phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development

Nathan D. Lawson,1,2 Joshua W. Mugford, Brigid A. Diamond, and Brant M. Weinstein

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

In this study, we utilize transgenic zebrafish with fluorescently labeled blood vessels to identify and characterize a mutant (y10) that displays specific defects in the formation of arteries, but not veins. We find that y10 encodes phospholipase C gamma-1 (plcg1), a known effector of receptor tyrosine kinase signaling. We further show that plcg1y10 mutant embryos fail to respond to exogenous Vegf. Our results indicate that Plcg1 functions specifically downstream of the Vegf receptor during embryonic development to govern formation of the arterial system.

Received January 3, 2003; revised version accepted April 14, 2003.

Historically, the structural differences between arteries and veins were thought to be the result of physiological factors such as the pressure and direction of blood flow [Lawson and Weinstein 2002b]. However, recent work demonstrates that a number of signaling pathways specifically induce the differentiation of arterial endothelial cells before circulation commences [Lawson et al. 2001, 2002; Mukouyama et al. 2002, Visconti et al. 2002]. In particular, vascular endothelial growth factor (Vegf) is required for the development of arteries [Mukouyama et al. 2002, Stalmans et al. 2002] and the differentiation of arterial endothelial cells [Lawson et al. 2002; Mukouyama et al. 2002] during embryonic development in both zebrafish and mouse. Despite the wealth of information concerning components of the Vegf signaling pathway in endothelial cell lines [Zachary and Gliki 2001], little is known about the downstream factors that are necessary in vivo during blood vessel formation and arterial development. In this study, we have taken advantage of transgenic zebrafish with fluorescently labeled blood vessels [Lawson and Weinstein 2002a] in conjunction with a forward genetic screen to identify a gene within the Vegf signaling pathway that is required for arterial development. We find that y10 mutant zebrafish embryos display defects in the formation of arteries, but not veins, and are deficient in the expression of artery-specific markers such as ephrin-B2a, similar to zebrafish and mouse embryos lacking Vegf function [Lawson et al. 2002; Mukouyama et al. 2002; Stalmans et al. 2002]. We show that y10 encodes the zebrafish homolog of phospholipase C gamma-1 (plcg1) a known downstream effector of numerous receptor tyrosine kinases [Rhee 2001]. Using plcg1y10 mutant embryos together with microinjection of vegf mRNA, we are able to demonstrate that Plcg1 is required for normal Vegf function. Taken together, our observations reveal a specific requirement for Plcg1 during formation and differentiation of arteries, and indicate that Vegf is the primary growth factor that utilizes Plcg1 signaling during embryonic development.

Results and Discussion

To identify putative regulators of Vegf signaling, we performed a screen for mutants lacking segmental blood vessels [N.D. Lawson, J.W. Mugford, B.A. Diamond, and B.M. Weinstein, unpubl.], a phenotype associated with loss of Vegf function in zebrafish embryos [Nasevicius et al. 2000]. As a screening tool, we utilized zebrafish harboring a fltl:enhanced green fluorescent protein transgene [TG(fli1:egfp)]1; Lawson and Weinstein 2002a] to enable fluorescent visualization of developing blood vessels in vivo. Using this strategy, we identified the zebrafish y10 mutant. In both wild-type and homozygous y10 mutant embryos, overall morphology is normal at 30 h post fertilization [hpf; Fig. 1a,d]. The somites and notochord, both of which are required for normal development of the trunk blood vessels [Brown et al. 2000, Parsons et al. 2002], appear normal by light microscopy [data not shown]. Primary segmental vessels, which sprout from the dorsal aorta, are formed by 30 hpf in wild-type TG(fli1:egfp)1 sibling embryos [Fig. 1b]. These vessels are absent in TG(fli1:egfp)1 embryos mutant for y10 [Fig. 1e]. This phenotype is not a general defect in sprouting blood vessel growth, or angiogenesis, as secondary segmental vessels, which sprout from the posterior cardinal vein by 50 hpf and contribute to parachordal vessels in wild-type embryos [Fig. 1c], are apparent in y10 mutant embryos [Fig. 1f]. The midcerebral vein, a blood vessel that forms adjacent to the midbrain–hindbrain boundary via angiogenesis, also forms normally in y10 mutant embryos [Fig. 1g,h]. In zebrafish embryos, the lateral dorsal aortae connect the outflow tract of the heart to the trunk circulatory network and fuse in the anterior trunk to give rise to a single dorsal aorta [Ishigai et al. 2001]. In wild-type embryos, the lateral dorsal aortae are lumenized by 30 hpf [Fig. 1i], whereas in y10 mutant embryos, these blood vessels appear thin and fail to lumenize [Fig. 1j]. In contrast, the branches of the posterior cardinal vein are normal in mutant embryos [Fig. 1, cf. i and j]. These observations indicate that y10 mutant embryos display a specific defect in the formation of arteries, whereas the development of veins appears unaffected. Consistent with these defects in blood vessel morphology, y10 mutant embryos fail to exhibit head or trunk circulation and have severe pericardial edema by 2 dpf, postfertilization [dpf, data not shown].
pression level of fli1 transcript (Thompson et al. 1998) in the dorsal aorta and posterior cardinal vein does not appear to be affected in y10 mutant embryos [Fig. 2i,j]; consistent with the equivalent level of fli1:egfp transgene expression in wild-type and mutant embryos [Fig. 1]. The semipenetrant nature of the loss in artery marker gene expression is consistent with our previous observations that numerous pathways likely contribute to the differentiation of arterial endothelial cells in the zebrafish dorsal aorta during embryogenesis (Lawson et al. 2002). In addition, the appearance of endothelial cells in the position of the dorsal aorta indicates a failure of these cells to undergo arterial differentiation rather than their inability to migrate to the correct location.

The phenotype of y10 mutant embryos is similar to zebrafish or mouse embryos lacking Vegf function (Lawson et al. 2002, Mukouyama et al. 2002, Stalmans et al. 2002). However, y10 does not map to vegf or its receptor, flk1 [data not shown]. To identify the gene responsible for the y10 mutant phenotype, we performed bulk segregant analysis on wild-type and mutant embryos and mapped y10 to within 0.9 cM of Z6376 on linkage group 23 [Fig. 3a]. By comparing the available zebrafish mei-

**Figure 1.** Vascular morphology in Tg(fli1:egfp)y10 wild-type and mutant embryos. (a) Transmitted light image of a wild-type sibling embryo at 30 hpf. Boxed region indicates views in b and c (wild type) and e and f (mutant). (b) Primary segmental vessels [white arrows] sprout from the dorsal aorta [red arrowhead] in a wild-type embryo at 50 hpf; the posterior cardinal vein is indicated with an asterisk. (c) A secondary segmental vessel [white arrow] sprouts from the posterior cardinal vein and contributes to the parachordal vessel [white arrowhead] at 50 hpf in a wild-type embryo. (d) Transmitted light image of a y10 mutant embryo at 30 hpf. (e) Primary segmental vessels fail to form in y10 mutant embryos, 30 hpf time point. (f) Secondary segmental vessels [white arrows] sprout from the posterior cardinal vein and give rise to the parachordal vessel [white arrowhead] in y10 mutant embryos at 50 hpf. (g) Midcerebral vein [white arrow] in a wild-type embryo at 30 hpf. (h) The midcerebral vein [white arrow] forms normally in 30 hpf y10 mutant embryos. (i) Dorsal view of the lateral dorsal aorta [red arrows] and branches of posterior cardinal vein in a wild-type embryo at 50 hpf, lumens of the lateral dorsal aorta [red brackets] and cardinal vein branches [white brackets] are indicated. (j) Dorsal view of lateral dorsal aorta in y10 mutant embryos. Note the poor lumenization and formation of lateral aorta branches [red arrows] compared with the posterior cardinal vein [white arrowheads, lumens indicated by white brackets]. [a–h] Lateral views; anterior is to the left, dorsal is at top. Images in b, c, e, and f were obtained by multiphoton laser scanning microscopy, whereas g–j were obtained using a confocal laser microscope. [g,h] Optical sections were limited to those containing the midcerebral vein to minimize fluorescence derived from the aortic arch mesenchyme.

In addition to defects in artery formation, y10 mutant embryos exhibit a loss of artery-specific gene expression in the dorsal aorta. By 24 hpf, wild-type embryos express ephrin-B2a in the dorsal aorta [Fig. 2a], whereas in approximately half of y10 mutant embryos, ephrin-B2a expression is strongly reduced or absent [Figs. 2b, 4g, below]. Dorsal aorta-specific expression of notch5 [Fig. 2c], an important regulator of arterial differentiation downstream of Vegf (Lawson et al. 2001, 2002), is also reduced or absent at a frequency similar to ephrin-B2a [Fig. 2d; data not shown]. We find that general endothelial cell markers, such as flk1 and tie1 are expressed in the major trunk blood vessels, and their expression appears to be mildly reduced in y10 mutant embryos [Fig. 2e–h]. Ex-

**Figure 2.** Loss of artery marker gene expression in y10 mutant embryos. (a) Normal ephrin-B2a in dorsal aorta [red arrow] of a wild-type sibling embryo. nt, neural tube; nc, notochord. (b) y10 mutant embryo with reduced dorsal aorta ephrin-B2a expression. (c) notch5 expression in dorsal aorta [red arrow] and neural tube [asterisk] of wild-type sibling embryo. (d) Reduced dorsal aorta notch5 expression in y10 mutant embryo. Neural tube expression in not affected. (e) flk1 expression in wild-type sibling embryo. Arrows indicate segmental vessels. (f) flk1 expression in a y10 mutant embryo. (g) tie1 expression in a wild-type sibling embryo. (h) tie1 expression in a y10 mutant embryo. Note absence of segmental vessels in f, h, and j. (a–h) Lateral views of 24 hpf zebrafish embryo trunk; anterior is to the left, dorsal is up.
plcg1 mRNA, scored for the presence or absence of trunk circulation or segmental vessels (SeV) at 30–32 hpf, and then genotyped using oligonucleotide primers 2182 and 2185. (b) Epifluorescence micrograph of a Tg(fli1:egfp)y1 embryonic mutant for plcg1 intron 1 and exon 2 splice junction (a G→T transition in the coding sequence, indicated by an arrow). (c) Transcriptase IVT (Telo-IVT) riboprobe at 24 hpf. (d) Wild-type Tg(fli1:egfp)y1 embryonic mutant implanted with 15 ng of control scrambled morpholino that displays fully formed segmental vessels (arrows) and dorsal longitudinal anastomotic vessel (arrowhead). (e) Tg(fli1:egfp)y1 embryonic mutant implanted with 15 ng of plcg1 morpholino with no segmental vessel sprouts. (f–j) Embryos derived from a plcg1y10–y10 cross were injected with 100 ng of myc-epitope-tagged plcg1 mRNA form segmental blood vessels (Fig. 3h,i) and dorsal longitudinal anastomotic vessel (arrowhead). (g) Plcg1 protein at amino acid 73 (Fig. 3e). The majority of these deletions result in a frame shift into a premature stop codon and truncate the Plcg1 protein at amino acid 73 (Fig. 3e). Less than 30% of the transcripts lack only three nucleotides and result in loss of I72 and change of D73 to N. Both of these amino acids are identical in rat, human, and zebrafish Plcg1 (data not shown) and are located in the pleckstrin homology domain, which is important for growth factor-induced membrane localization of Plcg1 (Falasca et al. 1998). To confirm that the plcg1 exon 1–exon 2 junction was affected in plcg1y10–y10 mutant embryos, we designed an antisense Morpholino oligonucleotide against the exon 1–intron 1 boundary sequence to interfere with plcg1 pre-mRNA splicing (Draper et al. 2001). Wild-type Tg(fli1:egfp)y1–y10 embryonic mutants injected with a scrambled control Morpholino displayed fully formed segmental blood vessels (Sv) by 32 hpf (Fig. 3f,j), whereas mutants displayed fully formed segmental blood vessels (Sv) by 32 hpf (Fig. 3f,i). Whole-mount in situ hybridization using a plcg1 riboprobe at 24 hpf. Lateral views; dorsal is up. (b) Plcg1 is expressed in the trunk blood vessels and diffusely in mesoderm, which is also shown in the boxed region.
circulation at 30 hpf (Fig. 3j; data not shown). Together, these results establish definitively that the gene responsible for the y10 mutant phenotype encodes the zebrafish homolog of Plcg1.

Biochemical studies have demonstrated that Plcg1 can be an effector of Vegf signaling in endothelial cell lines [Takahashi and Shibuya 1997; Takahashi et al. 2001], and evidence from mice lacking plcg1 indicates that it is required for blood vessel development [Liao et al. 2002]. However, there is no definitive genetic evidence to confirm that Plcg1 functions downstream of Vegf in vivo. The phenotype of zebrafish plcg1y10 mutant embryos and the similarity to mice [Mukoyama et al. 2002; Stalmans et al. 2002] or zebrafish [Nasevicius et al. 2000; Lawson et al. 2002] lacking Vegf function, suggests that Plcg1 is an important downstream effector of Vegf during artery development. The availability of plcg1y10 mutant zebrafish allowed us to address whether or not this was the case. To determine whether Plcg1 was required for Vegf function, we injected vegf121 mRNA into embryos derived from plcg1y10 heterozygous carriers. We then assayed for flk1 or ephrin-B2a expression in injected embryos, followed by PCR analysis to identify wild-type and mutant embryos. We have found previously that exogenous Vegf induces the expression of both of these markers, although induction of ephrin-B2a requires the Notch signaling pathway, whereas induction of flk1 does not [Lawson et al. 2002]. Consistent with previous observations [Liang et al. 2001; Lawson et al. 2002], we find that nearly all wild-type embryos injected with vegf121 mRNA display increased levels of flk1 expression in their trunk blood vessels [Fig. 4a,c]. In contrast, ectopic Vegf121 fails to induce flk1 expression in plcg1y10 mutant embryos [Fig. 4a,c]. Similarly, nearly all wild-type embryos injected with vegf121 mRNA also exhibit ectopic expression of ephrin-B2a in the posterior cardinal vein [Fig. 4f,h]. However, plcg1y10 mutant embryos injected with mRNA encoding Vegf121 display either normal or reduced ephrin-B2a expression [Fig. 4d,e,h] in proportions similar to uninjected mutant embryos [Fig. 4h]. These data indicate that Plcg1 function is required for induction of both Notch-dependent and Notch-independent signaling downstream of Vegf in vivo and is consistent with biochemical evidence that shows Plcg1 functions proximal to the Vegf receptor, Flk1 [Takahashi et al. 2001].

Although Plcg1 is known to function downstream of numerous receptor tyrosine kinases [Rhee 2001; Wilde and Watson 2001], we find that the primary defects in zebrafish plcg1y10 mutants are restricted to the vasculature within the embryo and are remarkably specific to a subset of blood vessels. The similarity of the plcg1y10 mutant phenotype to zebrafish embryos lacking Vegf [Nasevicius et al. 2000; Lawson et al. 2002] and the failure of plcg1y10 mutant embryos to respond to exogenous Vegf (this study) indicate that Plcg1 function is required downstream of Vegf to drive arterial development. This requirement for Plcg1 to mediate Vegf signaling during vascular development appears to be conserved in vertebrates, as mice lacking plcg1 also display severe defects in blood vessel formation [Liao et al. 2002] during embryogenesis, similar to those associated with loss of Vegf [Carmeliet et al. 1996]. However, mice lacking either Plcg1 or Vegf display a more severe phenotype, including failure to express general endothelial cell markers indicative of an early block in the formation of endothelial cells from mesodermal precursors, than in zebrafish embryos with comparable loss of function [Nasevicius et al. 2000; Lawson et al. 2002]. The reason for these differences is unknown at this time, but may reflect additional redundancy in the Vegf-signaling pathway in zebrafish that allows for early stages of endothelial progenitor cell development. Therefore, only defects in arterial development, which have been observed in mice lacking specific isoforms of Vegf [Stalmans et al. 2002], are observed in the blood vessels of plcg1y10 mutant embryos.

Despite apparent species-specific differences in Vegf sensitivity during embryonic development, recent evi
dence indicates that Vegf is an important signal that specifically drives development of the arterial system in both zebrafish and mice (Lawson et al. 2002, Mukouyama et al. 2002, Stalmans et al. 2002, Visconti et al. 2002) and does so through the action of the Notch signaling pathway (Lawson et al. 2001, 2002, Lawson and Weinstein 2002b). However, little is known about the downstream effectors of Vegf that mediate this effect on the developing vasculature in vivo. With the ability to perform screens for mutant blood vessel phenotypes using TG(fli1:egfp)y1 embryos, we are able to demonstrate that the zebrafish is an ideal model to dissect this signaling pathway. The studies presented here validate this forward genetic approach by describing the identification of plcg1 as a necessary component of the arterial differentiation pathway downstream of Vegf. It is likely that identification and characterization of additional mutants with similar phenotypes will yield further insight into the components of the Vegf pathway required for this particular aspect of blood vessel formation.

Materials and methods

Fish and handling
Zebrafish were maintained and bred as described elsewhere (Westerfield 1993). The TG(fli1:egfp)y1 transgenic line has been described previously (Lawson and Weinstein 2002a).

Mapping
To facilitate genetic mapping, the TG(fli1:egfp)y1plcg1y10 found male was crossed to the wild-type TL line. Identified mutant carriers of Z130, Z6376, and Z4003 are available at http://zebrafish.mgh.harvard.edu/

Cloning
Sequences encompassing the plcg1 3' UTR and start codon were obtained using available zebrafish EST [GenBank accession nos. AW510269, B979386, AW281801, BM778133, AW279908, AIJ38501, and AIJ379215] and genomic trace sequence. The plcg1 start codon was obtained through a BLAST search of zebrafish genomic traces using the rat Plcg1 amino acid sequence. The full-length coding sequence of zebrafish plcg1 was amplified by PCR using cDNA from wild-type embryos using the following oligonucleotides: 5'-TTACTAGTGAACAAACAGGGGAATCGTGGC-3' and 5'-TTTCTACACTGCTCGTTACGCTTGATATT-3' and TOPO-cloned into pcR2.1 (Invitrogen) to give pcRplcg1CDS. Additional plcg1 cDNA sequence was obtained or confirmed by cycle sequencing of reverse transcriptase PCR (RT-PCR) products using the BigDye sequencing kit and an ABI 310 capillary sequencer according to manufacturer's instructions (Applied Biosystems). A fragment containing the full-length plcg1 coding sequence was digested from pCRplcg1CDS and cloned into pcRS2+ to give pCSplcg1CDS. Subsequently, a 6x myc epitope tag was cloned in frame of the 5' end of plcg1 coding sequence in pCSplcg1 CDS to give pCSMTplcg1. pCSMTplcg1 was digested with NotI and used as template for mRNA synthesis using the mMessage mMachine kit according to manufacturer's protocols (Ambion).

Whole mount in situ hybridization
Antisense mRNA probes for ephrin-B2a, notch5, and fli1 were prepared as described (Lawson et al. 2001). To derive a plcg1 riboprobe, pCRplcg1CDS was linearized with HindIII and transcribed using T7 polymerase. Whole-mount in situ hybridization was performed as described elsewhere (Hauptmann and Gerster 1994).

GenBank accession numbers
Zebrafish plcg1 coding sequence, AY163168; plcg1 intron/exon containing CA repeat 2182–2185, AY163169; zebrafish plcg1 intron 1–exon 2 boundary, AY163170.

Acknowledgments
We thank the members of the "screen team": Michael Tsang, Neil Hukriede, Susan Lyons, Milton English, and Lin Li. We are grateful to Beth Roman, who provided advice on mapping. We thank the Wellcome Trust and Sanger Institute for making zebrafish genomic trace sequence readily available. We also thank Van Pham for helpful technical assistance and the members of the NIH zebrafish facility staff.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References