Key Regulators in Neuronal Polarity

Minireview

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Neurons are highly polarized cells, most of which develop a single axon and several dendrites. These two compartments acquire specific characteristics that enable neurons to transmit intercellular signals from several dendrites to an axon. A wealth of recent studies has shown that PI 3-kinase, Rho family GTPases, the Par complex, and cytoskeleton-related proteins participate in the initial events of neuronal polarization. Here, we review the role of polarity-regulating molecules and the potential mechanisms underlying the specification of an axon and dendrites.

Hippocampal neurons in culture, the most widely used culture system for examination of neuronal polarity, develop only one axon and several dendrites (as hippocampal neurons do in situ) and maintain their characteristics at structural and molecular levels. Banker and colleagues divided the morphological events of neuronal polarization into five stages (reviewed in Craig and Banker, 1994). Shortly after plating, the neurons form small protrusion veils and a few spikes (stage 1). These truncated protrusions then develop into several short neurites (stage 2). At this stage, because neurites are roughly equal in length, it is difficult to identify special characteristics that could allow us to predict which one will become an axon. One neurite then starts to break the initial morphological symmetry, growing at a rapid rate (Esch et al., 1999), and neurons immediately establish the polarity (stage 3). A few days after the axon has begun its rapid growth, the remaining neurites elongate and acquire the characteristics of dendrites (stage 4). About 7 days after plating, neurons form synaptic contacts and establish a neuronal network (stage 5). While initial axon formation is clearly a key event in neuronal polarization, the molecular mechanisms that underlie axon specification remained unclear for more than a decade.

PI 3-Kinase and Its Upstream Molecules in Neuronal Polarity

Because neurons develop polarity in culture without any directional gradients of extracellular cues, an internal polarization program appears to exist in neurons (reviewed in Craig and Banker, 1994). However, it has been revealed that extracellular substrate molecules can govern which neurite becomes an axon, depending on the substrate preference of neurite elongation (Esch et al., 1999). When neurons are plated on alternating stripes of poly-D-lysine and either laminin or neuron-glia cell adhesion molecule (NgCAM), neurons usually form an axon on laminin or NgCAM, suggesting that the signals produced by the attachment of laminin/NgCAM with adhesion molecules such as integrins cause the rapid neurite growth and are enough to induce axon formation. This rapid axon formation is also observed when an immature neurite contacts laminin-coated beads in stage 2 neurons (Menager et al., 2004). Given these findings, a signaling cascade accelerated by laminin through integrins may initiate the neurite growth and the subsequent axon formation, and certain extracellular cues may determine axon/dendrite fate during physiological development.

What are the downstream signals of the extracellular cues? Recent experiments have shown the importance of PI 3-kinase and its lipid product [PI(3,4,5)P3; PIP_3] in determining and maintaining internal polarity in neurotrophils and dictyostelium (reviewed in Iijima et al., 2002). Shi et al. (2003) reported that the PI 3-kinase activity is localized at the tip of a newly specified axon in stage 3 neurons, and PI 3-kinase inhibitor prevents the axon formation. Consistent with this, when an immature neurite at stage 2 contacts laminin-coated beads, it shows a rapid accumulation of Akt-pleckstrin homology domain-GFP, a monitoring tool of PIP_3, followed by the dramatic growth of the neurite as an axon (Menager et al., 2004, and references therein). These observations suggest that PI 3-kinase is specifically activated at the tip of the future axon and that PIP_3 is essential for neuronal polarization (Figure 1).

Da Silva et al. (2005) reported that the ganglioside-converting enzyme PMGS is necessary for axon outgrowth. PMGS asymmetrically accumulates at the tip of one neurite (future axon) at stage 2, and its depletion prevents axon generation. The activation of PMGS promotes elongation of a single neurite by accelerating the local activity of TrkA, a receptor of nerve growth factor (Da Silva et al., 2005). PMGS appears to restrict the TrkA-mediated signals at the tip of the future axon, which include PI 3-kinase and its product PIP_3. Although PMGS could serve as a landmark of the premature axon during the initial phase of polarization, the mechanism spatially restricting the PMGS accumulation remains unclear.

Recently, Dotti and colleagues presented evidence indicating that the centrosome may guide the neuronal polarity during the early stages (de Anda et al., 2005). In cultured hippocampal neurons, the centrosome, Golgi apparatus, and endosomes cluster together close to the area where the first neurite will form, which is opposite from the plane of the last mitotic division (de Anda et al., 2005). Moreover, directional microtubule assembly and membrane transport toward this one neurite are observed in neurite-emerging neurons. A neuron with more than one centrosome develops multiple axons, and the prevention of the centrosome's functions inhibits neurite formation. These results suggest that neurons have internally programmed polarization after cell division. It is well known, however, that the centrosome position is controlled by PI 3-kinase, Cdc42, microtubules, and dynein (reviewed in Etienne-Manneville and Hall, 2003), raising the possibility that the centrosome position is not the cause of axon formation but the outcome of PI 3-kinase- and Cdc42-mediated signals. Further studies

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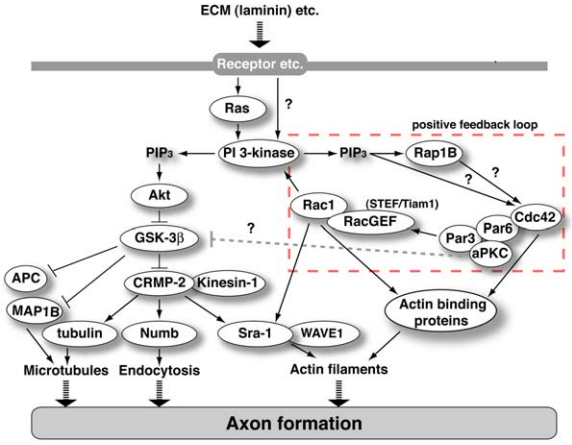

Figure 1. Signals and Polarity-Regulating Proteins in Neuronal Polarization

Extracellular matrix (ECM) activates PI 3-kinase through the interaction with adhesion molecules or receptors, thereby producing PIP_3. PIP_3 activates Akt, which inactivates GSK-3β, resulting in an increase in nonphosphorylated CRMP-2 at axonal growth cones. PIP_3 activates Cdc42 presumably through Rap1B and, in turn, recruits the Par6/Par3/aPKC complex. The Par complex stimulates STEF/Tiam1 and consequently activates Rac1. The relationship between the Akt/GSK-3β pathway and the Par3/Par6 pathway in neuronal polarity is not clear.

will be necessary to better understand the causal relation between the centrosome and axon formation.

Small GTPases and the Par Complex in Neuronal Polarity

A highly dynamic area is located at the tips of an axon, where drastic rearrangements of actin filaments and microtubules occur during neurite elongation (reviewed in Baas and Buster, 2004; Bradke and Dotti, 2000). This leads to the possibility that one of the major regulators of actin filaments and microtubules, the Rho family GTPases, are involved in axon specification. Among the Rho family GTPases, Cdc42, Rac1, and RhoA are the best characterized (reviewed in Govek et al., 2005). Cdc42 and Rac1 are involved in neurite extension in N1E-115 neuroblastoma cells through the action of their specific effectors, including Neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP family verprolin-homologous protein-1 (WAVE1), whereas RhoA is implicated in neurite retraction through the action of Rho-kinase (reviewed in Govek et al., 2005). Cdc42 is thought to act upstream of Rac1 for neurite extension. Tiam1 serves as a guanine nucleotide exchange factor for Rac1 downstream of Cdc42 (see below) and promotes neurite extension.

The signaling cascade of Cdc42 and Rac1 is also implicated in neuronal polarization. Expression of a constitutively active mutant of Cdc42 or Tiam1 induces multiple axon-like neurites (Nishimura et al., 2005; Schwamborn and Puschel, 2004) but impairs axonal maturation (Kunda et al., 2001; Nishimura et al., 2005), suggesting that cycling between GTP-bound and GDP-bound states of Cdc42 and Rac1 is needed for proper axonal maturation. Cytochalasin D application to stage 2 neurons causes multiple axon-like neurites, implying that the reorganization of actin filaments is necessary for axon formation (reviewed in Bradke and Dotti, 2000). In addition, Chuang et al. (2005) showed that the cytoplasmic dynein light chain Tctex-1 functions in initial neurite sprouting and axon outgrowth, acting through Rac1. Thus, it is likely that Cdc42/Rac1-mediated signal cascades regulate the reorganization of actin filaments in the prospective axon, thereby specifying axon. Given that Rac1 activates PI 3-kinase (reviewed in Govek et al., 2005), the signal initially evoked by PI 3-kinase appears to terminate at PI 3-kinase itself. This positive-feedback loop may be a driving force for axon specification and maturation (Figure 1).

A polarity complex of Par3, Par6, and atypical protein kinase C (aPKC) functions in various cell-polarization events (reviewed in Ohno, 2001; Wiggin et al., 2005), including axon formation (Nishimura et al., 2004; Shi et al., 2003). In hippocampal neurons, Par3 and Par6 are localized at the tips of axons. Suppression of Par3 or Par6 function inhibits axon formation. Ectopic expression of Par3 forms the multiple axon-like neurites but impairs axonal maturation, indicating that the proper localization and activity of Par3 and Par6 are necessary for axon maturation (Nishimura et al., 2005; Shi et al., 2003). PI 3-kinase activity is required for proper localization of the Par complex and Cdc42 at tips of the axon (Nishimura et al., 2005; Shi et al., 2003). Cdc42-GTP binds to Par6 and determines its localization, but little was known about the downstream signals from the Cdc42/Par complex until recently. Par3 directly interacts with STEF/Tiam1, guanine nucleotide exchange factors for Rac1 (Chen and Macara, 2005; Nishimura et al., 2005). The dominant-negative Par3 or knockdown of Par3 inhibits the Cdc42-induced Rac1 activation in N1E-115 neuroblastoma cells (Nishimura et al., 2005). Thus, the Par3/Par6 complex appears to mediate the signal from Cdc42 to Rac1 for axon formation (Nishimura et al., 2005). Chen and Macara (2005) found that Rac1 is slightly activated in MDCK epithelial cells lacking Par3. The knockdown of Par3 in MDCK cells might prevent the recruitment of Tiam1 to cell-cell contact sites and thereby promote the Tiam1-mediated Rac1 activation in cell periphery. On the other hands, it was reported that *Drosophila* Par6, aPKC, and Bazooka/Par3 are not involved in axon or dendrite specification in vivo (Rolls and Doe, 2004). The effects of Par deletion might be compensated by the extracellular circumstance and/or other signaling cascades linking Cdc42 and Rac1 in *Drosophila*. Further work will be required to elucidate the roles of Par proteins in polarization of mammalian neurons.

A member of the Ras subfamily of GTPases, Rap1B, functions as a positional signal and organizes cell architecture (Schwamborn and Puschel, 2004). Rap1 is a mammalian homolog of Bud1p/Rsr1p, which determines the position of incipient budding sites in *Saccharomyces cervisiae*. In hippocampal neurons, Rap1B is localized at the tips of axons preceding the accumulation of Cdc42 and the Par complex. Rap1B activation induces the multiple axon-like neurites and the accumulation of the Par complex in each axon, whereas the inactivation of Rap1B leaves the neurons without an axon. This reduction of axon formation is rescued by the coexpression of the active forms of Cdc42 and Par6, suggesting that Rap1B acts upstream of Cdc42 and the Par complex. Analysis using inhibitors revealed that PI 3-kinase functions upstream of Rap1B. Although it appears that the

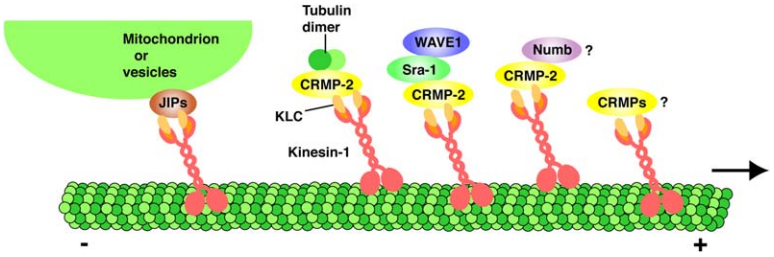

Figure 2. Kinesin-1 and Cargoes for Axonal Transport in Growing Axons

Kinesin-1 transports vesicles or mitochondria by interacting with JIPs. CRMP-2 associates with KLC of Kinesin-1 and links the Kinesin-1 to tubulin dimers or the Sra-1/WAVE1 complex. The soluble components responsible for axon formation and/or maturation appear to be transported via cargo receptors such as CRMP-2.

Rap1B localization at a single neurite is a decisive step in determining which neurite becomes the axon, by collecting the essential regulators of axon formation, the mechanism underlying how Rap1B is concentrated in one neurite remains unclear.

Akt/GSK-3β and Its Substrates in Neuronal Polarity

The signaling cascade from PI 3-kinase to Akt/GSK-3β participates in cell polarity in various cell types, including neurons (Cole et al., 2004; Jiang et al., 2005; Yoshimura et al., 2005a, and references therein). PI 3-kinase generates PIP_3, which is essential for the translocation of Akt at the plasma membrane. Akt is phosphorylated there and activated by phosphoinositide-dependent kinase (PDK) or other kinases, including integrin-linked kinase (ILK; reviewed in Hannigan et al., 2005). Activated Akt phosphorylates GSK-3β at Ser-9 and inactivates its kinase activity. Expression of constitutively active Akt or suppression of GSK-3β functions induce multiple axons, whereas expression of constitutively active GSK-3β prevents axon formation (Jiang et al., 2005; Yoshimura et al., 2005a). Thus, the signaling cascade from PI 3-kinase to Akt/GSK-3β appears to regulate axon formation.

Which substrates of GSK-3β are involved in axon specification? Collapsin response mediator protein (CRMP)-2 has been identified as a substrate of GSK-3β (Cole et al., 2004; Yoshimura et al., 2005a). CRMP-2 accumulates at the distal part of a growing axon (Inagaki et al., 2001). Overexpression of CRMP-2 induces multiple axons, and the inhibition of CRMP-2 functions impairs axon formation. Thus, CRMP-2 appears to play a critical role in axon specification.

Recently, several CRMP-2 binding proteins have been identified. CRMP-2 interacts with tubulin heterodimers and promotes microtubule assembly in vitro (Fukata et al., 2002). It binds and colocalizes with Numb at the central region of axonal growth cones and, in turn, regulates the endocytosis of L1, a neuronal cell adhesion molecule (Nishimura et al., 2003). CRMP-2 associates with the specifically Rac1-associated protein-1 (Sra-1)/WAVE1 complex and regulates actin filament stability (Kawano et al., 2005). Thus, CRMP-2 seems to promote neurite elongation and axon specification by regulating the microtubule assembly, the endocytosis of adhesion molecules, and the reorganization of actin filaments.

GSK-3β phosphorylates CRMP-2 at Thr-514 and thereby inactivates its binding to tubulin dimers/microtubules and Numb (Yoshimura et al., 2005a). A nonphosphorylated CRMP-2 is enriched in the axonal growth cones. The expression of constitutively active GSK-3β leaves some neurons with no axon, whereas the nonphosphorylated form of CRMP-2 counteracts the inhibitory effects of GSK-3β. Thus, GSK-3β regulates neuronal polarity through the phosphorylation of CRMP-2. In addition, GSK-3β phosphorylates MAP1B and the adenomatous polyposis coil gene product (APC), both of which are microtubule-associated proteins, suggesting that GSK-3β regulates microtubule dynamics and, in turn, neuronal polarity. In this regard, the SAD kinase/microtubule affinity-regulating kinase (MARK)/Par-1 is also implicated in neuronal polarity through the regulation of microtubule-associated proteins such as Tau (Kishi et al., 2005).

Possible Mechanism of Axonal Protein Trafficking

Intracellular trafficking is fundamental to the establishment and maintenance of neuronal polarity. Many proteins and vesicles are selectively transported along microtubules to either axon or dendrites by means of the kinesin and dynein families (reviewed in Hirokawa and Takemura, 2005). However, it is largely unknown how proteins, especially polarity-regulating proteins, are specifically transported to the future axon. Par3 is transported to the distal part of the growing axon by Kinesin-2 through the direct interaction with KIF3A (Nishimura et al., 2004). CRMP-2 links tubulin dimers or Sra-1 to Kinesin-1 through the interaction with kinesin light chain (KLC; Kimura et al., 2005; Kawano et al., 2005). The CRMP-2/Kinesin-1 complex regulates the transport of tubulin dimers and Sra-1/WAVE1 to the distal part of the growing axon. These observations suggest that CRMP-2 serves not only as a regulator of tubulin dimers, Numb, and Sra-1, but also as a cargo receptor, which links the partner proteins responsible for axon formation to motor proteins (Figure 2). A few cargo receptors have been identified for Kinesin-1, including c-*jun* NH2-terminal kinase (JNK)-interacting protein (JIP/SYD; reviewed in Hirokawa and Takemura, 2005). It remains unclear how a complex of Kinesin-1 with CRMP-2 or other cargo receptors is selectively transported to a growing axon.

Remaining Questions

Significant progress has been made toward understanding the intracellular events during neuronal polarization. However, more questions remain. For example, what extracellular cues govern neuronal polarity in situ? Extracellular matrix (ECM) is a good candidate, but its role in axon formation in situ must be examined. If certain receptors and adhesion molecules (such as integrins) participate in axon specification, how are signals conveyed to PI 3-kinase? The Ras family GTPases, including H-Ras and R-Ras, may become activated at the tip and stimulate PI 3-kinase (Figure 1). In this regard, H-Ras has been shown to regulate neuronal polarity acting through PI 3-kinase (Yoshimura et al., 2005b). Finally, how are the signals and polarity-regulating proteins spatially and temporally regulated, and how are they related to each other in axon specification? The analysis of spatial-temporal

dynamics of the signals and polarity-regulating proteins is required for a complete picture of neuronal polarization.

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