Developmental Biology 340 (2010) 22–29

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Syk and Zap-70 function redundantly to promote angioblast migration

Tara L. Christie, Alyson Carter, Evvi-Lynn Rollins, Sarah J. Childs *

Department of Biochemistry and Molecular Biology, and Smooth Muscle Research Group, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1

ARTICLE INFO

Article history:
Received for publication 25 March 2009
Revised 7 December 2009
Accepted 8 January 2010
Available online 22 January 2010

Keywords:
Syk
Zap-70
Vegf
Intersegmental vessel
Thoracic duct
Migration
Angioblast
Zebrafish

ABSTRACT

Spleen tyrosine kinase (Syk) plays critical roles in B-cell and T-cell development, the maintenance of vascular integrity, and proper partitioning of the blood vascular and lymphatic vascular system. Here, we utilize the zebrafish as an *in vivo* system to demonstrate novel roles for Syk and the related kinase Zeta associated protein (Zap-70) in promoting angioblast migration. Partial knockdown of either gene results in early angiogenic delay of the intersegmental vessels, dorsal intersegmental vessel patterning defects, and partial loss of the thoracic duct. Higher dose knockdown of both genes results in little to no angiogenic sprouting of the intersegmental vessels, a phenotype which resembles knockdown of *vegfa*. Di-phosphorylated ERK, an effector of the *vegfa* pathway, is also downregulated in the aorta of *syk:zap* double morphants. Over-expression of *syk* under the control of a blood-specific or vascular-specific promoter rescues sprouting defects after loss of *vegfa*. Together these results suggest that *syk* and *zap-70* function redundantly in an early progenitor to promote the migration of intersegmental vessel angioblasts and lymphangioblasts that contribute to the thoracic duct, either downstream of, or in parallel to *vegfa*.

Introduction

Regulation of vascular sprouting and growth involves multiple overlapping molecular pathways, and is still incompletely understood. Spleen tyrosine kinase (*Syk*) is a cytosolic kinase important for vascular, lymphatic, B-cell, and T-cell development (Abtahian et al., 2003; Cheng et al., 1995; Cornall et al., 2000; Sebzda et al., 2006; Turner et al., 1995; Yanagi et al., 2001). *Syk* is also critical for vascular development as most null mice die shortly after birth with systemic hemorrhage and edema (Cheng et al., 1995; Turner et al., 1995) due to improper partitioning of the developing lymphatic system from the blood vascular system, resulting in blood-filled lymphatics (Abtahian et al., 2003; Sebzda et al., 2006). *Slp-76* serves as a substrate for *Syk*, and null mice display a similar phenotype but also develop a network of dilated and tortuous blood vessels in the small intestine suggesting a role in vascular patterning and migration (Abtahian et al., 2003). In cell culture, *Syk* is involved in endothelial-cell proliferation and migration (Inatome et al., 2001), while lymphatic defects in *Syk* null embryos result from loss of signaling in circulating endothelial precursor cells (EPCs), which contribute to the developing lymphatic vasculature (Sebzda et al., 2006).

Zeta associated protein 70 (Zap-70) is a cytosolic tyrosine kinase, structurally related to *Syk*, that functions in hematopoietic activation and differentiation by signaling through T-cell antigen receptors (Arpaia et al., 1994). ZAP-70 deficiency in humans results in severe compromised immune deficiency syndrome due to the loss of signaling in T-cells (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1995; Elder et al., 1994; Gelfand et al., 1995). Although related in structure and function (as *syk* and *zap-70* are functionally interchangeable (Fallah-Arani et al., 2008; Gong et al., 1997)) lymphatic or blood endothelial defects have not been detected after loss of *zap-70*.

In B- and T-lymphocytes, Syk and Zap-70 bind to an 'immunoreceptor tyrosine-based activation' motif (ITAM) consisting of a consensus sequence with two tyrosines about 10–12 amino acids apart on immune receptors (Turner et al., 2000). Upon receptor activation these tyrosines become phosphorylated allowing cytoplasmic tyrosine kinases, including Syk and Zap-70, to bind the phosphorylated ITAM motifs through their SH2 domains and initiate downstream signaling. *In vitro*, Syk and Zap-70 have been shown to activate PLCγ1 (Law et al., 1996; Williams et al., 1999) and ERK (Griffith et al., 1998; Parsa et al., 2008; Shan et al., 2001; Slack et al., 2007). PLCγ1 and ERK are also important downstream components of the *vegfa* pathway in endothelial cells, and are involved in angiogenesis (Hong et al., 2006; Lawson et al., 2003). However, it is not known whether Syk or Zap-70 functions in endothelial cells *in vivo*, and if they function through similar pathways in endothelial cells as they do in B-cells and T-cells.

The external fertilization, optical clarity and availability of fluorescent transgenic animals make zebrafish an excellent model system for many developmental studies. The recent discovery of a functional lymphatic system in zebrafish has opened the door to take advantage of the unique benefits of zebrafish genetics and development to decipher the molecules and processes involved in blood–lymphatic vascular separation *in vivo* (Hogan et al., 2009; Kuchler et al., 2006; Yaniv et al., 2006). Although many of the genes involved in

* Corresponding author. Fax: +1 403 270 2211.
E-mail address: schilds@ucalgary.ca (S.J. Childs).

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.01.011

angiogenesis and lymphangiogenesis are conserved between mammals and zebrafish, the lymphatic vasculature of zebrafish develops by an alternative mechanism (Kuchler et al., 2006; Yaniv et al., 2006). In mammals, lymphatic endothelial cells bud off from the main cardinal vein and migrate to form the primary lymphatics (Wigle et al., 2002; Wigle and Oliver, 1999). While zebrafish lymphatics are still venous in origin, individual angioblasts do not directly pinch off from the major vein. Instead, they initially migrate as individual cells to the horizontal myoseptum before migrating dorsally to contribute to the dorsal longitudinal lymphatic vessels, or ventrally to contribute to the main thoracic duct (Hogan et al., 2009; Yaniv et al., 2006). This novel mechanism of zebrafish lymphatic development prompted us to analyze the function of zebrafish *syk* and *zap-70* in the developing vasculature to see if they play similar roles in fish angiogenesis and lymphangiogenesis as they do in mammals. Here we show that zebrafish *syk* and *zap-70* play unexpected and redundant roles to promote the migration of angioblasts to form both blood vascular and lymphatic vascular systems.

Materials and methods

Zebrafish husbandry

Embryos were raised at 28.5 °C in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.25 mg/l methylene blue, and staged by hours post fertilization (hpf) or days post fertilization (dpf) according to Kimmel et al. (1995). Chorions were removed by incubation in 20 mg/ml pronase (Sigma, St. Louis, MO) and pigmentation was blocked at 24 hpf by the addition of 0.003% 1-phenyl-2-thiourea (Sigma). *Tg(fli1a:egfp)^{y1}* (Lawson and Weinstein, 2002), *Tg(fli1a:nEGFP)^{y7}* (Roman et al., 2002) or *Tg(kdr-1:GFP)^{la116}* (Choi et al., 2007) embryos on a wild-type Tupfel long fin (TL) background were used for all experiments.

Identification, phylogenetic, and structural analysis of the zebrafish syk and zap-70 orthologs

The full length zebrafish (*Danio rerio*) *syk* ortholog has previously been identified: AF253046 or ZFIN:ZDB-GENE-040702-3. The full length *D. rerio zap-70* ortholog is *zgc:110383* (ZFIN:ZDB-GENE-050522-257). Nucleotide sequences of *D. rerio syk* (NP_998008.1), *Homo sapiens* SYK (NP_003168.2), *Mus musculus* SYK (NP_035648.2), *Tetraodon nigroviridis* Syk (CAF96564), *Gallus gallus* Syk (NP_001026601), *Xenopus tropicalis* Syk (NP_001086665), *Takifugu rubripes* Syk (SINFRUG00000143646), *D. rerio zap-70* (NP_001018425.1), *H. sapiens* ZAP-70 (NP_001070), *M. musculus* ZAP-70 (NP_033565.2), *T. nigroviridis* Zap-70 (CAG00734.1), *G. gallus* Zap-70 (NP_001026601.1), and *T. rubripes* Zap-70 (SINFRUG00000148610) were analyzed with the CLUSTAL W (1.81) multiple sequence alignment program.

Morpholino injection and angiography

The following splice site targeted morpholinos were synthesized by GeneTools LLC (Philomath, OR): *syk^{e2i2}* 5′-AGTGAAGAAGACTTACAGAA-ATTTG-3′; *syk^{e7i7}* 5′-TGAAGCACACCAACCTGAATCCAAG-3′; *zap-70^{i1e2}* 5′-TGGCATCCCCCTAAAAAGGTAACAA-3′; *zap-70^{e3i3}* 5′-TTGATGGG-CAAAACGTACCTGCCAC-3′; *vegfa3* 5′-TAAGAAAGCGAAGCTGCTG-GGTATG-3′ (Nasevicius et al., 2000). Morpholino efficiency was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) with gene specific primers over the morpholino target site (Supplementary Fig. 1). All primer sequences are listed in Supplementary Table 1. Equal loading of products was confirmed by RT-PCR with primers specific to elongation factor 1α (*eF1αF*/*eF1αR*, Supplementary Fig. 1). Off-target effects of morpholinos were assayed by incubation of live embryos in 5 μg/ml acridine orange (Sigma) for 30 min at 28 °C.

Blood flow in wild-type and morphant embryos was assessed by angiography through the injection of 2 MDa rhodamine dextran (Molecular Probes, Carlsbad, CA) into the sinus venosus of 5 dpf embryos. For fluorescent images embryos were mounted in 3% methyl cellulose (Sigma, St. Louis, MO), and photographed with a Stemi SV 11 microscope and an AxioCam HRc camera with AxioVision software (Carl Zeiss Canada Ltd., Toronto, ON). Confocal images were obtained by embedding embryos in 1.2% agarose, and photographed with an Olympus FV5-PSU. Some images taken at high resolution were pieced together with Adobe Photoshop.

Probe synthesis and whole-mount in situ hybridization

syk and *zap-70* probe templates were amplified from adult zebrafish fin cDNA with gene specific primers *sykF*/*T7sykR* and *zap-70F*/*T7zap-70R* (Supplementary Table 1). Products were purified with the QIAGEN PCR purification kit (QIAGEN, Mississauga, ON) prior to transcribing with T7 RNA polymerase (Invitrogen, Burlington, ON). Whole-mount *in situ* hybridization was performed on embryos as previously described (Oxtoby and Jowett, 1993). All post hybridization washes were performed with the aid of the BioLane™ HTI Robot (Holle and Huttner, Tübingen, Germany). The embryos were developed in BCIP/NBT at room temperature for 5–7 days (Roche Applied Science, Lavalle, QC). Whole-mount images were captured as above. A second set of full length antisense probes were also created against the *syk* and *zap-70* genes and resulted in the same expression patterns (data not shown).

Genetic rescue with gata1:syk and fli:syk

Multisite Gateway® cloning (Invitrogen), as developed by Kwan et al. (2007), was used to create full length, C-terminal myc-tagged transcripts of zebrafish *syk* under the control of a blood-expressed *gata1* promoter (Long et al., 1997) and endothelial-cell expressed *fli1* promoter (Buchner et al., 2007). Full length *syk* was amplified from wild-type cDNA with primers incorporating a portion of the attB1 and attB2 recombination sites required for Gateway cloning (*sykFB1*/*sykRB2*, Supplementary Table 1). A second round of PCR was performed to incorporate the entire attB1 and attB2 recombination sites, prior to Gateway cloning into the entry vector pDONOR221 with BP Clonase (Invitrogen). These middle entry constructs, plus a 5′ entry clone with either 5.4 kb of the *gata1* promoter (Long et al., 1997) or 2 kb of the *fli1* promoter (Buchner et al., 2007), and a 3′ entry clone with 6× C-terminal myc tag fusion and SV40 late polyA (p3E-MTpA), were combined with the multisite destination vector pDestTol2pA2 (Invitrogen) to obtain *gata1:syk* and *fli1:syk*. For rescue experiments, 50 pg of transposase and 10 pg of DNA were co-injected into single cell embryos.

Whole-mount immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 h at room temperature, permeabilized in methanol for 30 min at 4 °C, and then blocked in 10% normal sheep serum (NSS) in PBS with the addition of 0.1% Tween-20 (PBT). Immunostaining was performed overnight at 4 °C in 10% NSS/PBT with a 1:100 dilution of αphospho-p44/42 Map Kinase (Thr202/Tyr204) (αERK-P) polyclonal antibody (Cell Signaling, Danvers, MA), or a 1:20 dilution of αcMyc-9E10 (Developmental Studies Hybridoma Bank, Iowa City, IA). Embryos were washed thoroughly with PBT prior to incubation with a 1:250 dilution of goat α–rabbit Alexa555 secondary antibody (Invitrogen) in PBT. Embryos were imaged as for whole-mount *in situ* hybridization.