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Identification and characterization of an amphioxus matrix metalloproteinase homolog BbMMPL2 responding to bacteria challenge

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ABSTRACT

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases mainly involved in extracellular matrix (ECM) degradation. We have cloned and identified BbMMPL2 as homolog of MMPs from adult amphioxus. Recombinant BbMMPL2 proteins underwent self-processing during refolding *in vitro*. The final ~23 kDa polypeptide displayed proteolytic activity against ECM components like casein, gelatin, collagen IV and fibrinogen, but not laminin, fibronectin or α1-PI. This activity could be inhibited by GM6001 and TIMP-1/2. In addition, real-time RT-PCR analysis revealed that BbMMPL2 expressed in all issues/organs in adult amphioxus we tested. Its transcription was significantly up-regulated 12 h post immune challenge by *Escherichia coli* in epidermis and hepatic diverticulum but only slightly increased by *Staphyloccocus aureus* in epidermis. Furthermore, recombinant BbMMPL2-EGFP expressed in 293T and NIH/3T3 cells showed aggregation in cytoplasm and induced cell death. Our results provided new evidence that MMP was involved in immune response which could be conserved through evolution.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases which is evolutionarily conserved and structurally related among each other (Bode and Maskos, 2003; Nagase and Woessner, 1999). Most MMPs share a basic structure comprised of a signal peptide, a propeptide involved in enzyme latency maintenance (Freimark et al., 1994), a conserved catalytic domain (Bode et al., 1993), a flexible hinge region, and a C-terminal hemopexin-like domain involved in interactions with extracellular matrix (ECM) and other substrates, such as tissue inhibitors of metalloproteinases (TIMPs) (Brew et al., 2000; Burg-Roderfeld et al., 2007). In addition, individual MMP has specific domain structures, based on which they can be classified into four subfamilies: (1) archetypal MMPs with basic structure as mentioned above, including Collagenases, Stromelysins and other MMPs; (2) Gelatinases with fibronectin repeats in the catalytic domain; (3) Matrilysins which lacks C-terminal hemopexin-like domain, and (4) furin-activated MMPs with a furin-cleavage site in propeptide, including secreted MMPs, Type I, II transmembrane MT-MMPs and GPI anchored MMPs (Fanjul-Fernandez et al., 2010).

MMPs mainly function in ECM degradation and therefore are vital in many physiological processes involving in tissue remodeling like proliferation, migration, adhesion, angiogenesis, bone development and wound healing (Page-McCaw et al., 2007; Rowe and Weiss, 2008). Recently, a growing number of studies have shown that MMPs are also associated with innate immunity in mammals and insects as important modulators (Altincicek and Vilcinskas, 2006; Elkington et al., 2005; Manicone and McGuire, 2008; Nerusu et al., 2007; Parks et al., 2004; Van Lint and Libert, 2007). Studies on MMP-deficient mice further reveal that MMPs are necessary for normal immune response (Opdenakker et al., 2001). In addition, MMPs are also important to the host defense of traumatic, infectious, toxic or autoimmune insults (Nathan, 2002; Parks et al., 2004). However, the exact role of MMPs in inflammation and immunity has not yet been illuminated.

After the first MMP was identified from tadpoles (Gross and Lapiere, 1962), a burst of MMPs was reported in vertebrates and invertebrates. To date, at least 25 vertebrate MMPs have been identified (Löffek et al., 2011; Woessner, 2002) as well as various of MMPs in invertebrates and plants, such as sea urchin (Lepage and Gache, 1990), *Drosophila melanogaster* (Llano et al., 2000, 2002), *Caenorhabditis elegans* (Wada et al., 1998), *Hydra vulgaris* (Leontovich et al., 2000) and *Arabidopsis thaliana* (Maidment et al., 1999). However, no MMPs have been identified in amphioxus, which is the closest living relative of the vertebrate ancestor (Wada and Satoh, 1994) and serves as an important model for evolution of vertebrates from invertebrates.

Here we report cloning of an amphioxus MMP family member, *BbMMPL2*, which has the typical MMP structure. Recombinant BbMMPL2 proteins underwent self-processing like other MMPs

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and the final product, aBbMMPL2, showed proteolytic activity on gelatin, casein, collagen IV and fibrinogen. In addition, we found that *BbMMPL2* expression responded significantly to Gram-negative bacteria challenge, suggesting its role in innate immunity. Furthermore, when transfected into mammalian cells, BbMMPL2 tended to aggregate and cause cell death suggesting a possible mechanism in immune response.

2. Materials and methods

2.1. Animal

Adult amphioxus (*Branchiostoma belcheri tsingtauense*, also known as *Branchiostoma japonicum*) (Zhang et al., 2006) were collected from the sandy sea floor at Shazikou near Qingdao, China. They were starved to clear the gut for three days in sterilized seawater before experiments.

2.2. Bioinformatics analysis

Blast search was carried out in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and the DOE Joint Genome Institute (JGI, http://genome.jgi-psf.org/Brafl1/Brafl1.home.html) with known vertebrate MMPs for MMP homologs in amphioxus. Multiple protein sequence alignment was performed using DNAMAN package. Sequences used in this study are as following: *BbMMPL2*, HQ588917; MMP-1, NP_002412; MMP-2, NP_004521; MMP-3, NP_002413; MMP-7, NP_002414; MMP-8, NP_002415; MMP-9, NP_004985; MMP-10, NP_002416; MMP-11, NP_005931; MMP-12, NP_002417; MMP13, NP_002418; MMP-14, NP_004986; MMP-15, NP_002419; MMP-16, NP_005932; MMP-17, NP_057239; MMP-19, NP_002420; MMP-20, NP_004762; MMP-21, NP_671724; MMP23, NP_008914; MMP-24, NP_006681; MMP-25, NP_071913; MMP-26, NP_068573; MMP-27, NP_07 1405; MMP28, NP_077278.

Signal peptides prediction was performed by SignalP 3.0 Server at http://www.cbs.dtu.dk/services/SignalP/. TM prediction was performed by TNpred at http://www.ch.embnet.org/software/TMPRED_form.html. And GPI anchor prediction was performed using big-PI predictor at http://mendel.imp.ac.at/gpi/gpi_server.html.

2.3. Cloning of BbMMPL2

Adult amphioxus tissues/organs including gut, gill, hepatic diverticulum, muscle, epidermis, ovary and testis were dissected under stereo microscope and total RNA of each tissue/organ was extracted using TRIzol® Reagent (Invitrogen, USA) according to manufacturer's protocol. Complementary DNA was synthesized by reverse transcription using PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). *BbMMPL2* specific primers (forward primer 5′-CCATGAACAACGGAATGCTCTACC-3′, reverse primer 5′-CAGGTCAAGGCTCGATCTCTAGATCT-3′) or amphioxus *β-actin* primers (forward primer 5′-ACATCCGTAAGGACCTGTACGCCAAC-3′, reverse primer 5′-GATCGTCAGCAGAAGGAGATCACCTT-3′) (Huang et al., 2007) were used in RT-PCR amplification with *Hifi* DNA polymerase (Transgen, Beijing, China). PCR product was then cloned into T3 vector to generate BbMMPL2/T3 (Transgen, Beijing, China) and confirmed by DNA sequencing (BGI, China).

2.4. Expression, purification and refolding of recombinant BbMMPL2

Full-length cDNA of *BbMMPL2* was amplified by PCR with primers 5′-GGGATCCCATGAACAACGGAATG-3′ containing a *BamHI* site and 5′-CCGCTCGAGAGGCTCGATCTCTAGAT-3′ containing an *XhoI* site using BbMMPL2/T3 plasmid as template. PCR product was digested by *BamHI* and *XhoI* and subcloned into pET29b vector with a C-terminal 6× His-tag in frame to generate BbMMPL2-His/pET29b.

Recombinant BbMMPL2-His/pET29b construct was confirmed by DNA sequencing and transformed into *BL21* (*DE3*) cells (Transgen, Beijing, China). Protein expression was induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 37 °C. Bacteria were collected by centrifugation at 12,000×*g* for 5 min and sonicated before subjected to centrifugation at 15,000×*g* for 15 min at 4 °C. Recombinant proteins were then washed and dissolved from inclusion bodies with 20 mM Tris–HCl buffer (pH 8.0) containing 8 M urea, and purified through Ni–NTA column (Bio-Rad, USA) equilibrated with 6 M urea. Purified recombinant proteins were dialyzed first against refolding buffer I (50 mM Tris–HCl buffer, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$, 50 μM $ZnSO_4$, 20% glycerol and 0.05% Brij-35) (Llano et al., 2002) for 24 h at 4 °C and then against refolding buffer II (50 mM Tris–HCl buffer, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$) for another 24 h at 4 °C. Purified recombinant BbMMPL2 proteins (rBbMMPL2) or final dialysis product (aBbMMPL2) were concentrated by MICROCON (Millipore, USA) at 3500×*g* for 30 min at 4 °C and stored at −70 °C.

2.5. Enzymatic assay

Enzymatic activity of rBbMMPL2 or aBbMMPL2 was measured with fluorescein-conjugated DQ™ Gelatin or Collagen type IV (Invitrogen, USA) according to manufacturer's protocol. Protein was diluted in 1× reaction buffer (50 mM Tris–HCl buffer, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide), and incubated with 100 μg/ml DQ™ gelatin/college type IV in dark in the presence or absence of 0.5 mM 1,10-phenanthroline (1,10-PO) (Invitrogen, USA), 10 μM GM6001 (Santa Cruz, USA), 10 mM EGTA (Amresco, USA), 5 mM TIMP-1 (Invitrogen, USA) or 2 mM TIMP-2 (Sigma, USA) for 0–30 min at room temperature. Fluorescence was measured using a microplate reader set for excitation at 490 nm and emission at 530 nm. All values were corrected by subtracting background fluorescence from each value.

2.6. Zymography

Casein/gelatin zymography was performed using 15% SDS–PAGE containing 1 mg/ml casein/gelatin (Sigma, USA) as described previously (Troeberg and Nagase, 2003). In brief, after 50 μM purified proteins or refolded proteins were separated by electrophoresis, gels were washed four times for 15 min successively in wash buffer I (2.5% Triton X-100, 3 mM NaN_3), wash buffer II (2.5% (v/v) Triton X-100, 50 mM Tris–HCl, pH 7.5, 3 mM NaN_3), wash buffer III (2.5% (v/v) Triton X-100, 50 mM Tris–HCl, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) and wash buffer IV (50 mM Tris–HCl, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) at room temperature on shaker. Finally, gels were incubated in fresh washing buffer IV for 20–24 h at 37 °C. After that, gels were stained with Coomassie blue and destained till lytic bands were visible.

2.7. Proteolytic analysis

Degradation of ECM components was performed by incubating 20 μM aBbMMPL2 with 250 μg/ml fibronectin, 250 μg/ml fibrinogen, 250 μg/ml laminin or 5 μg/ml α1-PI (α1-proteinase inhibitor or α1-antitrypsin) in 1× reaction buffer (50 mM Tris–HCl buffer, pH7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, 0.02% NaN_3) for 16–18 h at 37 °C. Reaction was terminated by adding equal volume of 2× Laemmli sample buffer. Digested products were subjected to 10% non-reducing SDS–PAGE and bands were detected by Coomassie blue staining.