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A potent tilapia secreted granulin peptide enhances the survival of transgenic zebrafish infected by *Vibrio vulnificus* via modulation of innate immunity

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ABSTRACT

Progranulin (PGRN) is a multi-functional growth factor that mediates cell proliferation, survival, migration, tumorigenesis, wound healing, development, and anti-inflammation activity. A novel alternatively spliced transcript from the short-form *PGRN1* gene encoding a novel, secreted GRN peptide composed of 20-a.a. signal peptide and 41-a.a. GRN named GRN-41 was identified to be abundantly expressed in immune-related organs including spleen, head kidney, and intestine of Mozambique tilapia. The expression of *GRN-41* and *PGRN1* were further induced in the spleen of tilapia challenged with *Vibrio vulnificus* at 3 h post infection (hpi) and 6 hpi, respectively. In this study, we established three transgenic zebrafish lines expressing the secreted GRN-41, GRN-A and PGRN1 of Mozambique tilapia specifically in muscle. The relative percent of survival (RPS) was enhanced in adult transgenic zebrafish expressing tilapia GRN-41 (68%), GRN-A (32%) and PGRN1 (36%) compared with control transgenic zebrafish expressing AcGFP after challenge with *V. vulnificus*. It indicates tilapia GRN-41 is a potent peptide against *V. vulnificus* infection. The secreted tilapia GRN-41 can induce the expression of innate immune response-related genes, such as *TNFa*, *TNFb*, *IL-8*, *IL-1β*, *IL-6*, *IL-26*, *IL-21*, *IL-10*, *complement C3*, *lysozyme (Lyz)* and the hepatic antimicrobial peptide *hepcidin* (*HAMP*), in adult transgenic zebrafish without *V. vulnificus* infection. The tilapia GRN-41 peptide can enhance the innate immune response by further elevating *TNFb*, *IL-1β*, *IL-8*, *IL-6*, and *HAMP* expression in early responsive time to the *V. vulnificus* challenge in transgenic zebrafish. Our results suggest that the novel GRN-41 peptide generated from alternative splicing of the tilapia *PGRN1* gene is a potent peptide that defends against *V. vulnificus* in the transgenic zebrafish model by modulation of innate immunity.

1. Introduction

Progranulin (PGRN), also known as proepithelin [1,2], acrogranin [3,4], prostate cancer (PC) cell-derived growth factor (PCDGF) [5], and granulin/epithelin precursor (GEP) [6], is a multi-functional growth factor that mediates cell proliferation, survival, migration, tumorigenesis, wound healing, development, and anti-inflammation activity [7]. PGRN is a 68-kDa secreted glycoprotein and is expressed in various tissues and cell types, including epithelial cells, immune cells, hematopoietic cells, chondrocytes, neurons, skeletal muscle and adipose tissue [8–11].

In mammals, there is only one *PGRN* gene that encodes PGRN composed of 7.5 granulin (GRN) peptides, which were first purified from human leukocytes [7,12]. Each GRN unit consists of 12 conserved cysteines that form 6 disulfide bonds ($X_{2-3}CX_{5-6}CX_5CCX_8CCX_6CCX_5CCX_4CX_{5-6}CX_2$) [13,14]. GRN is a small, 6-kDa peptide that has been characterized in mammals, such as humans [12], rats [1], mice [4], and horses [15]. Moreover, GRN-like proteins have also been reported in non-mammalian organisms, including slime mold [16], plants [17,18], nematodes [19], the solitary sea squirt [20], insects [21,22], mussels [23], and teleosts [24,25]. A previous report revealed that PGRN and GRN exert opposite functions in inflammation [26]. PGRN inhibits inflammation by binding to tumor necrosis factor receptors (TNFR) to block TNFα/TNFR signaling [27]. Nevertheless, PGRN undergoes proteolysis by neutrophil elastase or proteinase 3 into GRN units to induce the expression of the pro-inflammatory cytokine

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Table 1
List of Mozambique tilapia primers used in this study.

Primer name	Primer sequence (5'→3')	Application
OmPGRN1-BamHI-F	GGATCCACCATGTTGAGGATCACTCTGTGTTTGTCAT	Cloning
OmPGRN1-SalI-R	GTCGACCTAGTTTTCTTTGTCCTCTGAAGTTGGG	Cloning
OmGRN-A-BamHI-F	GGATCCACCATGTTGAGGATCACTCTGTGTTTGTCAT	Cloning
OmGRN-A-ClaI-R	ATCGATTTATTTCTCACAGTTCATGGTAACC	Cloning
OmGRN-41-BamHI-F	GGATCCACCATGTTGAGGATCACTCTGTGTTTGTCAT	Cloning
OmGRN-41-SalI-R	GTCGACTCAAGTTACTGCACTGGCAG	Cloning
OmPGRN1-1F	TCTATCACATGTCCTGATGGGAGTAC	RT-PCR
OmPGRN1-1R	AGATCGGCACAGCACATGGCATGTGG	RT-PCR
OmGRN-41-1F	TCTATCACATGTCCTGATGGGAGTAC	RT-PCR
OmGRN-41-1R	AGTTACTGCACTGGCAGAAAG	RT-PCR
OmEF-1α-1F	GATGCCATCCTGCCACCTT	RT-PCR
OmEF-1α-1R	ATGTGGGCAGTGTGGCAAT	RT-PCR
GeneRacer™ 5′ Primer[a]	CGACTGGAGCACGAGGACACTGA	5′ RACE
GeneRacer™ 5′ Nested Primer[a]	GGACACTGACATGGACTGAAGGAGTA	5′ RACE
OmPGRN1 5′ RACE GSP-R	GCACAGCACATGGCATGTGGAAATGG	5′ RACE
OmPGRN1 5′ RACE GSP Nested-R	GCATGTGGAAATGGGCAGCAGCC	5′ RACE
OmGRN-41 5′ RACE GSP-R	CGGCACAGCACATGGCCTGCAAAG	5′ RACE
OmGRN-41 5′ RACE GSP Nested-R	GGCCTGCAAAGTAAGTAAGACCAACAGAGC	5′ RACE
GeneRacer™ 3′ Primer[a]	GCTGTCAACGATACGCTACGTAACG	3′ RACE
GeneRacer™ 3′ Nested Primer[a]	CGCTACGTAACGGCATGACAGTG	3′ RACE
OmPGRN1 3′ RACE GSP-F	GCCCATTTCCACATGCCATGTGCTG	3′ RACE
OmPGRN1 3′ RACE GSP Nested-F	TGCCATGTGCTGTGCCGATCTGC	3′ RACE
OmGRN-41 3′ RACE GSP-F	GCTGCCCATTTCCACATGTAAGTCAG	3′ RACE
OmGRN-41 3′ RACE GSP Nested-F	CATGTAAGTCAGAAACTTTCTGCCAGTGC	3′ RACE
OmPGRN1-2F	CCGCCACCTGCTGCAAGGC	qPCR
OmPGRN1-2R	CGGCACAGCACATGGCATGTGG	qPCR
OmGRN-41-2F	AGTGCAGTAACTTGATTGTTGGTTGT	qPCR
OmGRN-41-2R	GCAGATCGGCACAGCACAT	qPCR
OmEF-1α-2F	GAACCACCCCGGTCAGATC	qPCR
OmEF-1α-2R	ATGTGGGCAGTGTGGCAAT	qPCR

[a] The primer was provided by the GeneRacer™ Kit (Life Technologies, USA).

interleukin-8 (IL-8) [26,28]. GRN also functions as a critical soluble cofactor and contributes to innate immunity for toll-like receptor 9 (TLR9) signaling to produce TNFα in response to microbial DNA [29].

In the genome of teleost fish, there are long-form and short-form *PGRN* genes that encode PGRN composed of 9–11 GRN units and 1.5–3 GRN units, respectively [30]. Several fish *PGRN* cDNAs or genes have been identified from carp, goldfish, zebrafish, tilapia, flounder, medaka, fugu, etc. [31]. Three carp (*Cyprinus carpio*) GRN peptides (cGRN-1, cGRN-2 and cGRN-3) were purified from hematopoietic tissues (spleen and head kidney) [32]. Goldfish *PGRN* encodes a 1.5 GRN peptide, is expressed in hematopoietic tissues and can induce the proliferation of goldfish macrophages [33]. Two types of *PGRN* mRNAs, *f-PGRN* type 1 and type II, were identified in flounder (*Paralichthys olivaceus*) [34]. The expression level of the *f-PGRN* type I transcript was increased, whereas the level of the *f-PGRN* type II mRNA was decreased after lipopolysaccharide (LPS) treatment [34]. Four *PGRN* genes were discovered in the zebrafish (*Danio rerio*) genome: *PGRN-A*, *PGRN-B*, *PGRN-1* and *PGRN-2* [31]. Zebrafish long-form PGRN-A and −B consist of 10 and 9 GRN units, respectively, whereas short-form PGRN-1 and -2 are both composed of 1.5 GRN units [31]. Zebrafish PGRN-A, which is the orthologue of human PGRN, is involved in embryonic liver morphogenesis [35] and muscle growth and regeneration [36].

In tilapia, a short-form *PGRN* gene encoding a 206-amino acid (a.a.) PGRN protein composed of two GRN units was identified in Mozambique tilapia [37] and Nile tilapia (GenBank accession number: GQ241348), which we named *PGRN1* to differentiate it from a distinct tilapia short-form *PGRN* gene *PGRN2* encoding a 199-a.a. PGRN protein that we discovered in both Mozambique tilapia and Nile tilapia (unpublished data). In addition, we isolated a novel alternatively spliced transcript of the *PGRN1* gene that encodes a secreted, 41-a.a. GRN peptide, GRN-41, from both Mozambique and Nile tilapia. In this study, muscle-specific transgenic zebrafish lines expressing Mozambique tilapia secreted GRN peptides, GRN-A or GRN-41, or PGRN1 protein were established to investigate the function and molecular mechanism of tilapia GRN peptides and PGRN1 involved in regulation of fish immunity to defend against the bacterial pathogen *Vibrio vulnificus*.

2. Materials and methods

2.1. Experimental animals

Mozambique tilapia (*Oreochromis mossambicus*) obtained from the Institute of Cellular and Organismic Biology, Academia Sinica were maintained in indoor circular glass tanks at 28.5 °C, and fed commercial dry eel granular feed (Uni-president, Taiwan) twice daily. Wild-type AB strain zebrafish (*Danio rerio*) were purchased from the Taiwan Zebrafish Core Facility (TZCF) of the Zebrafish Core in Academia Sinica (ZCAS). Zebrafish were reared in a laboratory circulating system at 28.5 °C in a cycle of 14 h light and 10 h dark, and fed commercial dry ayu feed (Uni-president, Taiwan) two times per day. The animal use protocol of this research had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan Ocean University. The IACUC Approval No. is 101042. The 2-phenoxyethanol (2-PE; Sigma-Aldrich, USA) was used as anesthetic agent (100 ppm) before *Vibrio vulnificus* IP injection and euthanasia agent (400 ppm) for zebrafish and tilapia.

2.2. Cloning and analysis of the Mozambique tilapia PGRN1 transcripts by reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA was extracted from the spleen of Mozambique tilapia using TRIzol® reagent (Life Technologies, USA) and treated with DNase I (Qiagen, Germany) from the PureLink™ RNA Mini Kit (Life Technologies, USA), according to the manufacturer's protocol. The amount and quality of the RNA product were analyzed using a Nanodrop ND-1000 spectrophotometer (Thermo, USA) and agarose gel electrophoresis. The total RNA was reverse-transcribed into cDNAs using the SuperScript™ III First-Strand Synthesis System (Life