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Molecular cloning of three zebrafish *lin7* genes and their expression patterns in the retina

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Abstract

The vertebrate retina develops from an undifferentiated sheet of neuroepithelial cells, whose differentiation requires the generation and maintenance of the correct cellular polarity. To examine the role of cell polarity in retinal development, we cloned three zebrafish *lin7* genes (*lin7a*, *lin7b*, and *lin7c*), which each encodes a protein candidate that is required for generation/maintenance of neuroepithelial cell junctions. These three zebrafish Lin7 proteins share over 78% amino acid identity and contain both L27 and PDZ domains that are present in all Lin7 homologs. Immunoblots revealed that the Lin7b and Lin7c proteins were first expressed in the developing eye by 24 hr postfertilization (hpf), while Lin7a was not detected in the eye until 72 hpf. At 33 hpf, the Lin7 proteins localized at, or slightly apical of, the actin-associated adherens junctions in the retinal neuroepithelium. This subcellular distribution required the expression of the Nok protein. In the absence of Nok, the Lin7 proteins failed to localize to either the ectopic adherens junctions or the cell membrane. At 4 days postfertilization, in situ hybridisation revealed that all three *lin7* genes were expressed in both the ganglion cell layer and the bipolar cell region of the inner nuclear layer. The *lin7a* gene was also expressed in the amacrine and horizontal cell regions of the inner nuclear layer, while *lin7c* was also expressed in the outer nuclear layer. In the adult retina, where Lin7a is the predominant form expressed, the Lin7 proteins were localized to the outer and inner plexiform layers, the bipolar and horizontal cells of the inner nuclear layer, and the ganglion cells. These results suggest that the three zebrafish Lin7 proteins possess partially redundant, yet essential, roles in retinal development.

Keywords: Mals; Lin7; Nok; retina; retinal neuroepithelium; retinal development; zebrafish

1. Introduction

The mature vertebrate retina contains six major classes of neurons: photoreceptors, horizontal cells, amacrine cells, interplexiform cells, bipolar cells, and ganglion cells. These neurons are segregated into three cellular layers: the ganglion cell layer, the inner nuclear layer (INL), and the outer nuclear layer (ONL, Dowling, 1987). The Müller glia, the major class of retinal glial cells, are located in the INL and extend processes from the outer limiting membrane to the inner limiting membrane. All of these retinal cell classes are generated from a single sheet of epithelial cells as they differentiate in a specific temporal pattern (Hu and Easter, 1999). The development of this stratified retinal cell architecture is conserved in all vertebrates, which implies that a common fundamental mechanism is involved in the generation of these retinal layers. However, the detailed molecular mechanisms of retinal lamination are not well understood.

Recent genetic studies identified several genes that are essential for cellular pattern formation in the zebrafish retina (Horne-Badovinac et al., 2001; Peterson et al., 2001; Wei and Malicki, 2002; Erdmann et al., 2003; Malicki et al., 2003; Masai et al., 2003; Jensen and Westerfield, 2004; Wei et al., 2004). These genes encode proteins that are involved in the establishment and/or maintenance of epithelial cell polarity in a variety of organisms. One of these genes, *nagie oko* (*nok*), encodes a member of the MAGUK (membrane-associated guanylate kinase) protein family (Wei and Malicki, 2002).

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The Nok protein contains seven protein–protein interaction domains: a conserved amino terminus, L27N, L27C, PDZ, SH3, Hook, and GUK domains. Nok may serve as a scaffold to recruit other proteins to form a functional protein complex that is essential for retinal development (Wei and Malicki, 2002). In flies, Stardust, the Nok homolog, physically interacts with the Crumbs and Dlin7 proteins (Bachmann et al., 2001; Hong et al., 2001; Bachmann et al., 2004). Similarly, Pals1 (protein associated with Lin-7), the mammalian Nok homolog, interacts with the mammalian homologs of Crumbs and Discs Lost via its PDZ and L27N domains, respectively (Kamberov et al., 2000; Roh et al., 2002; Makarova et al., 2003; Roh et al., 2003). Furthermore, the conserved amino terminus of Pals1 interacts with the PDZ domain of the Par-6 protein (Hurd et al., 2003). Finally, an in vitro study revealed that the Pals1 L27C domain interacts with the mouse Lin-7 protein (Kamberov et al., 2000).

The *lin-7* gene was initially identified in *C. elegans* as a mutation that reduced, but failed to eliminate, vulva differentiation during development (Ferguson and Horvitz, 1985). The Lin-7 protein consists of approximately 300 amino acids that encode two different protein–protein interaction domains: L27 and PDZ. The Lin-7 protein forms a complex with both the Lin-2 and Lin-10 proteins through its L27 domain (Kaech et al., 1998). This complex is required for the basolateral membrane localization of the LET-23 receptor tyrosine kinase in *C. elegans* (Simske et al., 1996; Kaech et al., 1998). The Lin-7/-2/-10 complex is also conserved in mammals (Butz et al., 1998) and potentially plays an important role in the formation and function of synaptic junctions (Butz et al., 1998; Irie et al., 1999; Jo et al., 1999).

We cloned three zebrafish *lin7* genes (*lin7a*, *lin7b*, and *lin7c*) to analyse the roles of the zebrafish Lin7 proteins in retinal lamination. In the retinal neuroepithelium, the Lin7 proteins localize to the apical membrane in a Nok-dependent manner. As the wild-type retinal neuroepithelium develops into a mature laminated retina, the *lin7* genes and corresponding proteins exhibit a dynamically changing expression pattern. Our results suggest that three zebrafish Lin7 proteins possess different, but overlapping, functions in the developing and mature retina.

2. Materials and methods

Zebrafish and rabbits used in the experiments presented in this study were handled in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and experiments were approved by the University of Notre Dame IRB.

2.1. Molecular cloning of the zebrafish lin7 gene

We performed BLAST searches with the three rat *lin-7* homolog sequences (LIN-7-1, LIN-7-2, and LIN-7-3) (Jo et al., 1999) and identified seven zebrafish *lin7* EST clones: fj41b11, fj63g03, and fm75h02 for *lin7a*; fm63c12, fl94f08, and fm63c12 for *lin7b*; and fb75b09 for *lin7c*. 5′ and 3′RACE was performed on zebrafish embryonic mRNA isolated from 24 and 48 hpf embryos using the FastTrack 2.0 Kit (Invitrogen, Inc., Carlsbad, CA) to isolate the full-length *lin7* cDNA sequences. GenBank accession numbers have been assigned to *lin7a* (AY584747), *lin7b* (AY584746), and *lin7c* (AY584748).

2.2. Polyclonal anti-Lin7 antibody production

The cDNA sequence encoding the entire *lin7a* open reading frame was cloned into the pET-32a(+) vector (Novagen, Madison, WI) and the pMAL-c2 vector (New England Biolabs, Beverly, MA) to express a S-Tag fusion protein and a maltose-binding protein (MBP) fusion protein in *E. coli*, respectively. S-Tag and MBP-fusion proteins were affinity purified according to the manufacturers' protocols using S-protein agarose and amylose resin, respectively. Rabbits were immunized with purified S-Tag-Lin7 fusion protein as described (Vihtelic et al., 1999). The purified MBP-Lin7 fusion protein was coupled to an agarose column using the AminoLink Plus Immobilization Kit (Pierce, Rockford, IL). The anti-Lin7 rabbit serum was immunopurified over a MBP-Lin7 fusion protein column as described (Vihtelic et al., 1999).

2.3. Immunohistochemistry

Wild-type AB embryos were raised to the desired age and fixed in 4% paraformaldehyde in PBS at room temperature for 2 hr or 4 °C overnight. Fixed embryos were infiltrated with 40% sucrose in 1×PBS at room temperature for 2 hr, embedded in Tissue-Tek media (Sakura Finetek USA, Torrance, CA), and cryosectioned at 18 μm thickness. The sections were washed with PBS and incubated with blocking buffer (PBS containing 2% BSA and 0.5% Triton X-100) for 40 min at room temperature. The sections were incubated with the desired primary antibodies: immunopurified rabbit anti-Lin7 polyclonal serum (1:100 dilution), mouse monoclonal anti-PKCα (sc-17769, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-SV2 (Developmental Studies Hybridoma Bank, 1:100), rabbit anti-Pard3 polyclonal serum (Wei et al., 2004), mouse monoclonal anti-HuC/D (monoclonal antibody 16A11, 1:30, Molecular Probes, Inc., Eugene, OR) in blocking buffer at 4 °C overnight. After four washes (PBS containing 0.5% Triton X-100) at room temperature for 10 min each, sections were incubated with the desired secondary antibodies: Cy5-conjugated goat anti-rabbit IgG, Alexa 488-conjugated phalloidin or