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LIMK1 acts downstream of BMP signaling in developing retinal ganglion cell axons but not dendrites

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ABSTRACT

The actin cytoskeleton inside extending axonal and dendritic processes must undergo continuous assembly and disassembly. Some extrinsic factors modulate actin turnover through controlling the activity of LIM kinase 1 (LIMK1), which phosphorylates and inactivates the actin depolymerizing factor cofilin. Here, we for the first time examine the function and regulation of LIMK1 *in vivo* in the vertebrate nervous system. Upon expression of wildtype or kinase-dead forms of the protein, dendrite growth by *Xenopus* retinal ganglion cells (RGCs) was unchanged. In contrast, maintaining a low, but significant level, of LIMK1 function in the RGC axon is critical for proper extension. Interestingly, bone morphogenetic protein receptor II (BMPRII) is a major regulator of LIMK1 in extending RGC axons, as expression of a BMPRII lacking the LIMK1 binding region caused a dramatic shortening of the axons. Previously, we found that BMPRIIs stimulate dendrite initiation *in vivo*. Thus, the fact that manipulation of LIMK1 activity failed to alter dendrite growth suggests that BMPs may activate distinct signalling pathways in axons and dendrites.

Introduction

Retinal ganglion cells (RGCs) are the output neurons of the eye, receiving visual signals from the retina to then transmit to the brain. We and others have previously characterized some of the extrinsic factors important for the morphological development of RGCs, i.e. for the elaboration of a complex dendritic arbor and the extension/guidance of a long axon, followed by integration into the proper neural circuits (Erskine and Herrera, 2007; Hocking et al., 2008; Parrish et al., 2007; Sernagor et al., 2001). Yet, intrinsic factors also play a large role in the development of neuronal morphology. For example, the level of cyclic nucleotides can determine a growth cone's response to a guidance cue (Song et al., 1998). While the intracellular factors that modify the neuronal cytoskeleton in response to extracellular cues are beginning to be elucidated (Kalil and Dent, 2005), whether different signalling pathways mediate the responses of axons and dendrites to the same extracellular cues is not known.

One factor implicated in regulating the axonal and dendritic outgrowth of neurons *in vitro* is the cytoplasmic kinase LIM kinase 1 (LIMK1) (Endo et al., 2003; Hsieh et al., 2006; Lee-Hoeflich et al., 2004; Mizuno et al., 1994; Rosso et al., 2004; Tursun et al., 2005). The LIMK1 and LIMK2 proteins are ubiquitously expressed, though LIMK1 is particularly enriched in the brain as well as in the growth cones of cultured neurons (Acevedo et al., 2006; Foletta et al., 2004; Piper et al., 2006; Rosso et al., 2004). LIMK1 directly impacts the assembly of actin filaments in response to modulation of its activity by extrinsic signals (Sarmiere and Bamburg, 2004; Takahashi et al., 2003). Importantly, the addition of actin monomers to the fast-growing barbed ends of filamentous actin (F-actin) is thought to provide the framework needed to push the membrane forward and extend a lamellipodia or filopodia (Sarmiere and Bamburg, 2004). LIMK1 phosphorylates and inactivates the actin depolymerizing factors ADF and cofilin1/2 (referred to collectively as cofilin throughout). Cofilin regulates actin dynamics by removing actin monomers from the slow-growing pointed ends of F-actin, or by severing actin filaments (Sarmiere and Bamburg, 2004). Cofilin activity maintains a balance in cytoskeletal dynamics, both breaking down actin filaments where these are no longer needed and promoting filament growth. Interestingly, while cofilin, a crucial regulator of actin dynamics, is downstream of LIMK1, Rho GTPases, which are major players in axon and dendrite growth, act upstream of LIMK1 (Govek et al., 2005; Ruchhoeft et al., 1999). p21-activated kinase (PAK) and Rho-associated coiled-coil domain kinase (ROCK) are downstream effectors of Cdc42/Rac1 and RhoA, respectively, and both can activate LIMK1 through phosphorylation (Bernard, 2007).

The function of LIMK1 in process outgrowth has been investigated mainly in axons growing *in vitro*, in which the manipulation of LIMK

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expression has variable effects on axon extension and growth cone motility, apparently dependent on cell type and culture conditions (Endo et al., 2003; Rosso et al., 2004; Tursun et al., 2005). *In vivo*, we know only that overexpression and loss-of-function of LIMK in the mushroom body neurons of *Drosophila* each caused axon stalling and guidance errors (Ng and Luo, 2004). The involvement of LIMK1 in dendrite development is even less clear, although LIMK1 is expressed in dendritic growth cones (Lee-Hoeflich et al., 2004; Tursun et al., 2005). While hippocampal neurons in mice mutant for *limk1* do have impaired dendritic spine morphology and synaptic function, their dendritic arbors appeared to develop normally (Meng et al., 2002). Yet, *in vitro* experiments showed that LIMK1 interacts directly with the cytoplasmic tail of BMP receptor II (BMPRII) (Foletta et al., 2003), and mediates BMP-induced dendrite outgrowth of cortical neurons (Lee-Hoeflich et al., 2004). More recent work showed that the attraction of *Xenopus* spinal neuron axons to BMP7 in culture is also mediated by BMPRII and LIMK1 (Wen et al., 2007). Whether LIMK1 functions in the formation of vertebrate neuronal processes *in vivo*, either in response to BMP signalling or other extrinsic signals, remains largely unknown.

In this study, we investigate a role for LIMK1 in both axon and dendrite outgrowth of *Xenopus* RGCs *in vivo*. We show that *limk1* is expressed by these cells at the time of process outgrowth, and that it has a specific function in the morphological development of their axons. Dendrite initiation, growth, and branching were unaffected by overexpression of either wild type (wt) or kinase-dead (kd) LIMK1 within developing RGCs. RGC axon growth, however, requires tight control of the levels of LIMK activity as extension defects were observed with overexpression of wtLIMK1, kdLIMK1 and slingshot phosphatase, a negative regulator of LIMK1. Interestingly, a BMP receptor II (BMPRII) lacking the LIMK-binding region, but not the wildtype BMPRII, causes a similar shortening of axons, suggesting that BMPs in the brain signal through LIMK to promote RGC axon outgrowth. Since we found previously that BMP signalling promotes RGC dendrite initiation *in vivo* (Hocking et al., 2008), these data argue that LIMK1 function is necessary downstream of BMP receptor activation in axons, but not dendrites, indicating that distinct signal transduction mechanisms may act downstream of a receptor expressed in both axons and dendrites.

Experimental procedures

Animals

Xenopus laevis embryos were obtained by *in vitro* fertilization of eggs produced by females who had been primed with human chorionic gonadotrophin (Chorulon; Intervet). Embryos were reared in 0.1×Marc's Modified Ringer's solution (MMR; 0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) at 14–25 °C and staged according to a standard set of criteria (Nieuwkoop and Faber, 1994).

Expression constructs

Xenopus lim kinase 1 (*limk1*) constructs, both a wildtype (*wtlimk1*) and a kinase-dead version with a D435N mutation (*kdlimk1*) (Takahashi et al., 1997, 2001) were subcloned into the CS2-MT expression vector, with the myc tag at the 5′ end.

cDNA encoding a full-length *Xenopus* BMP receptor II (dnBMPRII) in the vector pCS2-ITR was obtained from Dr. Sylvia Evans (University of California at San Diego) (Shi et al., 2000). A dominant negative BMPRII construct that lacks the LIMK-binding region (BMPRIIΔLBR), but still has the kinase domain, was generated from the full-length BMPRII by designing PCR primers to amplify the entire coding sequence with the exception of 888 nucleotides at the 3′ end of the gene (full coding sequence is 3.1 kb). The primers used were BMPRIILBR_for: ATCGATCTCCTATGGTATTCTTGCTTATTCC, BMPRIILBR_rev: ATCGATGAACATCCTGGACAATGCAAGC. The dominant negative receptor was subcloned into CS2-MT such that a myc tag was added to the carboxyl terminus of the protein. A CS2 construct encoding green fluorescent protein (CS2-GFP) was used alone as a control in all experiments, and was co-injected with the experimental constructs to help screen for expressing embryos and for the analysis of cell morphology.

Transgene expression

The lipofection method used to create mosaic gene expression by injection of a mixture of a transfection agent (DOTAP) and cDNA constructs into the presumptive eye region of stage 19 *Xenopus* embryos has been described previously (Holt et al., 1990). Alternatively, electroporation was used as described (Chen et al., 2007) to target either the eye or brain of anaesthetized stage 27 embryos. Following gene transfer, embryos developed at room temperature in 0.1×MMR until stage 40, and were then fixed overnight at 4 °C in 4% paraformaldehyde and processed for wholemount or section immunochemistry.

Antibodies

Antibodies used include the following: anti-myc [9E10; Developmental Studies Hybridoma Bank (DHSB); 1:500], rabbit or mouse anti-GFP (Invitrogen; 1:500), rabbit anti-myc (Santa Cruz; 1:500), mouse anti-hemagglutinin (HA; Covance; 1:500), rabbit anti-HA (Covance; 1:500), rabbit anti-phosphocofilin1 (P-cofilin1; Santa Cruz; 1:3000), rabbit anti-phosphocofilin2 (Abcam; 1:100), and rabbit anti-phosphoLIMK1/2 (P-LIMK; Novus Biologicals; 1:50–1:150). Secondary antibodies included goat anti-mouse and anti-rabbit antibodies conjugated to peroxidase (1:500) or AMCA (1:250) from Jackson ImmunoResearch Laboratories Inc., and goat anti-mouse and goat anti-rabbit antibodies conjugated to Alexa 546 (1:1000) or Alexa 488 (1:1000) (Invitrogen).

Immunochemistry

Immunochemistry was performed on tissue sections, and whole embryos as previously described (Cornel and Holt, 1992; McFarlane et al., 1995). For tissue sections, fixed embryos were immersed in 30% sucrose in PBS prior to embedding in Optimal Cutting Temperature compound (OCT, Baxter) and quick freezing. Twelve micrometer transverse sections were cut on a cryostat (Leica) and collected on gelatin-coated slides. Slides or partially dissected embryos were incubated in primary antibodies diluted in PBT [PBS with 0.5% Triton (BDH) and 0.2% BSA] containing 5% goat serum (Invitrogen) for 1 to 3 h at room temperature or overnight at 4 °C. Samples were rinsed, and then in the case of slides, secondary antibodies conjugated to fluorescent tags and diluted 1:250–1:1000 in PBT-goat serum were added for 1 h. Slides were mounted in the antibleaching agent Polyaquamount (Polysciences Inc.). In contrast, immunolabelling in the wholemount brains was visualized with a peroxidase-conjugated secondary antibody followed by reaction with diamino benzidine (DAB; Sigma-Aldrich Co.). Anterograde axon labelling with horseradish peroxidase was performed as described (Chien et al., 1993), and brains were dissected prior to the DAB reaction. Brains were then removed and dehydrated in serial ethanol washes, cleared in xylene and mounted in Permount (Fisher Scientific Company) under coverslips supported with two plastic reinforcement rings.

Dendrite analysis

In vivo, RGCs were identified based on the location of the cell body in the ganglion cell layer of the retina. Dendritic arbors were assessed at stage 39/40, when the arbor is still relatively simple: on average