DISRUPTION OF ACVR1I INCREASES ENDOThelial CELL NUMBER IN ZEBRAFISH CRANIAL VESSELS

Beth L. Roman, Van N. Pham, Nathan D. Lawson, Magdalena Kulik, Sarah Childs, Arne C. Lekven, Deborah M. Garrity, Randall T. Moon, Mark C. Fishman, Robert J. Lechleider and Brant M. Weinstein

INTRODUCTION

The mechanism by which the embryonic vasculature forms can be divided into two major processes: vasculogenesis and angiogenesis. In vasculogenesis, mesodermally derived endothelial cell precursors or angioblasts migrate to future vessel sites and coalesce with neighbors to form endothelial cell cords, which lumenize and become ensheathed by supporting smooth muscle cells or pericytes. This primitive vascular network provides the substrate for angiogenic processes, which include remodeling of vasculogenic vessels, sprouting of new vessels, and intussusception, which facilitates vessel branching.

A number of ligand/receptor pairs have been identified that are required for both vasculogenesis and angiogenesis, whereas the rest play roles in angiogenic processes and/or perivascular sheath formation (for review, see Roman and Weinstein, 2000). TGFβ family signaling is also important in blood vessel development, although the precise ligand/receptor pairs and their specific roles are not well established. TGFβ family ligands bind to a heterodimeric complex consisting of a type II and a type I receptor, both of which are transmembrane serine/threonine kinases (for review, see Massague et al., 2000). Ligand binding stimulates the type II receptor to phosphorylate the type I receptor, which in turn phosphorylates a receptor-specific Smad. This phosphorylated Smad dimerizes with a common partner Smad (Smad4), forming a complex that translocates to the nucleus and directly regulates gene transcription. TGFβ family ligands can be divided into two groups based on Smad specificity: TGFβs, activins, and nodals signal through Smad1, Smad5, and Smad8.

SUMMARY

The zebrafish mutant violet beauregarde (vbg) can be identified at two days post-fertilization by an abnormal circulation pattern in which most blood cells flow through a limited number of dilated cranial vessels and fail to perfuse the trunk and tail. This phenotype cannot be explained by caudal vessel abnormalities or by a defect in cranial vessel patterning, but instead stems from an increase in endothelial cell number in specific cranial vessels. We show that vbg encodes activin receptor-like kinase 1 (Acvrl1; also known as Alk1), a TGFβ type I receptor that is expressed predominantly in the endothelium of the vessels that become dilated in vbg mutants. Thus, vbg provides a model for the human autosomal dominant disorder, hereditary hemorrhagic telangiectasia type 2, in which disruption of ACVR1I causes vessel malformations that may result in hemorrhage or stroke.

Movies available on-line

Key words: Acvrl1, Hereditary hemorrhagic telangiectasia, Endothelium, Angiogenesis, Zebrafish, violet beauregarde
development is illustrated by the following observations. Targeted deletion of Tgfb1 impairs yolk sac vasculogenesis but has no effect on vasculogenesis or angiogenesis within the embryo proper (Dickson et al., 1995). A similar extraembryonic vascular phenotype, characterized by dilated vessels that exhibit poor endothelial cell adhesion and impaired vascular smooth muscle development, results from targeted disruption of TGFβ receptor II (Tgfb2) or TGFβ receptor I (Tgfb1), which together constitute the canonical receptor pair for TGFβ1 (Larsson et al., 2001; Oshima et al., 1996). In addition to defects in yolk sac vasculogenesis, angiogenic defects are observed within the embryo proper in Tgfb2 null mice (R. J. L., unpublished observation) and within the embryonic portion of the placenta in Tgfb1 null mice (Larsson et al., 2001). Furthermore, disruption of endoglin (Eng), an accessory receptor that binds TGFβs, activins, and BMPs (Barbara et al., 1999), impairs primitive plexus remodeling and vascular smooth muscle differentiation both in the yolk sac and within the embryo proper (Li et al., 1999). Targeted disruption of Acvrl1, a TGFβ type I receptor that signals through Smad1/5/8 (Chen and Massague, 1999), or disruption of Smad5 itself, results in extraembryonic and intraembryonic vessel dilation, defects in vascular plexus remodeling, and impaired vascular smooth muscle differentiation (Oh et al., 2000; Urness et al., 2000; Yang et al., 1999). The ligand involved in this Acvrl1-Smad1/5/8 pathway is not clear but may be TGFB1, which has been shown to bind Acvrl1 and TGFβRII (Lux et al., 1999; Oh et al., 2000) and to stimulate Smad1 phosphorylation (Yue et al., 1999) in vitro. However, no downstream targets of TGFB1-stimulated Acvrl1 signaling have been identified.

In humans, mutations in ENG and ACVR1 are responsible for the autosomal dominant vascular dysplasias, hereditary hemorrhagic telangiectasia (HHT) type 1 and type 2, respectively (Johnson et al., 1996; McAllister et al., 1994), which together occur with a frequency of 1 in 10,000 (McDonald et al., 2000). These diseases present clinically in a similar manner, with symptoms including epistaxis (recurrent nosebleeds), mucocutaneous telangiectases (superficial vascular dilations that present as small red spots), and arteriovenous malformations (AVMs) (Guttmacher et al., 1995). Large AVMs, particularly in brain and lung, can lead to stroke if severe shunting or rupture occurs. The basis for the localized nature of these defects is not known, although it has been suggested that the appearance of pathological lesions is precipitated by some independent, site-specific event (Guttmacher et al., 1995). \[\text{Large AVMs in humans, in particular in brain and lung, can lead to stroke if severe shunting or rupture occurs. The basis for the localized nature of these defects is not known.}\]

**Microangiography and confocal imaging**

Microangiography was performed as described (Weinstein et al., 1995). For confocal microscopy, embryos were anesthetized using 0.016% Tricaine (Sigma), mounted on depression slides using 5% methylcellulose (Sigma), and Z-series of frame-averaged optical sections were generated using a Radiance 2000 confocal microscope (Bio-Rad). Two-dimensional and three-dimensional projections were generated using MetaMorph (Universal Imaging) software.

**Nuclei counting**

The basal communicating artery and posterior connecting segments (Isogai et al., 2001) were imaged by confocal microscopy at 2 and 3 days post-fertilization (dpf) in embryos derived from vbgft09e/+;TG(fli1:nEGFP)y7 and vbgft09e/+. Cell number within these vessels was analyzed in 12 wild-type and 12 mutant embryos by counting nuclei on three-dimensional, rotating projections using the ‘Manually Count Objects’ function in MetaMorph. Data were analyzed by repeated measures ANOVA.

**Meiotic and physical mapping**

Embryos used for meiotic mapping were digested overnight at 50°C in 100 μl of buffer containing 10 mM Tris, pH 8.0; 50 mM EDTA; 200 mM NaCl; 0.5 mg/ml proteinase K; and 0.5% SDS. Digests were spun through Sephacyr-S400 (Amersham Pharmacia), and the resulting preparation of genomic DNA was used for PCR. SSLP mapping was performed as described previously (Knappik et al., 1998) except primers were not radiolabeled and PCR reactions were resolved on 3% MetaPhor agarose gels (FMC). YAC clones were identified from DNA pools by PCR as described by (Kimmel et al., 1995; Westerfield, 1995). Embryo medium was supplemented with 0.003% phenylthiourea (Sigma) at 24 hours post-fertilization (hpf) to prevent melanin formation (Westerfield, 1995).
using BigDye reagents on an ABI310 capillary sequencer (Perkin-Elmer). PCR primers designed against non-repetitive regions of YAC and BAC DNA shown to be present on LG23 by radiation hybrid mapping (Hukriede et al., 1999) were used to establish physical contigs and to look for polymorphisms for use in mitotic mapping. To find polymorphisms, PCR products amplified from genomic DNA from individual wild-type, heterozygous and mutant embryos (genotype based on flanking markers) were sequenced. Single nucleotide polymorphisms were assayed as restriction fragment length polymorphisms (RFLPs), when possible, or were converted to RFLPs using derived cleaved amplified polymorphic sequence (dCAPS) analysis (Neff et al., 1998). One novel SSLP (bac156CA) was identified in BAC156f04 as described previously (Knapik et al., 1998).

**Cloning of zebrafish acvrl1 cDNA**

A blastn search uncovered a 3′ zebrafish EST, zehn1109, with homology to human ACVRL1. 5′ SMART RACE (Clontech) was performed using first strand cDNA synthesized from total RNA from 24 hpf embryos and a zehn1109-specific primer, 5′-CTTCTGCGCCA TGGTCCTCTG-3′. A single product was obtained, cloned into pCRII-TOPO (Invitrogen), and sequenced. The GGTGCCA TGTA TCGCTTG-3′ homology to human ACVRL1. A tblastn search uncovered a 3′-end region of the putative start codon (see Results). cDNA was synthesized from RNA obtained from wild-type, vbg-/-/vbg-/-, and vbg-/-/vbg-/-oocytes (kindly provided by M. Tsang). Whole-mount in situ hybridization was performed as described (Driever et al., 1996) and primers specific to the 5′ UTR of acvrl1 were assayed by dCAPS analysis using the following primers: 5′-ACCGTTGCTACTAACACAGTGAACAGA CT-3′ and 5′-TTGCCGCCCGTTATGAGAA T-3′. These primers contain a single mismatch (underlined), converting lysine 221 (AAG) to arginine (AGG). All constructs were verified by sequencing. Capped mRNA was synthesized from NotI-digested constructs using mMessage mMACHINE with SP6 RNA polymerase (Ambion). Morpholino-modified antisense oligonucleotides (Gene Tools) used were standard control (5′-CC-TCTTACTCAGTCAATTTTAAT-3′; MO1 (5′-CTGCAGAGCATCCTGAGGCTTT-3′), which targets +3 to +25 of the coding region of acvrl1; and MO2 (5′-CTCATTACTACACATAGAGT-GTA-3′), which targets −95 to −71 of the major acvrl1 splice variant containing noncoding exon1. Capped mRNAs and morpholinos were injected into 1- to 4-cell embryos either into a single blastomere or into the streaming yolk cytoplasm, just beneath the blastomeres, as described previously (Westerfield, 1995).

**RESULTS**

**vbg mutants exhibit abnormal circulation in the presence of normal vessel patterning**

In zebrafish, trunk circulation begins between 24 and 26 hpf. A few hours later, perfusion of the head is initiated (Isoegai et al., 2001). At these early stages, when heartbeat is weak and blood flow is slow, vbg mutants cannot be distinguished from their wild-type siblings. However, by 36-40 hpf, as the heartbeat quickens and strengthens and the number of circulating blood cells increases, most vbg mutants exhibit slightly weaker trunk circulation than wild-type siblings. By 2 dpf, although a few blood cells slowly traverse the trunk and tail in vbg mutants, most are restricted to a loop of dilated cranial vessels (Movies 1,2; http://dev.biologists.org/ supplemental/). The anterior limit of this vessel loop lies medial to the center of the eye, the dorsal limit lies medial to the otic vesicle, and the posterior limit lies just posterior to the otic vesicle. Homozygous vbg mutants become progressively edematous in the head, pericardium and yolk sac (Fig. 1A,B) and die between 7 and 10 dpf. Thus far, three vbg alleles have been identified: vbg-16 (Driever et al., 1996) and vbg-609e (Chen et al., 2001) were isolated in independent ENU mutagenesis screens, and vbg-40 (LG23) was isolated in a gamma ray mutagenesis screen and represents a deletion (Lekven et al., 2000). With respect to vascular phenotype, the three vbg alleles are essentially indistinguishable (see below).

In order to directly compare the patent vasculature in wild-type and vbg mutant zebrafish, we employed confocal microangiography (Weinstein et al., 1995). In the 2.25 dpf wild-type zebrafish (Fig. 1C,D), blood flows caudally through the lateral dorsal aortae, as well as rostrally, through the...
internal carotid arteries. Each internal carotid artery divides at the base of the eye into a cranial division, which continues rostrally, and a caudal division, which turns dorsally, behind the eye. The left and right caudal divisions of the internal carotid artery are connected beneath the midbrain via the basal communicating artery. From the basal communicating artery, blood flows through bilateral posterior connecting segments into the midline basilar artery, which runs beneath the hindbrain, and drains to the bilateral primordial hindbrain channels through central arteries, which penetrate the hindbrain. Confocal microangiography clearly highlights this network of cranial vessels in wild-type zebrafish (Fig. 1E). In contrast, angiograms of all three vbg mutant alleles reveal a cranial vasculature containing dilated major vessels and very few patent central arteries (Fig. 1F and data not shown) compared to wild-type siblings, although trunk and tail vasculature appear normal (data not shown). While there is no difference in CA number or pattern, almost all CAs are patent in wild-type embryos, whereas most are not patent in vbg mutants (see also gray arrows, J). AA1, first arch artery; LDA, lateral dorsal aortae; ICA, internal carotid artery; CaDI, caudal division of ICA; (A,B,D-J) Dorsal view, anterior to the left. (C) Dorsolateral view, anterior to the left.

To determine whether the defects noted in patent vessels in vbg mutants stem from defects in cranial vascular patterning, we used confocal microscopy to analyze the vasculature of transgenic fish [TG(fli1:EGFP)\(^{y1}\)] expressing EGFP under the control of the fli1 promoter (N. D. L. and B. M. W., unpublished). Simultaneous analysis of endothelial structures (EGFP) and patent vasculature (microangiography) in wild-type and vbg embryos reveals no difference in vessel patterning (Fig. 1I,J) despite striking differences in vessel caliber and patency (Fig. 1E,F). These data might suggest that the vbg mutation-associated change in vascular hemodynamics stems from a defect in central artery lumenization (Fig. 1G,H). However, enlarged cranial vessels in vbg mutants can be seen by angiography as early as 40 hpf, a time at which central arteries are not patent in wild-type fish. Therefore, it is likely that cranial vessel enlargement and not impaired central artery lumenization is the proximal vascular defect in vbg mutants.
**Fig. 2.** Disruption of *acvrl1* increases the number of endothelial cells in the basilar communicating artery (BCA) and posterior connecting segments (PCS) at 2-2.25 dpf. (A, B) Representative confocal micrograph of the central cranial vasculature of (A) a *vbg<sup>fli1:nEGFP</sup> y7* wild-type embryo, and (B) a *vbg<sup>fli1:nEGFP</sup> y7* mutant embryo. The greater than two-fold increase in endothelial cell number in the BCA/PCS in *vbg*0046 embryos compared to wild-type embryos is significant at *P*<0.00001. BA, basilar artery. Dorsal views, anterior to the left.

Cranial vessel enlargement in *vbg* mutants stems from an increase in endothelial cell number

In order to determine whether cranial vessel dilation in *vbg* mutants involves endothelial cell hypertrophy or an increase in endothelial cell number, we counted nuclei in the vascular triangle comprising the basilar communicating artery and posterior connecting segments. These vessels were chosen for analysis because they are consistently dilated in posterior connecting segments. These vessels were chosen for analysis because they are consistently dilated in *vbg* mutants and are easily imaged in *Tg(fli1:nEGFP)y7* embryos. (Strong expression of the *fli1:nEGFP* transgene in pharyngeal arch mesenchyme precludes visualization of endothelial nuclei in the first arch artery and the internal carotid artery/caudal division.) Within this vessel triangle at 2-2.25 dpf, wild-type embryos have 32.9±1.0 (mean±s.e.m., *n*=12) endothelial nuclei (Fig. 2A), whereas *vbg* mutants have 69.8±2.6 (mean±s.e.m., *n*=12) endothelial nuclei (Fig. 2B). This greater than 2-fold increase in endothelial cell nuclei in *vbg* mutants persisted at 3 dpf, at which time endothelial cell number was statistically unchanged compared to 2 dpf (data not shown).

The *vbg* gene encodes *Acvrl1*

In order to determine the molecular basis for the increase in endothelial cell number in specific cranial vessels in *vbg* mutants, we positionally cloned the *vbg* locus. Analysis of simple sequence length polymorphisms (SSLPs) in pooled genomic DNA from phenotypically wild-type and mutant embryos from *vbg*<sup>0046</sup> increases placed *vbg* on LG23, and fine mapping defined an approximately 1 centimorgan (cM) meiotic interval between z14967 and z4421 (Fig. 3). A physical contig anchored at the z14967-end of the interval was generated, and meiotic mapping of single nucleotide polymorphisms narrowed the critical interval to 0.035 cM: the distance between markers b82sp6 and *taram-a*. Analysis of 4256 mutant embryos (8512 informative meioses) failed to further narrow the interval, which was spanned by BAC177k23 and BAC157p19.

Because *Acvrl1* disruption causes vascular defects in mice and humans (Johnson et al., 1996; Oh et al., 2000; Urness et al., 2000), and because human *ACVR1L1* maps to chromosome 12q13, a region that shows conserved synteny with zebrafish LG23 (Postlethwait et al., 1998), *acvrl1* was tested as a candidate for *vbg*. A database search uncovered a 3′ zebrafish EST, *zechn1109*, with significant homology to the kinase domain of mammalian *Acvrl1*. Two independent PCR primer sets designed within the 3′UTR of this gene amplified fragments of the expected size from YAC35b1, BAC177k23, BAC157p19, and BAC143a24. The 5′ end of *zechn1109* was obtained by RACE and a primer made against the 5′UTR was successfully used in sequencing BAC177k23 but not BAC157p19 or BAC143a24. Therefore, the entire *zechn1109* gene is present within the critical interval, on BAC 177k23, and is absent from genomic DNA from *vbg<sup>df(LG23)w5</sup>* mutants (Fig. 3). It should be noted that, using RACE, two populations of 5′ clones were obtained that diverged at –5 with respect to the putative translational start codon. The most abundant fragment (5 out of 6 clones) contained a non-coding first exon, whereas the rare fragment (1 out of 6 clones) did not contain this exon. This phenomenon has also been reported for human *ACVRL1* (Berg et al., 1997).

Conceptual translation of the largest open reading frame of...
zehn1109 reveals a 499 amino acid protein with 55% identity to human and mouse Acvrl1 (Fig. 4). Homology to mammalian Acvrl1 is highest (75%) within the C-terminal kinase domain (from L196). Within this domain, the L45 loop (S254-S261), which confers Smad specificity to TGFβ type I receptors (Chen et al., 1998), contains only one conservative substitution. In contrast, the N-terminal ligand binding domain encoded by zehn1109 shares only 17% identity with mammalian Acvrl1, most of which can be accounted for by ten cysteines that are common to all TGFβ type I receptors. Despite the lack of homology in the ligand binding domain, we strongly believe that this gene is the zebrafish ortholog of mammalian Acvrl1 (see Discussion). Sequencing of acvrl1 cDNA synthesized from vbg y6 mutant embryos uncovered a point mutation within the kinase domain creating a leucine-to-phenylalanine substitution (L249F; Fig. 4). This leucine, which lies five residues N-terminal to the L45 loop, is conserved in all TGFβ type I receptors, although it has not previously been ascribed a specific function. When assayed for this polymorphism, each vbg y6 mutant embryo recombinant at either of the closest flanking genomic markers was shown to possess the mutant genotype. Furthermore, sequencing of the vbg y6 allele revealed a point mutation converting a tyrosine to a stop codon (Y88Stop; Fig. 4). This mutation stops translation within the ligand binding domain and therefore would be expected to produce a nonfunctional protein. To confirm that mutations in acvrl1 are responsible for the vbg phenotype, we attempted to phenocopy this mutant using morpholino-modified antisense oligonucleotides (morpholinos) (Summerton, 1999). Morpholinos have been used in zebrafish to phenocopy a number of early mutations (Nasevicius and Ekker, 2000), and their stability allows translation inhibition relatively late in development. When injected into 1- to 4-cell wild-type embryos, a total of 22.5 ng of a control morpholino had no effect on blood flow or vascular architecture at 2.25 dpf (Fig. 5A). In contrast, 22.5 ng of a 2:1 mixture of acvrl1 morpholinos directed against coding sequence (MO1) and noncoding exon1 (MO2) increased cranial blood flow relative to control in 86% (114/132) of embryos at 2.25 dpf. A 15 ng dose of morpholino mixture produced fewer embryos (11/50) with obvious phenotypes. Analysis of a random sample of affected embryos by confocal microangiography revealed a range of phenotypes, from essentially complete phenocopy of vbg mutants (Fig. 5B,C) to focal cranial vessel dilations (Fig. 5D). Affected vessels included the basal communicating artery, posterior connecting

Fig. 4. Alignment of human (Hs-), mouse (Mm-), and zebrafish (Dr-) Acvrl1 proteins. Zebrafish acvrl1 encodes a 499 amino acid protein with significant homology to mammalian Acvrl1 proteins in the C-terminal serine/threonine kinase domain. In contrast, although the ten cysteines common to all type I TGFβ receptors are conserved, the rest of the N-terminal ligand binding domain shows little homology. Black arrowhead, Y88Stop mutation in vbg y6; gray arrowhead, L249F mutation in vbg y6. GenBank accession numbers: Danio rerio acvrl1 mRNA, complete cds; includes noncoding exon 1: AF435024. Danio rerio acvrl1 mRNA, complete cds; no noncoding exon 1: AF435025.

Fig. 5 Antisense morpholino-modified oligonucleotides directed against acvrl1 phenocopy vbg mutants. Dorsal anterior angiograms of 2.25 dpf wild-type embryos injected at the 1- to 4-cell stage with 22.5 ng of a standard control morpholino (A) or 22.5 ng (C) or 15 ng (D) of a mixture of morpholinos directed against acvrl1. Note the strong resemblance of patent vessel architecture in C to that found in vbg mutants (B). The embryo shown in D is less severely affected but clearly shows dilated vessels and a decrease in the number of patent central arteries. BCA, basal communicating artery; PCS, posterior connecting segment; BA, basilar artery; PHBC, primordial hindbrain channel; CA, central artery. Dorsal views, anterior to the left.
segments, basilar artery, and primordial hindbrain channel, each of which can be dilated in vbg mutants.

**Zebrafish acvrl1 is expressed in endothelial cells**

We used RT-PCR and whole-mount in situ hybridization to determine the temporal and spatial distribution of zebrafish acvrl1 transcripts. By RT-PCR, acvrl1 transcripts are first detectable at tailbud stage (10 hpf) and continue to be expressed as late as 4 dpf (data not shown). However, in situ hybridization is not sensitive enough to reliably detect acvrl1 transcripts before 40-45 hpf. At this time, as in mammals, acvrl1 is expressed in endothelial cells. Rostral acvrl1 expression (Fig. 6A,B) is significantly weaker and more limited than that of the endothelial marker, tie1 (Fig. 6E,F), and predominates in vessels that become enlarged in vbg mutants. acvrl1 expression is strongest in the first aortic arch, the internal carotid artery and its caudal division, the basal communicating artery and the optic vein (Fig. 6A,B). The primordial hindbrain channels sometimes exhibit a weakly positive signal, whereas the basilar artery is consistently negative. The weak to undetectable staining of these latter vessels is also observed with tie1 (Fig. 6E,F) and may reflect the inherent difficulty in staining deep cranial vessels using whole-mount in situ hybridization. acvrl1 expression is moderate in the lateral dorsal aortae (Fig. 6B), weak in the trunk axial vessels, and undetectable in the trunk intersegmental vessels (Fig. 6C,D). Expression in these vessels is weaker but qualitatively identical to that of tie1 (Fig. 6F,G,H).

**Zebrafish Acvrl1 signals through the Smad1/5/8 pathway**

Mammalian Acvrl1 proteins signal through the Smad1/5/8, or BMP pathway, and not the Smad2/3, or TGFβ/activin/nodal pathway (Chen and Massague, 1999). To determine which Smad pathway is activated by zebrafish Acvrl1, we injected into 1- to 4-cell embryos activated acvrl1 mRNA (acvrl1*), which encodes a single amino change (Q193D) that renders Smad phosphorylation independent of ligand and type II receptor (Wieser et al., 1995). Injection of 5 pg acvrl1* mRNA induces Smad1 phosphorylation at shield stage (6 hpf), as detected by whole-mount immunohistochemistry using a phospho-Smading antibody (Fig. 7A,B). Five pg of acvrl1* also induces gata2 mRNA (Fig. 7D,E), a ventral ectodermal marker that is upregulated by signaling through the Smad1/5/8 pathway (Maeno et al., 1996). In contrast, up to 100 pg mRNA of the vbg6 allele of acvrl1* (Q193D; L249F) is ineffective in inducing Smad1 phosphorylation and only minimally effective in inducing gata2 mRNA expression (Fig. 7C,F) despite similar translation efficiencies of acvrl1* and acvrl1*(vbg6) mRNAs in vivo (data not shown).

BMPs, through the Smad1/5/8 pathway, play an important role in controlling dorsoventral patterning during early development. Thus, if zebrafish Acvrl1 does indeed signal through the Smad1/5/8 pathway, hyperactivation of the Acvrl1 pathway during gastrulation should ventralize embryos (Bauer et al., 2001; Kishimoto et al., 1997), whereas dominant negative inhibition should dorsalize embryos (Bauer et al., 2001; Hild et al., 1999; Mintzer et al., 2001). Of 116 surviving wild-type embryos injected with 1 pg acvrl1* mRNA, 83% exhibited a ventralized phenotype (Kishimoto et al., 1997) at 24-30 hpf. Complete loss of anterior structures and notochord concomitant with expanded blood islands was the most frequently observed phenotype in these ventralized embryos (Fig. 7H; compare to control, Fig. 7G). Injection of 10 pg mRNA of the vbg6 allele of acvrl1* mildly ventralized only 9% of embryos (n=128), whereas 100 pg ventralized 41% of embryos (n=181), most of which exhibited incomplete loss of anterior structures and complete loss of notochord (Fig. 7I). Conversely, of 252 surviving embryos injected with 100 pg mRNA encoding a kinase-dead, dominant-negative form of Acvrl1 (Bassing et al., 1994)
(acvrl1DN; K221R), 42% exhibited a dorsalized phenotype (Mullins et al., 1996) at 24 hpf. Severity of dorsalization ranged from embryos that lacked blood and exhibited a twisted trunk and no tail (Fig. 7J) to embryos in which the only abnormality noted was a small or absent tail fin (data not shown). In contrast, injection of 600 pg mRNA of the vbgy6 allele of acvrl1 (K221R; L249F) produced no phenotype (n=123; data not shown). These results provide further evidence that zebrafish Acvrl1, like mammalian Acvrl1, acts through the Smad1/5/8 pathway, and that the L249F mutation in the vbgy6 allele does indeed impair function of Acvrl1 protein.

The specificity of zebrafish Acvrl1 for the Smad1/5/8 pathway was confirmed in cultured cells. In P19 cells, luciferase transcription from the Smad2/3-responsive A3 promoter is induced by human TGFBR1*, but not human ACVRL1*, human BMPR1B* or zebrafish acvrl1* (Fig. 8A). In contrast, luciferase transcription from the Smad1/5/8-responsive TLX2 promoter is induced by human ACVRL1*, human BMPR1B*, and zebrafish acvrl1* but not by human TGFBR1* (Fig. 8B). These results confirm in vivo findings that zebrafish Acvrl1 signals through Smad1/5/8 and also demonstrate the inability of this receptor to signal through Smad2/3.

**DISCUSSION**

Using positional cloning and candidate gene testing, we have identified the molecular basis for the cranial vessel dilation observed in vbg mutants. We meiotically mapped the vbg locus to an interval of 0.035 cM on LG23 and established a physical contig across this region containing the acvrl1 gene. The vbgy6 allele of acvrl1 contains a missense mutation in the C-terminal serine/threonine kinase domain, whereas the vbgy6 allele contains a nonsense mutation in the N-terminal ligand binding domain. The former polymorphism exhibited no recombination in 4256 vbgy6 mutants (8512 informative meioses). Furthermore, injection of antisense, morpholino-modified oligonucleotides specific to acvrl1 phenocopies the vascular defect seen in vbg mutants. Finally, at 40 hpf, when the vbg mutant phenotype is first detectable, acvrl1 mRNA is expressed predominantly in vessels that are consistently dilated in vbg mutants: the first aortic arch, internal carotid artery/caudal division, and basal communicating artery. Taken together, these results support the conclusion that the
zebrafish \textit{vbg} gene is \textit{acvrl1}. It should be noted that injection of \textit{acvrl1} mRNA at the 1- to 4-cell stage was unable to rescue the \textit{vbg} mutant phenotype owing to instability of injected mRNA and/or translated protein; \textit{Acvrl1}-myc fusion proteins were undetectable by 24 hpf (data not shown).

Although the zebrafish gene residing at the \textit{vbg} locus is highly homologous to mammalian \textit{Acrvl1} in the kinase domain and in particular in the L45 loop, it shows little homology to any TGF\textbeta type I receptor in the ligand binding domain: only ten cysteines that are common to all TGF\textbeta type I receptors are conserved. However, we feel that it is appropriate to call the \textit{vbg} gene \textit{acvrl1} for a number of reasons. First, human \textit{ACVRL1} is located on chromosome 12q13 (Johnson et al., 1996), which shows conserved synteny with the region surrounding the \textit{vbg} locus on zebrafish LG23 (Postlethwait et al., 1998). Second, like mammalian \textit{Acrvl1} (Johnson et al., 1996; Oh et al., 2000; Roelen et al., 1997; Urness et al., 2000), the \textit{vbg} gene is expressed predominantly if not exclusively in endothelial cells, and \textit{vbg} disruption results in vascular defects. Third, both mammalian \textit{Acrvl1} (Chen and Massague, 1999) and the \textit{vbg} gene product signal through the Smad1/5/8 pathway, and not the Smad2/3 pathway. And fourth, a lack of homology within the ligand binding domain has also been observed for the zebrafish TGF\textbeta type I receptors, Alk8 (Mintzer et al., 2001; Payne et al., 2001; Yelick et al., 1998) and Taram-A (Renucci et al., 1996). Based on conserved synteny, L45 loop sequence, and Smad specificity, Alk8 and Taram-A seem to be orthologs of mammalian \textit{Acrvl1} and \textit{Acrvl1b}, respectively.

While the premature stop codon in the ligand binding domain generated by the \textit{vbg}^{600c} mutation most likely produces a nonfunctional Acrvl1 protein, the consequence of the L249F substitution produced by the \textit{vbg}^{65} mutation is not self-evident. However, the resulting phenotype, which is indistinguishable from \textit{vbg}^{600c}, suggests that L249 is critical for Acrvl1 protein function. Although in vivo assays using an activated form of the \textit{vbg}^{65} allele of \textit{acvrl1} suggest that it retains some activity, the artificial activation of this protein, allowing it to function independently of ligand and type II receptor, makes its true in vivo efficacy difficult to gauge. Activity of the non-activated \textit{vbg}^{65} allele could not be assayed because injection of either wild-type or \textit{vbg}^{65} mutant \textit{acvrl1} mRNA produced no phenotype in wild-type embryos (data not shown).

The lack of effect of injection of mRNA encoding a TGF\textbeta type I receptor has been previously reported (Bauer et al., 2001) and suggests that pathway activity is limited by ligand or type II receptor.

The three \textit{vbg} alleles analyzed in this study exhibit similar vascular phenotypes characterized by dilated cranial vessels that carry the bulk of blood flow. The cause of this vessel dilation is an increase in the number of endothelial cells within affected vessels, suggesting that Acrvl1 signaling may inhibit endothelial cell proliferation and promote vessel stabilization. While there is some in vivo evidence that TGF\textbeta family signaling in general (and Acrvl1 signaling specifically) plays a role in vessel stabilization via vascular smooth muscle cell recruitment and differentiation (Larsson et al., 2001; Li et al., 1999; Oh et al., 2000; Oshima et al., 1996; Urness et al., 2000; Yang et al., 1999), the dilated first aortic arch, internal carotid artery, and caudal division of the internal carotid artery in \textit{vbg} mutants normally express the vascular smooth muscle cell marker, \textit{sm22a}, at 2 dpf (data not shown). These data suggest that in zebrafish, the \textit{acvrl1} mutation-induced increases in endothelial cell number and vessel caliber are not correlated with vascular smooth muscle deficits. A similar phenomenon has recently been reported as a result of misexpression of Smad7, an inhibitory Smad that blocks signaling mediated by all receptor-specific Smads (Vargesson and Laufer, 2001).

The cross-species conservation of \textit{acvrl1} expression pattern and function suggests that the mechanism underlying vessel dilation in zebrafish \textit{vbg} mutants is most likely similar to that underlying vessel dilation—the first step in telangiectasia and AVM formation (Braverman et al., 1990)—in HHT2 patients. Thus, although the enlarged cranial vessels in \textit{vbg} mutants are not precursors of AVMs per se (the malformations seen in \textit{vbg} mutants appear to be normal, primitive connections that are aberrantly retained), an understanding of their etiology should lead to insight into the mechanism of HHT2-associated vascular lesions. Although it has been proposed (Urness et al., 2000) that the proximal event leading to AVMs in an \textit{Acvrl1} null mouse is loss of arterial identity, as assayed by loss of arterial \textit{ephrinB2} expression, vessel dilation in these mice (E8.0) precedes the onset of normal arterial \textit{ephrinB2} expression (E8.25) (Wang et al., 1998b) and AVM formation (E8.5), suggesting that in mice, too, the earliest manifestation of loss of \textit{Acvrl1} expression is vessel dilation. It should be noted that \textit{vbg} mutants express \textit{ephrinB2} normally in the dorsal aorta (data not shown); expression in cranial arteries, which are dilated in \textit{vbg} mutants, could not be assessed by whole-mount in situ hybridization because of intense staining of other cranial structures.

While it is clear that the genetic lesion responsible for HHT2 is a mutation in \textit{ACVRL1}, the age of onset, location, and severity of clinical manifestations of this autosomal dominant disorder vary greatly among heterozygous carriers. While it remains possible that loss of heterozygosity plays a role in determining lesion location, this phenomenon could not be demonstrated in lesions associated with the related disorder, HHT1 (Bourdeau et al., 2000a). Thus, in both diseases, additional genetic and/or environmental factors most likely come into play. While preliminary analysis of \textit{vbg}^{65+} fish has revealed no obvious superficial telangiectases or hemorrhages, it remains possible that such defects might manifest in certain genetic backgrounds [as in \textit{Eng} haploinsufficiency in mice (Bourdeau et al., 1999; Bourdeau et al., 2000b)], or that mutagenesis screening might help to define genetic modifiers that render \textit{acvrl1} haploinsufficiency symptomatic in zebrafish as well as in humans. Mutagenesis screens might also produce complementing mutations that may lead to discovery of upstream or downstream components of the Acrvl1 signaling pathway. Furthermore, \textit{vbg} mutants should prove useful in describing the consequences of \textit{acvrl1} deficiency in terms of endothelial cell behavior, as vessel formation can be documented in real time in transgenic lines such as \textit{TG(fli1:nEGFP)} \textit{cyg}3017. Thus, like a number of other zebrafish models of human diseases (Brownlie et al., 1998; Childs et al., 2000; Wang et al., 1998a), this zebrafish model of HHT2 provides a powerful tool that should complement established mouse models in the study of disease etiology and the development of treatment strategies.
We thank N. Hukriede for assistance with radiation hybrid mapping, supplying the SMART RACE library, and helpful discussions; S. Isogai for microangiography training; C. Abnet for statistical advice; R. Subramanian for critical review of the manuscript; A. Chin and P. Bennett for fish care; and M. Kawabata, K. Peters, M. Tsang, M. Whitman, J. Wrana, and L. Zon for plasmids. This work was funded by NIH Z01-HD-01011 (to B. M. W.) and HL65681 (to R. J. L.).

REFERENCES


