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Phospholipase C gamma-1 is required for granulocyte maturation in zebrafish

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ABSTRACT

The regulation of hematopoiesis is generally evolutionarily conserved from zebrafish to mammals, including hematopoietic stem cell formation and blood cell lineage differentiation. In zebrafish, primitive granulocytes originate at two distinct regions, the anterior lateral plate mesoderm (A-LPM) and the intermediate cell mass (ICM). Few studies in the zebrafish have examined genes specifically required for the granulocytic lineage. In this study, we identified the responsible gene for a zebrafish mutant that has relatively normal hematopoiesis, except decreased expression of the granulocyte-specific gene *mpx*. Positional cloning revealed that *phospholipase C gamma-1* (*plcg1*) was mutated. Deficiency of *plcg1* function specifically affected development of granulocytes, especially the maturation process. These results suggested that *plcg1* functioned specifically in zebrafish ICM granulopoiesis for the first time. Our studies suggest that specific pathways regulate the differentiation of the hematopoietic lineages.

Introduction

The ontogeny of the vertebrate hematopoietic system is characterized by two waves: primitive hematopoiesis and definitive hematopoiesis. Primitive hematopoiesis produces transitory cell populations, predominantly erythrocytes and some myeloid cells, while definitive hematopoiesis generates hematopoietic stem cells that are capable of self-renewal and giving rise to all blood lineages including lymphocytes (Moore and Metcalf, 1970; Morrison et al., 1995; Weissman, 2000). Every lineage plays its unique role in physiological processes throughout vertebrate life. The innate immune system, the first line of host defense against infections, includes granulocytes that respond to infections and tissue injury.

The maturation of granulocytes is a serial process that can be divided into six development stages m myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils and segmented neutrophils (Fiedler and Brunner, 2012). Different transcription factors regulating granulocyte specific gene activation (Fiedler and Brunner, 2012). The transcription factors PU.1 and CEBPα are important for fate decision of granulocytes by inhibiting monopoiesis (Friedman, 2002; Nakajima, 2011). GFI1 is critically required in the development of mature neutrophils by activating expression of granulocyte gene such as MPO and ELANE (Fiedler and Brunner, 2012; Horman et al., 2009). Blocked granulocytes maturation process may result in a disease named congenital neutropenia (Klein, 2009).

Zebrafish (*Danio rerio*) is an excellent model to study hematopoiesis because of many advantages over other model animals (Carradice and Lieschke, 2008). Hematopoiesis is highly conserved between mammals and zebrafish (Song et al., 2004). Primitive hematopoiesis of zebrafish initiates in two intra-embryonic sites known as the anterior lateral plate mesoderm (A-LPM) and posterior lateral plate mesoderm (P-LPM), the later subsequently forms the intermediate cell mass (ICM) (Bertrand and Traver, 2009). In A-LPM region, *pu.1* positive primitive macrophages are first detected at 12 hours post-fertilization (hpf). The ICM is the primary site of production of primitive erythrocytes marked by *gata1* expression and a few granulocytes marked by myeloperoxidase (*mpx*) expression (Bennett et al., 2001; Davidson and Zon, 2004). The origin of granulocytes in zebrafish occurs in two distinct sites (Bennett et al., 2001;

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Le Guyader et al., 2008). Expression of *mpx* is first detected at 18 hpf in the posterior ICM (p-ICM) region and *mpx*$^+$ granulocytes are subsequently observed on the anterior yolk sac at 20 hpf. Co-localization of *mpx* and *pu.1* suggests the cells arise from the A-LPM (Davidson and Zon, 2004; Warga et al., 2009). The *mpx*$^+$ cells in p-ICM also express *pu.1* and have been proved to share a common lineage with primitive erythrocytes (Galloway et al., 2005; Warga et al., 2009). Although both *mpx*$^+$ cells originate from *pu1*$^+$ progenitor cells, they may have separate regulatory mechanisms (Bennett et al., 2001; Yuan et al., 2011).

To investigate development of granulocytes in the p-ICM region, genetic mutants with specific aberrant *mpx* expression in this region may be informative (Keightley et al., 2011). From a forward genetic screen, we indentified a mutant line ldd239 that had decreased *mpx* expression specifically in the ICM. By positional cloning we identified that the gene *phospholipase C gamma-1*(*plcg1*) is responsible for the phenotype of ldd239. Plcg1 is known as a downstream effector of receptor or non-receptor tyrosine kinases mediated signaling pathways (Wilde and Watson, 2001). Plcg1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, thereby initiating and propagating numerous cellular signaling events that regulate many biological processes, such as fertilization, proliferation, differentiation, and chemotaxis (Gresset et al., 2010). There have been several plcg1 mutant lines identified in zebrafish and used for studying its roles in vasculogenesis (Covassin et al., 2009; Lawson et al., 2003). Our results demonstrated that depletion of *plcg1* specifically affected granulocyte maturation rather than differentiation process.

Materials and methods

Zebrafish maintenance and mutagenesis

Zebrafish in our lab were managed under standard conditions as described previously. For all experiments, zebrafish embryos were cultured in "egg water" consisting of 0.03% sea salt and 0.002% methylene blue as a fungicide. To inhibit the pigment formation and facilitate the in situ hybridization, embryos were incubated with 0.0045% 1-phenyl-2-thiourea (Sigma) (Kimmel et al., 1995). Zebrafish strain Tubegin (Tu) was mutagenized and WIK was used as the mapping strain. ENU mutagenesis was carried out as described (Mullins and Nusslein-Volhard, 1993). The zebrafish facility and zebrafish study were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Mapping and molecular analysis of ldd239

The ldd239 (Tu background) allele was mapped by outcrossing heterozygous Tu fish into the polymorphic wild-type strain WIK. We scanned the genome for linked SSLP markers by bulk segregation analysis using standard methods (Bahary et al., 2004). Fine mapping was carried out to narrow down the distance. The cDNAs of candidate genes were sequenced from pooled mutant RNA, and candidate mutation was confirmed by sequencing the genomic DNA. All primers used for the analysis are provided in supplemental data (Supplemental Table 1).

Plasmids construction

Pcs2–plcg1

The zebrafish *plcg1* was amplified from reverse transcription products and cloned into PCS2+ vector.

Tol2(flk1/mpx: plcg1-2a-mCherry)

Tol2 backbone including two arms was amplified with indicated primers and combined with *flk1*and *mpx* promoter to form primary constructs. Then the *plcg1*-2a-mCherry fragment was inserted into the primary constructs. All primers used in our experiments are supplied in supplemental data (Supplemental Table 1).

Morpholinos and mRNA microinjection

Morpholino oligonucleotides (MOs) were purchased from Gene Tools. *Plcg1* MO: GCGCTCGCAGCCATTTCCCCTGTTT, *vegfa*MO: GTATCAAATAAACAACCAAGTTCATA and control MO sequence was provided in supplemental Table 1. *Sumo123* MO is provided by Yuan et al. (2011). Capped mRNAs were transcribed from linearized PCS2+ plasmids (mMessage Machine; Ambion), purified, and diluted to 100 ng/μL for injection at one cell stage. Transient transgenic expression was carried according to the previous report (Suster et al., 2009).

Whole-mount in situ hybridization (WISH) and double fluorescent in situ hybridization

Digoxigenin-labeled RNA probes were transcribed using linearized constructs with T3 or T7 polymerase (Ambion). Whole-mount in situ hybridization (WISH) was performed as described (Fu et al., 2009; Thisse and Thisse, 2008; Zhang et al., 2008). The operation of double fluorescent in situ hybridization utilizes standard digoxigenin and fluorescein labeled probes along with tyramide signal amplification (TSA) (Brend and Holley, 2009). The TSA system used in our lab is TSA Plus Fluorescein system and TSA Plus Cyanine3 system (PerkinElmer), which, respectively couple with Anti-Fluorescein-POD, Fab fragments and Anti-Digoxigenin-POD, Fab fragments (Roche).

Phosphorylated histone H3 labeling and TUNEL assay

Terminal transferase UTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit, POD (Roche) according to the manufacturer's recommendations. Phosphorylated histone H3 labeling of fixed embryos was performed with the rabbit anti-phosphohistone H3 antibody (Santa Cruz) at 4 °C overnight and revealed with Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen).

Sudan black staining and O-dianisidine staining

Sudan black staining was used to detect the granules of granulocytes. After treatment with 4% formaldehyde, embryos incubated in Sudan Black for 20 min at 33 hpf. *O*-dianisidine staining was used to check hemoglobin and brown coloration indicated presence of hemoglobin in zebrafish embryos. Embryos were dechorionated at 36 hpf and stained for 15 min in the dark in *O*-dianisidine. And the two methods were detailed described as previously (Le Guyader et al., 2008; Ma et al., 2007).

Confocal scanning

Live fish were anesthetized with tricaine in embryo egg water, and observed in slides. Living scanning microscopy was performed on OLYMPUS-FV-1000 confocal microscope (under 40 × / 1.00 NA and 60 × /1.00 NA water-immersion objectives) (Le Guyader et al., 2008).