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Mechanisms of RhoA inactivation and CDC42 and Rac1 activation during zebrafish optic nerve regeneration

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ABSTRACT

When axons of the mammalian central nervous system (CNS) are injured, they fail to regenerate, while those of lower vertebrates undergo regeneration after injury. Wingless-type MMTV integration site family (Wnt) proteins play important roles in the CNS, and are reported to be activated after mammalian spinal cord or brain injury. Moreover, for axon growth to proceed, it is thought that small G-proteins, such as CDC42 and Rac1, need to be activated, whereas RhoA must be inactivated. However, the cell and molecular mechanisms involved in optic nerve regeneration remain unclear. In this study, we investigated axonal regeneration after injury using the zebrafish optic nerve as a model system. We sought to clarify the role of Wnt proteins and the mechanisms involved in the activation and inactivation of small G-proteins in nerve regeneration. After optic nerve injury, mRNA levels of Wnt5b, TAX1BP3 and ICAT increased in the retina, while those of Wnt10a decreased. These changes were associated with a reduction in β-catenin in nuclei. We found that Wnt5b activated CDC42 and Rac1, leading to the inactivation of RhoA, which appeared to be dependent on increased TAX1BP3 mRNA levels. Furthermore, we found that mRNA levels of Daam1a and ARHGEF16 decreased. We speculate that the decrease in β-catenin levels, which also further reduces levels of active RhoA, might contribute to regeneration in the zebrafish. Collectively, our novel results suggest that Wnt5b, Wnt10a, ICAT and TAX1BP3 participate in the activation and inactivation of small G-proteins, such as CDC42, Rac1 and RhoA, during the early stage of optic nerve regeneration in the zebrafish.

1. Introduction

In the mammalian central nervous system (CNS), axons fail to regenerate after injury. In contrast, the CNS axons of lower vertebrates are able to regenerate (Attardi and Sperry, 1963; Bahr and Bonhoeffer, 1994). We previously used the fish optic nerve as a model system to study regeneration after CNS axonal injury (Kato et al., 2013). CNS regeneration has been widely studied; however, many of the underlying mechanisms are not well understood (Matsukawa et al., 2004a; Becker and Becker, 2007; McCurley and Callard, 2010; Benowitz and Yin, 2010; Rasmussen and Sagasti, 2016).

The human wingless-type MMTV integration site family (Wnt) protein family consists of 19 secretory proteins. Wnts are ligands of Frizzled receptor transmembrane proteins and activate various signal transduction pathways, such as the canonical Wnt/β-catenin signaling pathway and the non-canonical β-catenin-independent signaling cascade (Logan and Nusse, 2004; Kato, 2005; Habas and Dawid, 2005). In addition, Wnt participates in the activation and inactivation of G-proteins in non-canonical pathways (Gomez-Orte et al., 2013). Moreover, Wnts are involved in many developmental

Abbreviations: ARHGEF, rhoA guanine nucleotide exchange factor; CDC42, cell division cycle 42; CNS, central nervous system; daam1, disheveled-associated activator of morphogenesis 1; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCL, ganglion cell layer; ICAT, inhibitor of β-catenin and T cell factor; INL, inner nuclear layer; JNK, c-Jun N-terminal kinase; ONL, outer nuclear layer; PAK, p-21 activated kinase; PtdInsP2, D-myo-Phosphatidylinositol 4,5-biphosphate; Rac, ras-related C3 botulinum toxin substrate; RhoA, ras homolog family member A; ROCK, rho kinase; RT-PCR, reverse transcription-polymerase chain reaction; Ryk, receptor-like tyrosine kinase; siRNA, small interfering RNA; TAX1BP3, Tax1-binding protein 3; TF/LEF, T-cell factor/lymphoid enhancer-binding factor; Wnt, wingless-type MMTV integration site family protein.

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(Logan and Nusse, 2004; Clevers, 2006; Cadigan, 2008; MacDonald et al., 2009; Zhang et al., 2016) and tissue repair processes (Whyte et al., 2012). In the CNS, they have been found to control synaptogenesis, adult neurogenesis, and mitochondrial dynamics (Rodriguez-Gil and Greer, 2008; Salinas, 2012; Hikasa and Sokol, 2013; Rosso and Inestrosa, 2013; Silva-Alvarez et al., 2013; Bengoa-Vergniory and Kypta, 2015).

Wnt proteins are activated after mammalian spinal cord or brain injury (Lambert et al., 2016), and the Wnt/β-catenin signaling pathway has been suggested to be involved in neurodegenerative diseases (Scott and Brann, 2013). In mice, the Wnt pathway is activated by retinal injury, and the Wnt signal causes a subset of Müller cells to proliferate and dedifferentiate into progenitor cells (Liu et al., 2013). After spinal cord contusion injury, mRNA levels of Wnt family members increase in adult rats (Fernández-Martos et al., 2011) and mice (González-Fernández et al., 2014). Following injury, Wnt1, Wnt4 and Wnt5a are quickly induced around the lesion site. Wnt1 and Wnt5a are strong repellents of corticospinal tract axons (Liu et al., 2008), and abrogation of β-catenin signaling promotes axonal regeneration after CNS injury (Rodriguez et al., 2014). Expression of the Wnt genes after injury inhibits axonal regeneration in mammals (Liu et al., 2008; Miyashita et al., 2009; Hollis, 2016). In contrast, in lower vertebrates, Wnt signaling is required for the regeneration of neural tissues. The non-canonical Wnt receptor, Ryk, mediates chemorepulsive axon guidance in the developing brain and spinal cord in response to Wnt5a (Keeble et al., 2006). Ryk has also been identified as a major suppressor of axonal regrowth after spinal cord injury in mouse cortical neurons (Clark et al., 2014). In comparison, Wnt/β-catenin signaling promotes regeneration after spinal cord injury in the adult zebrafish (Strand et al., 2016).

Wnt proteins participate in the activation and inactivation of small G-proteins, such as RhoA, CDC42 and Rac, which regulate neuronal morphogenesis (Li et al., 2002) and neurite outgrowth (Bromberg et al., 2011). Rac, CDC42 and RhoA have contrasting roles, with Rac and CDC42 promoting neurite outgrowth, while RhoA stimulates retraction (Koh, 2006–2007). After optic nerve injury, Rac and CDC42 are activated (Wells and Jones, 2010; Lorenzetto et al., 2013; Zheng et al., 2016). However, inactivation of RhoA is an important step in the regeneration of the optic nerve (Fischer et al., 2004; Fujita and Yamashita, 2014), and RhoA inhibitors have been shown to promote optic nerve regeneration in the mammalian retina (Benowitz et al., 2017) and CNS (Kubo et al., 2007). However, the mechanisms underlying the activation of Rac and CDC42 and the inactivation of RhoA in optic nerve injury are unclear, and it is not known whether Wnt signaling plays a role in optic nerve regeneration. Therefore, in this study, we investigated the role of Wnt signaling in zebrafish optic nerve regeneration, as well as the molecular mechanisms of CDC42 and Rac1 activation and RhoA inactivation.

2. Materials and methods

2.1. Animals and materials

Zebrafish (*Danio rerio*; 3–4 cm in body length) were purchased from local pet shops and were reared in water tanks at 28 °C with a 13:11-h light–dark cycle. Zebrafish were anesthetized with 0.0033% ethyl 3-aminobenzoate methanesulfonate (Fluka, Mexico City, Mexico), and optic nerves were transected and retinae were isolated as described previously (Tanaka et al., 2007). The majority of all chemicals were guaranteed reagent grade and purchased from Wako Jun-yaku (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). D-myo-phosphatidylinositol 4,5-biphosphate (P-4516, PtdInsP2; length of carbon chain: 14) was purchased from Echelon BioScience (Salt Lake City, USA); H-1152 was purchased from Enzo Life Science (Farmingdale, USA); calpeptin and IPA-3 were from Sigma-Aldrich Chem. (St. Louis, USA).; and JNK inhibitor II was from Santa Cruz Biotechnology (Santa Cruz, USA). PtdInsP2 was dissolved in methanol and then diluted with 20% methanol. H-1152, calpeptin, IPA-3 and JNK inhibitor II were dissolved in dimethyl sulfoxide (DMSO). The rabbit anti-β-catenin antibody and FITC-conjugated goat anti-rabbit IgG were purchased from Cosmo Bio (Tokyo, Japan), and AP-conjugated anti-digoxigenin antibody was purchased from Roche Diagnostics (Basel, Switzerland).

2.2. RNA isolation and RT-PCR

Total RNA was isolated from retinae using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Each sample contained RNA isolated from 10 to 12 retinae, and 4–6 samples were used for RT-PCR analysis. Semi-quantitative RT-PCR was performed using reverse transcriptase XL (AMV) (TaKaRa, Otsu, Japan) and KOD (TOYOBO, Osaka, Japan), according to the manufacturer's protocol, with primers obtained from FASMAC (Atsugi, Japan; primer sequences are shown in Table 1). The number of cycles for each transcript and primer set was determined as that sufficient to observe a clear band without saturating band intensity. The annealing temperature and PCR cycle numbers were shown in Fig. 1. The PCR products were electrophoresed and stained with ethidium bromide. The product bands were quantified with GeneGnome Image analyzer (Syngene, Cambridge, UK) and normalized to that of GAPDH.

2.3. Explant culture

Retinal explant culture was performed as described previously (Matsukawa et al., 2004b), with the exception that zebrafish were used instead of goldfish and six-well microplates instead of 3.5-cm dishes. Briefly, retinas were isolated from zebrafish in which the optic nerves were transected 4 days previously. The retinas were minced and suspended in L-15 medium supplemented with 10% fetal bovine serum, and retinal pieces were cultured in six-well microplates. Each well contained about 40 pieces prepared from about 6 to 8 retinas. The number of explants (pieces) that extended neurites in each well was counted and expressed as a percentage of the total number of explants in that well, as described previously (Matsukawa et al., 2004b). Briefly, explants were incubated at 28 °C for 2–6 days, and those extending more than five neurites, each longer than 150 μm, were counted. Three to five independent experiments were performed.

2.4. siRNA treatment of explants

siRNA (75 pmol/well) was mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. Isolated retinal explants were suspended in microtubes, and the siRNA-Lipofectamine mixtures were added to the medium and incubated at 28 °C for 5 h. Then, the microtubes were centrifuged briefly, the medium was removed, and fresh medium was added and washed once. The explants were resuspended in fresh medium, and transferred to the wells of a microplate. An inhibitor or an activator was added, at the same time the explants were transferred to the well.

Double-stranded synthetic siRNAs were obtained from FASMAC, and their sequences are as follows: siCDC42ss, GGGUAAAACCUGUCUAUUAtt; siCDC42as, UAAUAGACAGGUUUUACCCtt; siRac1ss, CCCUAACACUCCAAUAAUUtt; Rac1as, AAUUAUUGGAGUGUUAGGGtt; siTAX1BP3ss, UCGACCAAAUCCAAGCCAtt; siTAX1BP3as, UAGCCCACACCACGAUAGAtt; siWnt5bss,