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Sid4: A secreted vertebrate immunoglobulin protein with roles in zebrafish embryogenesis

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Abstract

The small members of the immunoglobulin superfamily (IGSF) are a molecularly diverse group of proteins composed solely of immunoglobulin domains. They may be secreted or tethered to the cell mebrane via GPI linkages and are proposed to have important functions in vivo. However, very few small IGSFs have been functionally characterized. During an ongoing in situ hybridization analysis of expressed sequence tags in zebrafish we identified *secreted immunoglobulin domain 4* (*sid4*), a gene encoding a soluble vertebrate protein composed solely of four immunoglobulin domains.

Throughout development, *sid4* is expressed in regions of the embryo undergoing active cell division and migration. Functional analysis using morpholino antisense oligonucleotides demonstrates that timing of gene expression is normal in morphants, but these embryos are smaller and exhibit defects in epiboly and patterning of axial and prechordal mesoderm. Analyses of *chordin*, *pax2*, *krox20*, and *dlx2* expression in morphants demonstrate that early brain patterning is normal but later organization of hindbrain neurons and development of cranial neural crest are perturbed.

Levels of apoptosis in morphants were normal prior to 90% epiboly, but were elevated after 10 h post-fertilization (hpf). Apoptosis does not account for early patterning defects of axial mesoderm, but likely contributes to overall reduction in embryo size. Phylogenetic analysis demonstrates that Sid4 is strikingly similar to the fibronectin binding Ig domains of Perlecan/HSPG2. Overall, our data demonstrate a fundamental role for *sid4*, possibly as a co-factor in extracellular matrix (ECM) interactions, in processes underlying tissue patterning and organogenesis in a vertebrate.
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Introduction

Proteins belonging to the IGSF are structurally diverse and have multiple evolutionary origins. All members utilize the immunoglobulin (Ig) domain to participate in processes that depend on protein interactions, such as co-receptors for growth factors (Mongiat et al., 2000), adhesion to cells and extracellular matrix (Rougon and Hobert, 2003; Vaughn and Bjorkman, 1996), pathfinding by axons (Panicker et al., 2003), and blood vessel branching (Rossant and Howard, 2002).

Unlike the large, multimeric members of the family, small IGSFs are composed solely of Ig domains. They may be secreted or tethered to the cell membrane via GPI linkages (Rougon and Hobert, 2003). The molecular diversity of the small IGSF subfamily has been documented in *Drosophila* (Nakamura et al., 2002) and *Caenorhabditis elegans* (Teichmann and Chothia, 2000) but very little is known about their in vivo functions. Two small Igs, *Drosophila* Beaten path (Beat) 1a and Hemolin, are secreted and act as anti-adhesives to disrupt cell–cell interactions

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during motorneuron axon migration (Fambrough and Goodman, 1996) and hemocytic aggregation in response to bacterial infection (Bettencourt et al., 1997, 1999; Kanost et al., 1994; Sun et al., 1990), respectively. BeatIa and Hemolin are also necessary for normal embryo development (Bettencourt et al., 2002; Pipes et al., 2001).

During an ongoing in situ hybridization screen of zebrafish expressed sequence tags (ESTs), we identified *secreted immunoglobulin domain 4* (after *Sid1*, (Yoder et al., 2002)). *sid4* is a novel vertebrate gene that, like moth Hemolin (Sun et al., 1990), encodes a secreted protein composed of four Ig domains and contains no other conserved motifs. *sid4* mRNA is maternally provisioned and zygotically expressed throughout development in regions of the embryo containing actively dividing and migrating cells.

Suppression of Sid4 by morpholino injection causes defects in morphogenesis but not cellular differentiation. Morphant embryos are reduced in size and exhibit impaired epiboly and morphogenesis of axial and somitic mesoderm. Despite morphological similarities to ventralized Wnt and dorsalized BMP mutants, expression of *bmp4* and *chordin* is normal in 6 hpf morphants. At 10 hpf, the conclusion of gastrulation in zebrafish, morphants first exhibit elevated levels of apoptosis, suggesting that decreased cell viability is secondary to the morphogenetic defect.

Although *sid4* mRNA is ubiquitously expressed through 16 hpf, morphants exhibit spatially and temporally restricted defects in morphogenesis of axial and prechordal mesoderm. For example, at similar stages of development, patterning of axial mesoderm is disrupted while anterior migration of prechordal mesoderm is normal. Additionally, while early hatching gland development appears normal, later anterolateral spreading of these cells is inhibited.

Phylogenetic sequence analysis demonstrates that Sid4 is closely related to mouse *Perlecan/HSPG2* and shares significant similarity with the fibronectin binding Ig domains of this large, multimeric protein. These data, together with our analyses of somitic mesoderm, cranial neural crest, and branchiomotor neurons suggest that this novel, vertebrate member of the IGSF may function as a secreted receptor or cofactor with important roles in some ECM interactions during embryogenesis.

Methods

Fish maintenance

Wild type and transgenic embryos expressing green fluorescent protein under the control of an *Islet-1* enhancer (Higashijima et al., 2000) were collected from natural matings and reared in 1/3 Ringer's (Westerfield, 2000). Embryos were staged using morphological criteria up to 24 h post fertilization (hpf) and by time of development at 28.5°C thereafter (Kimmel et al., 1995).

Cloning and molecular characterization of sid4

IMAGE clone 3719398 was initially identified as the zebrafish homolog of HSPG2/Perlecan. However, our preliminary BLAST analyses of this clone and human HSPG2 (gi184426) against the zebrafish genome demonstrated that these are distinct genetic loci. *sid4* was selected for functional characterization because it appeared to be a novel, developmentally expressed gene located in a region of the zebrafish genome containing an abundance of uncharacterized, predicted genes (Sanger, Zv4).

Full-length *sid4* (GENBANK AY494978) was isolated from 60 hpf total RNA (RNeasy, Qiagen). AMV-GeneRacer (Invitrogen) was used to amplify 5′ and 3′ cDNA ends with the oligonucleotides 5′CGCCTCGCTGGAGCCCACATGAT3′ and 5′TGACGGTGCCGTTCTGACCATCGCTAA3′, respectively. Amplification products were cloned into pCRII (Invitrogen) and multiple clones sequenced. A consensus cDNA sequence and conceptual translation were obtained using SeqMan (DNASTAR v. 4.0). ClustalW was used to produce nucleotide and amino acid sequence alignments (Macvector v. 7.2). Full-length *sid4* is identical to the available sequences in 64 recently deposited AGENCOURT ESTs (e.g gi46159009).

In situ hybridization

pSport containing *sid4* was linearized with *Sal1* and *Not1* and digoxigenin-labeled probes transcribed with Sp6 (antisense) and T7 (sense) RNA polymerases, respectively. Additional RNA probes used were zebrafish *flh* (Talbot et al., 1995), *bmp4* (Hwang et al., 1997), *chordin* (Schulte-Merker et al., 1997), *unc-45* (Etheridge et al., 2002), *pax-2* (Krauss et al., 1991), *hgg-1* (Vogel and Gerster, 1997), *goosecoid* (gsc) (Schulte-Merker et al., 1994; Thisse et al., 1994), *krox-20* (Oxtoby and Jowett, 1993), and *dlx-2* (Akimenko et al., 1994). In situ hybridization was carried out as described previously (diIorio et al., 2002; Willett et al., 1997).

Morpholino design and microinjection

Eight exons for *sid4* were localized to linkage group 25 (Sanger Zv4). We identified nucleotide sequence corresponding to the first 52 amino acids of *sid4*, but to date these have not been mapped. Exon 5 and flanking introns were amplified by standard PCR from genomic DNA using the oligonucleotides 5′CTAGATCACTTACGAGATGATCTTTGCGTGAA3′ and 5′CCACAATCAGA-GATCTGCAGCTCTGGA3′. Products were cloned into pCRII (Invitrogen) and sequenced. To verify the specificity of morpholino-induced phenotypes, two morpholinos were designed against distinct regions of *sid4* mRNA. The morpholino 5′GAGCTGCTGTCTGGAGCTTCATCAT3′ (t-mo) was designed against the *sid4* translation start site and 5′TGGTGATGGTGTGTTTACC-GGAGGC3′ (s-mo) targets

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