tris pH 7.5 and 150 mM NaCl. The stock solutions were aliquoted and then frozen at −80 °C until ready for use in an experiment. The day of an experiment, a solution of 0.1 M DTT was prepared. Immediately before the experiment, DTT was added to the PEDF aliquots so that the final solution used for the experiment was either 25 or 50 nM PEDF, 20 mM Tris pH 7.5, 150 mM NaCl, and 2 mM DTT.

The stock solution of streptavidin-Au nanoparticles was stored at 4 °C. The day of an experiment, an aliquot of the stock solution was diluted with buffer (5 mM sodium azide, 0.1% BSA, and 5 mM Tris pH 7.5) so that there would be an equimolar ratio of streptavidin and PEDF (i.e. 50 nM PEDF and 50 nM streptavidin-Au).

2.3. Binding experiments

Binding experiments performed in a stepwise fashion using separate incubations of *strep*-PEDF and Au_{NP} were carried out as follows (Scheme 1). 30 μL of a solution containing either 25 or 50 nM *strep*-PEDF in buffer (20 mM Tris, 150 mM NaCl, 2 mM DTT, pH 7.5) was incubated on a demineralized rabbit cortical femur for 10 min. The droplet was wicked away and the surface was rinsed with buffer. Then, 30 μL of a solution of Au_{NP} in buffer (5 mM sodium azide, 0.1% BSA, and 5 mM Tris pH 7.5) was incubated on the PEDF-treated rabbit femur for 10 min. The concentration of Au_{NP} was equivalent to the concentration of PEDF used in the previous step.

Binding experiments using pre-formed PEDF-*strep*-Au_{NP} were carried out as follows (Scheme 2). 15 μL of 50 nM *strep*-PEDF in buffer (20 mM Tris pH 7.5, 150 mM NaCl, and 2 mM DTT) was combined with 15 μL of 50 nM Au_{NP} in buffer (5 mM sodium azide, 0.1% BSA, and 5 mM Tris pH 7.5). The two solutions were gently mixed with a micropipette and then allowed to incubate at room temper-

Please cite this article in press as: Cauble, M., et al. Microstructure dependent binding of pigment epithelium derived factor (PEDF) to type I collagen fibrils. J. Struct. Biol. (2017), http://dx.doi.org/10.1016/j.jsb.2017.06.001