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phospholipase C, beta 3 is required for Endothelin1 regulation of pharyngeal arch patterning in zebrafish

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Abstract

Genetic and pharmacological studies demonstrate that Endothelin1 (Edn1) is a key signaling molecule for patterning the facial skeleton in fish, chicks, and mice. When Edn1 function is reduced early in development the ventral lower jaw and supporting structures are reduced in size and often fused to their dorsal upper jaw counterparts. We show that *schmerle* (*she*) encodes a zebrafish ortholog of Phospholipase C, beta 3 (Plcβ3) required in cranial neural crest cells for Edn1 regulation of pharyngeal arch patterning. Sequencing and co-segregation demonstrates that two independent *she* (*plcβ3*) alleles have missense mutations in conserved residues within the catalytic domains of Plcβ3. Homozygous *plcβ3* mutants are phenotypically similar to *edn1* mutants and exhibit a strong arch expression defect in Edn1-dependent Distalless (Dlx) genes as well as expression defects in several Edn1-dependent intermediate and ventral arch domain transcription factors. *plcβ3* also genetically interacts with *edn1*, supporting a model in which Edn1 signals through a G protein-coupled receptor to activate Plcβ3. Mild skeletal defects occur in *plcβ3* heterozygotes, showing the *plcβ3* mutations are partially dominant. Through a morpholino-mediated deletion in the N-terminal PH domain of Plcβ3, we observe a partial rescue of facial skeletal defects in homozygous *plcβ3* mutants, supporting a hypothesis that an intact PH domain is necessary for the partial dominance we observe. In addition, through mosaic analyses, we show that wild-type neural crest cells can efficiently rescue facial skeletal defects in homozygous *plcβ3* mutants, demonstrating that Plcβ3 function is required in neural crest cells and not other cell types to pattern the facial skeleton.

Keywords: Dlx; Endothelin1; Neural crest; *plcβ3*; *schmerle*

Introduction

Genetic analyses in mice support the hypothesis that during embryonic development the ligand Endothelin1 (Edn1) signals through a receptor [Endothelin receptor type A (Ednra)] that is coupled to G proteins of the G_q/G_{11} family (Clouthier et al., 2000; Dettlaff-Swiercz et al., 2005; Ivey et al., 2003; Kurihara et al., 1994). Mice carrying mutations in Edn1, Ednra, and G_q/G_{11} have similar facial skeletal defects in which the ventral lower jaw cartilages are severely reduced in size and are fused to their dorsal upper jaw counterparts. Zebrafish with mutations in *furinA* (an activator of the Edn1 ligand) and *edn1* (Piotrowski et al., 1996) also have facial skeletal defects resembling those of the mouse Edn1 pathway mutants, demonstrating that the Edn1 signaling pathway functions broadly within jawed vertebrates to give ventral character to the facial skeleton (Miller et al., 2000; Walker et al., 2006). In this paper, we expand the number of Edn1 pathway mutants so far known in either mouse or zebrafish by showing that the craniofacial gene *schmerle* encodes a zebrafish ortholog of Plcβ3 that *plcβ3* mutants have facial skeletal defects similar to *edn1* mutants, and that *plcβ3* and *edn1* genetically interact. We will refer to *schmerle* (*she*) as *plcβ3* for the remainder of the paper.

Phospholipase C enzymes are effectors of signal transduction pathways coupling an agonist-stimulated cell surface receptor to the intracellular production of the secondary

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messengers, IP3 and DAG (Rebecchi and Pentyala, 2000; Rhee, 2001). These messengers then promote the activation of protein kinase C and the release of Ca^{2+} from intracellular stores, inducing a wide variety of cellular functions. Plcβ3 is member of the beta class of Phospholipase C enzymes containing four family members, Plcβ1-4 (Rebecchi and Pentyala, 2000; Rhee, 2001). Plcβs are modular proteins containing an N-terminal PH (*p*leckstrin *h*omology) domain, EF-hand domains, catalytic X and Y domains, a C2 domain, and a C-terminal myosin tail. The PH domain binds membrane phosphoinositides and promotes targeting of Plcβ enzymes to the plasma membrane. The catalytic X and Y domains are both required for the hydrolysis of PIP2 to IP3 and DAG. The myosin tail domain is unique to Plcβ type enzymes. All four Plcβ family members are can be activated by G proteins of G_q/G_{11} family, and all but Plcβ4 are also activated by Gβγ (Lee et al., 1994; Smrcka and Sternweis, 1993).

Mice bearing mutations in Plcβ1, Plcβ2, and Plcβ4 family members are homozygous viable but exhibit specific defects (Chakrabarti et al., 2003; Li et al., 2000). Plcβ1 mutants die due to epileptic-like seizures, Plcβ2 mutants have enhanced responses to chemoattractants, and Plcβ4 mutants have defects in motor coordination and vision (Hashimoto et al., 2001). Two independent Plcβ3 mouse mutants have markedly different phenotypes (Wang et al., 1998; Xie et al., 1999). Xie et al. (1999) report that Plcβ3 deficient mice are homozygous viable with skin tumors and enhanced sensitivity to morphine, while Wang et al. (1998) report that Plcβ3 deficient mutants are early embryonic lethal. These discrepancies in Plcβ3 mutant mouse phenotypes may be due to differences in the deletion constructs used.

We report that independent zebrafish mutations in conserved X and Y catalytic domain residues of Plcβ3 yield facial skeletal phenotypes similar to *edn1* pathway mutants (Miller et al., 2000). Heterozygous *plcβ3* mutants have mild facial skeletal phenotypes resembling a partial loss of Edn1 function, while homozygous *plcβ3* mutants have severe facial skeletal phenotypes resembling a strong loss of Edn1 function (Miller and Kimmel, 2001). We provide evidence that the *plcβ3* catalytic domain mutations act in a dominant-negative manner, affecting the function not only of Plcβ3 but of other Plcβ family members as well. We further show through mosaic analyses that Plcβ3 function is autonomously required in neural crest cells specifically to pattern the facial skeleton.

Materials and methods

Fish stocks and maintenance

Fish were raised under standard conditions and occasionally with the addition of 0.0015% PTU (1-phenyl 2-thiourea) to inhibit melanogenesis (Westerfield, 1993). Stages are given in hours postfertilization (hpf) at 28.5 °C (Kimmel et al., 1995). *plcβ3*^{tg203e} and *plcβ3*^{th210} alleles, which are homozygous lethal, were generously provided by Drs. Tatjana Piotrowski and Christiane Nusslein-Volhard. The *sucker*^{tf216b} (*endothelin1*) mutants have been previously described (Miller et al., 2000). We obtained homozygous mutant embryos from natural matings of heterozygous carriers maintained on an inbred AB genetic background. *plcβ3*^{th210} was used for all phenotypic analyses unless noted otherwise. *fli1-GFP* albino transgenic fish, *TG(fli1:EGFP)*^{y1}*;alb*^{b4}, have been previously described (Lawson and Weinstein, 2002).

Tissue labeling procedures

Alcian green was used to stain the cartilage of 4–6 days postfertilization (dpf) fixed larvae. Facial cartilages were dissected out and prepared as flat mounts (Kimmel et al., 1998). Double Alcian blue (cartilage) and Alizarin red (bone) staining were done on 4–6 dpf fixed larvae using a modified "acid-free" protocol (Walker and Kimmel, in press). Whole-mount RNA in situ hybridizations were performed using digoxigenin-labeled riboprobes (Miller et al., 2000). *plcβ3* probe was made with a 2-kb c-terminal cDNA clone. References for other probes are: *dlx5a*, *dlx6a*, *barx1* (Walker et al., 2006), *dlx2a*, *dlx3b*, *gsc*, and *hand2* (Miller et al., 2000), *bapx1* (Miller et al., 2003), *runx2b* (Flores et al., 2004), *islet1* (Appel et al., 1995), and *sox9a* (Yan et al., 2002).

Mapping, sequence, and co-segregation analysis of plcβ3 Alleles

We first mapped the *schmerle* allele, *she*^{tg203e} to chromosome 7 on a hybrid *TF/AB* genetic background using bulked segregant analysis (Knapik et al., 1996). Individual diploid embryos were sorted into mutant and wild-type classes based on an open-mouth phenotype. The heads of these embryos were stained with Alcian green to confirm segregation of the mouth phenotypes with jaw skeletal defects, and DNA was prepared from the tails and amplified using primers flanking simple sequence-length polymorphisms. Finer mapping placed *she*^{tg203e} between the microsatellite markers Z7958 and Z8540 using a mapping panel containing 67 mutant and 29 wild-type diploid embryos. cDNAs were prepared from pools of mutant and wild-type sibling embryos by RT-PCR. Briefly, total RNA was isolated with Trizol (BRL) and primed for first strand cDNA synthesis using Superscript II reverse transcriptase (BRL) and oligo dT primer. Using sequence from the genomic clones Al772136 and BX511067, we designed primers to obtain overlapping cDNAs covering the full-length coding region of *plcβ3* as follows: RT-PCR using external primers 5′CCGTTGTTACACTGAAGGT3′/5′GTTTAGTGCCACCATCTG3′ followed by nested primers 5′GAAACTTCCAACCGAGAAG3′/5′CACGTTCCAGAAAAGCTGTG3′ yielded an n-terminal 2.0 kb *plcβ3* cDNA. RT-PCR using external primers 5′GCAAATGAGTCGCATTTACC3′/5′CGTACAACTTCTAGTAATAC3′ followed by nested primers 5′CAAAGGGACTCGTGTGGAC3′/5′GAGAATAGAAATCGATTTGAAG3′ yielded a c-terminal 2.0 kb cDNA. These PCR products were sequenced directly using an ABI automated sequencer.

Morpholino antisense oligonucleotide injections

Translation-blocking and splice site-blocking morpholinos (MOs) were purchased from Gene Tools, Inc. *plcβ3* splice site MO: 5′TTGTCGTGGTTACCTTGCAATAGCC3′ *plcβ3* translation MO: 5′*CAT*GGCTGCTGAATCGACGGGTGG3′ (sequence complementary to the start codon is underlined). Roughly 2 nl of MOs diluted to 0.5–10 mg/ml in 0.2 M KCl and 0.2% phenol red was pressure-injected into the yolk of 1–4 cell zebrafish embryos. For Table 1, the following MO amounts were used: WT+splice MO, 10 ng; WT+translation MO, 6 ng; WT+splice/translation MO, 10 ng and 3 ng, respectively; *plcβ3*$^{-/-}$+splice MO, 6 ng.

Tissue transplantation and confocal imaging

Tissue transplants were as described (Crump et al., 2004a,b). An 'Alexa568' mixture of 2% Alexa Fluor 568 dextran and 3% lysine-fixable biotin dextran (10,000 MW, Molecular Probes) was injected into the yolk of donor embryos at 1–4 cell stage. For neural crest transplants, donor tissue was taken from the animal cap at shield stage (6 hpf) and moved to shield stage host embryos to a position approximately 90° from the shield and 70° from the animal pole. For ectoderm transplants animal cap donor transplants were moved to shield stage host embryos to a position approximately 120° from the shield and 40° from the animal pole. For endoderm transplants donor embryos were additionally injected with TAR*RNA and margin donor tissue moved to 40% epiboly hosts (4 hpf) to margin position. All transplants were unilateral. Host embryos were screened at