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Identification and functional characterization of amphioxus Miple, ancestral type of vertebrate midkine/pleiotrophin homologues

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ABSTRACT

Midkine (MK) and pleiotrophin (PTN) are the only two members of heparin-binding growth factor family. MK/PTN homologues found from *Drosophila* to humans are shown to have antibacterial activities and their antibacterial domains are conserved during evolution. However, little is known about MK/PTN homologue in the basal chordate amphioxus, and overall, information regarding MK/PTN homologues is rather limited in invertebrates. In this study, we identified a single MK/PTN homologue in *Branchiostoma japonicum*, termed *BjMiple*, which has a novel domain structure of PTN-PTNr1-PTNr2, and represents the ancestral form of vertebrate MK/PTN family proteins. *BjMiple* was expressed mainly in the ovary in a tissue-dependent fashion, and its expression was remarkably up-regulated following challenge with bacteria or their signature molecules LPS and LTA, suggesting its involvement in antibacterial responses. Functional assays revealed that BjMiple had strong antimicrobial activity, capable of killing a panel of Gram-negative and Gram-positive bacteria via a membranolytic mechanism, including interaction with bacterial membrane via LPS and LTA, membrane depolarization and high intracellular levels of ROS. Importantly, strong antibacterial activity was localized in $PTN_{42\text{-}61}$ and $PTNr1_{42\text{-}66}$. Additionally, BjMiple and its derived peptides $PTN_{42\text{-}61}$ and $PTNr1_{42\text{-}66}$ were not cytotoxic to human RBCs and mammalian cells. Taken together, our study suggests that amphioxus Miple is the ancestral type of vertebrate MK/PTN family homologues, and can play important roles as innate peptide antibiotics, which renders it a promising template for the design of novel peptide antibiotics against multi-drug resistant bacteria.

1. Introduction

Midkine (MK), a small basic heparin-binding growth factor, was first identified as a gene induced by retinoic acid in murine carcinoma cells (Kadomatsu et al., 1988; Tomomura et al., 1990) and, independently, as the chicken homologue purified from chick basement membranes (Rauvala, 1989). The related factor, pleiotrophin (PTN), was initially recognized as a neurite outgrowth-promoting protein present in rat brain around birth and as a mitogen toward fibroblasts isolated from bovine uterus tissue (Milner et al., 1989; Rauvala, 1989). MK and PTN constitute a two-member family of heparin-binding growth factors with closely related structures (Muramatsu, 2002; Sorrelle et al., 2017). The proteins of MK/PTN family have a PTN domain (http://smart.embl-heidelberg.de/), which comprises two sub-domains, the N-terminally-located domain N-domain with six highly conserved cysteines and the C-terminally-located domain C-domain with four highly conserved cysteines, each forming three anti-parallel β sheets connected by disulfide bridges (Kadomatsu et al., 1988; Kaname et al., 1993; Muramatsu, 1993). The proteins of MK/PTN family are multifunctional. They have been implicated in various biological processes, such as cell proliferation, differentiation, survival and migration, neurogenesis, angiogenesis, oncogenesis and inflammation (Garver et al., 1993, 1994; Horiba et al., 2006). In addition, the proteins of MK/PTN family are also shown to have antibacterial activities and their antibacterial domains are conserved during evolution (Englund et al., 2006; Sorrelle et al., 2017; Svensson et al., 2010).

MK/PTN family homologues have been identified in many species, including human, mouse, fish, chicken, frogs, and insects (Kadomatsu and Muramatsu, 2004; Ezquerra et al., 2006; Obama et al., 1994; Sekiguchi et al., 1995). Interestingly, no MK/PTN family homologues have been reported, nor are obvious, in the *Caenorhabditis elegans* genome, suggesting their origin among insects (Svensson et al., 2010). However, the evolutionary origin of MK/PTN family is elusive. Moreover, information regarding MK/PTN family homologues remains

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rather limited within invertebrates. A search of the recently completed draft assembly and automated annotation of the Florida amphioxus *Branchiostoma floridae* genome (https://www.ncbi.nlm.nih.gov/genome/) and the Qingdao amphioxus *B. belcheri* genome (http://mosas.sysu.edu.cn/genome/index.php) both revealed the presence of a single MK/PTN family member: Miple. This abbreviation was coined by Muramatsu (2002) to name the *Drosophila* homologue, and comes from *mid*kine and *ple*iotrophin. To date, Miple has not been studied in any detail in amphioxus, an evolutionarily important animal transient from invertebrate to vertebrate. In this study, we show that *B. japonicum* Miple gene, *BjMiple*, indicates an ancestral type of vertebrate MK/PTN family homologues. We also show that BjMiple display a wide spectrum of antibacterial activities capable of killing both Gram-negative and positive bacteria. Finally, we demonstrate that BjMiple functions by a membranolytic mechanism including interaction with bacterial membrane via LPS and LTA, membrane depolarization and high intracellular levels of ROS.

2. Materials and methods

2.1. RNA extraction and cDNAs synthesis

Adult amphioxi *Branchiostoma japonicum* were collected during the breeding season in the vicinity of Qingdao, China and cultured in aerated seawater at room temperature for one week. Total RNAs were extracted with TRIzol (TaKaRa, Dalian, China) from *B. japonicum* according to the manufacturer's instructions. The cDNA was synthesized with reverse transcription system (Promega) using oligo (dT) primer after digestion with recombinant DNase I (RNase free) (TaKaRa) to eliminate the genomic contamination. The reaction was carried out at 42 °C for 50 min and inactivated at 75 °C for 15 min. The cDNAs synthesized were stored at −20 °C until use.

2.2. Cloning and sequencing of amphioxus heparin-binding growth factor gene (BjMiple)

Based on the sequence of a heparin-binding growth factor gene (No. 218220R.t1) in the database of *B. belcheri* genome (http://mosas.sysu.edu.cn/genome/index.php), a pair of primers P1 and P2 (Supplementary Table 1) was designed using Primer Premier 5.0 program. The PCR amplification reaction was carried out at 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplification products were gel-purified using DNA gel extraction kit (AXYGEN), cloned into the pGEM-T vector (Invitrogen), and transformed into Trans5α *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity. After determination of the partial cDNA sequence, rapid amplification of cDNA ends (RACE) was employed to obtain the full-length cDNA. The gene-specific primer pairs P3 and P4 as well as P5 and P6 (Supplementary Table 1) were used in RACE reactions for the cloning of 3′- end and 5′- end cDNAs, respectively. The 5′- and 3′-RACE-Ready cDNAs were synthesized from the total RNAs using the BD SMART™ RACE cDNA amplification kit (Clontech, Beijing, China) according to the manufacturer's instructions. The 5′- and 3′-RACE products were gel-purified, sub-cloned, sequenced and assembled.

2.3. Sequence analysis

The domain of deduced protein was analyzed using the SMART program (http://smart.embl-heidelberg.de/). Signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight (MW) and isoelectronic points (pI) of the mature protein were determined using ProtParam (http://www.expasy.ch/tools/protparam.html). Homology searches in the GenBank database were carried out by BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). The information of exon-intron organization was obtained from *B. belcheri* genome (http://mosas.sysu.edu.cn/genome/index.php) and NCBI database (http://www.ncbi.nlm.nih.gov/). Multiple protein sequences were aligned using the MegAlign program of the LASERGENE software suite (DNASTAR). Phylogenetic trees were constructed by MEGA6.0 using p-distance based on the neighbor-joining method (Tamura et al., 2013). The reliability of each node was estimated by bootstrapping with 1000 replications.

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to examine the transcriptional profile of *BjMiple* in the different tissues of *B. japonicum* (Shan et al., 2015; Yang et al., 2014). Total RNAs were extracted with Trizol (Invitrogen) from the different tissues including the hepatic caecum, hind-gut, gill, muscle, notochord, testis and ovary dissected out of *B. japonicum*. After digestion with RNase-free DNase (TaKaRa) to eliminate the genomic contamination, the cDNAs were synthesized with reverse transcription system using oligo d (T) primer, and used for qRT-PCR. The PCR primer pairs P7 and P8 as well as P9 and P10 (Supplementary Table 1) specific of *BjMiple* and *EF1α* were designed using primer 5.0 program. qRT-PCR was performed on ABI 7500 Real-time PCR system (Applied Biosystems, USA) as described by Wang et al. (2009). The *EF1α* gene was chosen as the reference for internal standardization. The analysis was performed using the statistical software package SPSS/PC.

The qRT-PCR was also performed to assay the transcriptional profile of *BjMiple* in response to challenge with the Gram-negative bacteria *Aeromonas hydrophila* (ATCC 35654) and *E. coli* (ATCC 25922), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633), as well as the bacterial signature molecules lipopolysaccharide (LPS) and lipoteichoic acid (LTA) according to the method described by Wang et al. (2009). Our previous studies showed that challenge with tolerable doses of bacterium (10^8 cells/ml), LPS (10 μg/ml) or LTA (10 μg/ml) induced a rapid and strong immune response in amphioxus (Fan et al., 2008; Wang et al., 2009; Wang and Zhang, 2011). Therefore, *B. japonicum* were cultured in 1 L of sterilized seawater containing 10^8 cells/ml of the bacteria *A. hydrophila*, *E. coli*, *S. aureus* or *B. subtilis*, or 10 μg/ml of the bacterial signature molecules LPS (Sigma, USA) or LTA (Sigma, USA), and sampled at 0, 2, 4, 8, 12, 24, 48, and 72 h after the exposure. *B. japonicum* cultured in sterilized seawater alone were used as control. Total RNAs were prepared with Trizol from the whole animals, and the extraction of total RNAs, cDNA synthesis and qRT-PCR were carried out as above.

2.5. Construction of expression vector

The sequences encoding mature BjMiple, PTN domain and PTNr1-PTNr2 (PTNr) domain were amplified by PCR using the primer pairs P11 and P12, P13 and P14 as well as P15 and P16 (Supplementary Table 1) with *EcoR* I and *Xho* I sites in the forward and reverse primers, respectively. The PCR products were sub-cloned into the plasmid expression vector pET-28a (Novagen) previously cut with the restriction enzymes *EcoR* I and *Xho* I. The identity of inserts was verified by sequencing, and the constructed plasmids were designated pET-28a/BjMiple, pET-28a/PTN and pET-28a/PTNr, individually.

2.6. Expression and purification of recombinant BjMiple (rBjMiple), PTN (rPTN) and PTNr (rPTNr)

The plasmids pET28a/BjMiple, pET28a/PTN and pET28a/PTNr were transformed into *E. coli* transetta (DE3), respectively, and the transformed *E. coli* cells were cultured overnight in LB broth containing kanamycin (50 μg/ml). The cultures were diluted 1:50 with LB broth and further incubated at 37 °C for about 6 h. The expression of recombinant proteins was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to the cultures at a final concentration of 0.1 mM. After