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Zebrafish *cypher* is important for somite formation and heart development

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Abstract

Mammalian CYPHER (Oracle, KIA0613), a member of the PDZ-LIM family of proteins (Enigma/LMP-1, ENH, ZASP/Cypher, RIL, ALP, and CLP-36), has been associated with cardiac and muscular myopathies. Targeted deletion of *Cypher* in mice is neonatal lethal possibly caused by myopathies.

To further investigate the role of cypher in development, we have cloned the zebrafish orthologue. We present here the gene, domain structure, and expression pattern of zebrafish *cypher* during development. Cypher was not present as a maternal mRNA and was absent during early development. Cypher mRNA was first detected at the 3-somite stage in adaxial somites, and as somites matured, *cypher* expression gradually enveloped the whole somite. Later, *cypher* expression was also found in the heart, in head and jaw musculature, and in the brain.

We further identified 13 alternative spliced forms of cypher from zebrafish heart and skeletal muscle tissue, among them a very short form containing the PDZ domain but lacking the ZM (ZASP-like) motif and the LIM domains.

Targeted gene knock-down experiments using cypher antisense morpholinos led to severe defects, including truncation of the embryo, deformation of somites, dilatation of the pericardium, and thinning of the ventricular wall. The phenotype could be rescued by a cypher form, which contains the PDZ domain and the ZM motif, but lacks all three LIM domains.

These findings indicate that a PDZ domain protein is important for normal somite formation and in normal heart development. Treatment of zebrafish embryos with cyclopamine, which disrupts hedgehog signaling, abolished *cypher* expression in 9 somite and 15-somite stage embryos. Taken together, our data suggest that cypher may play a role downstream of sonic hedgehog, in a late stage of somite development, when slow muscle fibers differentiate and migrate from the adaxial cells.

Keywords: Cypher; Somites; ZASP; Z-line; PDZ; Myopathy; Heart development; LIM domain

Introduction

PDZ motifs (Kennedy, 1995), previously known as GLGF repeats (Cho et al., 1992) or DHR domains (Woods and Bryant, 1991; Willott et al., 1993), are protein–protein interaction domains (Brenman et al., 1996; Kornau and Seeburg, 1997) composed of 80–120 amino acid residues, which can be present as single or multiple copies. PDZ domains are ubiquitous protein interaction modules, and their binding specificity includes recognition of the carboxyl-terminus of various proteins, PDZ–PDZ interactions and recognition of internal protein sequences or phosphatidylinositol lipids. PDZ domain-containing proteins play important roles in cellular signaling (Jelen et al., 2003), but currently there is a limited amount of knowledge on their role in development.

Recently, a group of proteins containing both a PDZ domain and LIM domains have been described. This PDZ-LIM family of proteins include Enigma/LMP-1, ENH, ZASP/Cypher, RIL, ALP, and CLP-36 in vertebrates (Wu and Gill, 1994; Zhou et al., 1999). It has been suggested that several members of the PDZ-LIM family of proteins act as adapters

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that direct other proteins to the cytoskeleton. Most interactions of PDZ-LIM proteins with the cytoskeleton have been identified in striated muscle, where several PDZ-LIM proteins are predominantly expressed (Faulkner et al., 1999; Zhou et al., 1999; Passier et al., 2000). An example is ZASP (Z-band alternatively spliced PDZ motif-containing protein) as it is referred to in humans (Faulkner et al., 1999) or Cypher (Zhou et al., 1999) (also Oracle (Passier et al., 2000)), the mouse orthologue.

Cypher is a PDZ-LIM protein which is predominantly expressed in cardiac and skeletal muscle and several alternatively spliced forms have been described in humans (Faulkner et al., 1999; Passier et al., 2000) and mice (Huang et al., 2003). Alternative splicing is an important mechanism for generating functional diversity for many proteins including PDZ domain-containing proteins (Sierralta and Mendoza, 2004). The *cypher* gene encodes several different functional domains. Its PDZ domain, LIM domains, and ZM motifs allow for alternative processing with different combinations of these interaction domains. In human cardiac and skeletal muscle, there are at least three different forms of ZASP, derived by alternatively splicing. A fourth form (KIAA0613) is present in brain, and other forms have been found in fetal lung tissue and in the pancreas (Faulkner et al., 1999). Six alternatively spliced forms of *Cypher*, the mouse orthologue, have been identified, and expression was observed predominantly in cardiac muscle and skeletal muscle and weak expression was observed in the lung (Zhou et al., 1999). In humans, ZASP was also found to be expressed in pancreas tissue and the brain (Faulkner et al., 1999). Three of the human forms KIAA0613, ZASP, and ZASP variant 3 are the counterparts of mouse Cypher1c (also Oracle1), Cypher2s, and Cypher3c (also Oracle2), respectively.

Cypher null mutations in mice were embryonic or perinatal lethal and mice died from functional failure in multiple striated muscle types (Zhou et al., 2001) displaying disorganized and fragmented Z-lines in skeletal and cardiac muscle (Zhou et al., 2001). The phenotype of Cypher null mutant mice suggested that Cypher is essential for maintaining Z-line structure and mice developed a severe form of congenital myopathy. The phenotype in mice could be partially rescued by a shorter splice form of Cypher lacking the LIM domains but containing the PDZ domain and the ZM motif (Huang et al., 2003). Recently, the structure of the ZASP PDZ domain has been solved, revealing that it is a classical class 1 PDZ domain (Au et al., 2004).

Mutations in *CYPHER* have been associated with dilated cardiomyopathy (DCM) in humans (Vatta et al., 2003; Arimura et al., 2004). Disorders of the myocardium with an unknown cause are generally termed “cardiomyopathy”. They can be roughly divided in three groups: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) (although hypertrophy occurs not only in HCM), and restrictive cardiomyopathy (Anderson and Becker, 1992). It was suggested that *CYPHER* is a gene causing DCM in humans, both the “pure” form of DCM and DCM associated with isolated left ventricular noncompaction of the myocardium (INLVM) (Vatta et al., 2003; Arimura et al., 2004). This disease is characterized by a hypertrophic dilated left ventricle, ventricular dysfunction, and deep trabeculations. The presence of multiple mutations in the *CYPHER* gene in patients with DCM and INLVM suggests that disruption of this gene is a common cause of left ventricular LV dysfunction and dilation. Recently, mutations in ZASP have been linked to a novel form of muscular dystrophy in humans (Selcen and Engel, 2005). However, a role in skeletal muscle development or somite development has so far not been attributed to Cypher.

The segmented bauplan of vertebrates is most evident in the metameric appearance of somites during embryogenesis. Somites are segmented divisions of the paraxial mesoderm in vertebrate embryos that pass through a transient epithelial stage before transforming into mesenchyme. Somites contribute cells to the musculoskeletal system and dermis of the trunk, tail, and fins. Cell determination of early somite cells is influenced by the neighboring notochord, neural tube, lateral plate mesoderm, and surface ectoderm. Somite formation, epithelization, and patterning involve many different mechanisms and signaling molecules. A complex series of events must occur correctly before the myotome is formed: including (1) paraxial mesoderm specification, (2) generation of periodic gene expression waves, (3) establishment of segment polarity and boundaries, (4) formation of morphological boundaries or furrows, (5) maintenance of these furrows, and (6) differentiation and migration of three different types of muscle cells: pioneers, slow twitch and fast twitch (Henry et al., 2005; Stickney et al., 2000). How adaxial cells influence these processes and affect somite morphology and boundary formation was well described in recent publications (Henry et al., 2005; Cortes et al., 2003; van Eeden et al., 1998).

The zebrafish has long been recognized as an ideal organism for studies of somite development. The overall process of somite development in zebrafish is similar to that in other vertebrates such as amphibians, birds, and mammals (Kimmel et al., 1995). The first somite forms shortly after the end of gastrulation. As somite formation continues, the trunk begins to lift off the yolk and the tail extends. At 24 h of development, somite formation is complete and somite patterning is close to completion. Genetic screens for zebrafish mutants defective in somite formation have identified over 50 complementation groups, and the majority of the genes responsible have not been identified (Haffter et al., 1996; van Eeden et al., 1996). Mutations in genes involved in the Notch pathway have been shown to cause failure of segmentation and absence or irregularity of somite boundaries (Takke and Campos-Ortega, 1999; Julich et al., 2005a; Sieger et al., 2004). It was further shown that somite patterning involves a biochemical oscillator, the segmentation clock, which operates in the cells of the presomitic mesoderm (Holley et al., 2000). Although some of the molecules crucial for somite formation have been identified, many of the molecular mechanisms and signaling molecules governing somite formation still remain elusive.

We have cloned the zebrafish *cypher* gene and investigated its role in zebrafish development. Our data suggest that *cypher* plays a role in somite formation. Furthermore, we observed defects in heart development which correlates with earlier