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Structural characterization and interaction of periostin and bone morphogenetic protein for regulation of collagen cross-linking

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ABSTRACT

Periostin appears to be a unique extracellular protein secreted by fibroblasts that is upregulated following injury to the heart or changes in the environment. Periostin has the ability to associate with other critical extracellular matrix (ECM) regulators such as TGF-β, tenascin, and fibronectin, and is a critical regulator of fibrosis that functions by altering the deposition and attachment of collagen. Periostin is known to be highly expressed in carcinoma cells, but not in normal breast tissues. The protein has a structural similarity to insect fasciclin-1 (Fas 1) and can be induced by transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP)-2. To investigate the molecular interaction of periostin and bone morphogenetic protein, we modeled these three-dimensional structures and their binding sites. We demonstrated direct interaction between periostin and BMP1/2 *in vitro* using several biochemical and biophysical assays. We found that the structures of the first, second, and fourth Fas1 domains in periostin are similar to that of the fourth Fas 1 domain of TGFBIp. However, the structure of the third Fas 1 domain in periostin is different from those of the first, second, and fourth Fas1 domains, while it is similar to the NMR structure of Fasciclin-like protein from *Rhodobacter sphaeroides*. These results will useful in further functional analysis of the interaction of periostin and bone morphogenetic protein.

1. Introduction

Periostin, which was originally known as osteoblast-specific factor 2, is a secretory protein expressed in collagen-rich fibrous connective tissues, such as periodontal ligament, the aorta, and heart valves [1,2]. Overexpression of periostin by adenoviral infection in cardiac valvulogenic tissue increases the overall viscosity, which is a measure of collagen cross-linking [3–6]. Periostin was first identified as a bone-specific protein and has more recently been implicated in heart valve morphogenesis, oncogenesis, and vascular smooth muscle cells [7–10]. Periostin expression was also found to be increased in head and neck squamous cell carcinoma (HNSCC) when compared with normal tissues [11]. This may be associated with the bone metastatic potential of lung cancers and the progression of breast cancer [12,13]. The molecular mechanism of periostin action in collagen cross-linking has investigated, and the results showed that periostin enhances the proteolytic activation of lysyl oxidase (LOX), which is an enzyme responsible for cross-link formation, and that it is caused by interacting with bone morphogenetic protein (BMP)-1 to promote the collagen cross-linking [14].

Bone morphogenetic proteins (BMPs) are cytokines that belong to the multifunctional transforming growth factor-β (TGF-β) superfamily, which controls growth, proliferation and differentiation of many cell types. In particular, BMPs have been found to be able to induce bone and cartilage formation at ectopic sites *in vivo* [15]. BMP-1 was also shown to provide the activity necessary for proteolytic removal of the C-propeptides of procollagens I–III and precursors of the major fibrillar collagens, as well as to be the prototype of a small group of extracellular metalloproteinases that play manifold roles in regulating formation of the extracellular matrix (ECM). Periostin is known to be induced by BMP2 in MC3T3 cells [16], and BMP2 and BMP signaling induce biological processes involved in early AV valvulogenesis; namely, mesenchymal cell migration and expression of periostin, indicating critical roles of BMP signaling in post-EMT AV cushion tissue maturation and differentiation [17].

Recent research suggested that PNDA-3 (Periostin-binding DNA Aptamer-3) selectively bound to the FAS-1 domain of periostin and disrupted the interaction between periostin and its cell surface

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receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [18]. The essential and minimal domain that is required for PNDA-3 binding has been shown to be the third FAS-1 domain, which is critical to integrin binding. To date, no structural information has been reported regarding periostin and its interaction. In this study, we demonstrated direct interactions between periostin and BMP1/2 *in vitro* using several biochemical and biophysical assays. The periostins first Fas1/third Fas1 and their mutants were successfully purified and analyzed by amino acid sequence alignment and circular dichroism (CD) measurement. We developed a molecular docking model using homology structures of periostin and BMP1/2. Furthermore, we modeled the structural differences among four periostin Fas 1 domains. Greater understanding of how the domains of periostin contribute to BMP1/2 interactions could provide important new information regarding pathological remodeling in the heart and new targets for therapy.

2. Materials and methods

2.1. Cloning, expression, and purification of periostin and BMP1/2

The periostin first Fas1 (97–234) and third Fas1 (368–492) genes were ligated into plasmid pET30a. BMP1 (114–321)/BMP2 (282–396) were subcloned into the N-terminal His-tagged fusion protein vector pET-28a for purification. BMP1/2 was also subcloned into a glutathione S-transferase (GST)-fused protein vector pGEX-4T1 to perform a pull-down assay. The periostin first Fas1 and third Fas1 were transformed into the overexpression host *Escherichia coli* BL21(DE3). A single colony was incubated in 5 ml Luria Betani (LB) medium containing 10 μg/ml of kanamycin at 37 °C overnight. The cell cultures were added to 2 l of LB medium with kanamycin and maintained at 37 °C until the OD_{600} reached 0.5–0.6. Protein expression was induced with 0.5 mM IPTG (isopropyl-thio-β-D-galactopyranoside) for 16 h at 25 °C, after which cells were harvested by centrifugation at 3660*g* for 25 min. The pellets were then resuspended with lysis buffer A [50 mM Tris–HCl (pH 8.0) and 200 mM NaCl] and sonicated on ice. Cell lysates were subsequently centrifuged at 20170*g* for 45 min to remove the supernatant, after which the periostin first Fas1/third Fas1 inclusion bodies were resuspended and sonicated in buffer [50 mM Glycine (pH 10.5)] on ice and centrifuged to remove the supernatant. Following the protein extraction step, samples were loaded onto a Ni-NTA (Amersham-Pharmacia Biotech) column and pre-equilibrated with buffer [50 mM Tris–HCl (pH 10.5) and 200 mM NaCl]. The bound protein was eluted by varying the concentration of imidazole from 20 mM to 200 mM, and the resulting fractions were analyzed by electrophoresis on a 15% SDS–PAGE gel. For the His pull-down assay, glutathione S-transferase (GST)-fused BMP1/2 constructs were transformed into the host *E. coli* BL21(DE3) for expression. Single colonies were then inoculated in 5 ml LB medium enriched with 50 μg/ml ampicillin. Following overnight culture at 37 °C, the cells were added to 2 l of LB medium containing ampicillin. The BMP1/2 cell pellets were then resuspended in lysis buffer (1× PBS) and sonicated on ice. The BMP1/2 pellets were then sonicated in buffer B [50 mM Tris–HCl (pH 8.0), 200 mM NaCl, and 6 M Urea] on ice and the resulting cell lysates were centrifuged. The BMP1/2 supernatants were subsequently loaded onto a glutathione-sepharose column at a flow rate 2.5 ml/min and washed in PBS buffer. The GST-BMP1/2 fusion proteins were then eluted in 5–30 mM glutathione. The GST-BMP1/2 proteins was loaded onto a Q-Sepharose fast-flow (Amersham-Pharmacia Biotech) anion exchange chromatography column pre-equilibrated with buffer A. Finally, the proteins were concentrated by centrifugation at 1320*g* using ultrafiltration devices.

2.2. Western blotting

The purified periostin first Fas1/third Fas1 and BMP1/2 proteins from 15% SDS–PAGE were transferred onto a nitrocellulose membrane at 105 V for 1 h on ice. The membrane was blocked with 5% skim milk in PBS buffer containing 0.1% Tween-20 (PBS-T) for 1 h. After blocking, the membrane was incubated in primary antibody [GST (B-14) diluted 1:2000, and His-probe (G-18) diluted 1:5000, Santa Cruz Biotechnology, Inc.] for 1 h. Following washing with PBS-T for 40 min, the membrane was incubated for 1 h with GST secondary antibody [goat anti-mouse IgG-HRP diluted 1:20000 (Santa Cruz Biotechnology, Inc.)] and His secondary antibody (goat anti-rabbit IgG-HRP diluted 1:100000) in blocking buffer for 1 h.

2.3. Mutagenesis of the periostin first Fas1 and third Fas1

Double-stranded oligonucleotides were used for site-directed mutagenesis of twelve different periostin first Fas1 (K129A, N171A, P186A, N191A, N201A, C208A, and T231A) and third Fas1 residues to alanine (Y444A, Q447A, G454A, Y462A, and R463A).

2.4. Far-ultraviolet circular dichroism spectroscopy analysis

Circular dichroism spectropolarimeter (JASCO J-715) measurements were taken using a 0.1 cm cell at 0.2 nm intervals and 25 °C. The CD (Far-UV circular dichroism) spectra of purified proteins were recorded in the 190–260 nm range. A far-UV CD spectrum was taken at a protein concentration of 0.5 mg/ml. The spectrum was obtained in milli-degrees, and the CD signal was converted to mean residue ellipticity (MRE) prior to secondary-structural analysis. Calculation of the secondary structural elements was performed using the CDNN program.

2.5. His-tagged pull down assay

A total of 50 μg of the purified proteins, periostin first Fas1/third Fas1 and GST-tagged BMP1/BMP2, were mixed with 50 μl Ni-NTA bead in buffer A for 4 h at 4 °C. The supernatants were removed via centrifugation at 805*g* for 3 min and the beads were washed five times with buffer A. Each time, the beads were then incubated with wash buffer on a rotator for 10 min, and then collected by centrifugation. The beads were subsequently eluted with elution buffer [50 mM Tris–HCl (pH 8.0), 200 mM NaCl, and 200 mM Imidazole], boiled in SDS–PAGE sample loading buffer for 5 min, resolved by SDS–PAGE, and visualized by immunoblotting assay using anti-GST and anti-His.

2.6. BIAcore biosensor analysis

Measurements of the apparent dissociation constants (K_D) between periostin and BMP1/2 were performed using a BIAcore 2000 biosensor (Biosensor, Sweden). Periostin first Fas1 and third Fas1 (100 μg/ml in 10 mM sodium acetate with a pH of 4.0) were covalently bound by an amine-coupling method to the carboxylated dextran matrix at a concentration corresponding to 160 RU (response units) according to the manufacturer's protocol. A flow path involving two cells was then employed to simultaneously measure the kinetic parameters from one flow cell containing the periostin first Fas1/third Fas1-immobilized sensor chip to another flow cell containing a blank chip. For kinetic measurements at room temperature, BMP1/2 samples were prepared by dilution with an HBS buffer [150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, and 10 mM HEPES (pH 4)] ranging in concentrations from 975 nm to 7800 nm. Each sample was injected with 70 μl of BMP1/2 solutions into the flow cells (association phase) at a rate

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