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Molecular and functional characterization of *kita* and *kitla* of the goldfish (*Carassius auratus* L.)

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ABSTRACT

Kit ligand and its type III tyrosine kinase receptor Kit promotes the survival, proliferation and differentiation of progenitor cells involved in mammalian myelopoiesis. In this study we report on the molecular and functional characterization of kit receptor A (*kita*) and kit ligand A (*kitla*) from the goldfish. Both *kita* and *kitla* were ubiquitously expressed in goldfish tissues, with higher mRNA levels observed in the kidney and spleen, the major hematopoietic organs of fish. Furthermore, both *kita* and *kitla* expressions decreased in a time-dependent manner in goldfish primary kidney macrophage (PKM) cultures, as progenitor to macrophage development progressed, and the highest expressions of both the receptor and ligand were observed in sorted progenitor cell populations. Activation of mature macrophage cultures increased both *kita* and *kitla* expressions. Kit ligand A induced chemotactic response, proliferation and survival of PKM cells in a dose-dependent manner, but did not induce differentiation of early PKM cells. These results are consistent with the role of *kita* and *kitla* during myelopoiesis of higher vertebrates and suggest a conserved mechanism of macrophage development throughout vertebrates.

1. Introduction

Kit ligand, also known as stem cell factor (SCF), mast cell growth factor, and steel factor in mammalian systems, and its type III tyrosine kinase receptor c-Kit are involved in hematopoiesis [1–3], spermatogenesis [4,5], and development of melanocytes [5–7] and mast cells [8–11]. Early studies reported that c-Kit was encoded by the White locus (*W*) in mice as the normal homologue of the Hardy-Zuckerman 4 feline sarcoma virus [12,13]. c-Kit is structurally similar to the type III tyrosine kinase receptors, colony stimulating factor-1 receptor (CSF-1R) and platelet-derived growth factor receptor (PDGFR) [14]. These receptors are characterized by an extracellular domain consisting of five immunoglobulin-like domains and by an insertion of ~70–100 amino acids in the middle of the intracellular tyrosine kinase signaling domain [15]. SCF was later identified by various groups [16–18] as short-chain four-helix bundle [19] encoded by the *Steel* locus in the mouse [12]. SCF is extensively glycosylated with both N- and O-linked sugars that make up approximately 30% of its molecular weight [20]. Two of these monomers non-covalently associate in a "head-to-head" manner to form a homodimer that binds to the second and third immunoglobulin domains of the kit receptor with a high affinity [20–25]. Binding of SCF to c-Kit results in receptor dimerization and conformational changes in the D4 and D5 domains of c-Kit resulting in autophosphorylation of the intracellular tyrosine kinase domains and downstream signaling [26]. Mice with mutations in the White or Steel loci exhibit hypopigmentation, mast cell deficiency, macrocytic anemia, and sterility, while complete loss of function of either of these genes results in mortality [12,27].

In lower vertebrates such as the zebrafish, genomic duplication has resulted in the expressions of kit ligand A (*kitla*) and B (*kitlb*) as well as kit receptor A (*kita*) and B (*kitb*) [28–30]. However, their function seems to be partitioned to *kita* and *kitla* with regards to melanocyte survival and cell migration and differentiation [30–34]. While studies in zebrafish have shown the importance of *kita* and *kitla* in melanocyte development, they did not observe obvious defects in hematopoiesis suggesting that *kita* and *kitla* were not essential to hematopoiesis in zebrafish. These studies on *kita* and *kitla* are in contrast to those performed in mammalian systems where signaling of SCF through c-Kit promotes the survival, proliferation and differentiation of progenitor cells involved in

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hematopoiesis. While zebrafish studies suggest that *kita* and *kitla* may not have an essential role in hematopoiesis, they do provide some evidence for their involvement in myeloid development as significant differences in proportions of myeloid cells, particularly promyelocytes, exist between wildtype and kit (*sparse*) mutant zebrafish [28]. To date, the role of *kita* and *kitla* during myeloid development in teleosts has not been fully examined, and further characterization of this receptor–ligand pair is required before its contribution to myelopoiesis can be defined.

In this study we report on molecular and functional characterization of *kita* and *kitla* from the goldfish. Both *kita* and *kitla* were ubiquitously expressed in goldfish tissues, with higher expressions observed in the kidney and spleen. The *kita* and *kitla* expressions decreased in a time-dependent manner in goldfish primary kidney macrophage (PKM) cultures as progenitor to macrophage development progressed. Indeed, characterization of sorted progenitors, monocytes and mature macrophages from PKM cultures revealed decreasing expressions of both receptor and ligand as progenitors differentiated into macrophages. Treatment of macrophages with lipopolysaccharide (LPS) or heat-killed *Aeromonas salmonicida* A449 increased the expressions of both *kita* and *kitla*. Functional analysis of goldfish *kitla* indicated that recombinant *kitla* protein promoted a chemotactic response of cells in both early and mature PKM cultures. Goldfish recombinant *kitla* also induced proliferation of early PKM cultures. Interestingly, *kitla* did not induce differentiation of early PKM cells but appeared to promote progenitor cell survival. These results are consistent with the role of *kita* and *kitla* during myelopoiesis of higher vertebrates and suggest a conserved role for this receptor/ligand pair in macrophage development of vertebrates.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were obtained from Mount Parnell (Mercersburg, PA). Fish were maintained in tanks with a continuous flow water system at 20 °C and with a 14 h light/10 h dark period in the aquatic facilities of the Biological Sciences building at the University of Alberta. Fish were fed until satiated daily and were acclimated for at least three weeks prior to use in the experiments. Prior to handling, fish were sedated using TMS (tricaine methane sulfonate) solution of 40–50 mg/L of water. The animals in the Aquatic Facility were maintained according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada).

2.2. Bacteria

A. salmonicida A449 was a kind gift from Dr. Jessica Boyd (NRC Institute, Halifax, Canada). This strain is virulent, possesses A layer and is aggregating. Colonies were stored at 4 °C on Tryptic Soy Agar (TSA) + 20 μg/mL chloramphenicol (Sigma) and a single colony was used to inoculate 5 mL of tryptic soy broth (TSB) + 20 μg/mL chloramphenicol that was grown to stationary phase for 24 h at 18 °C with shaking. Bacteria were washed twice and re-suspended in sterile 1× PBS, pH 7.4 to the original volume of the culture. *A. salmonicida* was heat killed for 45 min at 60 °C in a circulating water bath and stored at −20 °C until used.

2.3. Isolation and establishment of goldfish primary kidney macrophage (PKM) cultures

Goldfish (10–15 cm) were anesthetized with TMS, and killed. The isolation and cultivation of goldfish kidney leukocytes from the whole kidney in complete NMGFL-15 medium containing 5% carp serum and 10% newborn calf serum was performed as previously described [35,36]. Unlike other teleosts, the goldfish kidney cannot be clearly separated into anterior and posterior kidney. Cells were cultured at 20 °C in the absence of CO_2. These primary kidney macrophage (PKM) cultures were composed of three distinct cell populations consisting of early progenitors (R1), monocytes (R3) and mature macrophages (R2). These cell populations have been extensively characterized and differ in morphology, cytochemistry and function [35,36].

Table 1
List of primers.

Primer	Sequence (5′ to 3′)
Zebrafish *kitla* sense 1	TGCTGTACATCACAGTTGCTGCCT
Zebrafish *kitla* sense 2	AAAGAAGTGAGTGGCATGTGCTGGGT
Zebrafish *kitla* antisense 1	TTGCGCTGATGTTGTCGACGCTGTCGTAGT
Zebrafish *kitla* antisense 2	ACTGCGATTGCAAACGGGATGGTGAGGA
Goldfish *kitla* 3′ race	TGCCAATTTGTCAAGAGATGTCGAGGACTGCGAGC
Goldfish *kitla* nested 3′ race	GCAGAACAGTCTTATGTCGCTCCTCACCATCCCGT
Goldfish *kitla* 5′ race	ATTGGAGGATATGTTCCCAAACTTCTGCGCCAGG
Goldfish *kitla* nested 5′ race	CGCAGTCCTCGACATCTCTTGACAAATTGGCAGT
pSECTag2B *kitla* sense	AAGCTTTCCAGTGAAATAGGAAATCCCATTACA
pSECTag2B *kitla* antisense	CTCGAGCCACAACTTTCGGAAGGAATGCCCC
Zebrafish *kita* sense 1	ACCATCGTGTTCGACATTGTTGCGGCAGAA
Zebrafish *kita* sense 2	GTCGGAACTTTGGATGCCGCTACGGTTAAA
Zebrafish *kita* sense 3	CTGACGGAGCCCATTACTCAGGTGAGGACG
Zebrafish *kita* antisense 1	GTTCTCGCTGATAGAGAAGACTGTGTACACGAT
Zebrafish *kita* antisense 2	GGAGGACAGGATTCCCAGTACAGAGGGCTTCA
Zebrafish *kita* antisense 3	GTGGACTCTAAATTCTATAAGATGATCAAG
Goldfish *kita* 5′ race	AGGAGGCACGTCTGGAACGAGGCGAACA
Goldfish *kita* nested 5′ race	TGTTGGGCAGGGACTGTCCGTCACATTT
pCR2.1 forward sequencing	GTAAAACGACGGCCAG
pCR2.1 reverse sequencing	CAGGAAACAGCTATGAC
pSECTag2B forward sequencing	TAATACGACTCACTATAGGG
pSECTag2B reverse sequencing	TAGAAGGCACAGTCGAGG
QPCR *kita* sense	CAACTCATGTTCGCCTCGTT
QPCR *kita* antisense	CAGAGGCTGACCCAGTGTGA
QPCR *kitla* sense	TGGCTTGGAGGATTCAATGC
QPCR *kitla* antisense	TGGCCGTAAGCCACATCTC
QPCR TNFα-2 sense	TCATTCCTTACGACGGCATTT
QPCR TNFα-2 antisense	CAGTCACGTCAGCCTTGCAG
QPCR EF1α sense	CCGTTGAGATGCACCATGAGT
QPCR EF1α antisense	TTGACAGACACGTTCTTCACGTT

2.4. DNA sequencing and in silico analyses of goldfish kita and kitla

The sequences for goldfish *kita* and *kitla* were identified using homology based PCR using primers (IDT) designed against zebrafish *kita* and *kitla* sequences in the NCBI database, accession numbers NM131053 and AY929069, respectively. The complete list of primers used for homology based PCR, RACE PCR, Q-PCR sequencing and recombinant protein expression are listed in Table 1. From this initial fragment, RACE PCR (BD Sciences, Clonetech) was performed to obtain a partial open reading frame for goldfish *kita* and the full open reading frame for goldfish *kitla* according to manufacturer's specifications. Generated amplicons were gel purified using the QIA Gel Extraction kit (Qiagen) and cloned into the TOPO TA pCR2.1 vector (Invitrogen). Positive colonies were identified by colony PCR using the vector specific M13 forward and reverse primers, isolated using the QIAspin Miniprep kit (Qiagen) and sequenced using an ET terminator cycle sequencing dye and a PE Applied Biosystems 377 automated sequencer. Single pass sequences were analyzed using 4peaks software (http://mekentosj.com/4peaks/) and sequences aligned and analyzed using BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Protein sequences, conserved motifs, secretion signals, transmembrane domains and potential O- and N-glycosylation sites were predicted using programs from the ExPASy website (http://ca.expasy.org/) and the conserved domains program on NCBI