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Identification and expression of a new Ly6 gene cluster in zebrafish *Danio rerio*, with implications of being involved in embryonic immunity

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ABSTRACT

Lymphocyte antigen-6 (Ly6) superfamily is a large family of proteins and characterized by precisely spaced cysteine motifs, termed the three-finger fold. To date, a large number of members of the Ly6/uPAR family were identified among many species. In this study, we first report the identification and characterization of the secreted Ly2.1-3 proteins on the chromosome 2 in zebrafish and determine the expression pattern. Ly2.1-3 all possess a conserved LU domain and adopt similar three-finger structure with human CD59, SLURP1 and other Ly6 family members. Ly2.1-3 cluster on chromosome 2 and share high homology, possibly originated from chromosomal gene duplication. Ly2.1-3 exhibit distinct expression pattern in the endoderm, they were found abundantly and specifically in the digestive tract, liver and pancreas respectively. The differential expression pattern may suggest Ly2.1-3 acquire new function during gene duplication. The expression level of Ly2.1-3 were up-regulating challenged with LPS indicated that they have a role in innate immune responses of the digestive system during endotoxin challenge in early stage.

1. Introduction

Members of Ly6 superfamily are characterized by LU domain, also known as three-finger protein domain (TFPD), which is defined by a distinct disulfide bonding pattern formed by 8–10 precisely spaced cysteine residues, yielding a three-finger structure involved in protein binding [1,2]. Three-finger domain, also known as three-finger snake toxin motif, was first identified in the sea-snake erabutoxin b about 40 years ago [3]. Ly6 superfamily members can be divided into two subfamilies: one includes secreted proteins, and the other comprises glycosylphosphatidylinositol (GPI)- anchored membrane proteins [4].

Members of Ly6 superfamily have been identified in a variety of species ranging from invertebrates such as honeybee and fruit-fly to vertebrates including fish, amphibians, reptiles and mammals. More than 14 genes and 36 genes of Ly6 superfamily have been documented in the honeybee and fruit-fly, respectively [5]. In human genome at least 45 genes are identified to code for proteins containing one to three TFPDs [6]. GPI-anchored Ly6 superfamily genes usually show distinct expression patterns. *Drosophila* Ly6-like genes are usually selectively expressed in the ectoderm, the central nervous system, the mid-gut and the peripheral nervous system [5]. In addition, some of *Drosophila* Ly6-like genes show overlapping expression pattern. For example, Crok, Cold, Crim and Boudin and CG2813, CG6038 and CG6583 are all expressed in the ectoderm, functioning cooperatively in the assembly of SJ components [7,8]. By contrast, vertebrate Ly6-like proteins exhibit diverse substrate specificity and tissue expression pattern. Ly6a (Sca-1), Ly6d, Ly6e (Sca-2, RIG-E), the PSCA, CD59 or lynx1 and uPAR are glycosylphosphatidylinositol (GPI) -anchored Ly6-like proteins. Since their anchorage to the cell surface presents opportunities for novel and dynamic mechanisms of receptor modulation and regulation, they have been shown to be involved primarily in cell

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activation, adhesion and migration [5,9], and contribute to neuronal excitability regulation [10]. E48 gene is known to be expressed in human keratinocytes and modulates desmosomal cell–cell adhesion of keratinocytes [11]. Lynx1 colocalizes and directly associates with neuronal nicotinic receptors in the central nervous system, modulates the cellular calcium permeability, and affects the responses of the receptors to nicotine [10,12,13]. In adult mouse, Ly6 superfamily members, such as ThB, Ly6A/E, TSA-1, and Ly6C, are primarily expressed in immune system [14].

The functions of secreted Ly6 members lacking a typical GPI-anchoring sequence such as non-mammalian secreted neurotoxins from snake and frog, and the secreted proteins of SLURP-1, SLURP-2, and prostate and testis expressed Pate proteins, seem vary greatly from species to species. For example, the biological activity of cytotoxins and neurotoxins secreted from snake and frog can interact specifically with certain receptors (α-neurotoxins), they can also induce non-specific cell lysis (cytotoxins) [15]. SLURP-1 is the first described mammalian member of the Ly6/uPAR protein family [4,16], it potentiates the human α7 nicotinic acetylcholine receptors that are present in keratinocytes [17]. Mutations in human SLURP1 gene are the cause of the disease Mal de Meleda (MDM), and impair T-cell activation in a family with MDM [17–19], while SLURP1 deficiency in mice elicits metabolic and neuromuscular abnormalities in addition to PPK [20]. As a key immunomodulatory peptide, SLURP1 is abundantly expressed in healthy corneas and is down-regulated by Klf4 in proinflammatory conditions [21]. SLURP-2, a homolog to SLURP-1, has also been isolated from human epidermal and oral keratinocytes [22].

Knowledge regarding the expression and function of Ly6 superfamily members has been increasingly accumulated in recent years. However, few studies were conducted to investigate their expression and function in the process of early development of zebrafish *Danio rerio*. Zebrafish is a widely used model organism, whose digestive tract is similar to that of mammals in terms of development, organization, and function [23,24]. Here, we report the identification of three novel secreted protein genes of Ly6/uPAR superfamily and their specific expression patterns in zebrafish digestive system. We also show that they are possibly involved in the immune responses of the digestive system.

2. Materials and methods

2.1. Zebrafish farming

Wild-type AB strain zebrafish (*Danio rerio*) were reared in Zebrafish farming system of ESEN at 28 ± 1 °C under a 14 h/10 h light/dark photoperiod and fed twice daily. Sexually mature *D. rerio* were placed together in the late evening at a female to male ratio of 2:1, and the embryos were collected early in the next morning, and transferred to Holtfreter solution (MgSO4·7H2O 0.163 mg/ml, KCl 0.03 mg/ml, NaCl 1 mg/ml, CaCl2 0.04 mg/ml). All the embryos were staged as described by Kimmel [25] and mounted with RNAiso Plus (Takara, Japan) for RNA extraction or fixed in 4% Paraformaldehyde/PBS (PFA) for whole-mount in situ hybridization.

Table 2
Primers used for specific antisense probe.

Gene	GeneBank accession no.	Forward primer (5′–3′) reverse primer (5′–3′)
Ly2.1	XM_009298910	GTGTTTGCGAATGGCTCCTC GTCCGTCTGACAGCATCTGG
Ly2.2	XM_009298911	TGATGGCTCTGCCCTGAAAT TCTGACAGCACTTGGCCTTA
Ly2.3	XM_009298912	CTGTGTCCCAAAGAAAGCCG CGAAGGTGTTGCAGAAGTCC

Table 3
Primers used for qRT-PCR.

Gene	GeneBank accession no.	Forward primer (5′–3′) Reverse primer (5′–3′)
Ly2.1	XM_009298910	TCTGCTGCTGGCTCTTGTTCTCG CGGAAGTAGGAATAGGGTTGTATGGTG
Ly2.2	XM_009298911	CTCTTGTGTTTGCTGATGGCTCTGC GTAGCCAAAGGGAGGACTGGTGAATC
Ly2.3	XM_009298912	TATTGGCTGTTGTTCTTATGCTGGC CACTTGATGTTGATGTAAGAGTTGCG
β-actin	FJ915059.1	CGAGCAGGAGATGGGAACC CAACGGAAACGCTCATTGC

2.2. Gene structure and protein sequence analysis

The predicted Ly6 sequences were obtained from the zebrafish genome at NCBI website (http://www.ncbi.nlm.nih.gov/). A gene cluster containing 3 Ly6 genes located on chromosome 2 were chosen to study in detail because no information is available about them presently. The three genes were designated as Ly2.1, Ly2.2, Ly2.3, respectively, according to their location on chromosome 2. The exon-intron structures of Ly2.1-3 were performed by the online program Gene Structure Display Server (http://asia.ensembl.org/index.html). All the exons and introns were mapped and then the relative intron lengths were compared.

Prediction of GPI-anchored site and signal peptide were performed by PreGPI (http://gpcr2.biocomp.unibo.it/gpipe/index.htm) and SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), respectively. Multiple sequence alignments were performed using the ClustalX algorithm and DNASTAR. Three-dimensional (3D) structure of the LU domain of Ly2.1-3 was predicted by homology modeling methods, SWISS-MODEL (http://www.swissmodel.expasy.org/). The Prediction of antimicrobial peptide (AMP) of Ly2.1-3 proteins was realized by Antimicrobial Peptide Calculator and Predictor (http://aps.unmc.edu/AP/main.php) (Table 1).

2.3. Whole-mount in situ hybridization (WISH)

Whole-mount in situ hybridization was performed following the protocol [26] (Primers used for specific antisense probe are listed in Table 2). The vectors habor Ly2.1-3 fragment were digested by NcoI, SalI, SalI (Promega, USA) restriction enzymes, then Digoxigenin (DIG)-labeled Ly2.1-3 antisense RNA probes were synthesized in vitro using T7, Sp6, and Sp6 RNA polymerases, respectively, and the vectors harboring Ly2.1-3 fragment were

Table 1
Antimicrobial Peptides prediction.

Gene	Protein-ID	Cysteine number	Total net charge	pI	Hydrophobic ratio
Ly2.1	XP_009297185	11	+6	8.5	46%
Ly2.2	XP_009297186	11	+4	8.2	48%
Ly2.3	XP_009297187	11	+4	8.2	47%