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Involvement of retinoic acid signaling in goldfish optic nerve regeneration

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ABSTRACT

Recently, we identified a retina-specific retinol-binding protein, purpurin, as a trigger molecule in the early stage of goldfish optic nerve regeneration. Purpurin protein was secreted by photoreceptors to injured ganglion cells, at 2–5 days after optic nerve injury. Purpurin bound to retinol induced neurite outgrowth in retinal explant cultures and retinoic acid (RA) had a comparable effect on neurite outgrowth. These results indicate that purpurin acts as a retinol transporter and facilitates conversion of retinol to RA. Intracellularly, RA is transported into the nucleus with cellular retinoic acid-binding protein IIb (CRABPIIb) and binds with retinoic acid receptor α (RARα) as a transcriptional regulator of target genes. Here, we investigated the RA signaling through RA synthesis to RARα in the goldfish retina during optic nerve regeneration by RT-PCR. Retinaldehyde dehydrogenase 2 (RALDH2; an RA synthetic enzyme) mRNA was increased by 2.7-fold in the retina at 7–10 days and then gradually decreased until 40 days after nerve injury. In contrast, cytochrome P450 26a1 (CYP26a1; an RA degradative enzyme) mRNA was decreased to less than half in the retina at 5–20 days and then gradually returned to the control level by 40 days after nerve injury. CRABPIIb mRNA was increased by 1.5-fold in the retina at 10 days after axotomy, RARαa mRNA was increased by 1.8-fold in the retina at 10 days after axotomy. The cellular changes in the RA signaling molecules after optic nerve injury were almost all located in the ganglion cells, as evaluated by *in situ* hybridization. The present data described for the first time that RA signaling through RALDH2 and CRABPIIb to RARα was serially upregulated in the ganglion cells at 7–10 days just after the purpurin induction. Therefore, we conclude that the triggering action of purpurin on optic nerve regeneration is mediated by RA signaling pathway.

1. Introduction

Retinoic acid (RA) is a metabolite of retinoids and has several physiological functions, including roles in cell differentiation, neurite outgrowth and cell survival. Synthesis of RA contains two oxidation processes (Duester, 1996; Napoli, 1996). The first involves oxidation of retinol to retinaldehyde by alcohol dehydrogenases and short-chain dehydrogenases/reductases, while the second involves oxidation of retinaldehyde to RA by retinaldehyde dehydrogenases (RALDHs). Intracellularly, synthesized retinol and RA are transported in bound forms with cellular retinol-binding proteins (CRBPs) and cellular retinoic acid-binding proteins (CRABPs), respectively (Liu et al., 2004, 2005; Sharma et al., 2005). In particular, CRABPII, but not CRABPI, mediates transport of RA to the nucleus (Dong et al., 1999; Bastie et al., 2001). In the nucleus, RA regulates gene transcription via ligand-activated transcription factors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) heterodimers (Hale et al., 2006). RA is metabolized to its inactive forms of 4-hydroxy-retinoic acid and 4-oxo-retinoic acid by cytochrome P450 26al (CYP26a1) (McCaffery and Dräger, 2000). These retinoid metabolizing enzymes and binding proteins are considered to act as RA signaling molecules and their expression levels are tightly regulated by RA itself (Balmer and Blomhoff, 2002).

Recently, we identified fish purpurin as a trigger molecule for optic nerve regeneration (Matsukawa et al., 2004). Purpurin contains β-barrel structures that are characterized as retinol-binding proteins (Schubert et al., 1986). During fish optic nerve regeneration, purpurin mRNA was synthesized in photoreceptor cells and the protein was secreted to injured retinal ganglion cells

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Abbreviations: CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; CYP26a1, cytochrome P450 family 26 subfamily A polypeptide 1; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PFA, paraformaldehyde; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RGC, retinal ganglion cell; RXR, retinoid X receptor.

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(RGCs) at 2–5 days after axotomy (Matsukawa et al., 2004; Tanaka et al., 2007). In fact, purpurin combined with retinol drastically induced neurite outgrowth in retinal explant cultures. Disulfiram, an inhibitor of RALDH2, completely blocked the neurite outgrowth evoked by purpurin with retinol. These results strongly suggest that the action of purpurin in triggering neurite outgrowth in the early stage of optic nerve regeneration may be mediated by RA synthesis. To confirm this possibility, in the present study, we determined the mRNA expression levels of RALDH2, CYP26a1, CRABPs and RARs in the goldfish retina after optic nerve injury. The levels of RALDH2, CRABPIIb and RARs mRNAs were significantly increased in the RGCs after optic nerve transection. In contrast, the expression levels of CYP26a1 mRNA was decreased in the RGCs after optic nerve transection. These results strongly indicate that RA signaling molecules are activated in the RGCs during the early stage of optic nerve regeneration in goldfish.

2. Experimental procedures

2.1. Animals and surgery

Common goldfish (*Carrassius auratus*; 6–7 cm in body length) were used throughout this study. The goldfish were anesthetized with ice-cold water and the optic nerves were transected with scissors at a distance of 1 mm from the eyeballs. After surgery, the goldfish were maintained in water tanks at 22 ± 1 °C for appropriate intervals.

2.2. RNA isolation and sequence analysis

Total RNA was isolated from the goldfish retinas at appropriate times after transection using Sepasol RNA I (Nacalai tesque, Japan). Next, cDNA fragments were obtained by RT-PCR using an RNA PCR kit (AMV) Ver. 3 (TaKaRa, Japan) with specific primers (Table 1). The amplified PCR products were subcloned into the pGEM-T Easy Vector (Promega, USA). The cDNA fragments were sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) and a dye terminator kit with Ampli Tag DNA polymerase (Applied Biosystems).

2.3. RT-PCR

RT-PCR was performed with the above-described RNA PCR kit (AMV) Ver. 3. The PCR products were electrophoresed and stained with ethidium bromide. The product bands were quantified with Scion Image software (Scion Corporation, USA) and normalized by the corresponding bands for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

2.4. Tissue preparation

Isolated goldfish eyes were immediately fixed in 4% paraformaldehyde (PFA) solution containing 0.1 M phosphate buffer (pH 7.4) and 5% sucrose for 2 h at 4 °C. After gradually increasing the sucrose concentration from 5 to 20%, the eyes were embedded in an OCT compound and cryosectioned at a thickness of 12 μm.

2.5. In situ hybridization

In situ hybridization was performed as described previously (Sugitani et al., 2006). Briefly, retinal sections were rehydrated, acetylated and permeabilized with 10 μg/ml proteinase K at room temperature for 10 min. After refixation in 4% PFA solution, the sections were prehybridized in hybridization buffer for 30 min. Hybridization was performed with 20 ng of cRNA probes labeled with digoxigenin in 100 μl of hybridization solution overnight at 42 °C. On the following day, the sections were washed and treated with 20 μg/ml RNase A at 37 °C for 30 min. To detect the signals, the sections were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Germany) overnight at 4 °C, and visualization was performed using tetrazolium-bromo-4-chloro-3-indolyl-phosphate (Roche) as the substrate.

2.6. Statistical analysis

Changes in mRNA levels after optic nerve transection were expressed as percent increases of the control values (no treatment). All data were expressed as means ± S.E.M for three experiments. The significance of differences among values was evaluated by one-way ANOVA followed by Fisher's PLSD multiple comparison test. Statistical significance was accepted for values of $P < 0.05$.

3. Results

To evaluate the expression levels of RA signaling molecules during optic nerve regeneration, we constructed specific primers for RALDH2, CYP26a1, CRABPIa, CRABPIIb and RARs by referring to the Ensemble database of zebrafish cDNAs (www.ensemble.org). The primer sequences are shown in Table 1. We obtained clear single bands with the precise lengths expected for the individual primers by RT-PCR. To confirm the primer specificities, we sequenced fragments of the PCR products and compared them with the zebrafish cDNA sequences using the FASTA program. All cDNAs isolated from goldfish retina total RNA exhibited high homologies of more than 80% with the zebrafish sequences. Thus, we concluded that the primers used in this study correctly amplified goldfish cDNA products corresponding to the zebrafish cDNAs.

3.1. Increase of RALDH2 mRNA expression in the goldfish retina

RALDHs are synthetic enzymes for RA. In particular, the expression of RALDH2 mRNA is well characterized in zebrafish retinal development (Marsh-Armstrong et al., 1994; Prabhudesai et al., 2005). To investigate the level of RALDH2 mRNA following optic nerve injury, we performed RT-PCR. The level of RALDH2 mRNA was significantly increased by 2.7-fold in the goldfish retina at 7–10 days after optic nerve transection (Fig. 1A). The expression gradually declined to the control value at 40 days after injury. To determine the localization of the increased RALDH2 mRNA in the

Table 1
PCR primer design.

Primer	Sequences (5′–3′)	Ensemble gene ID number
RALDH2	AACTGCCAGGAGAGGTGAAG (upper) TCTGCAAAAATGATGTTGGG (lower)	ENSDARG00000053493
CYP26a1	GCTTGCCGTTCATTGGAGAAAC (upper) TCGTCCGTCTTTATTTGCTCTGG (lower)	ENSDARG00000033999
CRABPIa	TTCTGAAAGCTCTCGGCGTAAAC (upper) GTACGTAAATCCGCGTGCAAAC (lower)	ENSDARG00000045926
CRABPIIb	GAAAGAAAGACACGCAGAAA (upper) GTTGAGTTGAGATGAAGTGG (lower)	ENSDARG00000030449
RARαa	CAGACCTGGTGTTCGCTTTC (upper) AGAGGGAGGGGCTACAACTG (lower)	ENSDARG00000056783
RARαb	TTAGCGAGCTCTCCACCAAA (upper) CGAACTGGGAGACAAACTGG (lower)	ENSDARG00000034893
RARγ	CACCCGCCCTGCTCACGA (upper) GAACCCGTTGAAAGTACACTGTTAAAAG (lower)	ENSDARG00000034117